TRYPANOPAIN: A POSSIBLE TARGET FOR ANTI-TRYPANOSOMAL AGENTS?

Linda Troeberg

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Linda Troeberg
BSc (Hons) (Pietermaritzburg)

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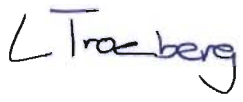
PREFACE

The experimental work described in this thesis was carried out in the Department of Biochemistry, University of Natal, Pietermaritzburg from January 1994 to November 1997 under the supervision of Dr Theresa H. T. Coetzer and Prof. John D. Lonsdale-Eccles. These studies represent original work by the author and have not been submitted in any other form to another University. Where use was made of the work of others, it has been duly acknowledged in the text.



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ABSTRACT

The protozoan parasite *Trypanosoma brucei brucei* causes nagana in cattle and is a widely used model for human sleeping sickness. The major lysosomal cysteine proteinases (trypanopains) of African trypanosomes may contribute to pathogenesis by degrading proteins in the mammalian bloodstream and also appear to be essential for the viability of *T. cruzi* and *T. congolense* . This study describes the first purification to electrophoretic homogeneity of trypanopain-Tb from *T. b. brucei* and the first reported characterisation of its enzymatic properties. Trypanopain-Tb was purified from bloodstream forms of *T. b. brucei* by a combination of three phase partitioning (between ammonium sulfate and tertiary butanol), and chromatography on quaternary amine or pepstatin A-Sepharose resins.

Trypanopain-Tb was found to be a typical cysteine proteinase, in that it is inhibited by typical cysteine proteinase inhibitors and requires reducing agents for full activity. Trypanopain has cathepsin L-like specificity for synthetic substrates and readily degrades various proteins. *In vitro* analysis of the kinetics of trypanopain interaction with cystatins suggested that these are likely to inhibit any trypanopain released into the mammalian bloodstream. Furthermore, no trypanopain-like activity was detectable in the blood of infected hosts, so it appears that trypanopain is unlikely to contribute directly to pathogenesis by degrading bloodstream host proteins.

Antibodies against a peptide corresponding to a region of the trypanopain active site were produced in rabbits and chickens. Both enzyme activity-enhancing and enzyme activity-inhibiting antibodies were produced and these effects varied with the substrate tested. Thus, the *in vivo* effects of anti-trypanopain antibodies will only become clearly understood once the physiological substrates of trypanopain have been identified.

Various cysteine proteinase inhibitors, including peptidyl diazomethylketones, killed cultured bloodstream forms of *T. b. brucei.* Use of biotinylated derivatives of peptidyl diazomethylketone and fluoromethylketone inhibitors suggested that trypanopain is the likely intracellular target of these inhibitors, indicating that the enzyme is essential for parasite viability. Furthermore, chalcones (a class of reversible cysteine proteinase inhibitors) killed *in vitro* cultured parasites and also prolonged the life of *T. b. brucei*-infected mice. Thus, trypanopain-Tb seems to be a possible target for new anti-trypanosomal drugs.

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ABBREVIATIONS

A_x	absorbance at x nm
α_2-M	α_2-macroglobulin
ABTS	2,2-azino-di-[3-ethylbenzthiazoline sulfonate]
ACE	angiotensin converting enzyme
AEBSF	4-(2-aminoethyl)-benzenesulfonyl fluoride
AIDS	aquired immunodeficiency syndromw
AMC	7-amino-4-methylcoumarin
AMT	acetate-MES-Tris
AP	alkaline phosphatase
BCA	bicinchoninic acid
BCDS	bathocuproine disulfonate
Bis	N, N'-methylenebisacrylamide
Boc	butoxycarbonyl
BSA	bovine serum albumin
Bz	benzoyl
c	concentration
CAMOR	carrier-modified residues
C-terminal	carboxy terminal
cDNA	copy deoxyribonucleic acid
CMK	chloromethylketone
C1	Complement factor 1
C3	Complement factor 3
BCIP/NBT	5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium
DDT	dichlorodiphenyl trichloroethane
DCI	3, 4-dichloroisocoumarin
DEAE	diethylaminoethyl
DFMO	DL-α-difluoromethylornithine
DFP	di-isopropyl fluorophosphate
DMF	dimethyl formamide

DMK	diazomethylketone
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTNB	5,5'-dithiobis(2-nitrobenzoic acid)
DTT	dithiothreitol
ε	extinction co-efficient
ES-MS	electrospray mass spectroscopy
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
$[E]_0$	initial enzyme concentration
[E]	enzyme concentration
F_{ab}	fragment, antibody binding
F_c	fragment, crystallisable
FITC	fluorescein isothiocyanate
FMK	fluoromethylketone
FCS	foetal calf serum
Hepes	*N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid
HIV	human immunodeficiency virus
H-kininogen	high molecular mass kininogen
HRPO	horseradish peroxidase
[I]	inhibitor concentration
IAA	iodoacetic acid
IAN	iodoacetamide
IC_{50}	50% inhibitory concentration
IgG	immunoglobulin G
IgG1	immunoglobulin G, subclass 1
IgM	immunoglobulin M
IgY	immunoglobulin Y
ILRAD	International Laboratory for Research on Animal Diseases
L-kininogen	low molecular mass kininogen
k_{ass}	association rate constant
k_{cat}	maximum catalytic rate

kDa	kiloDalton
KLH	keyhole limpet haemocyanin
K_i	equilibrium constant for inhibition
K_m	Michaelis constant
k_{obs}	observed association rate constant
k_{diss}	dissociation rate constant
L	length of the light path
mAbs	monoclonal antibodies
MeOSuc	methoxysuccinyl
m/v	mass per volume
MBS	m-maleimidobenzoyl-N-hydroxysuccinimide ester
MEM	Minimal Essential Medium
MES	acetate-2(N-morpholino)ethanesulphonic acid
MHC	major histocompatability complex
mRNA	messenger ribonucleic acid
NEM	N-ethylmaleimide
N-terminal	amino terminal
PAGE	polyacrylamide gel electrophoresis
pepstatin A	isovaleryl-Val-Val-Statine-Ala-Statine
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonyl fluoride
pNa	para-nitroanilide
PSG	phosphate saline glucose
OP-Tb	oligopeptidase-Tb
Q	quaternary amine
RT	room temperature
rTM	rat trypanopain modulator
[S]	substrate concentration
SBTI	soybean trypsin inhibitor
SDS	sodium dodecyl sulfate
Suc	succinyl

$t_{½}$	half life
TBS	Tris buffered saline
TCA	trichloroacetic acid
TEMED	N, N, N´, N´-tetramethyl ethylene diamine
TPCK	N-tosyl-L-phenylalanyl chloromethylketone
TLCK	N-tosyl-L-lysyl chloromethylketone
TMB	3,3',5,5'-tetramethyl benzidine
TPP	three-phase partitioning
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
UNP	University of Natal (Pietermaritzburg)
v_o	initial velocity of an uninhibited enzyme reaction
v_i	initial velocity of an inhibited enzyme reaction
V_{max}	maximum velocity
v/v	volume per volume
VSG	variant surface glycoprotein
WHO	World Health Organisation
Z	carbobenzoxy

Chapter 1

African trypanosomiasis

1.1 Introduction

Trypanosomes are protozoan parasites that infect a variety of animals including fish, amphibians, reptiles, birds and mammals (Chandler and Read, 1961; Hall, 1977). Almost all domestic animals and many wild animals can serve as hosts for one or more species of trypanosome, with the severity of infection and symptoms varying widely. The primary features of mammalian trypanosomiasis are intermittent fever, progressive anaemia and general loss of condition. Human African trypanosomiasis, which is also called African sleeping sickness, is often fatal if left untreated and cattle trypanosomiasis, also known as nagana, is of great economic importance in many parts of Africa. Cattle trypanosomiasis is "unique among diseases in that it is the only one which by itself has denied vast areas of land to all domestic animals other than poultry" (Hornby, 1950). About one quarter of Africa is completely unsuitable for livestock farming and at least one third of the continent is affected by trypanosomiasis to some degree. Most African trypanosomes complete part of their life cycle in the tsetse fly vector (Fripp, 1983) with the distribution of this vector thus limiting the general distribution of trypanosomiasis in Africa to some 11 million km^2 between 14°N and 29°S (Kuzoe, 1993). African trypanosomiasis is thus a predominantly tropical disease (Hall, 1977). Approximately 55 million people in 36 countries are at risk of contracting African sleeping sickness [Kuzoe, 1993; International Laboratory for Research on Animal Diseases (ILRAD), 1994], of which there are two forms: Gambian sleeping sickness occurring in West Africa and Rhodesian sleeping sickness occurring in East Africa.

1.2 Major African trypanosomiases

1.2.1 Gambian sleeping sickness

Trypanosoma brucei gambiense causes Gambian sleeping sickness in humans. Initial symptoms include an intermittent fever, headaches, glandular enlargement and oedema (Chandler and Read, 1961; Fripp, 1983; see Section 1.7). Although the infection may spontaneously

disappear, the disease usually becomes chronic. When parasites enter the cerebrospinal fluid, neurological symptoms occur. Patients express a desire to sleep continuously, experience apathy, problems with co-ordination, tremors of the tongue and limbs, slow and mumbling speech and paralysis (Ukoli, 1984). Starvation, pyrexia and/or opportunistic diseases cause the patient to fall into a coma, which is followed by death.

Before tsetse fly control was instituted, an epidemic in the Great Lakes region of Uganda reduced the population from 300 000 in 1901 to 100 000 in 1908. Effective control measures were subsequently instituted, with the result that in the 1950's sleeping sickness was largely under control. Recent political upheavals in many areas of Africa have led to relaxation of tsetse fly control and a resultant increase in the incidence of Gambian sleeping sickness in Congo, Cameroon, Sudan, Uganda, Tanzania, Angola, Malawi and Chad (Kuzoe, 1993; CDR News, 1994). In the late 1980's, some 5-10 000 cases were treated annually in Congo while in 1994, 150 000 cases were suspected. In some areas, up to 70% of the population is thought to be infected (CDR News, 1994), but poor medical surveillance complicates estimation of the true scope of the disease.

T. b. gambiense is transmitted between mammalian hosts by the tsetse flies *Glossina palpalis* and *G. tachinoides*, which breed in wooded areas near lakes and rivers (Swellengrebel and Sterman, 1961). In areas where water supplies are limited and where man and the fly are dependent on the same water supplies, contact between man and fly is common and the disease easily transmitted. The disease is thus endemic in many areas, and while the prevalence is commonly less than 1%, foci of higher transmission occur (Markell *et al.*, 1992). No animal reservoirs of the parasite are known.

1.2.2 Rhodesian sleeping sickness

Human infection with *T. b. rhodesiense* causes Rhodesian sleeping sickness, which is a highly acute illness causing death within a few weeks or months of infection (Chandler and Read, 1961; Hall, 1977; Tizard *et al.*, 1978; Fripp, 1983). Exceptionally high levels of parasitaemia induce a high fever and rapid enlargement of the liver and spleen (see Section 1.7). The rapidity of disease progression prevents the development of neurological symptoms such as those seen in Gambian sleeping sickness (Ukoli, 1984). *G. morsitans*, the primary vector, differs from the

riverine *G. palpalis* in that it lives mainly in open woodland and so there is less contact between the vector and hosts. Despite its higher virulence, *T. b. rhodesiense* is thus less of a health problem than *T. b. gambiense* since outbreaks are sporadic and contained. In southern Africa, this disease still occurs in the Okavango Delta of Botswana, where wildlife such as antelope are asymptotically infected and serve as important reservoirs for the parasite.

1.2.3 Cattle trypanosomiasis

Cattle trypanosomiasis, also known as nagana, is by far the most economically important of the African trypanosomiases (Chandler and Read, 1961). Thirty percent of Africa's cattle and small ruminant population are at risk of contracting trypanosomiasis; with losses in meat production, milk yield and tractive power estimated to cost in the region of US$ 500 million per year. If lost potential in livestock and crop production is also considered, the disease costs Africa an estimated US$ 5 billion per year (ILRAD, 1994). Some of the most potentially productive agricultural land in Africa is under-utilised because of the threat of nagana (ILRAD, 1992).

Cattle trypanosomiasis is caused primarily by *T. congolense*, *T. vivax* and *T. brucei*, which are transmitted between cattle by various *Glossina* species (Soulsby, 1982; Kaufmann, 1996). In addition to cattle, horses, camels, goats, chickens, dogs, cats and pigs can become infected with various species of trypanosome (Kaufmann, 1996).

1.4 Trypanosome morphology

Trypanosomes have a variety of morphological stages, but the trypanosomal cell is characteristically elongated (15-40 μm long) and slender (1½-3½ μm wide), with a single flagellum extending along the length of the cell and beyond (Kreier and Baker, 1987). The flagellum arises from an intracellular basal body called the kinetosome and emerges through the flagellar pocket. Trypanosomes swim actively using this flagellum, which is attached to the cell by an undulating membrane (Ukoli, 1984). Most of the remaining trypanosomal cellular structures (e.g. nucleus, Golgi, endoplasmic reticulum) are similar to those found in higher organisms (Figure 1). Trypanosomes do, however, contain an unusually large amount of mitochondrial deoxyribonucleic acid (DNA) that is packaged into a body called the kinetoplast inside the single, large trypanosomal mitochondrion (Kreier and Baker, 1987). The kinetoplast

is as a small, rounded, nucleus-like body near the base of the flagellum (Kreier and Baker, 1987). Its function is unclear.

1.5 Classification of trypanosomes

Members of the family Trypanosomatidae that spend their life partly in the blood or tissue of vertebrates and partly in the alimentary system of blood-sucking insects are known as haemoflagellates (Ukoli, 1984). This group includes the genera *Trypanosoma* and *Leishmania*, both of which are of medical and veterinary importance (Figure 2, Hoare, 1972; Markell *et al.*, 1992). The family is thought to have arisen from an ancestral insect parasite and some species (e.g. *Leptomonas* and *Crithidia*) are still exclusively insect parasites. Other than *Trypanosoma*, the only other genus of this family that parasitises humans is *Leishmania*. Both these species have two hosts during their life cycle, the one being an insect vector and the other a mammalian host.

On the basis of their transmission by such insect vectors, mammalian-infecting trypanosomes may be divided into two main groups: salivarian and stercorial. The salivarian trypanosomes are transmitted from the salivary glands of infected insect vectors and are thus said to develop at the anterior station of the vector. Stercorial trypanosomes, on the other hand, are transmitted in the faeces of infected insect vectors and are thus said to develop at the posterior vector station (Hoare, 1972). African trypanosomes are salivarian parasites, while American trypanosomes are stercorial.

South American trypanosomes differ most importantly from African trypanosomes in that they multiply intracellularly (most importantly, in cardiac muscle) and have as their vector a blood-sucking insect of the family Reduviidae (Swellengrebel and Sterman, 1961; Soulsby, 1982; Fripp, 1983). Parasites develop in the hindgut of the vector and are transmitted to mammalian hosts when infected faeces are rubbed into a bite wound. Various domestic animals, especially dogs, serve as important reservoirs of the disease. The most important member of this group is *T. cruzi*, which causes Chagas' disease in humans. Up to 18 million people are thought to be affected by the disease, while millions more are at risk (Cazzulo *et al.*, 1997). In some areas of Central and South America, the prevalence of this fatal disease may exceed 50%, with young children and infants most susceptible. The initial symptoms of infection include fever,

cardiomyopathy and enlargement of the colon. Interestingly, *T. cruzi* is thought to be the cause of the debilitating illness experienced by Charles Darwin for over half his life (Medawar, 1996). At the time, Darwin's doctors were baffled by his illness, suggesting that Darwin's symptoms were psychosomatic. *T. cruzi* was not identified until 27 years after Darwin's death, and modern authors have suggested that his symptoms fit those of Chaga's disease and postulate that Darwin's chances of escaping *T. cruzi* infection during his travels in and around South America on the Beagle were negligible (Medawar, 1996). *T. rangeli* (also called *T. ariari* or *T. guatemalensis*) is another interesting South American trypanosome, that infects humans apparently asymptomatically. In Panama, approximately 75% of people under 16 years old are thought to be infected with this parasite (Markell *et al.*, 1992).

T. b. brucei , *T. b. gambiense* and *T. b. rhodesiense* are morphologically indistinguishable and cannot be differentiated by serological means (Markell *et al* 1992). The three species differ only in host specificity and the severity of the disease they cause. The classification of this sub-group of parasites is thus controversial; with some authors proposing a division into three species and others into three sub-species (Ukoli, 1984). The latter is more widely accepted (Tait *et al.*, 1984), with the sub-species divided along geographical and host lines. *T. b. brucei* is defined as being found throughout Africa and infecting wildlife and domestic animals, but not man.

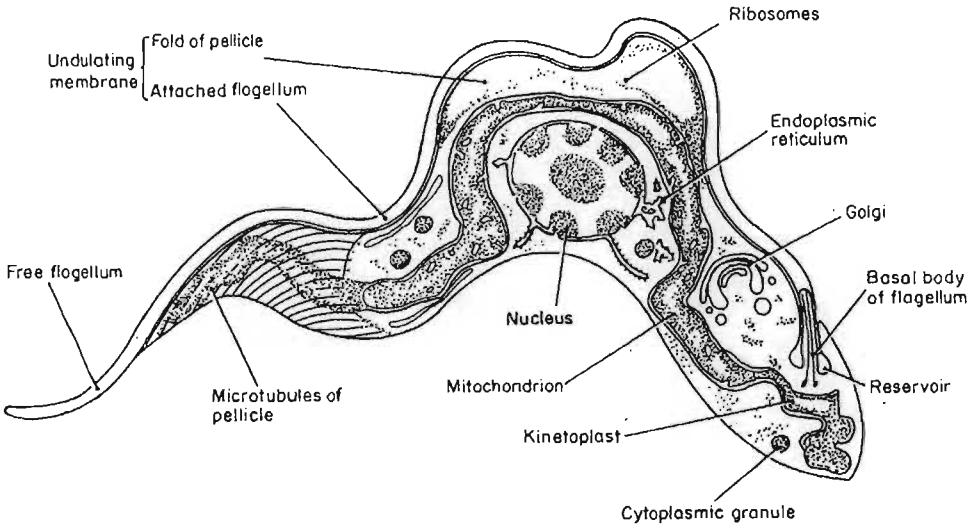


Figure 1: Diagrammatic representation of generalised *T. b. brucei* morphology.

T. b. brucei is a unicellular organism that actively swims with a single flagellum that extends along the length of the cell and beyond. Trypanosomes have a variety of morphological stages, but the trypanosomal cell is characteristically elongated (15-40 μm long) and slender (1½-3½ μm wide). Adapted from Vickerman (1970).

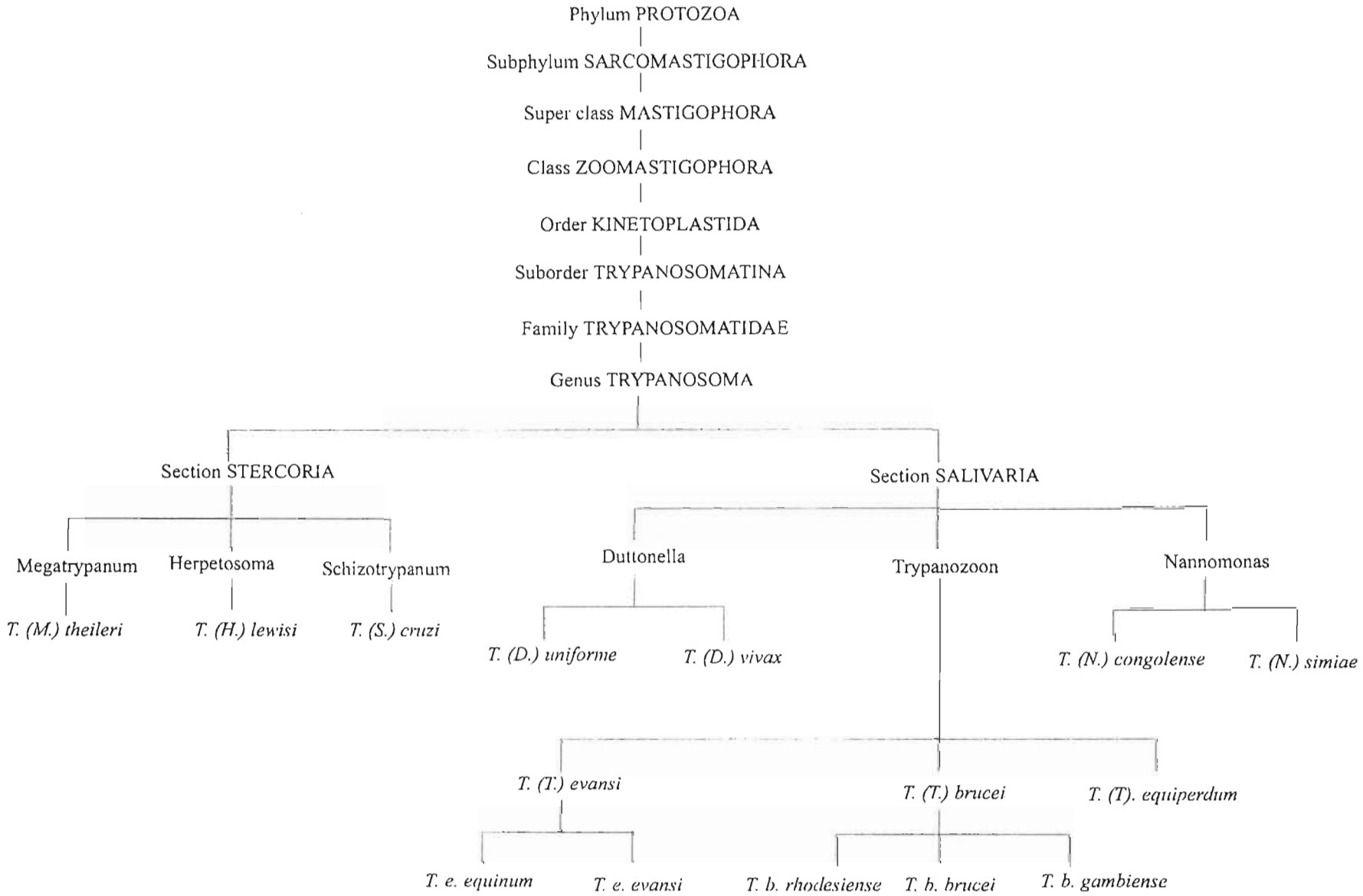


Figure 2: Schematic representation of the *T. b. brucei* phylogenetic tree.

1.6 Life cycle of African trypanosomes

African trypanosomes spend part of their complex life cycle (Figure 3) in a mammalian host and the rest in an insect vector, usually the tsetse fly (Chandler and Read, 1961; Swellengrebel and Sterman, 1961). The species of fly that serves as a vector varies with the genus of trypanosome. Cyclic transmission occurs when non-dividing metacyclic forms, present in the salivary glands or mouth parts of infected flies, infect susceptible mammals when the flies bite. Once inside the mammalian host, the metacyclic forms differentiate into bloodstream tryptomastigote forms that undergo rapid multiplication by binary fission in the bloodstream, lymphatics and cerebrospinal fluid (Markell *et al.*, 1992). The long and slender, rapidly dividing bloodstream forms stop dividing and become short and stumpy later in the infection. Some of the biochemical changes between the bloodstream forms suggest pre-adaptation for tsetse fly infection. Some trypanosome species, especially *T. b. brucei* and *T. b. gambiense*, are highly pleomorphic, with both morphological forms occurring simultaneously in the host.

When a fly bites the infected mammal, it takes up trypanosomes in the bloodmeal. These parasites differentiate in the midgut of the tsetse fly into dividing procyclic forms before differentiating further into dividing epimastigote forms in the proboscis or salivary glands of the fly. The final stage of the 20-30 day life cycle is the differentiation of the epimastigote form into the non-dividing, mammalian-infective metacyclic form.

The mechanism for the transformation from long, slender to short, stumpy forms is poorly understood. Various possibilities have been proposed, including that transformation is a programmed event in the cell cycle or that depletion of a key nutrient initiates transformation, but it seems most likely that a host-derived growth inhibitor triggers transformation into short, stumpy forms (Seed and Sechelski, 1989).

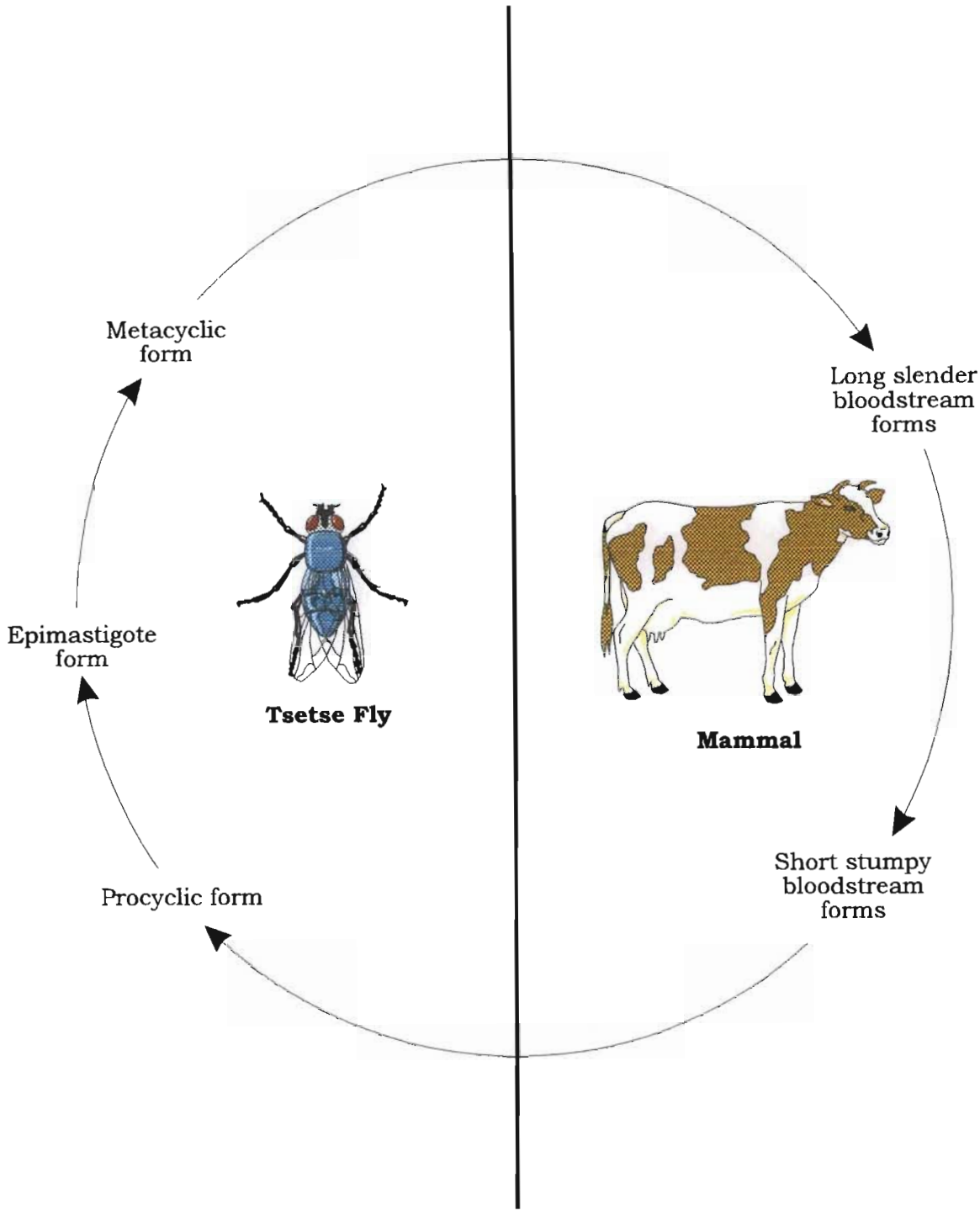


Figure 3: Life cycle of *T. b. brucei* showing morphological stages present in mammalian and tsetse fly hosts.

1.7 Symptoms of African trypanosomiasis

In the case of Gambian sleeping sickness, a bite by an infected insect is followed by an asymptomatic incubation period of a few days to several weeks, while onset of Rhodesian sleeping sickness is acute (Markell *et al.* 1992). The area of the bite may become ulcerated to form a painful "trypanosomal chancre", from which trypanosomes can be aspirated. At this stage, the infected person appears outwardly healthy, although trypanosomes can be detected in the bloodstream. This stage can persist for several months and the infection may disappear without the development of further symptoms (Markell *et al.* 1992). However, if the trypanosomes multiply and invade the lymphatic system, the patient begins experiencing fevers, headaches and joint pains, possibly accompanied by anorexia, generalised weakness, nausea and vomiting. An asymptomatic phase then follows, with the levels of trypanosomes in the bloodstream dropping. This is followed by further attacks of intermittent fever as the trypanosome population repeatedly rises and falls. Large numbers of trypanosomes are detectable in the bloodstream during periods of fever.

Infection of the lymphoid system is particularly common in the early stages of the disease. While any lymph nodes may become infected, those in the posterior cervical region are particularly susceptible. Inflammation of these nodes is called "Winterbottom's Sign", and was used by slave traders to identify potentially infected Africans. Proliferation of parasites in the lymphoid tissue causes generalised lymphoid hyperplasia. Anaemia, thrombocytopaenia, hypergammaglobulinaemia [especially of Immunoglobulin M (IgM)] and disseminated intravascular coaggulation are common symptoms. While congenital transmission is uncommon, stillbirths and abortions are common during this stage of the disease.

Neurological symptoms, such as lassitude, fatigue, confusion and apathy, develop later in the course of the infection when trypanosomes enter the brain (Markell *et al* 1992). Such symptoms are much more marked in Gambian than Rhodesian sleeping sickness. Motor changes include fibrillation of the muscles of the face, lips and fingers, and inco-ordination leading to slurred speech and an ataxic gait. Sensory changes are less marked, but pressure on the palms or ulnar nerve may be followed by severe pain a short time after pressure is removed (Kerandel's Sign). Neurological symptoms become severe in the final stages of the disease.

Convulsions, paraplegia or hemiplegia and incontinence are commonly experienced, with the patient eventually slipping into a coma.

Cattle trypanosomiasis is characterised by similar symptoms to human trypanosomiasis. After a one to three week incubation period, infected cattle develop an intermittent fever, increased pulse rate and shallow respiration; after which emaciation, severe anaemia, lymph node enlargement, splenomegaly and oedema are experienced. Wasting of both body fat and muscle and impaired reproductive ability are important from an economic standpoint (Urquhart *et al.*, 1987; Kaufmann, 1996). Although the severity of the disease varies with the nutritional status and stress levels of an animal, the slow, progressive anaemia is often fatal. In very acute cases, death can occur within 1 week of infection.

1.8 Pathogenesis of trypanosomiasis

Trypanosome-infected individuals show a wide variety of clinical signs and since no obvious gross lesions are present, there has been some debate as to the pathogenesis of the disease (Tizard *et al.*, 1978). Severe anaemia is a common characteristic of trypanosomal infection and contributes to the fatigue and loss of condition experienced by infected hosts. Although multi-factorial, anaemia is now thought to be caused primarily by extensive lysis of red blood cells. Although the mechanisms whereby haemolysis occurs are unclear, some possible contributory mechanisms are listed below (Boreham, 1974; Tabel *et al.*, 1978; Tizard *et al.*, 1978; Murray and Dexter, 1988):

- Soluble trypanosomal antigens may adsorb onto circulating erythrocytes, where they can become opsonised by circulating anti-trypanosome antibodies. Such surface-bound antibodies initiate antibody-dependent cytotoxic reactions against erythrocytes by recruiting F_c (fragment, crystallisable)-receptor bearing phagocytes and eosinophils and by triggering the classical complement pathway.
- Trypanosomal surface glycoproteins can also stimulate the classical complement pathway directly and so initiate erythrocyte lysis independently of circulating antibodies.
- The presence of trypanosomes in the blood appears to damage capillary walls, leading to diffuse intravascular coagulation and the development of microthrombi in capillary beds. Passing erythrocytes are trapped and damaged by these microthrombi and must

subsequently be removed from circulation. Such microangiopathic anaemia is thus a secondary feature of damage to capillary walls.

- Trypanosomal phospholipase A acts on phosphatidyl choline to release free fatty acids which may have detergent-like effects on erythrocyte membranes.
- Trypanosomes release a 12 kiloDalton (kDa) protein that appears to directly mediate haemolysis.

Once haemolysed, erythrocytes are thought to be removed from the bloodstream primarily by mononuclear phagocytes. Spleen enlargement is a common early feature of trypanosomiasis and is thought to be caused partly by increased sequestration of phagocytosed red blood cells. After prolonged stimulation by the massive parasite load, the expanded mononuclear phagocyte system is thought to remain active once trypanosomes have been eliminated from the blood. Non-specific phagocytosis of erythrocytes may thus continue, contributing to further anaemia (Murray and Dexter, 1988).

Factors other than haemolysis are also thought to contribute to trypanosome-induced anaemia. For example, erythropoeisis and haemopoeisis seem to be inadequate in relation to the extent of anaemia (Murray and Dexter, 1988). Trapping of iron in reticuloendothelial cells is thought to exacerbate abnormal haemopoeisis, so administration of testosterone, cobalt or erythropoeitin has been suggested to increase iron release from storage sites in the body.

In addition to inducing severe anaemia, trypanosome infection commonly causes disturbances in functioning of the host's immune system. Parasites endeavour to evade the host immune system by regularly changing their surface antigens, which are known as variable surface glycoproteins (VSG). A single trypanosome may have as many as 1 000 different VSG varieties (Hadjuk, 1984) and the persistent presence of new trypanosomal antigens in the blood results in the production of high levels of circulating antibodies, particularly IgM (Tizard *et al.*, 1978). Some of these antibodies are directed against trypanosomal antigens but many are directed against heterophils, antiglobulins and other self antigens, thus initiating autoimmune complications (Tizard *et al.*, 1978). Cross-reactivity between host and parasite proteins may also contribute to autoimmune reactions (Müller *et al.*, 1993). Serum complement levels are depleted by trypanosome infection, with the early components such as complement factors 1

(C1) and 3 (C3) being most affected (Tizard *et al.*, 1978). The increase in antibody levels described above leads to an increase in the number of circulating immune complexes, which fix complement and so contribute to its depletion. Depletion of complement is thought to favour parasite survival by hampering complement-mediated lysis of parasites.

Reports on the effects of trypanosomal infection on T-cell responses are varied. While some authors state that only susceptible cattle experience T-cell suppression (Longstaffe and Terry, 1982), others conclude that there is no such correlation (Flynn and Sileghem, 1993).

Trypanosome-infected hosts experience a wide variety of symptoms other than the anaemia and immune system malfunctioning discussed above. While *T. congolense* is found almost exclusively in the bloodstream *T. b. rhodesiense*, *T. b. gambiense* and *T. b. brucei* also infect tissues (Ukoli, 1984). These organisms thus also cause degeneration, necrosis and inflammation of tissues, producing a complex set of symptoms. Many of these symptoms are thought to be caused by toxic trypanosomal metabolites (Alafiatayo *et al.*, 1993). For example, the neurological symptoms especially common in Gambian sleeping sickness may be caused by transamination of tryptophan and other related indoles to produce tryptophol (indole-3-ethanol, Tizard *et al.*, 1978). In experimental animals, tryptophol produces a comatose-like state, alters body temperature and initiates immunosuppression and is thus thought to be at least partially responsible for the "sleepiness" symptoms of Gambian sleeping sickness. The headache often experienced during the early stages of the disease may be caused by parasites blocking capillaries in the choroid plexus, which causes localised oedema and obstructs the flow of cerebrospinal fluid (Markell *et al.*, 1992).

1.9 Diagnosis of African trypanosomiasis

Since the initial symptoms (e.g. fever, headaches, joint pains) of trypanosomiasis are fairly non-specific, diagnosis of trypanosomiasis requires identification of parasites in the blood, lymphatic or cerebrospinal fluid (Chandler and Read, 1961; Tizard *et al.*, 1978; Fripp, 1983). In cases where only a few parasites are present, blood can be centrifuged to concentrate the parasites in the buffy coat.

Diagnostic complement fixation and fluorescent antibody tests are also available, with extremely sensitive monoclonal antibody-based antigen-detection enzyme-linked immunosorbent assays (ELISAs), DNA hybridisation assays and polymerase chain reaction (PCR)-based techniques under development for future early diagnosis (ILRAD, 1994). Histological analyses show localised accumulation of trypanosomes in the micro-vasculature, accompanied by extensive endothelial damage. Inflammatory responses to this damage, such as localised infiltration of lymphocytes and phagocytes, are also seen.

1.10 Current prevention and treatment of trypanosomiasis

Most of the countries that regularly report cases of trypanosomiasis have an annual per capita health budget of less than US$10 (De Raadt, 1989). It is thus clear that preventative measures and treatments for sleeping sickness must be cheap to be of any significance in the affected areas.

1.10.1 Control of tsetse fly

Prevention of trypanosomiasis has centred mainly on control of tsetse fly populations using insecticides such as dichlorodiphenyl trichloroethane (DDT), dieldrin and endosulfan (Hall, 1977; Grosvenor, 1986). These are still used to control tsetse fly numbers despite their deleterious effects on the environment (Wooff and Phillemon-Motsu, 1993). Alternative methods for controlling tsetse fly such as clearing bush around human settlements and the use of cattle odour-baited tsetse targets and traps, have met with some success (Wooff and Phillemon-Motsu, 1993), but domestic animals should be frequently sprayed or dipped in insecticide and stables screened to prevent re-entry of the fly into the area (Grosvenor, 1986). Many wild animals serve as reservoir species for trypanosomes and food for *Glossina* species but elimination of entire populations of reservoir species is not ecologically acceptable.

An alternative method of controlling the disease is to protect susceptible animals from infection by parasites, instead of from biting by the fly. The prophylactic drug pentamidine is relatively expensive and the period of protection decreases with successive use. Thus, none of the currently available prevention methods is cost effective or efficient enough to allow cattle farming in the tsetse fly belt of Africa without close supervision (ILRAD, 1993).

Some species of cattle, such as the N'Dama (*Bos taurus*) are naturally more resistant to infection than others and are more suited to areas where trypanosomiasis is endemic. Resistant cattle seem able to recognise and mount an effective immune response against parasite factors contributing to pathogenesis and so control disease progress (Authié *et al.*, 1993). While introduction of resistant N'Dama into tsetse-supporting areas is attractive, there are some drawbacks. For example, while meat production by N'Dama is good, they produce little milk and are generally too small for draught work. Also, their trypanotolerance is reduced when the animals are stressed (ILRAD, 1992; 1993).

1.10.2 Current treatment methods

Highly toxic arsenic and antimony-based drugs were commonly used for treatment of trypanosomiases until the 1960's but have now been largely replaced by less toxic alternatives (Chandler and Read, 1961; Swellengrebel and Sterman, 1961; Soulsby, 1982; Fripp, 1983). One arsenical, melarsoprol, is still commonly used to treat the later, neurological stages of sleeping sickness. While the mechanism of action remains unclear, enzymes involved in energy-generation are thought to be primary targets (Gutteridge, 1985).

Suramin is a sulfated naphthylamine first introduced in the 1920's as a treatment for the early stages of Gambian and Rhodesian sleeping sickness (Pépin, 1994). The drug is thought to exert its effect primarily through inhibition of essential trypanosomal glycolytic enzymes, but its exact mode of action remains unclear (Markell *et al.*, 1992). While the drug is taken up selectively by trypanosomes and not mammalian cells, suramin can be extremely toxic in certain patients. Side effects include loss of consciousness, seizures, renal failure, fever, hepatitis, rash, pruritis, oedema, pain, conjunctivitis and photophobia (Markell *et al.*, 1992). The drug does not cross the blood-brain barrier and is thus ineffective against the neurological stages of the disease (Gutteridge, 1985).

Pentamidine (Lomidine or 4-4'-diaminodiphen-oxypentane di-β-hydroxyethane sulfonate) is a diamine compound that is thought to kill trypanosomes by interacting specifically with kinetoplast DNA (reviewed by Pépin, 1994). Trypanosomes are thought to be more sensitive to pentamidine than mammalian cells because they possess a pentamidine transport system that raises the intracellular concentration of the drug several fold above the plasma concentration

(Gutteridge, 1985). The drug, which was introduced in the 1940's, is particularly effective as a treatment for the early, haemolytic stages of Gambian sleeping sickness (Markell *et al.*, 1992). The main advantage of pentamidine over suramin is that a full course of the drug can be given over 7-10 days, while suramin treatment takes several weeks (Gutteridge, 1985). Pentamidine does not cross the blood-brain barrier, so is ineffective against the neurological stages of the disease (Gutteridge, 1985). There are relatively few side-effects to treatment, but light-headedness, renal damage and pain at the site if injection have been reported. Berenil is an aromatic diamine similar to pentamidine, but is used only in livestock (Gutteridge, 1985; Kinabo, 1993).

Melarsoprol is a trivalent arsenic-based compound that is thought to inhibit various sulfur-containing trypanosomal enzymes. The drug is of particular use in the neurological stage of the disease since it can cross the blood-brain barrier (Markell *et al.*, 1992). This advantage must be weighed against the often extreme toxicity of melarsoprol, with symptoms such as encephalopathy experienced in 5-10% of patients (Kuzoe, 1993). Certain strains of both *T. b. gambiense* and *T. b. rhodesiense* are resistant to melarsoprol (Kuzoe, 1993).

DL-α-difluoromethylornithine (DFMO, eflornithine) is a highly effective treatment for both the early and late stages of trypanosomiasis. The compound is a potent inhibitor of ornithine decarboxylase, which is involved in polyamine (most importantly, trypanothione) biosynthesis (McCann *et al.*, 1981; Bacchi *et al.*, 1983; Fairlamb *et al.*, 1987). However, limited host toxicity has been observed. Additionally, the drug must be administered intravenously four times daily for 14 days, making it an expensive treatment (Kuzoe, 1993). Variants of DFMO are being tested in the hope that they will prove to be more effective or easier to administer than the parent compound (Bitonti *et al.*, 1985).

None of the currently available drugs is thus entirely satisfactory. Major limitations are a lack of oral activity, side-effects and an inability to treat all stages of the disease (Gutteridge, 1985). While there are few reports of drug resistant human trypanosomes, resistant cattle strains limit the veterinary efficacy of the current drugs. This, together with the time and cost involved in controlling tsetse fly populations, has led to a search for novel means of disease therapy and prevention.

1.11. Parasite proteinases

Parasitic protozoa cause numerous important human and animal diseases, including malaria, leishmaniasis and amoebiasis. The proteinases of these pathogens are though to be essential for parasite viability and/or contribute to pathogenesis. They are thus the subject of intense study as potential chemotherapeutic targets (McKerrow, 1989; North *et al.*, 1990a). The discovery (or synthesis) of specific proteinase inhibitors has made it possible to consider proteinase inhibition as a new chemotherapeutic approach for treating various parasite infections (McKerrow, 1989; North *et al.*, 1990a). The cysteine proteinases of numerous parasites are under investigation as potentially contributing to pathogenesis or as possible targets for novel anti-parasite drugs. Some of the cysteine proteinases thus studied include those of the protozoa *Leishmania* (Hunter *et al.*, 1992); *Plasmodium* (Rosenthal *et al.*, 1993); *Entamoeba histolytica* (Luaces and Barrett, 1988; Avila and Calderon, 1993); *Theileria parva* (Nene *et al.*, 1990), *Trichomonas* (North *et al.*, 1990b) and *Cryptosporidium parvum* (Nesterenko *et al.*, 1995) as well as the helminths *Spirometra mansoni* (Yong Song and Chappell, 1993), *Trichinella spiralis* (Todorova *et al.*, 1995), *Fasciola hepatica* (Dowd *et al.*, 1995), *Necator americanus* (Brown *et al.*, 1995); the trematode *Schistosoma* (Dresden and Deelder, 1979; Klinkert *et al.*, 1989) and the nematodes *Haemonchus contortus* (Cox *et al.*, 1990) and *Caenorhabditis elegans* (Ray and McKerrow, 1992). Various functions have been proposed for these proteinases, including:

- parasite protein catabolism, including activation or degradation of parasite peptide hormones, enzymes or regulatory molecules
- parasite remodelling during transition from one life cycle stage to another
- invasion of host tissue by degradation of connective tissue
- evasion of the host immune system by degradation or activation of various molecules of the immune system
- disturbance of host hormonal system by degradation of host peptide hormones.

While these proteinases have been shown to degrade various host proteins (including complement components, cell surface proteins, immunoglobulins and haemoglobin) *in vitro*, their *in vivo* role(s) and substrate(s) remain largely speculative. Compared with their mammalian counterparts, namely cathepsins B, H and L, protozoan cysteine proteinases are

often larger and more stable in alkaline conditions (North *et al.*, 1990a). They are usually lysosomal and have similar substrate specificities to homologous mammalian enzymes.

1.11.1 *E. histolytica* cysteine proteinase

E. histolytica is the causative agent of amoebiasis, characterised by colitis, dysentery and amebic abscess. Two of the virulence factors identified are a metalloproteinase (Scholze and Schulte, 1990) and a cysteine proteinase [called histolysain (Luaces and Barrett, 1988)]. The major cysteine proteinase of the parasite has been identified as a cytotoxin by virtue of the fact that addition of purified proteinase to tissue cultured monolayers of host cells induces cytotoxicity (Keene *et al.*, 1986, Luaces and Barrett, 1988). Furthermore, specific irreversible inhibitors of the proteinase prevent this damage (Keene *et al.*, 1990). A correlation also exists between the pathogenicity of field isolates and their level of proteinase secretion (Reed *et al.*, 1989). The purified enzyme (23-27 kDa) is a typical cysteine proteinase, being activated by reducing agents and inhibited by diagnostic cysteine proteinase inhibitors (Scholze and Schulte, 1988). A similar enzyme has recently been identified in *E. invadens* (Sharma *et al.*, 1996) and fragments of a homologous gene have been found in non-pathogenic *E. histolytica* (also known as *E. dispar)* (Mirelman *et al.*, 1996). *E. histolytica* additionally possesses a membrane-associated metallocollagenase thought to be involved in host invasion (Luaces and Barrett, 1988; Muñoz *et al.*, 1990).

1.11.2 *Leishmania* proteinases

Leishmaniasis is classified by the World Health Organisation (WHO) as one of the six major parasitic diseases, with over 12 million people infected by species of the parasite. The disease is characterised by symptoms varying from self-healing cutaneous ulcers to non-resolving mucocutaneous lesions and hepatosplenomegaly leading to death. Promastigotes express a surface zinc metalloproteinase at high density (Bordier, 1987; Bouvier *et al.*, 1985) which is not inhibited by any physiological inhibitors (including α_2-macroglobulin) (Heumann *et al.*, 1989). Use of this proteinase as a vaccine has met with varying success (Russell and Alexander, 1988; Handmann *et al.*, 1990; Jaffe *et al.*, 1990; Lopez *et al.*, 1991). Additionally, *Leishmania* amastigotes have been shown to express several biochemically distinct cysteine proteinases encoded by at least 19 genes (Robertson and Coombs, 1992). Three main groups of cysteine proteinases have been identified in *L. mexicana* and *L. pifanoi* (reviewed in Robertson *et al.*,

1996). Type I cysteine proteinases are cathepsin L-like enzymes encoded by developmentally regulated multi-copy genes (e.g. *lmcpb* in *L. mexicana*; *lpcys2* in *L. pifanoi*) and contain the long C-terminal extension seen in cruzipain and trypanopain-Tb (Robertson and Coombs, 1990). Type II leishmanial cysteine proteinases are also cathepsin L-like, but are encoded by single copy genes (e.g. *lmcpa* in *L. mexicana*; *lpcys1* in *L. pifanoi*) and lack the C-terminal extension found in Type I enzymes. Type III leishmanial cysteine proteinases are cathepsin B-like enzymes encoded by single copy genes (e.g. *lmcpc* in *L. mexicana*). Cysteine proteinase inhibitors inhibit growth of *L. mexicana* in mouse macrophages by 95% (Coombs and Baxter, 1984) and there is a correlation between the strain pathogenicity and the level of cysteine proteinase expression (Bonaldo *et al.*, 1991). Gene knockout of the *lmcpb* group of proteinases from *L. mexicana* has shown that these are not essential for viability, since double knockout mutants can be cultured *in vitro* (Mottram *et al.*, 1996). Knockouts were, however, less able to infect host cells, showing that the enzymes are virulence factors. The various isoenzymes in the *lmcpb* group differ in their ability to restore virulence to null mutants (Mottram *et al.*, 1997), indicating that they have different functions in the parasite.

1.11.3 *Schistosoma* proteinases

Schistosoma mansoni and *S. japonicum* contain a variety of cathepsin-like cysteine proteinases, including enzymes resembling cathepsin B (Sm31, Dresden and Deelder, 1979; Klinkert *et al.*, 1989; Ghoneim and Klinkert, 1995; Dalton *et al.*, 1996), cathepsin L (Sm24, Smith *et al.*, 1994; Michel *et al.*, 1995; Dalton *et al.*, 1996), cathepsin D (Becker *et al.*, 1995; Wong *et al.*, 1997) and cathepsin C (Butler *et al.*, 1995). A membrane-anchored serine proteinase has also been described (Ghendler *et al.*, 1996). Additionally, schistosomes encode an asparaginyl endopeptidase called Sm32, which cleaves on the carboxy (C-) terminal side of asparagine residues that are not at either of the two positions closest to the amino (N-) terminal and that are not glycosylated (Davis *et al.*, 1987; Ishii, 1994, Becker *et al.*, 1995). Sequence analysis has revealed that there are no known mammalian homologues of this enzyme (Davis *et al.*, 1987), and if this enzyme is shown to be vital for parasite viability, it is a very attractive target for drug design.

1.11.4 Malarial proteinases

Malaria is the most important protozoan infection worldwide, with about 1 million reported deaths annually (Walsh, 1989). Numerous studies have implicated both cysteine and serine proteinases in invasion and rupture of erythrocytes, as well as processing of proteins required for this process (reviewed in McKerrow *et al.*, 1993). Additionally, aspartic and cysteine proteinases are thought to be involved in haemoglobin degradation, which is the major source of parasite amino acids. A *P. falciparum* aspartic proteinase has been shown to cleave native haemoglobin at a specific site on the α-chain, suggesting that it might initiate haemoglobin digestion (Goldberg *et al.*, 1991), with a cysteine proteinase thought to be responsible for further degradation (Rosenthal *et al.*, 1988). Incubation of parasites with proteinase inhibitors blocks parasite development (Rosenthal *et al.*, 1988; Bailly *et al.*, 1992), suggesting that these enzymes might be suitable chemotherapeutic targets. In an animal model of malaria, morpholine urea-Phe-hPhe-fluoromethylketone (FMK) cured 80% of mice infected with murine malaria (*P. vinckei*) when administered subcutaneously for 4 days at 100 mg/kg body weight (Rosenthal *et al.*, 1993).

1.12 Trypanopains

At the outset of the present study in 1994, zymogram profiles of the proteolytic components of *T. b. brucei* identified a 28 kDa cysteine proteinase (Robertson *et al.*, 1990, Huet *et al.*, 1992). The copy DNA (cDNA) of this enzyme, named trypanopain-Tb (referred to as trypanopain throughout this thesis) had been sequenced (Mottram *et al.*, 1989), but the enzyme had not been isolated or enzymatically characterised. The homologous enzymes from *T. cruzi* and *T. congolense* (called cruzipain/cruzain and trypanopain-Tc respectively) had been more extensively studied, and various lines of evidence, outlined below, suggested these proteinases might have pathogenic relevance.

1.12.1 Correlation between disease resistance and production of anti-trypanopain-Tb IgG1 antibodies

Different breeds of cattle have varying resistance to trypanosome infection. For example, N'Dama cattle are resistant to *T. congolense* infection, while Boran cattle are susceptible. N'Dama cattle are termed trypanotolerant because they have the ability to control trypanosome infection, while susceptible Boran cannot. While parasite numbers in resistant and susceptible

cattle are comparable for the first 30-40 days post-infection, parasitaemia then drops in resistant cattle but continues to increase in susceptible cattle (Authié *et al.*, 1993). While there are likely to be many differences between trypanotolerant and susceptible cattle, one difference between the breeds is in their responses to the major cysteine proteinase of *T. congolense*, namely trypanopain-Tc. Authié *et al.* (1993) showed that while trypanotolerant cattle produce anti-trypanopain Immunoglobulin G subclass 1 (IgG1), susceptible cattle do not. Also, African buffalo, which are known to be trypanotolerant, produce high levels of anti-trypanopain-Tc immunoglobulins during primary infection. Both susceptible and resistant cattle produce IgM, so the type of antibody elicited seems to be of significance.

Trypanopain-Tc has been detected in the plasma of susceptible and resistant cattle (Authié *et al.*, 1993) where it may contribute to pathogenesis by degrading various host proteins (Rautenberg *et al.*, 1982; Mbawa *et al.*, 1992). If active trypanopain exerts a pathogenic effect, then anti-trypanopain antibodies may conceivably be protective if they inhibit enzyme activity or target circulating enzyme for removal by phagocytic cells. Therefore, animals failing to mount an effective immune response against trypanopain may have higher levels of active enzyme in their plasma, which may increase pathogenesis in these animals (Authié *et al.*, 1993).

Crosses between Boran and N'Dama cattle have intermediate resistance to trypanosomiasis compared with the parent breeds, but even within a breed, individual animals have differing susceptibility to the disease. The breed-restricted nature of anti-trypanopain responses suggests that recognition of the enzyme is somehow genetically restricted (Authié *et al.*, 1992). Trypanopain-Tc shares high homology with mammalian cathepsin L (Fish *et al.*, 1995) and so, like cathepsin L, trypanopains may carry only a small number of T cell epitopes (Authié *et al.*, 1992). Since T cell recognition is dependent on interaction with major histocompatability complex (MHC) molecules, T cell responses to the few T cell epitopes of trypanopains may be strongly genetically restricted.

While differences in responses to trypanopain are unlikely to be the sole difference between resistant and susceptible cattle, the observations of Authié *et al.* (1993) do suggest that further investigation of the role of anti-trypanopain immunoglobulin G subclass 1 (IgG1) in the control of trypanosomiasis is warranted. Promisingly, susceptible cattle repeatedly experimentally infected and cured of infection by drug intervention were found to develop high titres of

protective anti-trypanopain antibodies, suggesting that susceptible breeds can develop immunity against infection (Authié *et al.*, 1993).

1.12.2 Cysteine proteinase inhibitors kill live trypanosomes

Live *T. congolense* are killed *in vitro* 24-48 h after incubation with micromolar concentrations of specific peptidyl diazomethylketones (DMK) (Mbawa *et al.*, 1992). The most trypanocidal inhibitors were those that best inhibit purified trypanopain-Tc. Similar results were obtained with live *T. cruzi* treated with DMK and acyloxymethylketone inhibitors of the major *T. cruzi* cysteine proteinase, cruzipain (Franke de Cazzulo *et al.*, 1994).

1.12.3 General features of trypanopains

Trypanopain-Tc and cruzipain are located primarily in the trypanosomal lysosome (Lonsdale-Eccles and Grab, 1987a; Bontempi *et al.*, 1989; Murta *et al.*, 1990; Mbawa *et al.*, 1992), and the same is thought to be true of trypanopain. Small amounts of trypanopain-Tc and cruzipain are found on the surface of trypanosomes (e.g. the flagellar pocket) and in flagellar pocket-associated vesicles, suggesting that the proteinases may be targeted for secretion (Murta *et al.*, 1990; Souto-Padrón, 1990; Mbawa *et al.*, 1991a).

The molecular masses of trypanopain-Tc (32 kDa, Rautenberg *et al.*, 1982) and trypanopain (27 to 30 kDa, Lonsdale-Eccles and Grab, 1987a; Pamer *et al.*, 1989; Boutignon *et al.*, 1990) are similar to those of mammalian cysteine proteinases. Cruzipain, however, is an unusually large (60 kDa) two-chain proteinase (Rangel *et al.*, 1981; Murta *et al.*, 1990). The reason for its large size is unclear.

Different amounts of trypanopain are present at different stages in the trypanosome life cycle (Lonsdale-Eccles and Mpimbaza, 1986; Murta *et al.*, 1990; Souto-Padròn *et al.*, 1990; Mbawa *et al.*, 1991b). Mammalian-infective forms have higher levels of active trypanopain than do insect-infective forms (Mbawa *et al.*, 1992). Additionally the differentiation of *T. b. brucei* bloodstream forms from the long slender to short stumpy forms is accompanied by an 8-fold increase in trypanopain activity (Pamer *et al.*, 1989; Mbawa *et al.*, 1991b). The last insect stage, namely the metacyclic form, has increased enzyme activity over the other insect forms, possibly reflecting preparation for mammalian infection. Cruzipain and some of the *Leishmania*

cysteine proteinases are also expressed at different levels during the parasite life cycle (Campetella *et al.*, 1990; Souza *et al.*, 1992; Franke de Cazzulo *et al.*, 1994; Tomás and Kelly, 1994).

These changes in trypanopain levels may reflect a change in the nutritional requirements of the parasite at different stages of its life cycle. As the differentiation from long slender to short stumpy forms is accompanied by many metabolic changes, it has been suggested that trypanopain activity increases to cope with the catabolism of obsolete proteins (Pamer *et al.*, 1989). Alternatively, the enzyme could aid initial survival in the insect midgut (Pamer *et al.*, 1989).

1.12.4 Primary sequence of trypanopains

On the basis of their primary sequence data (Mottram *et al.*, 1989) and their catalytic activity, trypanopains belong to the papain family (C1) of cysteine proteinases in the CA clan (Barrett and Rawlings, 1996). Most cysteine proteinase fit into this group, which also contains the mammalian lysosomal proteinases cathepsins L, B, S, C, K and H, the plant proteinases bromelain, ficin and actinidin and cysteine proteinases from numerous other parasites and bacteria (*Fasciola*, *Schistosoma*, *Leishmania*, *Plasmodium*, *Theileria*, *Streptococcus*, *Lactococcus* and *Porphyromonas*).

Trypanopain is encoded as a pre-pro-enzyme by multicopy genes that are arranged in tandem arrays on one or more chromosomes (Mottram *et al.*, 1989). More than 20 copies of the trypanopain gene are present in *T. b. brucei*, while as many as 130 copies of the cruzipain gene are found on 2-4 different chromosomes in certain *T. cruzi* strains (Campetella *et al.*, 1992; Eakin *et al.*, 1992).

Similarly, *L. mexicana* cysteine proteinases are encoded by 19 genes located in a tandem array on a single chromosome (Souza *et al.*, 1992; Mottram *et al.*, 1996). The reason for this multiplication is unclear and it is not known whether all copies of this gene are transcribed or if some are pseudogenes. Studies of cruzipain isoforms suggest that the genes may encode enzymes with slightly differing substrate specificity. For example, the isoform cruzipain-2 has the novel and potentially physiologically important ability to cleave bradykinin from kininogen

(Lima *et al.*, 1994). Gene knockout has also shown that the various lmCPb enzymes of *L. mexicana* also differ in their substrate specificity (Mottram *et al.*, 1997).

Trypanopain is synthesised as a 48 kDa precursor that is processed to a 28-31 kDa mature form (Mottram *et al.*, 1989). The transcript can be divided into a hydrophobic pre-region, a hydrophilic pro-region, a central catalytic domain and a C-terminal extension. The pre-pro, catalytic domain and C-terminal extension are 30, 60 and 70% homologous to the corresponding regions of cruzipain (Campetella *et al.*, 1992). A SwissProt search (Bairoch and Boeckmann, 1994, accession number P14658) reveals that trypanopain shares highest sequence homology with cysteine proteinases of other *Trypanosoma* and protozoan parasites of the family Trypanosomatidae (i.e. *Leishmania*, Markell *et al.*, 1992). Of the mammalian cathepsins, trypanopain is most similar to rat and mouse cathepsin L (Table 1).

Trypanopain, trypanopain-Tc, cruzipain and the *L. mexicana* lmCPb cysteine proteinase have unusually large C-terminal extensions consisting of about 130 amino acid residues in cruzipain (Campetella *et al.*, 1992; Eakin *et al.*, 1992), 110 in trypanopain (Mottram *et al.*, 1989) and 100 in *L. mexicana* (Souza *et al.*, 1992). While there are no known equivalents of this extension in mammalian cysteine proteinases, a cold-induced messenger ribonucleic acid (mRNA) found in tomatoes shows 22% homology to the trypanosomal extension (Schaffer and Fischer, 1988). The sequences are most closely related in a region thought to function as a proteolytically-sensitive hinge between the extension and the central domain, where trypanopain and cruzipain have consecutive proline and threonine residues respectively (Åslund *et al.*, 1991) and the tomato mRNA has a proline/serine string. From knowledge of the size of active trypanopains, it is predicted that about 50 residues of the extension remain in the mature enzyme.

Table 1: Degree of homology between trypanopain from *T. b. brucei* and other cysteine proteinases.

Parent organism	Enzyme	% Homology	Reference
T. congolense	trypanopain-Tc	69	Fish *et al.* (1995)
T. cruzi	cruzipain	60	Cazzulo *et al.* (1989)
L. pifanoi	cysteine proteinase A-2	22	Traub-Cseko *et al.* (1993)
L. mexicana	lmCPb (Type I)	21	Souza *et al.* (1992)
L. mexicana	lmCPa (Type II)	23	Mottram *et al.* (1992)
L. pifanoi	cysteine proteinase A-1	23	Traub-Cseko *et al.* (1993)
Rattus norvegicus	cathepsin L	11	Ishidoh *et al.* (1987)
Mus musculus	cathepsin L	11	Troen *et al.* (1987)
Homo sapiens	cathepsin L	11	Gal and Gottesman (1988)
Ovis aries	cathepsin L	9	Ritonja *et al.* (1996)
P. falciparum	falcipain	9	Rosenthal *et al.* (1992)
P. vivax	falcipain	6	Rosenthal *et al.* (1994)
P. vinckei	falcipain	6	Rosenthal (1993)
Carica papaya	papain	11	Cohen *et al.* (1986)

Since the extension has limited occurrence in distantly related proteins, North (1991) suggested that the extension arose in a distant ancestral enzyme where it served an unknown function that is obsolete in most modern cysteine proteinases. In enzymes where it remains, it may confer unusual and beneficial properties. Various functions have been proposed for the extensions including mediation of trypanopain targeting to the lysosome (Cazzulo *et al.*, 1990a; Martìnez *et al.*, 1991; McKerrow, 1991), interaction with membranes (Souto-Padrón *et al.*, 1990) or maintainance of trypanopains in a zymogen state until the mature proteinase is cleaved off (McKerrow, 1991). However, the extension has subsequently been shown to be unnecessary for the activity of a trypanopain homologue from *T. b. rhodesiense* (Pamer *et al.*, 1991), cruzipain (Eakin *et al.*, 1993) and *L. pifanoi* (Duboise *et al.*, 1994). Additionally, Lpcys1 from *L. pifanoi* lacks the extension but is still correctly targeted within the cell (Duboise *et al.*, 1994) and *L. mexicana* lmCPb isoenzymes lacking the extension are also active and correctly targeted (Mottram *et al.*, 1997).

Cruzipain has been identified as one of the major antigens (called GP 57/51) recognised by humans infected with *T. cruzi* (Murta *et al.*, 1990; Malchiodi *et al.*, 1993; González *et al.*, 1996) and cruzipain's dominant B-cell epitopes are located primarily in the C-terminal extension of the enzyme. Binding of antibodies to this region does not affect enzyme activity, leading to the suggestion that cruzipain contains defined antigenic and catalytic domains, with the C-terminal extension acting as an immune decoy to protect cruzipain against host attack (Cazzulo and Fransch, 1992; Martìnez *et al.*, 1993). Trypanopain-Tc is known to be an important cattle antigen (Authié *et al.*; 1992) and it would be interesting to determine whether this enzymes shares a division into catalytic and antigenic domains.

1.12.5 Catalytic mechanism of trypanopain

While the catalytic mechanism of trypanopain has not been directly investigated, sequence data places the enzyme firmly in the papain-like family of cysteine proteinases (Mottram *et al.*, 1989). The catalytic mechanism of this class has been the subject of a number of review papers, including those by Brocklehurst (1987), Dunn (1989a), Storer and Ménard (1994) and Turk *et al.* (1997). Cysteine proteinases contain, by definition, a reactive cysteine residue in their active sites, and they additionally contain a conserved reactive site histidine residue. The enzymes are only active when these residues are ionised, when they are said to form the thiolate-imidazolium ion pair.

All known papain-like cysteine proteinase (with the exception of cathepsin C) are monomers folded into two domains (called L- and R-), between which is the active site cleft that binds substrate at many points. The active site cysteine residue is located on the L-domain and the active site histidine on the opposite R-domain. Determination of the cruzipain crystal structure confirmed that trypanosomal cysteine proteinases are folded according to this typical papain blueprint (Figure 4) (McGrath *et al.*, 1995).

Substrate hydrolysis begins with non-covalent association of the enzyme and substrate to form the Michaelis complex. The thiolate group of the active site cysteine acts as a nucleophile and attacks the carbonyl carbon of the scissile bond to form a postulated transient tetrahedral intermediate (Figure 5). The active site histidine donates its proton to the amide nitrogen of the

scissile bond, promoting formation of the acyl-enzyme intermediate and release of the C-terminal portion of the substrate. The acyl-enzyme intermediate undergoes nucleophilic attack by a water molecule to release the free enzyme and the N-terminal portion of the substrate.

Various other tetrahedral intermediates are postulated to form between the stages shown in Figure 5Figure, but they are thought to be too short-lived to have been directly detected (reviewed by Storer and Ménard, 1996). The initial nucleophilic attack on the carbonyl carbon of the scissile bond is facilitated in part by binding of the carbonyl oxygen into the oxyanion hole formed by neighbouring cysteine and glutamine residues. While the oxyanion hole is crucial for serine proteinases, various site-directed mutagenesis studies suggest that it is not as important in cysteine proteinases.

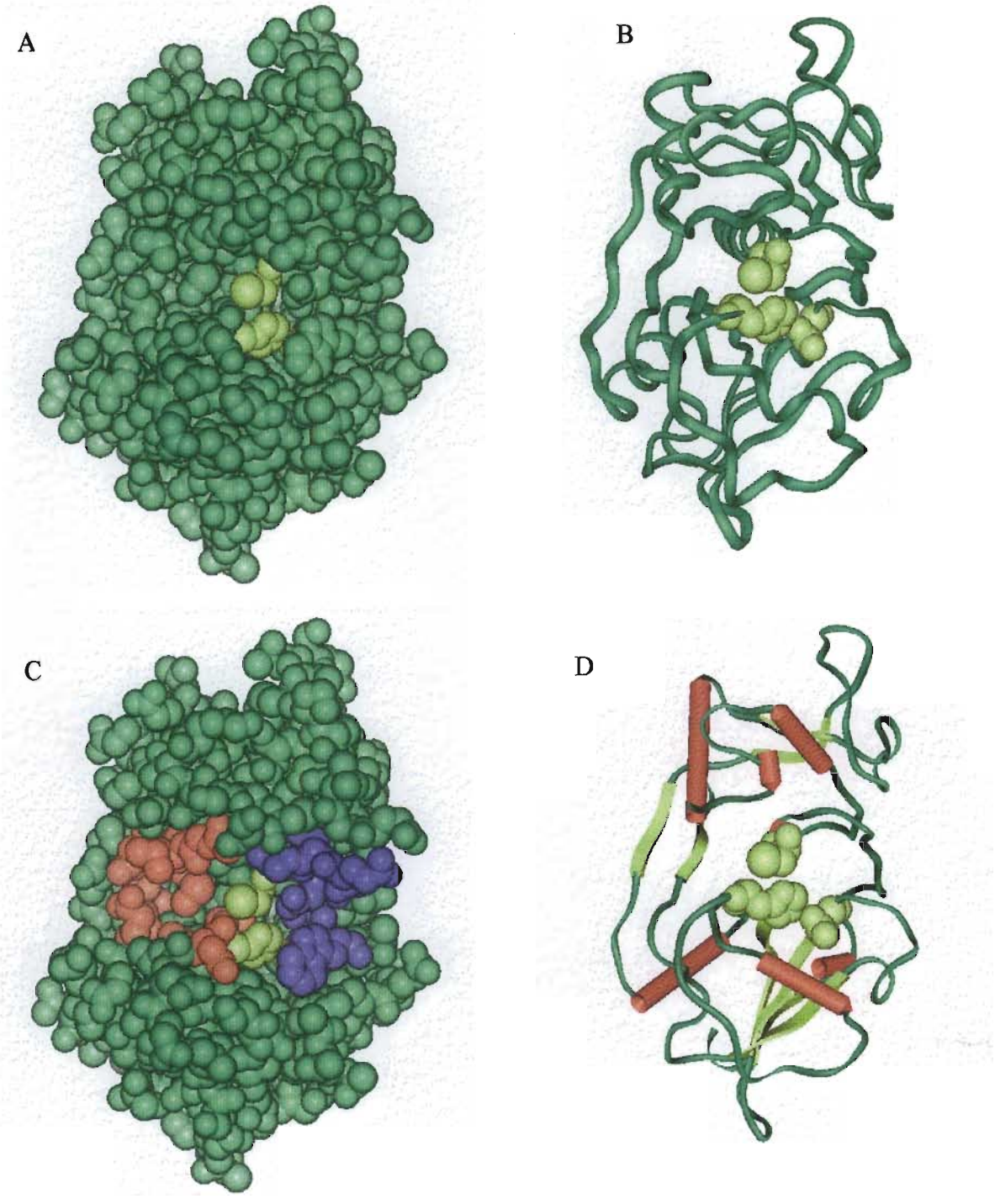


Figure 4: Cartoon representations of cruzipain three-dimensional structure.

A and B show space fill and ribbon models respectively of cruzipain, with catalytic residues highlighted in yellow. C shows a space fill model of cruzipain, with the catalytic residues in yellow, the S_2 and S_3 subsites in red and the S_1' subsite in blue. D shows a ribbon model of cruzipain, with α-helices in red and β-pleated sheets in yellow. The orientation of all representations is such that the active site cysteine residue is on the upper portion of the active site and the histidine/aspartate active site residues on the lower portion. Figures were prepared by Xiaowu Chen of the Department of Pharmaceutical Chemistry at the University of California, San Francisco, using the co-ordinates of McGrath *et al.* (1995).

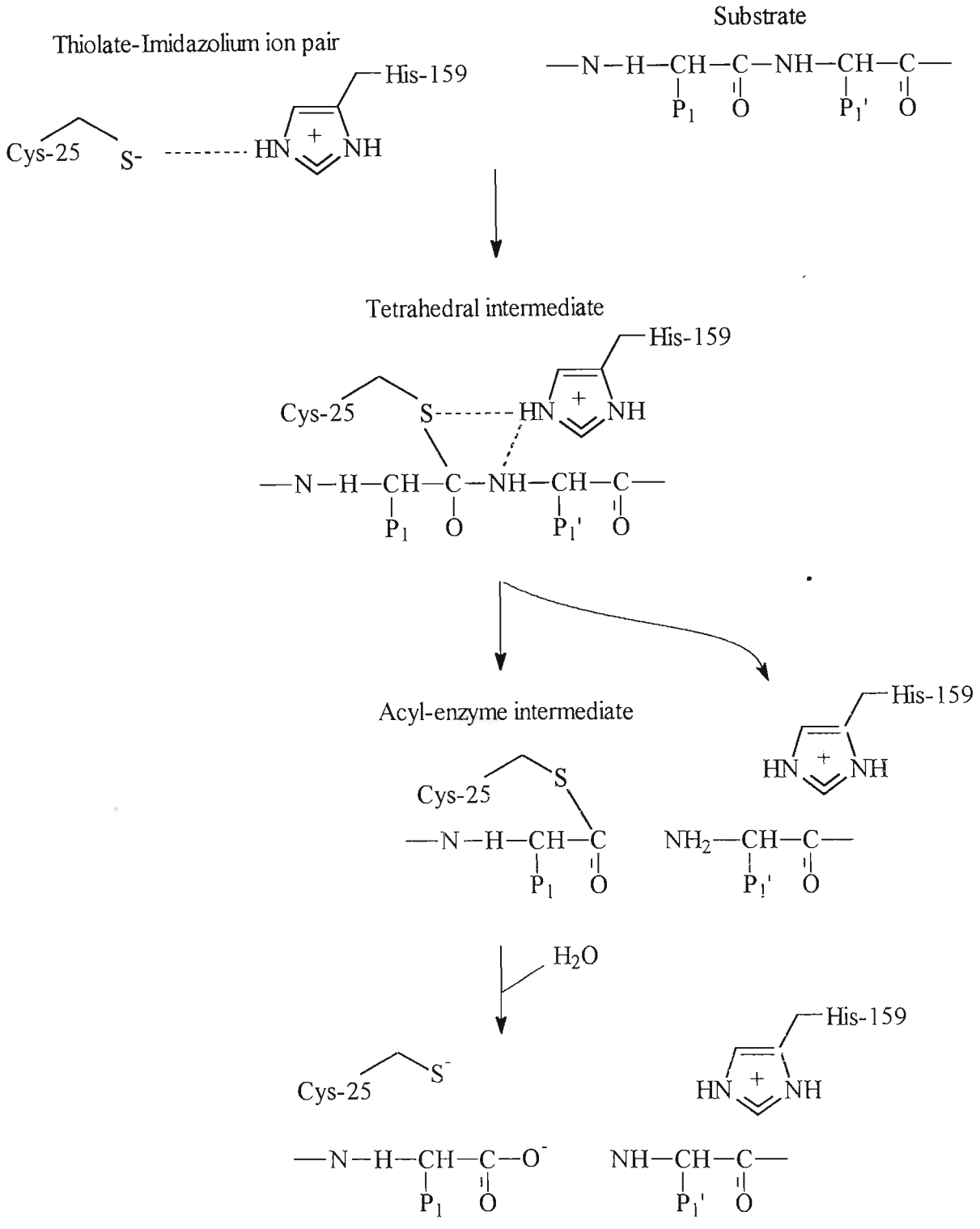


Figure 5: Proposed catalytic mechanism of cysteine proteinases.

The active site cysteine residue attacks the carbonyl carbon of the scissile bond to form a tetrahedral intermediate that rearranges to form the acyl-enzyme intermediate and release the first portion of the substrate. Nucleophilic attack on the acyl-enzyme intermediate by H_2O releases the second portion of the substrate. After Dunn (1989a).

1.12.6 Possible physiological functions of trypanopains

Although the functions of the trypanopains remain unclear, various possibilities have been proposed. The enzymes appear to be essential for parasite viability because specific cysteine proteinase inhibitors kill isolated parasites *in vitro* (Mbawa *et al.*, 1992; Franke de Cazzulo *et al.*, 1994). Their lysosomal locations suggest that these enzymes are centrally involved in the catabolism of parasite proteins, but the enzymes are also thought to digest host proteins as a source of nutrition. Haemoglobin digestion is thought to be carried out by the cysteine proteinases of numerous parasites, e.g. *S. mansoni* (Dalton *et al.*, 1995), *P. falciparum* (Rosenthal *et al.*, 1988), and *Haemonchus* (Cox *et al.*, 1990). In *S. mansoni*, cysteine proteinase gene expression is controlled at a temporal and spacial level, being up-regulated in the gut during the feeding stages of the parasite life cycle (Ray and McKerrow, 1992).

Trypanopains have been proposed to help the parasite evade the host immune system. Trypanopains appear to interact with various components of the host immune system and may protect parasites against immune system attack in various ways. Firstly, fluorescence microscopy indicates that surface-bound anti-VSG antibodies are endocytosed and apparently degraded by live *T. brucei*, as only small amounts of labelled antibody are present within parasites after VSG clearance (Russo *et al.*, 1994). However, if a cocktail of proteinase inhibitors is added, larger amounts of antibody are detectable in intracellular organelles. This suggests that lysosomal proteinases, possibly trypanopain, are involved in degradation of host antibodies. If this were the case, then trypanopain would help the parasite escape the opsonising effects of antibodies.

Cruzipain also has IgG-degrading properties (Bontempi and Cazzulo, 1990). The enzyme extensively degrades the F_c region of human IgG at pH 5.0 *in vitro*, destroying complement-activating and phagocyte-recruiting ability (Bontempi and Cazzulo, 1990). However, the F_{ab} (fragment, antibody binding) region remains relatively undegraded and may still be able to bind to the relevant antigen, in effect protecting it from binding to other whole, opsonising antibodies (Bontempi and Cazzulo, 1990). Furthermore, trypanosome-specific T-cells are reportedly not primed following infection (Paulnock *et al.*, 1988) and it has been postulated that trypanopain interferes with T cell responses to trypanosomal infection (Pamer *et al.*, 1991).

Anti-cruzipain $F(ab)_2$ fragments and cruzipain inhibitors partially prevent *T. cruzi* entry into mammalian cells (Piras *et al.*, 1985; Souto-Padrón *et al.*, 1990), suggesting that cruzipain plays a role in cell penetration. Since neither *T. b. brucei* nor *T. congolense* are intracellular, trypanopain and trypanopain-Tc cannot have similar functions.

Parasite cysteine proteinases are thought be involved in the transition between morphologically distinct stages in the parasite life cycle. Inhibition of cruzipain prevents maturation of epimastigotes to metacylic forms, as well as the maturation of tryptomastigotes to amastigotes (Mierelles *et al.*, 1992; Harth *et al.*, 1993; Franke de Cazzulo *et al.*, 1994). Cruzipain may be involved in remodelling of necessary cytoskeletal elements or catabolism of obsolete proteins from the previous stage. Addition of cysteine proteinase inhibitors also inhibits maturation of *L. mexicana* promastigotes to amastigotes (Coombs *et al.*, 1982).

Trypanopains may contribute to pathogenesis by degrading various host proteins. Trypanopains degrade haemoglobin, IgG, oxidised insulin A and B chains and serum albumin *in vitro*, but it is not known if these are substrates *in vivo* (Rautenberg *et al.*, 1982; Raimondi *et al.*, 1991; Mbawa *et al.*, 1992). Proteinase activity, thought to be due to trypanopain, is released from isolated *T. brucei* (Nwagwu *et al.*, 1988) but it is not clear whether the enzyme is actively secreted by live parasites or merely released upon death of the parasites. Even if released in an active form, the effect of host proteinase inhibitors on the enzyme has not been reported.

1.13 Objectives of the present study

Enzymes essential for parasite survival or involved in disease progression are receiving increasing attention as possible immuno- and chemotherapy targets. Although the pathogenic function(s) of trypanopain remain unclear, various studies suggest that inhibition of the enzyme may have therapeutic benefits. While trypanopain genomic DNA and cDNA have been sequenced, relatively little is known about the active enzyme. The aim of this study was to purify trypanopain from *T. b. brucei* to electrophoretic homogeneity (Chapter 3) and to investigate some of the enzyme's fundamental properties, such as its pH activity and stability, its response to inhibitors and reducing agents and its specificity for synthetic substrates (Chapter 4). The interactions between trypanopain and various potential inhibitors [both natural cystatins

(Chapter 5) and anti-active site antibodies (Chapter 6)] were investigated to determine whether the enzyme is likely to be effectively inhibited should it be released into the host bloodstream. Additionally, the effects of synthetic trypanopain inhibitors on purified trypanopain and parasite viability were investigated to determine whether trypanopain is a potential target for novel anti-trypanosomal agents (Chapter 7).

Chapter 2

General material and methods

A variety of fundamental biochemical techniques were used throughout the study and are described here for convenience. More specific experimental procedures are described in their appropriate chapters. The suppliers of all materials used in this study are listed below.

2.1 Materials

Buffer salts and other common chemicals were from BDH (UK), Merck (Germany), Riedel deHaën (Germany), UniLab (SA) or Boehringer Mannheim (Germany) and were of the highest purity available. Unless otherwise specified, distilled water was used throughout this study. HiLoad™ Q-Sepharose and Resource-Q™ columns were from Pharmacia (Sweden). Acrylamide, bis-acrylamide, Triton X-100 and bovine serum albumin (BSA) were from BDH Limited (UK). Papain, 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF) and 2,2 azino-di-[3-ethylbenzthiazoline sulphonate] (ABTS) were from Boehringer Mannheim (Germany). Percoll, *m*-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), Minimal Essential Medium (MEM), penicillin/streptomycin, bathocuproine disulfonate (BCDS), 3,3',5,5'-tetramethyl benzidine (TMB), 5-bromo-4-chloro-3-indolyl phosphate/ nitroblue tetrazolium (BCIP/NBT), goat anti-rabbit-alkaline phosphatase (AP), fluorescein isothiocyanate (FITC)-albumin, commonly available enzyme inhibitors and fibrinogen were from Sigma Chemical Co. (USA). Dry dimethyl formamide (DMF) and di-isopropylfluorophosphate (DFP) were from Fluka (Switzerland). Nunc-Immuno MaxiSorp and PolySorp F96 ELISA plates were from Weil Organisation (SA). Chymostatin, leupeptin and antipain were from Cambridge Research Chemicals (UK). Freund's complete and incomplete adjuvants and gelatin were from Difco (USA). Rabbit anti-human high molecular weight (H-) kininogen antibodies were from CalBiochem (USA). Foetal calf serum (FCS) was from Delta BioProducts (SA). Sterile disposable cell culture plasticware and filters were from Corning (UK), Millipore (USA) and Bibby Sterilin (UK). Diethylaminoethyl (DEAE)-cellulose and Whatman No.1 filter paper were from Whatman International Ltd (UK). Lumax™ scintillation cocktail was from Lumac (Netherlands).

2.2 Bicinchoninic acid (BCA) protein assay

The biuret protein assay is based on the reduction of alkaline Cu^{2+} to Cu^{+} by the peptide bonds of a protein (or any other biuret-containing polymer) to form a concentration-dependent blue complex. The Folin-Ciocalteau reagent (a phospho-molybdic/phosphotungstic acid complex) is commonly used to enhance the sensitivity of the biuret assay (Lowry *et al.*, 1951; Hartree, 1972). However, this reagent is unstable at the alkaline pH values required for Cu^{2+} reduction, necessitating careful timing of reagent addition. Smith *et al.* (1985) described the use of BCA as an alternative, more stable reagent to detect Cu^{+} and so enhance the sensitivity of the biuret assay (Figure 6). BCA is a stable, water-soluble compound that forms purple complexes with Cu^{+} in an alkaline environment. The BCA assay is more convenient than the Lowry assay in that it is completed in a single step, is less sensitive to interference by buffer salts and ionic or non-ionic detergents and shows less protein-to-protein variation than the Lowry assay (Smith *et al.*, 1985). Reducing agents and copper chelators do, however, interfere with the assay.

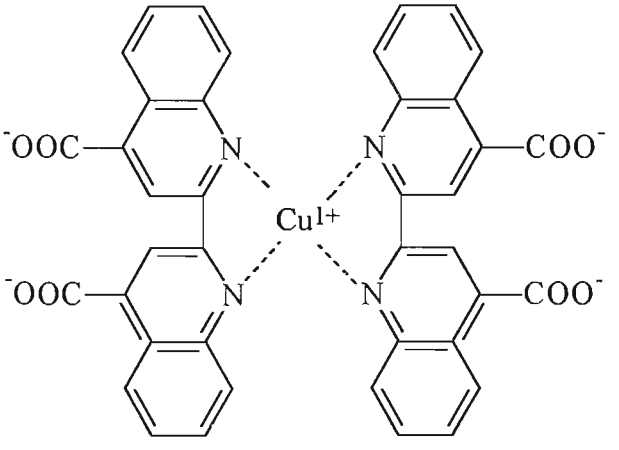


Figure 6: Complex formation between Cu^{+} and BCA.

The N atoms of two molecules of BCA interact with a Cu^{+} ion in an alkaline environment to produce a stable, purple, H_2O-soluble complex that can be quantified by its absorbance at 562 nm. After Smith *et al.* (1985).

2.2.1 Materials

Reagent A [1% mass per volume (m/v) Na-BCA, 2% (m/v) Na_2CO_3, 0.16% (m/v) Na-tartrate, 0.4% (m/v) NaOH, 0.95% (m/v) $NaHCO_3$]. Sodium-BCA (1 g), Na_2CO_3

(2 g), Na-tartrate (0.16 g), NaOH (0.4 g) and $NaHCO_3$ (0.95 g) were dissolved in 90 ml of H_2O, adjusted to pH 11.25 with NaOH and made up to 100 ml.

Reagent B [4% (m/v) $CuSO_4.5H_2O$]. $CuSO_4$ (4 g) was dissolved in 90 ml of deionised H_2O and made up to 100 ml.

Standard working solution. Reagent A (100 volumes) was mixed with Reagent B (2 volumes) to produce an apple-green standard working solution that was stable for approximately 1 week.

2.2.2 Method

Protein samples (0.2-50 µg) were diluted to 50 µl with a suitable buffer in 1½ ml polyethylene microfuge tubes. Standard working reagent (1 ml) was added and the solution thoroughly mixed. Depending on the degree of sensitivity required, the mixture was incubated according to one of the following protocols:

(i) Room temperature (RT) for 2 h (suitable for detecting 20-120 µg protein),

(ii) 37°C for 30 min (intermediate sensitivity), or

(iii) 60°C for 30 min (5-25 µg protein).

After incubation, samples were cooled to RT and the absorbance at 562 nm (A_{562}) read. Buffer alone added to dye reagent served as an absorbance blank. A standard curve for each incubation protocol was prepared from quintiplicate assays carried out at 6 concentrations of ovalbumin (5-120 µg protein). The protein concentrations of samples were calculated from equations generated by linear regression of the standard protein results.

2.3 Concentration of proteins

During enzyme purification, it was often necessary to concentrate dilute protein solutions [e.g. before sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) or before further purification]. Samples were concentrated by dialysis against sucrose or polyethylene glycol (PEG, 20 kDa), with the sample placed inside a closed dialysis bag and surrounded by a layer of either dry sucrose or PEG (20 kDa) polymer at 4°C. Water moved along a concentration gradient from the dilute solution inside the membrane to the

concentrated polymer outside of the membrane. The pores of the dialysis membrane used were small enough to prevent loss of proteins greater than 12 kDa from the sample. Gloves were worn during handling of the dialysis membrane to prevent introduction of skin keratins into samples. These proteins are troublesome contaminants which cross-react non-specifically with many antibodies in Western blots and appear as contaminants at about 68 kDa on reducing SDS-PAGE (Ochs, 1983; Shapiro, 1987). Once a sample was sufficiently concentrated (usually 5-10 fold in 2-4 h), the membrane was briefly rinsed in H_2O and the membrane cut open to release the sample.

Pores in the dialysis membrane used (cut-off limit 12 kDa) were small enough to allow sucrose to enter the dialysis bag and mix with the sample. In cases where such contamination was unacceptable (e.g. prior to electrophoresis), samples were dialysed against PEG (20 kDa), which is too large to pass through the membrane and so does not contaminate the sample. PEG is, however, considerably more expensive than sucrose, so dialysis against PEG was only carried out during the final stages of enzyme purification.

2.4 Tricine SDS-PAGE

SDS-PAGE is an analytical technique used to evaluate the purity and molecular mass of protein samples (Laemmli, 1970). The method involves the separation of proteins on the basis of size during migration through a polyacrylamide gel placed in an electric field. The effects of a protein's innate charge on its migration are counteracted by addition of the anionic detergent SDS to mask the protein's innate charge. This reagent converts native, globular, amphoteric proteins into highly negatively charged rod-like complexes; the length of which is dependent on the molecular mass of the protein. Binding of SDS thus renders the charge to mass ratio of all proteins in the sample equal, allowing proteins to be separated on the basis of size alone. An inverse relationship exists between the log of a protein's molecular weight and the distance it migrates from the cathode (starting point) (Dunn, 1989b), so SDS-PAGE is commonly used to estimate the molecular masses of sample proteins by comparing their migration to that of standard proteins of known molecular mass.

Polyacrylamide gels are formed by the co-polymerisation of acrylamide monomers with a cross-linking agent, such as N, N'-methylenebisacrylamide (Bis) (Figure 7). Gel

polymerisation is initiated by agents such as N, N, N', N'-tetramethylethylenediamine (TEMED) and accelerated by agents such as ammonium persulfate (Dunn, 1989b). The pore size of the gel is dependent on the concentration of the total monomer (acrylamide plus Bis, %T) and on the concentration of the cross-linker, Bis (%C). The %T determines the effective separation range of the gel.

Although tricine SDS-PAGE is a modification of the original SDS-PAGE method described by Laemmli (1970), it operates on the same fundamental principles. In contrast to the Laemmli system, different buffers are placed in the upper and lower buffer reservoirs to create a discontinuous buffer system. Once electrophoresis starts, proteins stack between the leading chloride ion and the trailing tricine ion (Schägger and von Jagow, 1987). This concentrates samples into thin starting zones in the stacking gel and thus ensures good separation in the separating gel. Once the samples enter the separating gel, the decreased pore size of the separating gel "unstacks" the proteins, causing them to separate on the basis of size alone (Dunn, 1989b). The system has various advantages over the Laemmli system, such as increased resolution of smaller proteins (5-20 kDa) and uniformity of stacking and separating gel buffers. In addition, the gel buffer used is more stable than the Laemmli stacking gel buffer and so can be stored for longer periods of time (Schägger and von Jagow, 1987). The tricine SDS-PAGE method was routinely used for electrophoretic analysis throughout this study.

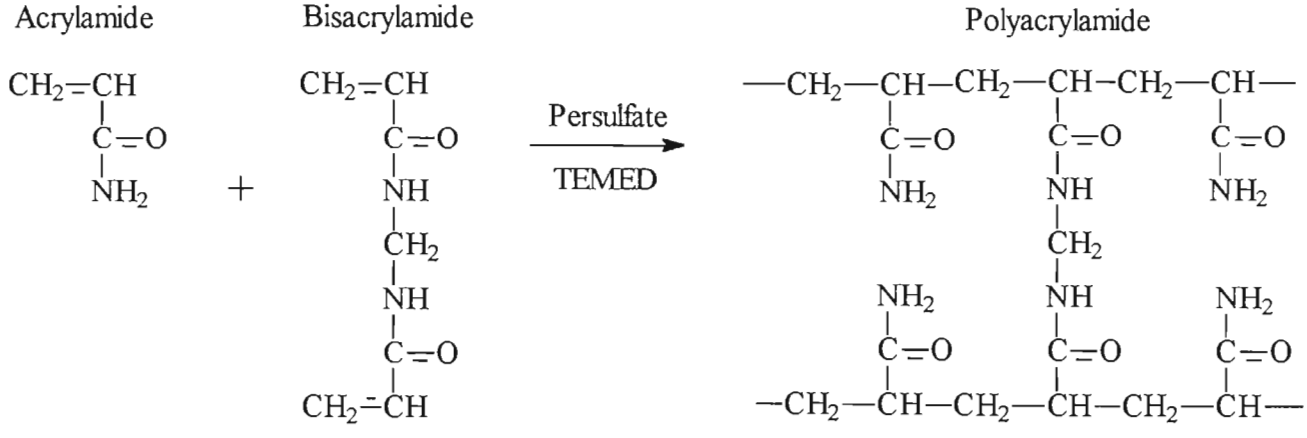


Figure 7: Polymerisation of acrylamide by Bis, TEMED and ammonium persulfate.
Acrylamide monomers are cross-linked by Bis in a reaction initiated by TEMED and accelerated by ammonium persulfate.

2.4.1 Materials

Anode buffer [0.2 M 2-amino-2-(hydroxymethyl)-1,3-propandiol (Tris)-Cl, pH 8.9]. Tris (24.22 g) was dissolved in 950 ml H_2O, titrated to pH 8.9 with HCl and made up to 1 l.

Cathode buffer [0.1 M Tris, 0.1 M tricine, 0.1 % (m/v) SDS, pH 8.25]. Tris (12.2 g) and tricine (17.9 g) were dissolved in 950 ml of H_2O and 10% (m/v) SDS stock solution (10 ml) added. The pH was adjusted to 8.25 and the buffer made up to 1 l.

Gel buffer [3.0 M Tris-Cl, 0.3% (m/v) SDS, pH 8.45]. Tris (72.7 g) and SDS (0.6 g) were dissolved in 150 ml of H_2O, titrated to pH 8.45 with HCl and made up to 200 ml.

Monomer [49.5% (m/v) acrylamide, 3% (m/v) Bis]. Acrylamide (48 g) and Bis (3 g) were dissolved in 50 ml of H_2O and the solution made up to 100 ml.

10% (m/v) Ammonium persulfate. Ammonium persulfate (0.1 g) was dissolved in H_2O (1 ml) just before use.

Reducing sample buffer [125 mM Tris-Cl, 4% (m/v) SDS, 20% volume per volume (v/v) glycerol, 10% (v/v) β-mercaptoethanol, pH 6.8]. Gel buffer (2.5 ml), 10% SDS (4 ml), glycerol (2 ml) and β-mercaptoethanol (1 ml) were made up to 10 ml with H_2O. β-Mercaptoethanol was added to the treatment buffer to reduce any disulfide bonds in the sample and thus allow unhindered binding of SDS to disulfide-bonded regions of sample proteins and to permit the dissociation of disulfide-linked subunits.

Non-reducing sample buffer [125 mM Tris-Cl, 4% (m/v) SDS, 20% (v/v) glycerol, pH 6.8). Gel buffer (2.5 ml), 10% SDS (4 ml) and glycerol (2 ml) were made up to 10 ml with H_2O.

2.4.2 Method

Tricine SDS-PAGE was carried out as described by Schägger and von Jagow (1987) using a BioRad Mini-Protean® II electrophoresis cell, assembled as described in the manufacturer's manual. For gels that were to be silver stained, extra care was taken to clean the plates thoroughly before gel assembly by soaking them in nitric or chromic acid. Separating and

stacking gels were prepared as described in Table 2and allowed to polymerise for at least 2 h and a half hour respectively.

Samples (containing at least 1-2 μg of protein per band for Coomassie staining or 100-300 ng of protein per band for silver staining) were mixed with either an equal volume of reducing sample buffer or a half volume of non-reducing sample buffer. More uniform binding of SDS to proteins was effected by boiling samples (90 s) to fully denature proteins. Bromophenol blue marker dye, which migrates at the buffer front, was added to each sample before loading to allow monitoring of protein migration through the gel. Gels were run at 70 V until the bromophenol blue tracker dye entered the separating gel, and then at 100 V until the bromophemol blue reached the bottom of the gel. Throughout this study, Pharmacia (Sweden) molecular mass markers were used. These are phosphorylase b (96 kDa), BSA (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa) and soybean trypsin inhibitor (20 kDa).

Table 2: Preparation of two tricine SDS-PAGE gels for electrophoresis on the BioRad Mini-Protean® II electrophoresis cell.

Type of gel	**Gel buffer** (ml)	**Acrylamide** (ml)	**$(NH_4)_2S_2O_8$** (μl)	**TEMED** (μl)	**H_2O** (ml)
Separating	6	3.6	60	6	8.4
Stacking	1.5	0.5	30	12	4

2.5 Substrate SDS-PAGE

Heussen and Dowdle (1980) described a modification of SDS-PAGE that allows visualisation of proteinases after electrophoretic separation by incorporation of a suitable protein substrate into the separating gel prior to polymerisation. Following electrophoresis, gels are incubated in a relevant assay buffer to allow proteinases immobilised in the gel to digest the incorporated substrate. Areas of proteinase activity can be seen as clear bands of digestion against a dark staining protein background. Variations of this method have been successfully used to investigate the proteinases of various pathogens, including *T. brucei* (Robertson *et al.*, 1990), *T. congolense* (Mbawa *et al.*, 1992), *F. hepatica* (Smith *et al.*, 1993), *T. spiralis* (Todorova *et al.*, 1995), *S. mansoni*

(Smith *et al.*, 1994; Dalton *et al.*, 1996), *L. mexicana* (Robertson and Coombs, 1990; Hunter *et al.*, 1992; Ilg *et al.*, 1994) and *E. histolytica* (Sharma *et al.*, 1996). Fibrinogen and gelatin have been successfully used as substrates for trypanopains (Lonsdale-Eccles and Grab, 1987a; Lonsdale-Eccles *et al.*, 1995).

The method should, however, not be relied upon for an accurate estimate of proteinase molecular mass. Hummel *et al.* (1996) reported that incorporation of a protein substrate into the SDS-PAGE gel can increase the apparent molecular mass of proteins by 15-20%, with the degree of increase differing for different proteins. They thus recommend that estimates of molecular mass made from substrate SDS-PAGE be viewed with caution.

2.5.1 Materials

Protein solution [1% (m/v) gelatin or fibrinogen in gel buffer]. Gelatin or fibrinogen (0.1 g) was dissolved in gel buffer (10 ml, Sections 2.4.1) with gentle heating.

2.5% (v/v) Triton X-100. Triton X-100 (5 ml) was diluted to 200 ml with H_2O.

Assay buffer [100 mM sodium acetate, 1 mM ethylenediaminetetra-acetic acid (EDTA), 30 mM cysteine, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (5.725 ml), $Na_2EDTA.2H_2O$ (0.37 g) and NaN_3 (0.2 g) were dissolved in 950 ml of deionised H_2O, adjusted to pH 5.5 with NaOH and made up to 1 l with deionised H_2O. Cysteine (0.53 g/100 ml assay buffer) was added just before use.

0.1% (m/v) Amido black. Amido black (0.1 g) was dissolved in methanol:acetic acid:H_2O in a 30:10:60 ratio (100 ml) and filtered through Whatman No. 1 filter paper.

2.5.2 Method

Separating gels were cast as per Section 2.4.2, except that the gel buffer (6 ml) was replaced by a mixture of the protein substrate solution [3 ml of 1% (m/v) gelatin or fibrinogen in gel buffer] and gel buffer (3 ml), giving a final concentration of 0.16% (m/v) of protein in the separating gel. Stacking gels were cast as described in Section 2.4.2. To ensure that enzyme activity was maintained, samples were never boiled prior to

electrophoresis. After electrophoresis, the gels were soaked in two changes of 2.5% (v/v) Triton X-100 in 1 h to remove SDS and re-nature any proteinases present. Gels were incubated in assay buffer (3 h, 37°C) to allow digestion of the incorporated substrate and stained in amido black for 1 h. Gels were destained in several changes of methanol:acetic acid:H_2O (30:10:60) until clear bands were visible against a dark background.

2.6 Coomassie blue R-250 protein stain

Coomassie Brilliant Blue R-250 is a non-polar, sulfated triphenylamine dye used to visualise at least 1 μg of protein per band (Dunn, 1989b). This method provides a simple way of visualising proteins separated by SDS-PAGE.

2.6.1 Materials

Stain stock solution [1% (m/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in 100 ml of H_2O and filtered through Whatman No. 1 filter paper.

Staining solution [0.125% (m/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml) and made up to 500 ml with H_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) and acetic acid (100 ml) were made up to 1 litre with H_2O.

Destaining solution II [7% (v/v) methanol, 5% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (70 ml) were made up to 1 litre with H_2O.

2.6.2 Method

After removal from the electrophoresis unit, gels were placed in stain solution for 4 h. The gels were rinsed in H_2O and immersed in destain I (4 h), followed by destain II (until the background had cleared fully). Gels were stored in polyethylene zip-seal bags and kept well hydrated until photographed. They were stable for long periods of time in this form.

2.7 Silver staining of proteins in polyacrylamide gels

Silver staining is a highly sensitive method of detecting proteins in electrophoresis gels, based on the reduction of silver ions to metallic silver (Blum *et al.*, 1987). As little as 100 ng of protein per band can be detected, making this technique second only to radioactive labelling in terms of sensitivity. The method described by Blum *et al.* (1987) differs from previous methods in that gels are pre-treated in a thiosulfate solution to increase sensitivity. Thiosulfate is also included in the developing solution to complex with insoluble silver salts and thus prevent their accumulation on the surface of the gels (Blum *et al.*, 1987).

2.7.1 Materials

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) 37% (v/v) formaldehyde]. Methanol (100 ml), acetic acid (24 ml) and formaldehyde [100 µl of 37% (v/v) solution] were made up to 200 ml with H_2O.

Thiosulfate solution [0.02% (m/v) sodium thiosulfate]. $Na_2S_2O_3.5H_2O$ (40 mg) was dissolved in H_2O (200 ml).

Silver nitrate solution [0.2% (m/v) $AgNO_3$, 0.075% (v/v) 37% (v/v) formaldehyde]. $AgNO_3$ (400 mg) was dissolved in 150 ml of H_2O, formaldehyde [150 µl of a 37% (m/v) solution] added and the solution made up to 200 ml with H_2O.

Developing solution [6% (m/v) $NaCO_3$, 0.05% (v/v) 37% formaldehyde, 0.0004% (m/v) $Na_2S_2O_3$). $NaCO_3$ (12 g) was dissolved in 150 ml of H_2O, formaldehyde [0.1 ml of 37% (m/v) solution] and $Na_2S_2O_3.5H_2O$ (4 ml of thiosulfate solution above) added, and the solution made up to 200 ml with H_2O.

Stopping solution (50% (v/v) methanol, 12% (v/v) acetic acid). Methanol (50 ml) and 12% (v/v) acetic acid (12 ml) were diluted to 100 ml with water.

2.7.2 Method

For each step, 50 ml of reagent was required to immerse each gel. Deionised water was used throughout. Gels (1.5 mm thickness) were fixed for at least 1 h (preferably overnight) and stained according to the following protocol:

(i) 50% (v/v) ethanol (3×20 min)

(ii) thiosulfate solution (1×30 min)

(iii) H_2O (3×20 s)

(iv) silver nitrate solution (20 min)

(v) H_2O (2×20s)

(vi) developing solution (until bands were visible)

(vii) H_2O (until bands were fully developed)

(viii) stop solution (at least 20 min).

2.8 Isolation of chicken antibodies from egg yolks

Immunoglobulin Y (IgY) was isolated from egg yolk by PEG precipitation, as described by Polson *et al.* (1980). The method is essentially a two-step procedure, with vitellin and fats removed in the first precipitation step and antibodies precipitated in the second. PEG is a water-soluble, high molecular weight polymer that concentrates proteins in the extra-polymer space by steric exclusion, until they exceed their solubility limit and precipitate.

2.8.1 Materials

100 mM Na-phosphate buffer, 0.02% (m/v) NaN_3, pH 7.6. $NaH_2PO_4.2H_2O$ (15.60 g) and NaN_3 (0.2 g) were dissolved in 950 ml of H_20, titrated to pH 7.6 with NaOH and made up to 1 l with H_2O.

2.8.2 Method

Eggs produced by chickens immunised with identical antigens were kept separate to determine whether individual responses differed. Egg yolks were separated from the whites and gently washed under running tap water. The yolk membranes were pierced, the yolk contents collected and mixed with two volumes of sodium phosphate buffer. Solid PEG (6 kDa) was added to 3.5% (m/v) and dissolved with gentle stirring. The precipitated

vitellin fraction was removed by centrifugation (4 420×*g*, 30 min, RT) and the supernatant filtered through a plug of absorbent cotton wool in a glass funnel.

The PEG concentration of the clear filtrate was increased to 12% (m/v) by adding 8.5% (m/v) solid PEG. The solution was centrifuged (12 000×*g*, 10 min, RT) and the antibody pellet re-dissolved in a volume of sodium phosphate buffer equivalent to that obtained after filtration. PEG was added to 12% (m/v) and the solution centrifuged (12 000×*g*, 10 min, RT). To ensure gentle re-suspension, the antibody pellet was dislodged from the walls of the centrifuge tube with a glass rod and left overnight to slowly re-suspend in sodium phosphate buffer of $^1/_6$ the original yolk volume. The final antibody solution was centrifuged to remove undissolved material (27 000×*g*, 10 min, RT) and stored at 4°C with NaN_3 [0.02% (m/v)] as a preservative.

To determine the concentration of purified antibody, the equation $A = \varepsilon Lc$ (Dawson *et al.*, 1968) was used,

where: A represents absorbance at 280 nm (arbitrary units),

ε, the extinction co-efficient ($mg^{-1}.cm^{-1}.ml^{-1}$),

L, the length of the light path (cm) and

c, the concentration of the absorbing solution (M).

For IgY, ε is equal to 1.25 $mg^{-1}.cm^{-1}.ml^{-1}$ (Coetzer, 1985).

2.9 Isolation of rabbit antibodies

A method similar to that used for IgY isolation from egg yolk was used for isolation of rabbit antibodies. PEG (6 kDa) was used to precipitate IgG from rabbit serum, using conditions optimised by Polson *et al.* (1964) in their studies on PEG precipitation of serum components.

2.9.1 Materials

Borate buffered saline (35 mM boric acid, 37 mM NaCl, 17.5 mM NaOH, 8 mM HCl, pH 8.6). Boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% (v/v) HCl (0.62 ml) were dissolved in about 950 ml of H_20, titrated to pH 8.6 and made up to 1 l with H_2O.

2.9.2 Method

One volume of serum was mixed with 2 volumes of borate buffered saline and solid PEG (6 kDa) added to 14% (m/v). After dissolving the PEG by gentle stirring, the mixture was centrifuged (12 000×*g*, 10 min, RT) and the supernatant discarded. The antibody-containing pellet was re-suspended in the original serum volume of sodium phosphate buffer and PEG added to 14% (m/v) as before. The solution was centrifuged (12 000×*g*, 10 min, RT), the pellet resuspended in a volume of sodium phosphate buffer containing 30% (m/v) glycerol equivalent to $^1/_2$ the original serum volume and the preparation stored at -20°C with NaN_3 [0.02% (m/v)] as preservative. To determine the concentration of purified antibody, the equation $A = \varepsilon Lc$ was used as above, with ε for IgG equal to 1.43 $mg^{-1}.cm^{-1}.ml^{-1}$ (Hudson and Hay, 1980).

2.10 Enzyme-linked immunosorbent assays (ELISAs)

An ELISA is a technique exploiting the highly specific nature of the antigen/antibody interaction to detect or quantify either component in a test sample (Nakane *et al.*, 1966; Engvall and Perlmann, 1971). For example, an antigen can be immobilised on an inert plastic support (microtitre plate) and overlaid with test solution containing the complementary antibody. Antibody present in the test solution will only bind to the immobilised antigen, and the amount of bound antibody can be quantified. This is usually done using a second antibody directed specifically against the first, and conjugated to an enzyme that has a virtually colourless substrate and a coloured product that can be quantified spectrophotometrically. The absorbance of the final solution is a measure of the amount of antibody in the test solution. ELISAs were used in the present study to monitor antibody production in experimental animals and to evaluate the ability of anti-peptide antibodies to cross-react with whole trypanopain and cathepsin L.

Horseradish-peroxidase (HRPO, 40 kDa) was used as the reporter enzyme in all cases. This enzyme catalyses the transfer of electrons from the substrate to peroxide, generating a coloured product (Kemeny and Chantler, 1988). HRPO and its chromogens are economical and offer a good combination of sensitivity and speed of reaction. The choice of HRPO substrate used depends on the sensitivity required for the assay (Kemeny and Chantler, 1988). ABTS generates a blue-green product, which is measured at 405 nm, and

is suitable for most routine ELISAs. For more sensitive assays, TMB is a more suitable substrate. TMB generates an initial blue reaction product that is converted into a yellow product (which is monitored at 450 nm) by addition of the acidic stop reagent (Josephy *et al.*, 1982). TMB is more quickly oxidised than other HRPO substrates, so colour development is faster. The increased sensitivity of this method can, however, lead to increased background, so the plate must be thoroughly washed between incubations.

2.10.1 Materials

50 mM carbonate coating buffer. $NaHCO_3$ (0.21 g) was dissolved in 45 ml of H_2O, titrated to pH 6.0 with HCl and made up to 50 ml with H_2O just before use.

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in 950 ml of H_2O and made up to 1 l with H_2O.

0.5% (m/v) Bovine serum albumin in PBS (BSA-PBS). BSA (0.5 g) was dissolved in 90 ml of PBS and made up to 100 ml with PBS.

0.1% (v/v) Tween-20. Tween-20 (1 ml) was dissolved in 1 l of PBS.

0.15 M Citrate-phosphate buffer, pH 5.0. An aqueous solution of citric acid (21 g/l) was titrated with a solution of $Na_2HPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

0.05% (m/v) ABTS and 0.0015% (m/v) H_2O_2 in 0.15 M citrate-phosphate buffer. ABTS (7.5 mg) was dissolved in 0.15 M citrate-phosphate buffer, pH 5.0 (15 ml) and H_2O_2 (7.5 µl) added just before use.

ABTS stopping buffer [0.15 M citrate-phosphate buffer containing 0.1% (m/v) NaN_3]. NaN_3 (0.1 g) was dissolved in 100 ml of 0.15 M citrate-phosphate buffer, pH 5.0.

TMB. One TMB tablet was dissolved in 1 ml of dimethylsulfoxide (DMSO) and diluted to 10 ml with 3 ml 0.15 M citrate-phosphate buffer (pH 5.0) and 6 ml H_2O. H_2O_2 (2 µl) was added just before use.

TMB stopping solution (2 M H_2SO_4). H_2SO_4 (19.6 g) was diluted to 100 ml with H_20.

2.10.2 Method

Plates were washed three times with 0.1% (v/v) Tween-20 between each stage of all ELISAs. The volume of reagents given applied unless otherwise stated. Test antigens (150 µl/well) were coated to the wells of multititre ELISA plates in carbonate buffer (16 h, 4°C) and remaining sites in the wells blocked with BSA-PBS (200 µl/well, 1 h, 37°C). Test primary antibodies (commonly titrated between 200 and 1 µg/ml in BSA-PBS, 100 µl/well) were incubated in the wells (2 h, 37°C), followed by anti-species-HRPO secondary antibodies (120 µl/well in BSA-PBS, 1 h, 37°C) and ABTS/H_2O_2 or TMB/H_2O_2 substrate (150 µl/well). Once colour had adequately developed, ABTS or TMB stop solution (50 µl/well) was added and the absorbance at 405 nm or 450 nm respectively read on an EL 312 Bio-Kinetics Reader (Bio-Tek Instruments).

2.11 Western Blotting onto nitrocellulose membranes

Western blotting is a method devised to allow identification of electrophoretically separated bands using specific antibodies. To facilitate binding of antibodies to complementary antigens, the separated protein bands are transferred by an electric current from the SDS-PAGE gel in which they were separated to a supporting nitrocellulose membrane. The gel and nitrocellulose are placed adjacent to one another to form a "sandwich" which is arranged in the blotting apparatus so that the gel is placed on the cathode side and the nitrocellulose on the anode side. Negatively charged SDS-coated protein anions migrate from the negative cathode towards the positive anode. They migrate out of the polyacrylamide gel and bind to the adjacent nitrocellulose, maintaining the separation achieved in the SDS-PAGE step. Remaining unoccupied sites on the nitrocellulose are blocked with a protein solution to prevent non-specific adherence of antibodies (which are proteins themselves) to the nitrocellulose. Diagnostic primary antibodies are incubated with the blot and bind to any complementary antigens present on

the nitrocellulose. An enzyme-linked secondary antibody (also called the reporter antibody) is incubated with the blot to detect the bound primary antibodies, after which an appropriate substrate is used to visualise the bound secondary antibodies.

Numerous stages of the procedure are open to optimisation. The composition of the blocking agent can be varied to achieve low levels of non-specific background colour. Gelatin, low fat milk powder and BSA are commonly used. The composition of the blotting buffer can be varied to maximise protein mobility or adherence to the nitrocellulose (Spinola and Gannon, 1985). The Towbin buffer (Towbin *et al.*, 1979) described below was routinely used in the present study, but the renaturing buffer described by Dunn (1986) was occasionally used (e.g. Section 6.6). HRPO and alkaline phosphatase (AP) detection systems were commonly used. HRPO conjugates and substrates (e.g. 4-chloro-1-naphthol) are economical, but are less sensitive and are susceptible to fading upon exposure to light. AP systems (e.g. BCIP/NBT) are more sensitive and the precipitate does not fade.

2.11.1 Materials

Towbin blotting buffer [25 mM Tris, 192 mM glycine, 20% (v/v) methanol, 0.1% (m/v) SDS, pH 8.3]. Tris (9.08 g) and glycine (43.2 g) were dissolved in 2 l of H_2O. Methanol (600 ml) and SDS (3 ml of a 10% solution) were added and the solution was made up to 3 l with H_2O. The pH of this buffer should not be adjusted, since this increases buffer conductivity, resulting in a higher initial current and decreased initial resistance. The pH will vary between pH 8.1 and 8.5 depending on the quality of the reagents. This buffer should only be used once, since it loses its ability to maintain a stable pH during transfer. SDS and methanol increase protein mobility, but may also reduce protein binding to nitrocellulose.

Tris-buffered saline (TBS, 25 mM Tris-Cl, 200 mM NaCl, pH 7.4). Tris (2.42 g) and NaCl were dissolved in 950 ml of H_2O, adjusted to pH 7.4 with HCl and made up to 1 l with H_2O.

Ponceau S stain [0.1% (m/v)]. Ponceau S (0.1 g) was dissolved in 1% (v/v) glacial acetic acid in H_2O.

Blocking reagent I [0.5% (m/v) BSA-TBS]. BSA (0.5 g) was dissolved in 100 ml of TBS.

Blocking reagent II [5% (m/v) low-fat milk powder]. Low fat milk powder (5 g) was dissolved in TBS (90 ml) and made up to 100 ml with TBS.

4-Chloro-1-naphthol/H_2O_2. A stock solution of 0.3% (m/v) was prepared by dissolving 4-chloro-1-naphthol (0.03 g) in methanol (10 ml). This was diluted to a working solution by diluting 2 ml to 10 ml with TBS. H_2O_2 (10 µl) was added to the working solution.

BCIP/NBT. One BCIP/NBT tablet was dissolved in H_2O (10 ml).

500 mM NaOH. NaOH (20 g) was dissolved in 900 ml of H_2O and the solution made up to 1 l with H_2O.

2.11.2 Method

Gloves were worn throughout to prevent development of keratin bands on the blot (Ochs, 1983; Shapiro, 1987). Nitrocellulose was cut to the approximate size of the gel and pre-soaked in blotting buffer for 15 min. After completion of electrophoresis, gels were removed from the electrophoresis apparatus and rinsed in blotting buffer. The Western blot "sandwich" apparatus was submerged in chilled blotting buffer. A fibre pad and three sheets of blotting paper were soaked in blotting buffer and placed on the anode side of the Western blot "sandwich" apparatus. Pre-soaked nitrocellulose was placed on top of the blotting paper, followed by the gel and three further sheets of wet blotting paper. The sandwich was closed and inserted into the blot apparatus (half filled with blotting buffer) with the gel facing towards the cathode side of the apparatus and the nitrocellulose towards the anode side of the apparatus. A stirrer bar was placed in the bottom of the apparatus to maintain uniform conductivity and temperature during blotting. The apparatus was attached to a cooling water bath to maintain a low temperature during blotting. Gels were either blotted for 2 h (200 mA, unlimiting volts) or overnight (30 V,

unlimiting current). Overnight blotting was empirically determined to be most successful for trypanopain transfer.

On completion of blotting, the sandwich was disassembled and the blot stained with Ponceau S (1 min) and washed in H_2O to reveal the molecular mass markers. These were gently marked with a blunt pencil and the blot cut into smaller sections if required. The blot was destained by drop-wise addition of 500 mM NaOH to the H_2O. Free sites on the membrane were blocked using block I or II (1 h) and washed in TBS (5 min, 3 times). Primary antibody in blocking reagent was applied (2h) and the blot washed in TBS (5 min, 3 times). Secondary antibody in blocking reagent was applied (1 h) and the blot washed in TBS (5 min, 3 times). Substrate was added, with 4-chloro-1-naphthol used for HRPO-linked secondary antibodies and BCIP/NBT used for AP linked secondary antibodies.

Chapter 3

Isolation of trypanopain from *T. b. brucei*

3.1 Introduction

To obtain sufficient amounts of pure trypanopain for kinetic characterisation, large numbers of *T. b. brucei* were required. This chapter describes established techniques for expansion of cryo-preserved parasites in suitable mammalian hosts. Additionally, trypanopain had not been purified to electrophoretic homogeneity at the outset of this study, so this chapter describes the modified cathepsin L assay used to monitor initial trypanopain isolations, and the subsequent development of the first reported isolation protocol for trypanopain.

3.2 Initial enzyme assay

At the outset of this study, no optimised assay for trypanopain activity was available. To conveniently monitor the activity of proteolytic enzymes *in vitro*, synthetic substrates containing di- or tri-peptides linked to chromogenic or fluorogenic leaving groups are commonly used. The chemistry of the chromophore or fluorophore must be such that it only emits a strong signal once cleaved from the peptide portion, so that the light or fluorescence emitted is a measure of the amount of substrate hydrolysis (Knight, 1995). The peptide portion is designed with the substrate specificity of the target enzyme in mind, thereby conferring selectivity for a particular enzyme or group of enzymes. The leaving group occupies the proteinase subsite immediately C-terminal of the scissile bond. These substrates are thus most effective for enzymes whose specificity is governed by the S subsites (notation of Schechter and Berger, 1967) which bind residues on the N-terminal side of the peptide cleavage site, rather than the S' subsites which bind residues on the C-terminal side of the scissile bond (Knight, 1995).

Para-nitroanilide (pNa)-based substrates are commonly used colourimetric proteinase substrates, while many fluorometric proteinase substrates are based on 7-amino-4-methylcoumarin (AMC). AMC is an aromatic amine that fluoresces at different wavelengths depending on whether it is linked to an amino acid or not (Zimmerman *et al.*, 1976). It is regarded as a "sensitive, safe and convenient leaving group" for proteinase substrates (Barrett and Kirschke, 1981). Carbobenzoxy (Z)-Phe-Arg-AMC is commonly used as a simple and

sensitive assay for mammalian cathepsin L (Barrett and Kirschke, 1981), cruzipain (Murta *et al.*, 1990; Lima *et al.*, 1992) and trypanopain-Tc (Mbawa *et al.*, 1992), so this substrate was chosen for initial monitoring of trypanopain activity.

3.2.1 Materials

Assay buffer [340 mM sodium acetate, 60 mM acetic acid, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, 4 mM dithiothreitol (DTT), pH 5.5]. Sodium acetate.$3H_20$ (23.13 g), glacial acetic acid (1.72 ml), $Na_2EDTA.2H_2O$ (1.49 g) and NaN_3 (0.1 g) were dissolved in 450 ml of H_2O, adjusted to pH 5.5 with NaOH and made up to 500 ml with H_2O. DTT (6 mg) was added to 10 ml of buffer just before use. After purification and characterisation of trypanopain, this assay buffer was modified to achieve increased sensitivity. The modified assay buffer is described in Section 4.8.6.

Stock substrate solution (1 mM Z-Phe-Arg-AMC). Z-Phe-Arg-AMC (1 mg) was dissolved in DMSO (1.5 ml) and stored at 4°C.

Working substrate solution (20 µM Z-Phe-Arg-AMC). Stock substrate solution (100 µl) was diluted to 5 ml with H_2O.

Brij-35 diluent [0.1% (m/v)]. Brij-35 (0.1 g) was dissolved in 90 ml of H_2O and made up to 100 ml with H_2O.

Stopping reagent (100 mM monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3). Monochloroacetate (9.45 g), sodium acetate.$3H_2O$ (4.08 g) and glacial acetic acid (4 ml) were dissolved in 950 ml of H_2O, titrated to pH 4.3 with NaOH and made up to 1 l with H_2O.

1 mM AMC standard. AMC (1.8 mg) was dissolved in DMSO (10 ml). The standard was used at 0.5 µM working solution by diluting the stock solution (5 µl) in a 1:1 mixture of the assay buffer and stopping reagent (10 ml).

3.2.2 Method for standard enzyme assay

For standard continuous assays, samples (containing approximately 1-5 ng trypanopain) were diluted with Brij-35 diluent (to 250 µl) and equilibrated to 37°C in a temperature controlled cell and pre-warmed assay buffer (500 µl) added. After 1 min activation in assay buffer, substrate (250 µl of working solution) was added and product fluorescence monitored on a Hitachi F-2000 spectrofluorimeter (excitation 370 nm, emission 460 nm). Standard stopped time assays were performed in the same manner, except that stop solution (1 ml) was added 10 minutes after substrate addition and the fluorescence measured.

3.2.3 Method for enzyme micro-assays

When numerous samples were to be assayed, a modification of the above assay was performed using a 7600 Microplate Fluorimeter (Cambridge Technology, Inc.). This method proved to be of particular use for assaying column eluates, since many samples could be assayed simultaneously. Micro-continuous assays were performed by diluting trypanopain (1-5 ng) with Brij-35 (to 25 µl) and activating the enzyme for 1 min in pre-warmed assay buffer (50 µl, 37°C). Substrate (25 µl) was added and the fluorescent product monitored (excitation 370 nm, emission 460 nm). Micro-stopped time assays were performed in the same manner, except that stop reagent (100 µl) was added 10 min after substrate addition. Trypanopain activity was calibrated by quantifying the moles of AMC released from the substrate using an AMC standard (50-250 pmol). A standard curve was prepared for each assay method, taking the different final volumes into account. Unless otherwise stated, trypanopain activity is expressed as picomoles of AMC produced per second.

3.3 Growth and harvesting of trypanosomes

Cryo-suspensions of field-isolated trypanosomes can be kept indefinitely by freezing the parasites in a cryo-protective solution in liquid nitrogen. Cryo-preserved parasite populations can be expanded when large numbers of parasites are required by injecting the cryo-suspension into suitable susceptible mammalian hosts such as rats. Parasites can be purified from the blood of the infected animals using Percoll gradients (Grab and Bwayo, 1982) and anion exchange chromatography on DEAE-cellulose (Lanham and Godfrey, 1970).

3.3.1 Materials

Phosphate saline glucose (PSG) (57 mM NaH_2PO_4, 57 mM Na_2HPO_4, 46 mM NaCl, 56 mM glucose, 0.1 mM hypoxanthine, pH 8.0). $NaH_2PO_4.2H_2O$ (8.89 g), Na_2HPO_4 (8.09 g), NaCl (2.68 g), glucose (10.08 g) and hypoxanthine (0.014 g) were dissolved in 950 ml of H_2O, adjusted to pH 8.0 with HCl and made up to 1 l with H_2O. Hypoxanthine has been found to help maintain the viability of parasites during the isolation procedure (Lonsdale-Eccles and Grab, 1987b).

Sodium citrate anticoagulant [3% (m/v)]. Trisodium citrate.$2H_2O$ (6 g) was dissolved in PSG (200 ml).

Percoll solution [0.25 M sucrose, 2% (w/v) glucose in Percoll, pH 7.4]. Sucrose (17.1 g) and glucose (4 g) were dissolved in 150 ml Percoll, adjusted to pH 7.4 with solid *N*-2-hydroxyethylpiperazine-N-2-ethane sulfonic acid (Hepes) and made up to 200 ml with Percoll.

Elution buffer [100 mM Na-acetate, 1 M NaCl, pH 5.0]. NaCl (58.44 g) was dissolved in 900 ml of H_2O and glacial acetic acid (5.7 ml) added. The pH of the solution was adjusted to pH 5.0 with NaOH and the volume made up to 1 l.

3.3.2 Method

Cryo-suspensions of *Trypanosoma brucei brucei* (clone IL-Tat 1.1) were thawed from liquid nitrogen and diluted as required in PSG. The viability of the parasites was microscopically confirmed and the parasites injected intraperitoneally into adult Wistar rats. For *T. b. brucei*, an inoculum of about 10^6 parasites per rat ensured the development of high parasitaemia in less than 1 week. Blood parasitaemia was monitored daily by microscopic examination (at 400× magnification) of blood taken from the tail vein and when high parasitaemia was evident, rats were sacrificed using ether and the infected blood collected into anticoagulant by cardiac puncture.

Parasites were purified from blood components by a combination of isopycnic density centrifugation on a Percoll gradient (Grab and Bwayo, 1982) and anion exchange

chromatography on DEAE-cellulose (Lanham and Godfrey, 1970). Blood was mixed 1:1 with Percoll and centrifuged (30 000×*g*, 30 min, 4°C). The opaque trypanosome layer was carefully aspirated, washed once with 3 volumes of cold PSG (3 000×*g*, 20 min, 4°C) and the trypanosome-containing pellet re-suspended in a minimum volume of PSG. Parasites were applied to DEAE-cellulose pre-equilibrated in a sintered glass funnel with cold PSG. The pH of the sample and the gel (pH 8.0) was found to be critical for effective separation, so solid Tris was added to the sample at this stage to re-establish the pH at 8.0, if necessary. A 4 to 5-fold excess of gel over blood ensured good separation, with trypanosomes passing through the gel unhindered and erythrocytes and other blood components binding to the gel (Lanham and Godfrey, 1970). The eluted parasites were concentrated by centrifugation (1 000×*g*, 10 min, 4°C) and re-suspended in a small volume of PSG. Purified parasites were counted using a Neubauer haemocytometer and frozen at -70°C until required. Material bound to the DEAE-cellulose was eluted by washing the gel with 5 column volumes of PSG containing 1 M NaCl, followed by 5 column volumes of elution buffer. Regenerated resin was stored in 1% (m/v) thiomersal in PSG (with glucose omitted) between purifications. Cryo-suspensions of *T. b. brucei* were prepared by mixing infected blood 1:1 with PSG containing 20% (m/v) glycerol, and freezing the solution slowly in a polystrene container submerged in liquid nitrogen.

3.4 Determination of active enzyme concentration by E-64 titration

The concentration of active enzyme in a preparation can be determined by titration with an irreversible enzyme inhibitor that reacts only with active enzyme molecules in an equimolar manner. Since such an inhibitor binds covalently to the enzyme, the concentration of active enzyme present can be calculated from the minimum concentration of inhibitor required for full inhibition of the enzyme. L-*trans*-epoxysuccinyl-leucylamido (4-guanidino)butane (also known as E-64) is an irreversible inhibitor of most thiol proteinases and binds covalently to the active site cysteine residue of susceptible enzymes (Barrett *et al.*, 1982). Members of the papain superfamily of cysteine proteinases are particularly susceptible to E-64, facilitating the use of this reagent as an active site titrant for this family of enzymes. The reagent does not react with free reducing agents, non-active site cysteine residues or other classes of proteinases (Barrett *et al.*, 1982).

E-64 was first isolated by Hanada *et al.* (1978a) from a solid culture of *Aspergillus japonicus*, but various analogues of E-64 (e.g. E-64c, E-64d, Ep-475, Ep-453 and Ep-459) containing the active L-*trans*-epoxysuccinic acid moiety have since been synthesised and characterised (Hanada *et al.*, 1983). E-64c is of particular interest because it penetrates cells more readily than the parent molecule (Mason and Wilcox, 1992). The exact mechanism of E-64 inactivation of cysteine proteinases is not understood, but the carboxylate groups of E-64 are thought to position and activate the epoxide of E-64, causing it to alkylate the active site cysteine residue (Figure 8) (Hanada *et al.*, 1978b; Meara and Rich, 1996). The E-64 leucyl-isobutyl side chain may bind to the S_1' or S_2' enzyme subsite (Barrett *et al.*, 1982; Rich, 1986, nomenclature of Schechter and Berger, 1967).

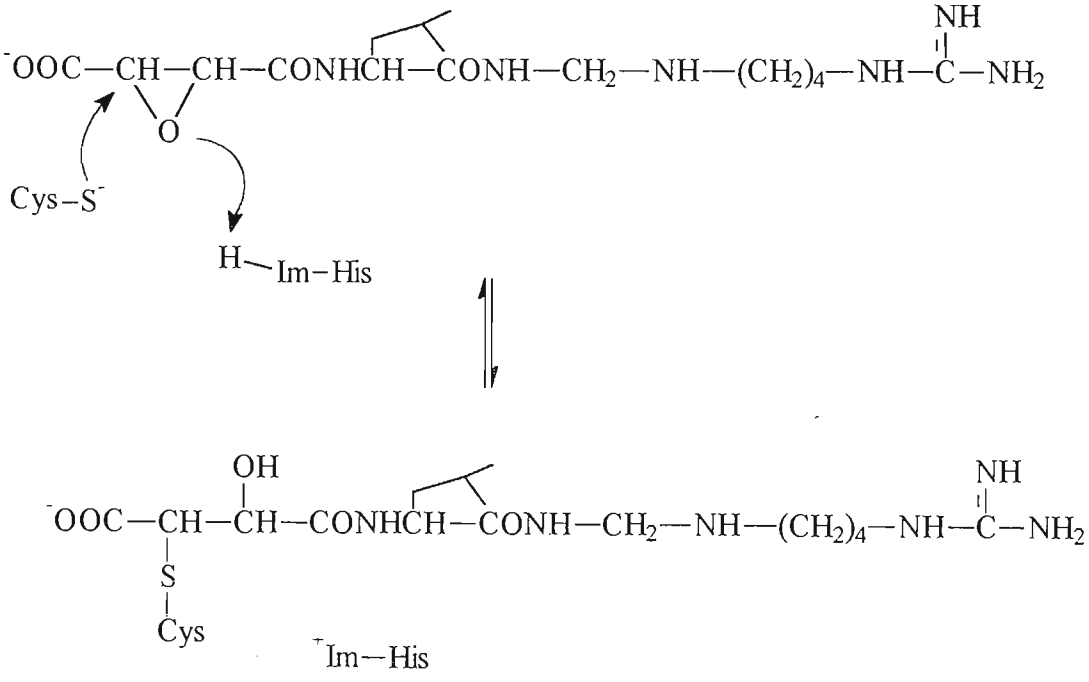


Figure 8: Proposed mechanism for E-64 inactivation of cysteine proteinases.

Rich (1986) proposes that the thiol of the active site cysteine residue (Cys-S^-) of the target proteinase attacks the C-3 carbon of the epoxysuccinyl group of E-64, allowing protonation of the E-64 epoxide oxygen by the active site histidine (H-Im-His).

3.4.1 Materials

As per Section 3.2.1.

Stock E-64 (10 mM). E-64 (3.8 mg) was dissolved in DMSO (100 µl) and diluted to 1 ml with H_2O. This stock solution was diluted to a 3 µM working solution with H_2O when required.

3.4.2 Method

Trypanopain (2-10 ng, 25 µl) was incubated with E-64 (25 µl of a 1-10 µM solution) for 30 min at 30°C in assay buffer (50 µl). After 30 min, the mixture was diluted to 250 µl with Brij-35 diluent and assayed in a stopped-time assay as described in Section 3.2.3 to determine residual enzyme activity. Linear regression was performed on a plot of trypanopain activity *vs* E-64 concentration. The minimum concentration of E-64 that completely inactivated all trypanopain activity was taken as the active concentration of the enzyme.

3.5 Rate of inactivation with E-64

Barrett *et al.* (1982) suggested that full inhibition of a target proteinase by E-64 occurs in 30 min. The rate of trypanopain inactivation by E-64 was determined to confirm that this 30 min incubation period was sufficient for full trypanopain inactivation and thus sufficient for accurate enzyme titration.

3.5.1 Materials

As per Section 3.4.1.

3.5.2 Method

A molar excess of E-64 (10 µM, 30 µl) was incubated with trypanopain (150 nM, 30 µl) in assay buffer (60 µl) for various lengths of time before the enzyme was assayed against Z-Phe-Arg-AMC in stopped-time assays as described in Section 3.2.2. Dilution into the substrate and assay buffer is sufficient to halt association of trypanopain and E-64 (Salvesen and Nagase, 1989).

3.6 Three-phase partitioning as an initial purification method

No purification of trypanopain to electrophoretic homogeneity had been reported at the outset of the present study, so a new purification method was devised. Three-phase partitioning (TPP) was chosen for initial purification of the enzyme. TPP is a relatively crude fractionation procedure in which *t*-butanol [30% (v/v) of total mixture volume] is mixed with the sample solution and various percentages [(m/v) of the total mixture volume] of ammonium sulfate added. *t*-Butanol is infinitely miscible with pure water, but becomes increasingly less miscible

when salt is added. Consequently, addition of ammonium sulfate to a *t*-butanol-protein mixture causes precipitation of proteins as a concentrated, dehydrated button between the salt and butanol layers (Pike and Dennison, 1989). Since different proteins precipitate out at different salt concentrations, incremental addition of ammonium sulfate allows for crude fractionation of protein mixtures. Compared with ammonium sulfate precipitation, greater purification may be obtained using TPP since lipids, pigments and other organic solvent-soluble materials remain in the butanol layer. The re-dissolved TPP pellet also contains low amounts of salt, enabling the resulting sample to be loaded onto ion exchange columns without prior desalting.

3.6.1 Materials

1% (m/v) Triton X-100. Triton X-100 (1 g) was diluted to 100 ml with H_2O.

Zero NaCl buffer [20 mM sodium acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (1.145 ml), $Na_2EDTA.2H_2O$ (0.37 g) and NaN_3 (0.2 g) were dissolved in 950 ml of deionised H_2O, adjusted to pH 5.5 with NaOH and made up to 1 l with deionised H_2O. The buffer was filtered through a 0.22 μm filter before use.

3.6.2 Method

Lysis of *T. b. brucei* (approx. 1×10^8 cells) was effected by addition of Triton X-100 to 0.1% (m/v) final concentration and the lysate diluted 10-fold in zero NaCl buffer. *t*-Butanol was added to 30% (v/v) of the total volume, i.e. 11 ml *t*-butanol added per 25 ml of sample. Increasing percentages of ammonium sulfate were added to optimise trypanopain isolation by sequential precipitation of trypanosomal proteins. The solution was centrifuged (10 000×*g*, 10 min, RT) after each round of ammonium sulfate addition and the protein precipitates re-dissolved in zero NaCl buffer before being assayed against Z-Phe-Arg-AMC in stopped-time assays (Section 3.2.3). The Z-Phe-Arg-AMC-hydrolysing activity from *T. b. brucei* was shown to precipitate between 10 and 25% (m/v of the total volume) ammonium sulfate.

For subsequent large scale purification of trypanopain, trypanosomes (approximately 5.5×10^9 parasites) were lysed as described above. *t*-Butanol was added to 30% (v/v) of the total volume, i.e. 11 ml *t*-butanol added per 25 ml of sample. Ammonium sulfate was added to 10% (m/v) (i.e. 3.6 g/25 ml sample), the mixture centrifuged (10 000×*g*, 10 min, RT) and the

membranous pellet discarded. Further ammonium sulfate was added to the supernatant to give a final concentration of 25% (m/v), i.e. 5.4 g/25 ml sample. The trypanopain-containing precipitate was collected by centrifugation (10 000×*g*, 10 min, RT) and re-suspended in a minimal volume of zero NaCl buffer. The final trypanopain-containing sample was centrifuged (15 000×*g*, 30 min, 4°C) to remove any insoluble material.

3.7 Q-Sepharose chromatography

Following the initial isolation with TPP, further trypanopain purification was effected using anion exchange chromatography on HiLoad™ quaternary amine (Q)-Sepharose® High Performance and Resource™ Q columns (both from Pharmacia, Sweden). The HiLoad™ Q-Sepharose® High Performance column was used in the initial chromatography step, since it combines excellent resolution, high flow rates (up to 100 cm/h) and a high protein-binding capacity (up to 30 mg of protein per ml of medium). This step was followed by chromatography on Resource™ Q, which is more suited for removal of trace contaminants. The small volume of this polyetheretherketone-based column (1 ml) allows for fine separation by enabling maintenance of a plateau in the salt gradient in response to elution of a protein.

3.7.1 Materials

All buffers were filtered through 0.22 μm filters before use to prevent clogging of the high performance columns. All columns were run on the GradiFrac™ Chromatography System (Pharmacia, Sweden), facilitating reproducible gradient formation.

Q-Sepharose equilibration buffer [20 mM sodium acetate, 1 mM Na_2EDTA, 100 mM NaCl, 0.02 % (m/v) NaN_3, pH 5.5]. Glacial acetic acid (1.145 ml), $Na_2EDTA.2H_2O$ (0.37 g), NaN_3 (0.2 g) and NaCl (5.844 g) were dissolved in 950 ml of deionised H_2O, adjusted to pH 5.5 with NaOH and made up to 1 l with deionised H_2O.

Q-Sepharose elution buffer [20 mM sodium acetate, 1 mM Na_2EDTA, 1 M NaCl, 0.02% (m/v) NaN_3, pH 5.5]. Glacial acetic acid (1.145 ml), $Na_2EDTA.2H_2O$ (0.37 g), NaN_3 (0.2 g) and NaCl (46.752 g) were dissolved in 950 ml of deionised H_2O, adjusted to pH 5.5 with NaOH and made up to 1 l with deionised H_2O.

Z-Arg-Arg-AMC stock solution (1 mM). Z-Arg-Arg-AMC (1.2 mg) was dissolved in 2 ml of DMSO.

Z-Arg-Arg-AMC working solution (20 µM). Stock Z-Arg-Arg-AMC (100 µl) was diluted to 5 ml with H_2O.

Oligopeptidase-Tb (OP-Tb) assay buffer [100 mM Tris-Cl, 10 mM DTT, pH 8.0]. Tris (12.11 g) was dissolved in 900 ml of H_2O and the pH adjusted to pH 8.0 with HCl. DTT (15.4 mg/10 ml) was added just before use.

3.7.2 Method

The trypanopain-containing sample from Section 3.6 was loaded onto HiLoad Q-Sepharose (dimensions 26×110 mm, flow rate 1 ml/min) pre-equilibrated in Q-Sepharose equilibration buffer. After elution of the unbound material, a gradient of 100 mM to 1 M NaCl in Q-Sepharose equilibration buffer was applied over 5 column volumes, followed by one column volume of Q-Sepharose elution buffer. The eluted fractions were assayed against Z-Phe-Arg-AMC and Z-Arg-Arg-AMC in stopped-time assays as described in Section 3.2.3. Fractions active against Z-Phe-Arg-AMC but not Z-Arg-Arg-AMC were identified as trypanopain, while fractions active against both substrates were identified as OP-Tb. OP-Tb fractions were pooled for further purification (Troeberg *et al.*, 1996).

Active trypanopain fractions were pooled, dialysed against zero NaCl buffer (2×45 min, 1 l each) and loaded onto Resource Q (dimensions 6.4×30 mm, flow rate 1 ml/min) equilibrated in Q-Sepharose equilibration buffer. After elution of the unbound material, a NaCl gradient (0.1-0.5 M in Q-Sepharose equilibration buffer) was applied over 15 column volumes (15 ml) with the salt concentration held constant during the elution of each protein peak to enhance separation. Active fractions (determined from stopped-time assays against Z-Phe-Arg-AMC as described in Section 3.2.3) were dialysed against three changes of zero NaCl buffer (500 ml each) overnight at 4°C to remove salt and against PEG (Section 2.3) to concentrate the sample.

3.8 Pepstatin A chromatography

The Q-columns used in Section 3.7 were highly efficient, allowing purification of trypanopain in three short steps. However, the Resource Q column proved to have a short life span, clogging irreparably after three or four runs despite efforts to prevent this (i.e. filtration of buffers and samples through 0.22 µm filters, and the manufacturer's recommended cleaning procedures). At R2 000 per column, this highly effective purification step was not cost-ineffective, necessitating the search for an alternative chromatographic purification strategy. Chicken egg white cystatin affinity chromatography proved ineffective, since trypanopain failed to elute from the column in a variety of elution buffers. This is presumably because trypanopain binds too tightly to the immobilised inhibitor. Since pepstatin A was known to be a weak trypanopain inhibitor (Pamer *et al.*, 1989), pepstatin A-Sepharose was tested as an affinity matrix.

3.8.1 Materials

Pepstatin A-Sepharose. This resin was a gift from Dr Philip Fortgens, Department of Biochemistry, University of Natal, Pietermaritzburg (UNP).

Pepstatin A-Sepharose equilibration buffer [20 mM sodium acetate, 1 mM Na_2EDTA, 500 mM NaCl, 0.02 % (m/v) NaN_3, pH 5.5]. Glacial acetic acid (1.145 ml), $Na_2EDTA.2H_2O$ (0.37 g), NaN_3 (0.2 g) and NaCl (29.22 g) were dissolved in 950 ml of deionised H_2O, adjusted to pH 5.5 with NaOH and made up to 1 l with deionised H_2O.

Pepstatin A-Sepharose elution buffer [20 mM sodium acetate, 1 mM Na_2EDTA, 500 mM NaCl, 5% (m/v) Brij-35, 0.02 % (m/v) NaN_3, pH 5.5]. Glacial acetic acid (1.145 ml), $Na_2EDTA.2H_2O$ (0.37 g), NaN_3 (0.2 g), Brij-35 (50 g) and NaCl (29.22 g) were dissolved in 950 ml of deionised H_2O, adjusted to pH 5.5 with NaOH and made up to 1 l with deionised H_2O.

HiTrap™ Blue Sepharose equilibration buffer [50 mM Tris-Cl, 0.02 % (m/v) NaN_3, pH 8.0]. Tris (6.055 g) and NaN_3 (0.2 g) were dissolved in 900 ml of water, adjusted to pH 8.0 with HCl and made up to 1 l with H_2O.

HiTrap™ Blue Sepharose elution buffer [50 mM Tris-Cl, 1 M NaCl, 0.02 % (m/v) NaN_3, pH 8.0]. Tris (6.055 g), NaCl (58.44 g) and NaN_3 (0.2 g) were dissolved in 900 ml of water, adjusted to pH 8.0 with HCl and made up to 1 l with H_2O.

3.8.2 Method

The trypanopain fraction eluted from HiLoad Q-Sepharose was loaded onto pepstatin A-Sepharose (dimensions 13×10 mm, flow rate 0.26 ml/min) pre-equilibrated in pepstatin A-Sepharose equilibration buffer. After elution of the unbound material, trypanopain bound to the column was eluted step-wise in pepstatin A-Sepharose elution buffer. Fractions active against Z-Phe-Arg-AMC (stopped-time assays conducted as described in Section 3.2.3) were pooled, dialysed against three changes of zero NaCl buffer (500 ml each) overnight at 4°C to remove salt and concentrated using PEG (Section 2.3). Trace contamination with albumin was occasionally observed after pepstatin A-Sepharose chromatography. This was removed by passing the sample over HiTrap Blue Sepharose (dimensions 9×23 mm, flow rate 1 ml/min) equilibrated in HiTrap Blue Sepharose equilibration buffer. Trypanopain was recovered in the unbound fraction, with bound albumin subsequently removed from the column with HiTrap Blue Sepharose elution buffer.

3.9 Production of anti-trypanopain antibodies

After the purification of trypanopain, anti-trypanopain antibodies were produced, primarily as tools for evaluation of trypanopain presence in various samples (Section 5.5). The effects of these antibodies on enzyme activity were also evaluated. Because of the extremely limited amount of purified enzyme available, only one experimental animal could be immunised. A chicken was chosen for this task because of the ease with which large quantities of IgY can be isolated from the yolk, where IgY is concentrated to provide passive immunity for the developing chick (Polson *et al.*, 1980).

Trypanopain (purified on Resource Q, Section 3.7.1) was triturated with adjuvant until a thick emulsion formed. A chicken was injected intramuscularly at 4 to 6 sites on either side of the sternum at weeks 0 (10 µg trypanopain), week 1 (10 µg), week 2 (5 µg) and week 6 (5 µg) and eggs collected from the start of the immunisation protocol. Freund's complete adjuvant was

used for the initial inoculation and Freund's incomplete adjuvant for subsequent inoculations (Freund and McDermott, 1942).

3.10 ELISA evaluation of anti-trypanopain antibody production

ELISAs were conducted to assess the progression of antibody production in the immunised chicken. Reagent volumes were reduced throughout the assay to minimise the amount of purified trypanopain required to complete the assay.

3.10.1 Materials

As per Section 2.10.1.

3.10.2 Method

Trypanopain (20 pmol/ml, in 50 µl carbonate coating buffer) was adsorbed to the wells of microtitre plates (16 h, 4°C) and remaining sites blocked with BSA-PBS (200 µl, 1 h, RT). Plates were washed in PBS-Tween (3 times) and test primary antibodies (50 µl, 1-200 µg/ml in BSA-PBS) incubated (2 h, 37°C). The plate was washed as before and incubated with rabbit anti-IgY-HRPO secondary antibodies (50 µl, 1 h, 37°C). After washing of the plate, ABTS/H_2O_2 substrate was added, colour development stopped and the A_{405} monitored as described in Section 2.10.2.

3.11 Evaluation of antibodies on a Western blot

The ability of the anti-trypanopain antibodies to recognise denatured trypanopain on a Western blot was investigated using lysed *T. b. brucei* samples.

3.11.1 Materials.

As per Sections 2.11.1.

3.11.2 Method

Proteins separated by tricine SDS-PAGE were electroblotted onto nitrocellulose at 30V for 16 h and blocked with block II (Section 2.11.2). The nitrocellulose was incubated with IgY anti-trypanopain antibodies in block II (200 µg/ml, 2 h), followed by rabbit anti-IgY-HRPO

secondary antibodies in block II (1/300 dilution, 45 min) and 4-chloro-1-naphthol/H_2O_2 substrate.

3.12 Results

3.12.1 Rate of inactivation with E-64

Trypanopain activity against Z-Phe-Arg-AMC decreased rapidly within the first minute of incubation with E-64 (Figure 9). After this, residual activity decreased slowly. The 30 min E-64 incubation period recommended by Barrett *et al.* (1982) was thus considered sufficient for accurate enzyme titration.

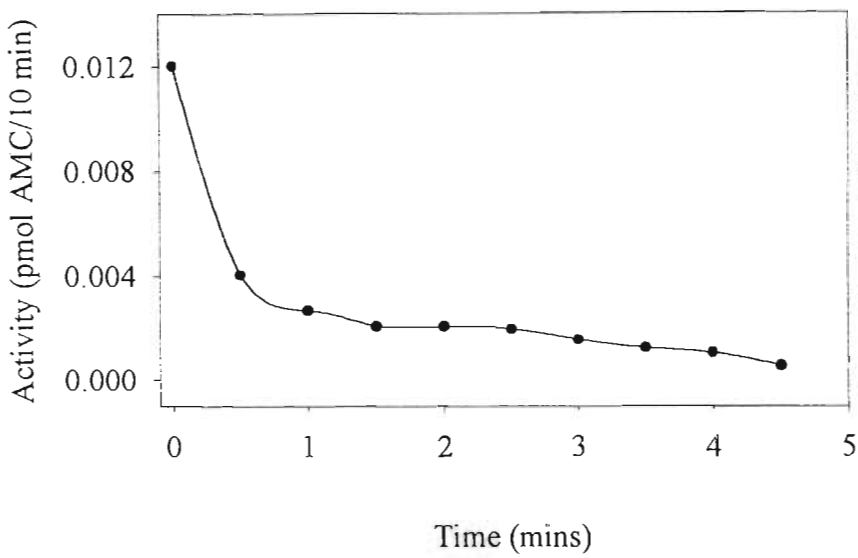


Figure 9: Rate of trypanopain inactivation by a molar excess of E-64.

Trypanopain (150 nM) was pre-incubated for various lengths of time in assay buffer with a molar excess of E-64 (10 μM). The residual enzyme concentration was determined from quintuplicate stopped-time assays against Z-Phe-Arg-AMC (5 μM) as described in Section 3.2.2.

3.12.2 Isolation of trypanopain

Optimisation of TPP indicated that the Z-Phe-Arg-AMC-hydrolysing activity of *T. b. brucei* precipitates between 10 and 25% (m/v) ammonium sulfate (Figure 10).

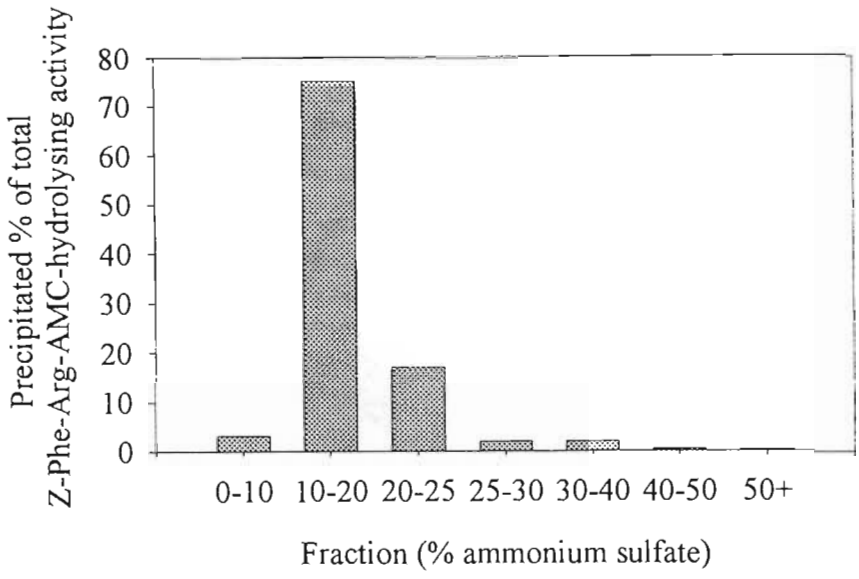


Figure 10: Optimisation of TPP for the isolation of trypanopain from *T. b. brucei*.

Increasing percentages of ammonium sulfate were sequentially added to triplicate samples of lysed *T. b. brucei* containing *t*-butanol added to 30% (v/v) of the total volume. After centrifugation (10 000×*g*, 10 min, RT), each protein precipitate was re-dissolved and assayed against Z-Phe-Arg-AMC in triplicate stopped-time assays (Section 3.2.3).

The 10-25% ammonium sulfate cut from TPP was loaded onto HiLoad Q-Sepharose and eluted in a 5 column volume salt gradient. All fractions were assayed against both Z-Phe-Arg-AMC (hydrolysed by trypanopain and the trypanosomal oligopeptidase OP-Tb) and Z-Arg-Arg-AMC (hydrolysed only by OP-Tb) to monitor the separation of these two enzymes. OP-Tb eluted in the unbound fraction, while trypanopain eluted in the bound fraction (Figure 11). This chromatographic procedure thus allowed separation of these enzymes early in the purification protocol, allowing purification of two enzymes of interest from one batch of starting material.

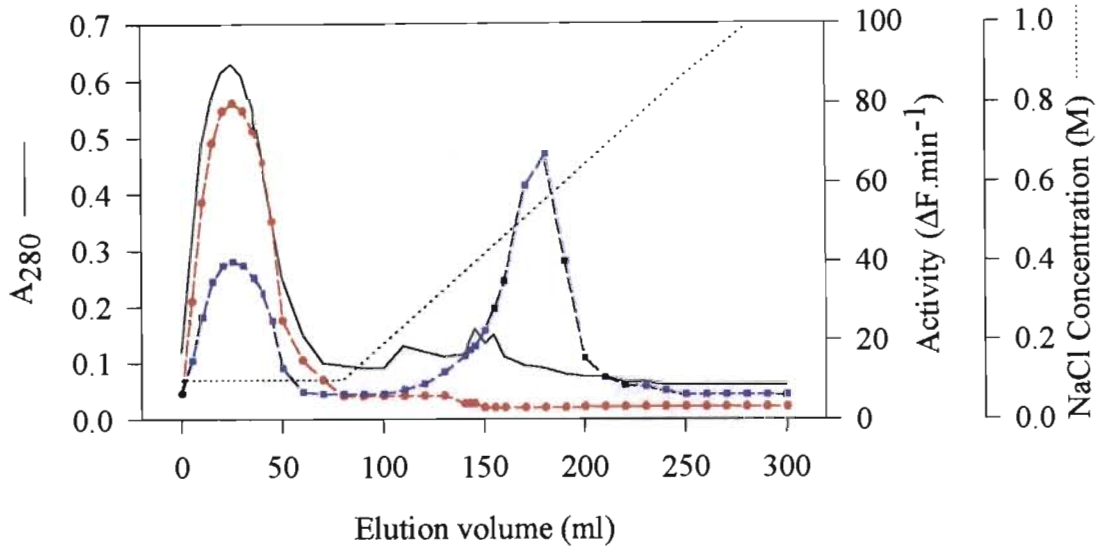


Figure 11: Chromatogram of HiLoad Q-Sepharose separation of trypanopain and OP-Tb from *T. b. brucei*.

The 10-25% $(NH_4)_2SO_4$ cut of TPP was loaded onto HiLoad Q-Sepharose (dimensions 26×110 mm, flow rate 1 ml/min) pre-equilibrated in Q-Sepharose equilibration buffer [100 mM NaCl in 20 mM Na-acetate, 1 mM Na_2EDTA, 100 mM NaCl, 0.02 % (m/v) NaN_3, pH 5.5] and the unbound material eluted. A NaCl gradient (0.1-1 M) was applied over 5 column volumes and the fractions (4 ml) assayed against Z-Phe-Arg-AMC (■) and Z-Arg-Arg-AMC (●) in duplicate stopped time assays as described in Section 3.2.3.

Active HiLoad Q-Sepharose fractions were dialysed and applied to a Resource Q column. The increased resolving power of the Resource Q column enabled separation of the HiLoad Q-Sepharose sample into 3 active peaks (Figure 12). Silver staining of these Resource Q peaks revealed that the middle active peak contained a single protein of 30 kDa. Gelatin-containing tricine SDS-PAGE showed that only the 30 kDa band contained in these peaks was proteolytically active (results not shown).

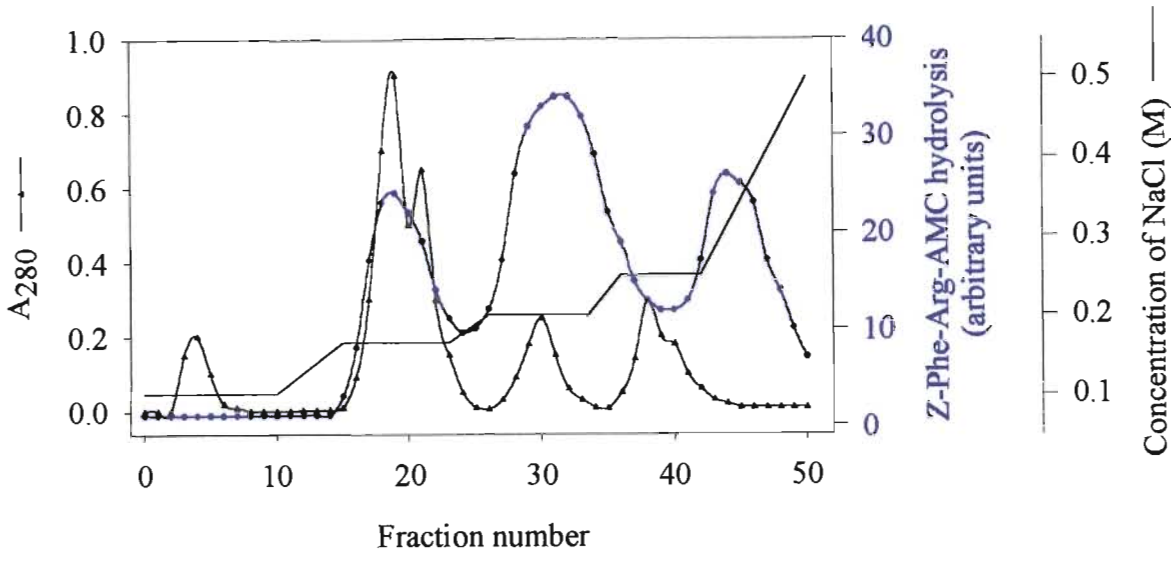


Figure 12: Resource Q chromatography of trypanopain.

Active fractions from HiLoad Q-Sepharose were loaded onto Resource Q (dimensions 6.4×30 mm, flow rate 1 ml/min) pre-equilibrated in Q-Sepharose equilibration buffer [20 mM Na-acetate, 1 mM Na_2EDTA, 100 mM NaCl, 0.02 % (m/v) NaN_3, pH 5.5]. After elution of the unbound material, a NaCl gradient (0.1-0.5 M) was applied, with the salt concentration held constant during the elution of each protein peak (at 0.17 M, 0.2 M and 0.25 M) to enhance separation. Active fractions (●) were determined from duplicate stopped-time assays as described in Section 3.2.3.

Trypanopain was thus effectively purified into three component parts by HiLoad and Resource Q chromatography. However, the crucial Resource Q column was not cost effective (Section 3.8). Pepstatin A-Sepharose chromatography thus replaced this step in later trypanopain purifications. A single peak of Z-Phe-Arg-AMC activity eluted from the pepstatin A-Sepharose column. The elution of trypanopain could not be monitored using the A_{280} trace, since the Brij-35 in the elution buffer absorbs strongly at 280 nm and obscured the increase in A_{280} occurring with elution of the enzyme (Figure 13). Trace albumin contamination was occasionally observed after this step. This was easily removed by passing the sample over HiTrap Blue Sepharose. SDS-PAGE showed that trypanopain was pure after HiTrap Blue Sepharose chromatography (Figure 14).

Trypanopain was found to be most stable if stored at –20°C in 50% glycerol. However, even when stored in this manner, the enzyme lost activity over time. Irrespective of the purification protocol, non-reduced, boiled trypanopain had an apparent molecular mass of 30 kDa, in agreement with previous reports (Mottram *et al.*, 1989). However, boiled, reduced enzyme had an apparent mass of 35-39 kDa (Figure 14). It was noted that upon storage, the apparent molecular mass of trypanopain also increased in this manner, with the increase correlating with loss of activity and age of the preparation. For example, trypanopain purified on Resource Q initially had an apparent molecular mass of 30 kDa, but when inactive enzyme was electrophoresed some months later, this same sample of purified enzyme had an apparent molecular mass of 39 kDa (Figure 15).

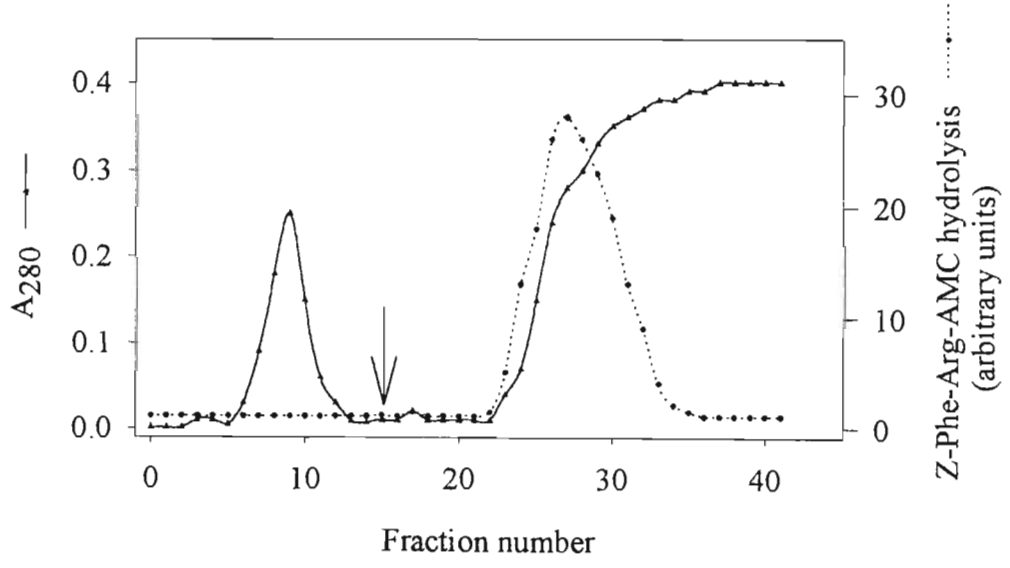


Figure 13: Pepstatin A-Sepharose chromatography of trypanopain.

The bound trypanopain fraction from HiLoad Q-Sepharose was loaded onto pepstatin A-Sepharose (dimensions 13×10 mm, flow rate 0.26 ml/min) equilibrated in pepstatin A-Sepharose equilibration buffer [20 mM Na-acetate, 1 mM Na_2EDTA, 500 mM NaCl, 0.02 % (m/v) NaN_3, pH 5.5]. After elution of the unbound material, trypanopain bound to the column was eluted in pepstatin A-Sepharose elution buffer [equilibration buffer plus 5% (m/v) Brij-35, applied from the position of the arrow]. Active fractions were identified from duplicate assays against Z-Phe-Arg-AMC performed as in Section 3.2.3.

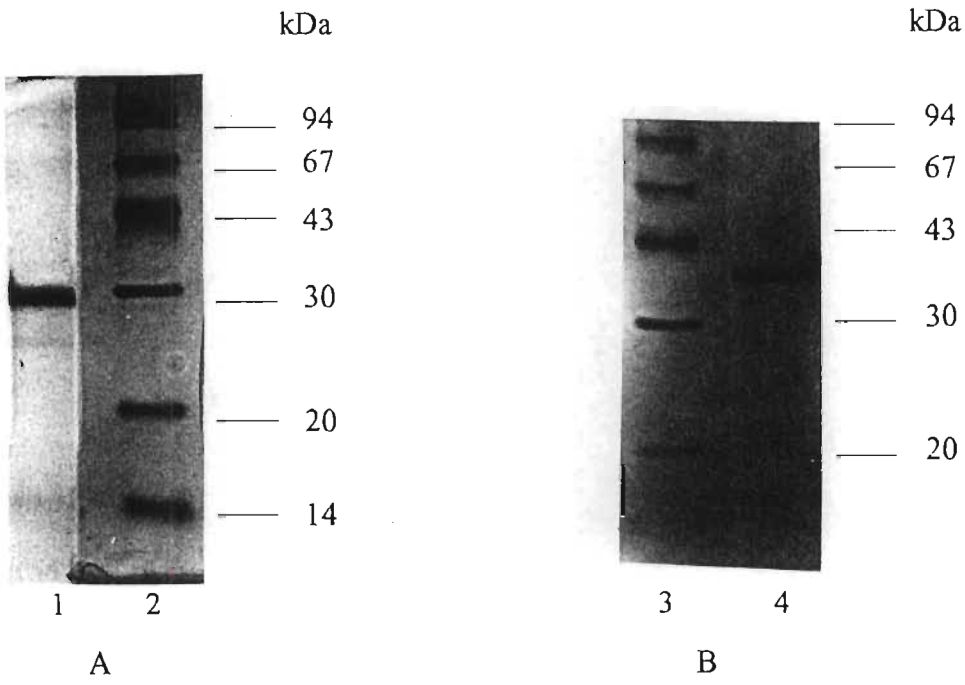


Figure 14: SDS-PAGE of trypanopain after pepstatin A-Sepharose chromatography.

A: Trypanopain (300 ng, 30 kDa, lane 1) was mixed with an equal volume of non-reducing treatment buffer and boiled for 2 min prior to electrophoresis and silver stained after electrophoresis. Pharmacia molecular mass markers (Section 2.4) are shown in lane 2.

B: Trypanopain (300 ng, 39 kDa, lane 3) was boiled in an equal volume of reducing treatment buffer for 2 min prior to electrophoresis and silver stained after electrophoresis. Pharmacia molecular mass markers (Section 2.4) are shown in lane 4.

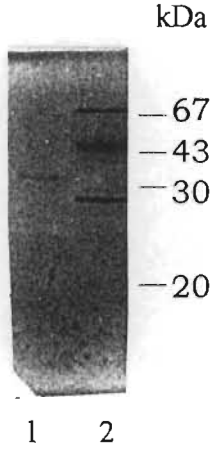


Figure 15: Tricine SDS-PAGE of inactive trypanopain after Resource Q chromatography.

Inactive trypanopain (300 ng, 35 kDa, lane 1) was mixed with an equal volume of non-reducing treatment buffer and boiled for 2 min prior to tricine SDS-PAGE and silver stained after electrophoresis. Pharmacia molecular mass markers (Section 2.4) are shown in lane 2.

Gelatin zymogram analysis of trypanopain purified by either Resource Q or pepstatin A-Sepharose (Figure 16) chromatography also showed that trypanopain was the only proteinase present in purified samples, with a molecular mass of approximately 30 kDa. Many bands of activity were visible in lysed *T. b. brucei*, with molecular masses ranging from approximately 30 kDa to above 94 kDa. These may either represent many different proteinases or, as suggested by Lonsdale-Eccles *et al.* (1995), they may show various activated forms of trypanopain.

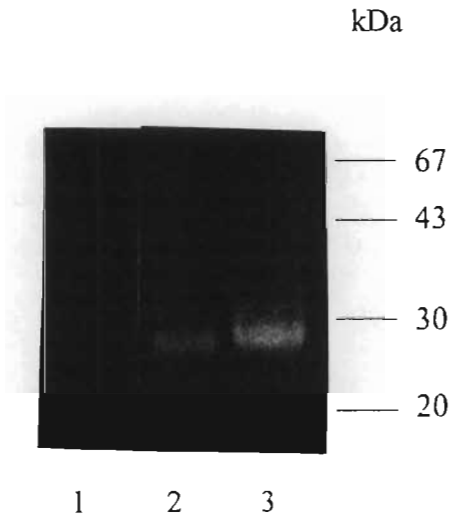


Figure 16: Gelatin-containing tricine SDS-PAGE of crude and purified trypanopain.

Tricine SDS-PAGE gels containing gelatin were prepared and electrophoresed as in Section 2.5. After electrophoresis, the gels were soaked in Triton X-100 to remove SDS and re-nature any proteinases present. Gels were incubated in assay buffer (3 h, 37°C, 100 mM Na-acetate, 1 mM Na_2EDTA, 30 mM cysteine, 0.02% (m/v) NaN_3, pH 5.5) to allow digestion of the incorporated substrate and stained as in Section 2.5. Lane 1 shows Pharmacia molecular mass markers (Section 2.4). Lane 2 shows gelatin digestion by pepstatin A-Sepharose-purified trypanopain (30 kDa) and lane 3, digestion by lysed *T. b. brucei*.

Table 3 compares the two methods used for trypanopain purification. OP-Tb readily cleaves Z-Phe-Arg-AMC (K_m=0.73 μM; Kornblatt *et al.*, 1992; Troeberg *et al.*, 1996) and E-64 titration showed that OP-Tb accounts for 50% of the Z-Phe-Arg-AMC-hydrolysing activity in samples taken from lysed *T. b. brucei* and following TPP (Troeberg *et al.*, 1996). Values given for trypanopain activity in lysed cells and after TPP are thus half of the total Z-Phe-Arg-AMC-hydrolysing activity present in these samples. OP-Tb and trypanopain are separated on HiLoad Q-Sepharose, so trypanopain activity values given for this and subsequent steps are calculated

from the total Z-Phe-Arg-AMC-hydrolysing activity present in these samples. Table 3 shows that the two purification methods were comparable in terms of yield and purification.

Table 3: Comparison of purification of trypanopain from *T. b. brucei* using Resource Q and pepstatin A-Sepharose chromatography.

Purification with Resource Q					
Fraction	*Total protein (mg)*	*Total activity (pmol.s^{-1})*	*Specific activity (pmol.s^{-1}.mg^{-1})*	*Purification (fold)*	*Yield (%)*
Lysed cells	210	2 400	11	1	100
TPP	7	850	121	11	35
HiLoad Q-Sepharose	0.24	405	1 688	153	17
Resource Q	0.01	200	20 000	1 818	8
Purification with pepstatin A-Sepharose					
Fraction	*Total protein (mg)*	*Total activity (pmol.s^{-1})*	*Specific activity (pmol.s^{-1}.mg^{-1})*	*Purification (fold)*	*Yield (%)*
Lysed cells	213	2281	11	1	100
TPP	9	572	64	6	25
HiLoad Q-Sepharose	0.2	397	1 985	180	17
Pepstatin A-Sepharose	0.04	252	6 300	573	11
HiTrap Blue Sepharose	0.01	245	24 500	2 227	11

3.12.3 ELISA evaluation of anti-trypanopain antibodies

High concentrations of anti-trypanopain IgY antibodies were produced in the immunised chicken against very low levels of immunogen (30 µg total over 4 weeks), showing that the enzyme is highly immunogenic in chickens. The highest titre antibodies were produced between weeks 4 and 6 (Figure 17).

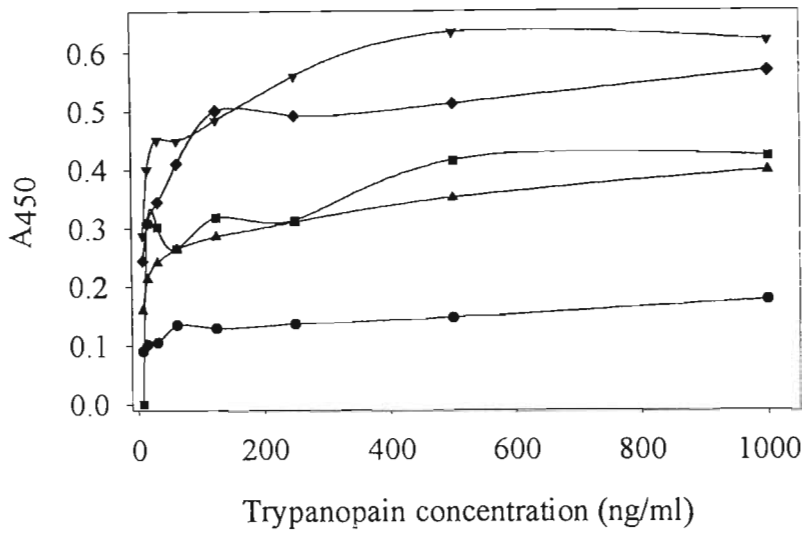


Figure 17: ELISA monitoring antibody titre in a chicken immunised with trypanopain. Trypanopain was coated onto ELISA plates (20 pmol/ml in Na-carbonate buffer, 50 µl/well, 16 h, 4°C) and test primary chicken antibodies titrated between 200 and 1 µg/ml (in BSA-PBS, 50 µl/well, 2 h, 37°C). Rabbit anti-chicken-HRPO secondary antibodies (50 µl/well in BSA-PBS, 1 h, 37°C) and ABTS/H_2O_2 substrate (100 µl/well) were used as the detection system. Absorbance readings at 405 nm of non-immune antibodies (●) and antibodies from week 3 (▲), week 4 (■), week 5 (♦) and week 6 (▼) represent the average of duplicate experiments.

3.12.4 Western blot evaluation of anti-trypanopain antibodies

Anti-whole enzyme antibodies recognised both purified trypanopain and enzyme present in lysed cells (Figure 18). As expected, only one band was targeted in purified trypanopain samples. In lysed cells, however, the antibodies targeted 3 bands, at 30, 35 and 38 kDa. Pamer *et al.* (1991) have previously reported this pattern of recognition by anti-trypanopain antibodies for *T. b. rhodesiense* and suggested that the additional bands are processing intermediates of the enzyme.

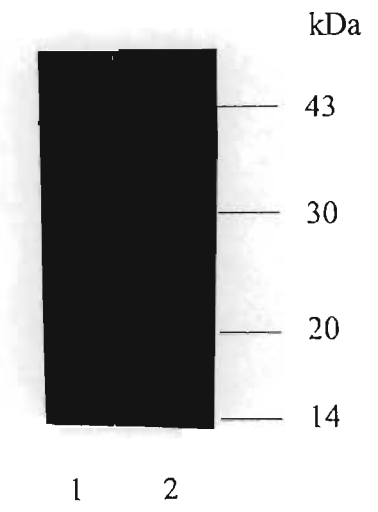


Figure 18: Western blots showing recognition of trypanopain by chicken anti-whole trypanopain IgY antibodies.

The tricine SDS-PAGE gel was electroblotted onto nitrocellulose (30 V, 16 h) and blocked with 5% (m/v) low-fat milk powder. The blot was incubated with chicken anti-trypanopain IgY (200 µg/ml, 2 h), followed by rabbit anti-IgY-HRPO secondary antibodies (1/300 dilution, 45 min) and 4-chloro-1-naphthol/H_2O_2 substrate. Lane 1 shows recognition of purified trypanopain (200 ng) and lane 2 of lysed *T. b. brucei* (containing approximately 200 ng trypanopain). No proteins were recognised by non-immune antibodies (results not shown).

3.13 Discussion

E-64 inhibited trypanopain fully within 4½ minutes, with most of the inhibition occurring in the first 30 seconds. The rate of association was too fast to enable calculation of the association rate constant, k_{ass}, without a stopped-flow apparatus. As expected, this shows that E-64 is a suitable titrant for trypanopain and that trypanopain is similar to cathepsins B, H and L and papain in this respect (Barrett *et al.*, 1982). While 10 min pre-incubation with E-64 would probably have been sufficient for full inactivation, all titrations were carried out over 30 min as suggested by Barrett *et al.* (1982) to allow for direct comparison with the reported method and to compensate for the lower concentrations of E-64 routinely used to titrate trypanopain.

At the outset of this study, trypanopain had not previously been purified to electrophoretic homogeneity. Previous studies on this enzyme have used either partially purified enzyme (Huet *et al.*, 1992) or lysed *T. b. brucei* (Boutignon *et al.*, 1990; Robertson *et al.*, 1990). However, purification of trypanopain-Tc and cruzipain is well documented. Ammonium sulfate

precipitation is commonly used for crude fractionation of these enzymes (Murta *et al.*, 1990; Lima *et al.*, 1992), while affinity chromatography procedures have been extensively used for further purification, with ligands including *p*-aminophenylmercuric-acetate (Rangel *et al.*, 1981), thiopropyl groups (Rautenberg *et al.*, 1982; Mbawa *et al.*, 1992), cystatin (Mbawa *et al.*, 1991a) and monoclonal antibodies (Authié *et al.*, 1993). Anion exchange chromatography on Q-resins (Cazzulo *et al.*, 1989) and molecular exclusion chromatography (Rangel *et al.*, 1981; Rautenberg *et al.*, 1982; Mbawa *et al.*, 1992) have also been employed for purification. In the present study, trypanopain was purified from *T. b. brucei* by TPP between tertiary butanol and ammonium sulfate (Pike and Dennison, 1989), anion exchange chromatography on Q-resins and affinity chromatography on pepstatin A-Sepharose. No other group has reported the use of TPP or pepstatin A-Sepharose chromatography for isolation of trypanopains.

TPP was an effective crude isolation procedure, successfully removing large amounts of contaminating protein (97% of the starting material). This step was also useful in that the re-dissolved TPP pellet contained low amounts of salt, enabling the trypanopain sample to be loaded onto Q-Sepharose columns without prior desalting. While the concomitant loss of enzyme activity (25-35% yield) was also high, similar losses were recorded using ammonium sulfate precipitation (results not shown), indicating that this would not have been a better initial step. Furthermore, the yield after this step was highly variable (20-60%) for apparently identical isolations. *T. b. brucei* undergoes various biochemical changes during its transition from the short to stumpy bloodstream form, and it is likely that the relative numbers of each form differed from preparation to preparation, depending on the immune response of the host animal, the number of parasites inoculated, etc. Thus the biochemical composition of the starting material probably varied between isolations, possibly accounting for the variable yield.

The success of the purification reported here hinged on the fact that trypanopain adheres to quaternary amine resins at pH 5.5, while most other trypanosomal proteins do not. This implies either that the pI of trypanopain is below pH 5.5, or that a particular domain of the enzyme is strongly negatively charged below the pI. SDS-PAGE showed that only trace contaminants remained after the first chromatography step. Additionally, this step enabled separation of trypanopain from OP-Tb early in the purification protocol, allowing isolation of the two enzymes from the same starting material. This minimised the number of experimental animals killed and protected OP-Tb from digestion by trypanopain.

The increased resolving power of the Resource Q column over the HiLoad Q column enabled purification of trypanopain from remaining trace contaminants. The small volume of this column (1 ml) precluded its use as an initial chromatography step, but facilitated finer separation of trypanopain than was possible with the HiLoad column. This was done by maintaining constant salt concentrations in response to elution of a protein using the GradiFrac chromatography system. The single trypanopain peak from HiLoad Q-Sepharose was separated into three active peaks on Resource Q. Interestingly, cruzipain also separates into three active peaks on Mono Q columns (Cazzulo *et al.*, 1989). No such separation was seen when eluting pepstatin A-Sepharose with a Brij-35 gradient, presumably due to the lower resolving power of this column. In the present study, only the middle trypanopain peak from Resource Q proved to be pure, with the other two peaks shown by SDS-PAGE to contain various contaminants in addition to the predominant 30 kDa trypanopain band. Separation is thought to have occurred because of the high resolution of the Resource Q column and the non-linear gradient applied.

Trypanopain is encoded by many tandemly arranged genes (Mottram *et al.*, 1989), as are the major cysteine proteinases of *T. cruzi* (Campetella *et al.*, 1992; Eakin *et al.*, 1992) and *Leishmania* (Souza *et al.*, 1992; Mottram *et al.*, 1996). In the case of cruzipain, the different gene products vary slightly in their substrate specificity (Lima *et al.*, 1994), possibly indicating that the gene products fulfil slightly different roles in the organism. Preliminary kinetic analysis of the three Resource Q peaks obtained in the present study revealed no such variation in trypanopain specificity, but a more comprehensive search using more substrates may reveal some differences. While the variation between the enzyme populations from Resource Q is likely to be slight, their separation must reflect some underlying variability in trypanopain. The enzyme may be differentially glycosylated *in vivo* or the enzyme population may consist of three different gene products. Further analysis of the translation of trypanopain-encoding genes is required to unravel the cause and significance of this chromatographic separation, as well as to understand why trypanopain is encoded by multiple genes. However, since no variation in kinetic behaviour could be detected for the three Resource Q peaks, no distinction was made between the use of the middle Resource (pure) Resource Q fraction and trypanopain (eluting as a single fraction) from pepstatin A-Sepharose in future kinetic experiments.

Despite filtering all buffers and samples through 0.22 μm filters, two individual Resource Q columns became blocked after only three or four runs, so an alternative chromatographic step was sought. The high affinity with which cystatin B and chicken egg white cystatin inhibit trypanopain rendered these ligands unsuitable for affinity chromatography since trypanopain bound essentially irreversibly to these resins (results not shown). Chicken egg white cystatin has, however, been used as an effective ligand for one-step affinity chromatography purification of cruzipain (Labroila *et al.*, 1993) and trypanopain-Tc (Mbawa *et al.*, 1991a), indicating that trypanopain has a higher affinity for this inhibitor than other trypanopains. While pepstatin A is traditionally considered an inhibitor of aspartic proteinases, it has been shown to inhibit trypanopain from *T. b. rhodesiense* fairly weakly (Pamer *et al.*, 1989) and was thus considered a potentially suitable affinity chromatography ligand. Pepstatin A-Sepharose provided a highly effective chromatography step, removing all but a 68 kDa contaminant from the trypanopain preparation. Removal of this contaminant by HiTrap Blue Sepharose confirmed the suspicion that it was albumin. In preliminary studies, trypanopain was eluted from pepstatin A-Sepharose using a linear Brij-35 gradient of 0-10% (m/v). Since the single peak eluted in this manner contained either only pure enzyme or enzyme contaminated with a small amount of albumin, a step gradient of 5% Brij was considered time-effective and sufficient for trypanopain elution from pepstatin A-Sepharose. In addition to its lower cost, the pepstatin A-Sepharose purification method was slightly more effective than the Resource Q method. The former gave an 11% yield of enzyme, while the latter gave an 8% yield. While not particularly high, the final yield achieved compares favourably with isolation of other parasite cysteine proteinases (Cazzulo *et al.*, 1989; Smith *et al.*, 1993; Yong Song and Chappell, 1993).

It was repeatedly observed during the course of this study that successful purification of trypanopain was only possible if the entire purification was completed in 1 day. The high flow rates possible with the GradiFrac system were thus essential, and the step gradient elution from pepstatin A-Sepharose additionally helpful. Attempts to utilise molecular exclusion chromatography were unsuccessful because all trypanopain activity was lost over the protracted time required for elution. All enzyme activity was also lost on hydrophobic columns (butyl- and phenyl-Sepharose). Similar low enzyme stability has been reported for the cathepsin L-like cysteine proteinase of *F. hepatica* (Smith *et al.*, 1993).

Substrate SDS-PAGE (Lonsdale-Eccles and Grab, 1987a) and primary sequence analysis (Mottram *et al.*, 1989) suggest that trypanopain has a molecular mass of 28-31 kDa. In the present study, non-reduced, non-boiled trypanopain had an apparent molecular mass of 30 kDa, while the boiled, reduced enzyme had an apparent mass of 35-39 kDa (Figures 14 and 15). Cruzipain has also been shown to migrate differently on SDS-PAGE depending on reduction and boiling prior to electrophoresis (Martínez and Cazzulo, 1992a,b). Amino-acid sequence data suggests the true size of cruzipain to be 36.3 kDa (Campetella *et al.*, 1992), but reduction and boiling increase the apparent molecular mass from 33 to 55 kDa. Martínez and Cazzulo (1992a, b) suggested that N-glycosylation plays a minor role in this increase, since the enzyme contains only 3 potential N-glycosylation sites which should only account for a variation of about 5 kDa. They proposed that the increase in mass following reduction and boiling is due primarily to reduction of disulfide bridges in the enzyme and resultant altered binding of SDS. They noted that the apparent molecular mass of cruzipain in substrate SDS-PAGE gels is low (approximately 33 kDa) and suggest that this is a consequence of not boiling the enzyme rather than of substrate incorporation into the gel.

The results of the present study suggest that trypanopain exhibits similar electrophoretic behaviour to cruzipain. Again, since mature trypanopain also only has three potential N-glycosylation sites (Mottram *et al.*, 1989), the variation in mass is more likely to be a function of reduction of disulfide binds than of increased glycosylation. Interestingly, old, completely inactive preparations of trypanopain showed a similar increase in molecular mass to that seen with reduced samples, suggesting that these disulfide bonds are essential for trypanopain activity. This suggests that oxidation of disulfide bonds, rather than auto-digestion, is the primary cause of the loss of activity in stored trypanopain.

Only 30 µg of trypanopain over 4 injections was required to elicit high titre antibodies in a single immunised chicken. This is much less than is normally considered necessary to evoke an effective immune response. For example, Thorpe (1994) recommends that chickens be immunised with at least 30 µg of antigen per inoculation. The small amount of trypanopain available for immunisation prevented a statistically valid study of the immunisation protocol, but the result suggests that trypanopain is highly immunogenic in chickens, which is likely to be a function of the evolutionary distance between chickens and *T. b. brucei*. Far less immunogen was used in the present study than has been used by other researchers to produce antibodies

against related parasite cysteine proteinases (Table 4). This suggests that antibodies can be successfully produced using smaller amounts of immunogen than are traditionally considered necessary. This is of particular significance where the required immunogen is unstable in its purified form or starting material is limited, as is commonly the case with parasite enzymes.

Table 4: Comparison of immunisation protocols used to produce antibodies against various parasite cysteine proteinases.

Parasite species	**Total immunogen used** (μg)	**Immunisation period** (weeks)	**Experimental animal**
T. b. brucei [1]	30	6	chicken
S. mansoni [2]	500	4	rabbit
L. mexicana [3]	100	4	rabbit
T. congolense [4]	180	4	rabbit
T. cruzi [5]	200	9	rabbit
F. hepatica [6]	250	nr	rabbit
Ancylostoma caninum [7]	200	6	rabbit

nr Not reported.

References:

[1] Present study [2] Michel *et al.* (1995) [3] Ilg *et al.* (1994)

[4] Mbawa *et al.* (1991a) [5] Souto-Padron *et al.* (1990) [6] Smith *et al.* (1993)

[7] Harrop *et al.* (1995)

These antibodies produced against whole trypanopain recognised the enzyme in ELISAs and Western blots, paving the way for the use of these antibodies as tools for various studies. As previously reported for *T. b. rhodesiense*, (Pamer *et al.*, 1991), three bands were recognised by anti-trypanopain antibodies in lysed *T. b. brucei*. Pamer *et al.* (1991) suggest from analysis of the trypanopain gene coding region that these are likely to be processing intermediates of the enzyme. Pulse-chase studies are required to confirm this suggestion.

While the cDNA of trypanopain from *T. b. brucei* and *T. b. rhodesiense* has been sequenced (Mottram *et al.*, 1989; Pamer *et al.*, 1990), the enzyme has not previously been purified to

electrophoretic homogeneity and hence little is known about trypanopain's basic enzymatic properties. To this end, the pH profile for the hydrolysis of synthetic and protein substrates, the effect of various reducing agents, and the enzyme's specificity for synthetic and protein substrates were investigated and are reported in the following chapters.

Chapter 4

Enzymatic characterisation of trypanopain-Tb

4.1 Effect of pH on trypanopain activity against Z-Phe-Arg-AMC

When assessing the effect of pH on enzyme activity, it is important to maintain a constant ionic strength across the pH range investigated. Changes in ionic strength can affect ionisation of protein side chains and so, in turn, can affect enzyme activity independently of pH changes (Dehrmann *et al.* 1995; 1996). Maintenance of a constant ionic strength across the range of pH values tested ensures that changes in activity can be ascribed to changes in pH alone (Ellis and Morrison, 1982). For this study, an acetate-2(N-morpholino)ethanesulphonic acid (MES)-Tris (AMT) buffer system with an ionic strength of 0.1 was chosen, covering the pH range from 4.0 to 9.0 (Ellis and Morrison, 1982).

Trypanopain-Tc is optimally active against Z-Phe-Arg-AMC between pH 5.5 to 6.0 (Lonsdale-Eccles and Mpimbaza, 1986; Mbawa *et al.*, 1992) while cruzipain is optimally active against this substrate between pH 5.0 and 7.0 (Murta *et al.*, 1990). Studies on partially purified trypanopain from *T. b. brucei* indicated that the enzyme has a pH optimum against Z-Phe-Arg-AMC of between 6.0 and 8.0 (Huet *et al.*, 1992).

4.1.1 Materials

As per Section 3.2.1.

AMT buffers (100 mM Na-acetate, 200 mM Tris-Cl, 100 mM MES, 6 mM DTT and 4 mM Na_2EDTA). Glacial acetic acid (1.72 ml), MES (5.86 g), Tris (7.27 g) and $Na_2EDTA.2H_2O$ (0.37 g) were dissolved in H_2O (200 ml). This solution was divided into 10 aliquots (20 ml each) which were titrated to pH values in the range 4.0 to 9.0 using HCl or NaOH and each diluted to 25 ml with H_2O. DTT (6 mM, 1.4 mg/1.5 ml) was added just before use.

4.1.2 Method

Trypanopain (1.5 ng) hydrolysis of Z-Phe-Arg-AMC at 37°C was measured using stopped-time

assays as described in Section 3.2.3, except that assay buffer was replaced by AMT buffers in the pH range 4.0 to 9.0.

4.2 Effect of pH on activity against ^{14}C-gelatin

For a more accurate estimate of trypanopain's *in vivo* pH requirements, the optimal pH for digestion of a protein substrate by the enzyme was investigated. Zymogram analysis of lysed *T. b. brucei* suggests that trypanopain has a pH optimum of 5.0 to 6.0 for digestion of fibrinogen and collagen (Lonsdale-Eccles and Mpimbaza, 1986). Since only small amounts of purified trypanopain were available, a highly sensitive assay was required. Radio-labelled gelatin was found to be the most suitable of the potential substrates investigated, since preliminary studies showed that its sensitivity far surpassed that of other available methods e.g. azocasein (Barrett and Kirschke, 1981) and biotin- (Koritas and Atkinson, 1995) or fluorescein-labelled proteins (De Lumen and Tappel, 1970; Twining, 1984).

4.2.1 Materials

AMT buffers. As for Section 4.1.1

^{14}C-Gelatin (1 mg/ml) was a gift from Dr Theresa Coetzer, Department of Biochemistry, UNP. Briefly, ^{14}C-type IV collagen was prepared according to the method of Cawston and Barrett (1979) using [1-^{14}C] acetic anhydride, which reacts primarily with the ε-amino groups of lysine residues within the target protein. ^{14}C-type IV collagen was converted to ^{14}C-gelatin by incubation at 44°C for 2 h.

Stop solution [15% (m/v) trichloroacetic acid (TCA), 0.75% (m/v) tannic acid]. TCA (1.5 g) and tannic acid (0.075 g) were dissolved in 7 ml of H_2O and made up to 10 ml with H_2O.

Scintillation cocktail [40% (v/v) Lumax in toluene]. Lumax (400 ml) was mixed with toluene (600 ml).

4.2.2 Method

Trypanopain (6 ng) was diluted to 375 µl with 0.1% (m/v) Brij-35 and incubated with AMT buffers in the pH range 4.0 to 9.0 (375 µl, 10 min, 37°C). ^{14}C-Gelatin (100 µl) was added and

digestion allowed to proceed for 15 h at RT. Stop solution (100 µl) was added and the mixture maintained on ice for 30 min. Following centrifugation (10 000×*g*, 15 min, 25°C), the supernatant (500 µl) was mixed with scintillation cocktail (5 ml) and the counts per minute read in a Packard Tri-Carb Liquid Scintillation counter.

4.3 Effect of reducing agents on trypanopain activity against Z-Phe-Arg-AMC

Since cysteine proteinase activity is dependent on reduction of the active site cysteine residue, these enzymes are usually pre-incubated with reducing agents before activity is measured (Dunn, 1989a). The effects of various concentrations of the reducing agents DTT, cysteine, β-mercaptoethanol and reduced glutathione on trypanopain hydrolysis of Z-Phe-Arg-AMC were investigated to determine which is the most effective activator of trypanopain activity.

4.3.1 Materials

As for Section 3.2.1, except that various reducing agents were used in the assay buffer.

DTT (1 M). DTT (1.4 mg) was dissolved in H_2O (200 µl) on the day of use.

Cysteine (1 M). Cysteine.HCl (0.035 g) was dissolved in H_2O (200 µl) on the day of use.

β-mercaptoethanol (1 M). β-mercaptoethanol (14 µl) was diluted to 200 µl with H_2O on the day of use.

Reduced glutathione (1 M). Reduced glutathione (0.064 g) was dissolved in H_2O (200 µl) on the day of use.

4.3.2 Method

Trypanopain activity against Z-Phe-Arg-AMC was measured using stopped-time assays as described in Section 3.2.2, except that various reducing agents were used in the assay buffer. Trypanopain (1.5 ng) was thus activated for 1 min with assay buffer containing DTT, cysteine, reduced glutathione or β-mercaptoethanol (dilutions of stock solutions above giving 1-250 mM final concentration) before substrate addition.

4.4 Optimal time of activation by cysteine

In order to reproducibly assay cysteine proteinases using stopped-time assays, the enzyme must be fully reduced when substrate is added so that total substrate hydrolysis during the assay is not limited by time taken for enzyme activation. The optimal time required for activation of trypanopain by cysteine (at its optimal final concentration of 30 mM, determined in Section 4.3) was thus investigated.

4.4.1 Materials

As for Section 3.2.1, except that the revised trypanopain synthetic substrate assay buffer [400 mM sodium phosphate, 60 mM cysteine, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 7.0] described in Section 4.8.6 was used, giving a final cysteine concentration of 30 mM.

4.4.2 Method

Trypanopain (1.5 ng) was assayed as described in Section 3.2.2 except that the enzyme was pre-incubated in assay buffer for various lengths of time (0-7 min) before addition of Z-Phe-Arg-AMC.

4.5 Effect of pH on trypanopain stability

The stability of an enzyme is pH-dependent and the pH at which an enzyme is optimally stabile may differ from the pH at which it is optimally active. There are three main approaches to the study of pH stability. The enzyme under study can be pre-incubated at various pH values and then assayed at those values, which assesses a combination of pH stability and pH activity. Secondly, after pre-incubation at various pH values, the enzyme can be assayed at its optimal pH, giving a better reflection of the effect of pH on stability without the influence of pH on activity. Thirdly, hydrolysis of the substrate can be monitored continuously at various pH values with excess substrate and the decrease in velocity over time used to calculate the half-life of the enzyme at each pH. To assess the influence of pH on trypanopain stability alone, the second method was used. Trypanopain was thus incubated at different pH values in the range pH 4.0 to 9.0 and then assayed at pH 7.0 against Z-Phe-Arg-AMC.

4.5.1 Materials

AMT buffers. As per Section 4.1.1.

Assay buffer [400 mM sodium phosphate, 60 mM cysteine, 4 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 7.0]. As described in Section 4.8.6.

4.5.2 Method

To determine the pH stability of trypanopain in the absence of reducing agents, trypanopain (15 ng) was incubated in AMT buffers (100 mM, pH 4.0 to 9.0, 25 µl) without cysteine for 1 h at 37°C. Assay buffer (50µl) was added and residual trypanopain assayed using stopped-time assays as described in Section 3.2.3. The molarity of the second assay buffer (400 mM) was high enough to ensure that an optimum pH of 7.0 was maintained during this section of the assay.

To determine pH stability in the presence of reducing agents, trypanopain (15 ng) was incubated in AMT buffers (100 mM, pH 4.0-9.0, 25 µl) containing cysteine (30 mM final concentration) for 1 h at 37°C. The enzyme was assayed using stopped time assays as described in Section 3.2.3. The assay buffer (50 µl) subsequently added contained additional cysteine, maintaining a final cysteine concentration of 30 mM.

4.6 Specificity for synthetic substrates

At the outset of this study, no analysis of the specificity of trypanopain for synthetic substrates had been reported. However, since both trypanopain-Tc (Mbawa *et al.*, 1992) and cruzipain (Cazzulo *et al.*, 1990b; Murta *et al.*, 1990) are cathepsin L-like (Barrett and Kirschke, 1981), the same is likely to be true of trypanopain from *T. b. brucei*. Once trypanopain had been purified to electrophoretic homogeneity, this hypothesis was tested by examining trypanopain hydrolysis of a range of synthetic substrates.

The active site of a proteinase can be divided into subsites, each of which interacts with a single amino acid residue of the substrate (Schechter and Berger, 1967; Figure 19). Schechter and Berger (1967) devised a nomenclature system that is still widely used to describe these subsites. They proposed that the proteinase subsites on the N-terminal side of the susceptible peptide bond be referred to as the S_1, S_2 etc. sites and those on the C-terminal side of the susceptible bond as the S'_1, S'_2 etc. sites. The corresponding amino acid residues of the substrate are

referred to as P_1, P_2, P'_1, P'_2 etc. For papain-like cysteine proteinases, specificity is primarily governed by the S_2 and S_1' binding sites (reviewed in Turk *et al.*, 1997).

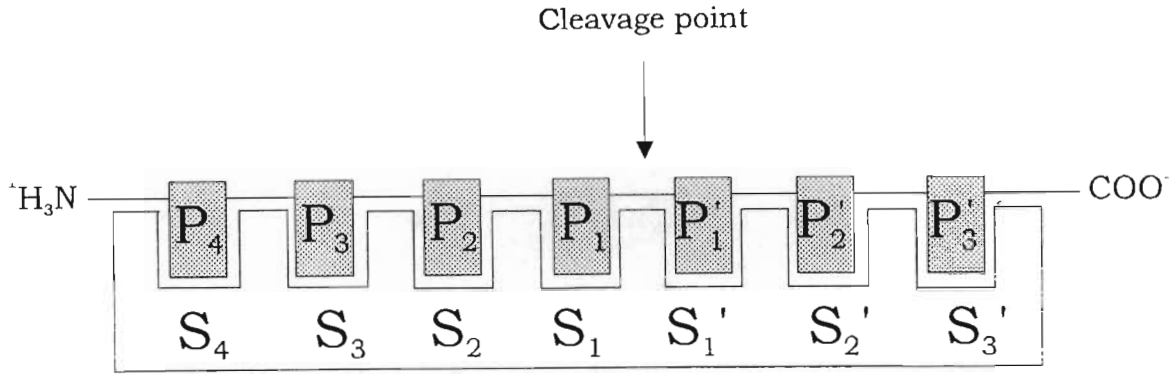


Figure 19: Schechter and Berger notation for proteinase subsites.

This notation is used to describe subsites of a proteinase active site and the corresponding substrate amino acid residues. Amino acid residues on the N-terminal side of the scissile bond are termed P_1, P_2 etc, while those on the C-terminal side are denoted P_1', P_2' etc. The corresponding proteinase subsites are denoted S_1, S_2...S_1', S_2' etc. After Schechter and Berger (1967).

The steady-state kinetics of enzyme hydrolysis of a single substrate can be considered in terms of the Briggs and Haldane (1925) revision of the Michaelis-Menten equation, which states that the initial velocity (v_o) of such a reaction is hyperbolically related to the initial substrate concentration, [S], so that

$$v_o = \frac{V_{max.}[S]}{[S] + K_m}$$

where v_o is the initial rate of substrate hydrolysis ($M.s^{-1}$)

V_{max} is the maximum reaction velocity ($M.s^{-1}$)

[S] is the initial substrate concentration (M), and

K_m is the Michaelis constant (M).

The reaction between a substrate and enzyme can be described by the two kinetic constants V_{max} and K_m. K_m is defined as the substrate concentration required to reach half V_{max} and is a measure of the affinity between the enzyme and a particular substrate. k_{cat}, the turnover number, is equal to $V_{max}/[E]_0$ where $[E]_0$ is the initial enzyme concentration (expressed in M). k_{cat} (with units of s^{-1}) represents the maximum number of substrate molecules that can be turned over by the enzyme per second. (Pamer, 1981) K_m and k_{cat} can be determined experimentally

by measuring v_o at various [S] and fitting the data to a hyperbolic curve (Price and Stevens, 1989). Since it is mathematically easier to fit such experimental data to a straight line, various straight-line transformations of the Michaelis-Menten plot have been derived. These include the Lineweaver-Burk plot (Lineweaver and Burk, 1934), the Eadie-Hofstee plot (Eadie, 1942; Hofstee, 1952), the direct linear plot of Eisenthal & Cornish-Bowden (Eisenthal and Cornish-Bowden, 1974; Cornish-Bowden and Eisenthal, 1974) and the Hanes plot (Hanes, 1932). While the Lineweaver-Burk is the most commonly used, it is the least statistically valid (Cornish-Bowden, 1995). For small values of v_o, small errors lead to large errors in $1/v_o$, while for large v_o, these same small errors lead to small errors in $1/v_o$. This transformation thus gives misleading impressions of experimental error. The Eadie-Hostee and Hanes plots also have various statistical limitations, albeit less marked than the Lineweaver-Burk plot (Cornish-Bowden, 1995). The direct linear plot is considered by many authors as the most reliable of the methods listed above.

4.6.1 Materials

The following peptide AMC substrates were all dissolved in DMSO to make 1 mM stock solutions:

Arg-AMC. HCl (Cambridge Research Chemicals); Benzoyl (Bz)-Arg-AMC. HCl. ½ H_2O (Bachem); Z-Arg-AMC.HCl (Bachem); Z-Arg-Arg-AMC. 2 AcOH (Bachem); Z-Ala-Arg-Arg-AMC. 2 acetate (Bachem); Z-Phe-Arg-AMC (Bachem); Pro-Phe-Arg-AMC. 2 HCl. 2½H_2O (Bachem); Glu-Gly-Arg-AMC (NovaBiochem); Z-Gly-Gly-Arg-AMC.HCl (Cambridge Research Chemicals); Butoxycarbonyl (Boc)-Val-Gly-Arg-AMC. HCl (Bachem); Boc-Leu-Gly-Arg-AMC. HCl. 1½ H_2O (NovaBiochem); Boc-Ile-Glu-Gly-Arg-AMC (Peninsula Laboratories); Z-Pro-Arg-AMC (Bachem); Bz-Phe-Val-Arg-AMC. 2 H_2O (Bachem); D-Val-Leu-Lys-AMC. 2 AcOH. 1.6 H_2O (Bachem); D-Ala-Leu-Lys-AMC. 1½ H_2O (Bachem); Succinyl (Suc)-Ala-Phe-Lys-AMC. 1½ TFA. H_2O (Bachem); Ala-Phe-Lys-AMC (Bachem); Ac-Ala-Ala-Pro-Ala-AMC (Bachem); Suc-Leu-Tyr-AMC (Sigma); Ac-Ala-Ala-Tyr-AMC (Bachem); Leu-AMC. H_2O (Bachem); Gly-AMC. HBr (Bachem); Z-Gly-Pro-AMC (Bachem); Methoxysuccinyl (MeOSuc)-Asp-Tyr-Met-AMC (Bachem); MeOSuc-Gly-Leu-Phe-AMC (Bachem); MeOSuc-Gly-Trp-Met-AMC (Bachem); Suc-Ala-Ala-Phe-AMC (Sigma); Glu-Gly-Gly-Phe-AMC (Bachem).

4.6.2 Method

The concentration of active trypanopain was determined by titration against E-64 as described in Section 3.4. Various concentrations of readily hydrolysed substrates (25-100 μM final concentration) were incubated with trypanopain (0.05 pmol, 1.5 ng) in stopped-time assays for 10 minutes at 37°C as described in Section 3.2.3, while poorly hydrolysed substrates (25-100 μM final concentration) were incubated with trypanopain (0.05 pmol, 1.5 ng) in stopped-time assays as described in Section 3.2.3 for 4 h at 37°C to allow increased substrate hydrolysis.

With excess substrate, the reaction velocity for the poorly hydrolysed substrates was assumed to be constant over the 4 h assay period and so stopped-time assays (Section 3.2.3) were considered adequate to approximate v_o. However, for the most rapidly hydrolysed substrates, v_o was determined more accurately from continuous assays performed over 10 min at each substrate concentration. Kinetic constants calculated for Z-Phe-Arg-AMC from continuous and stopped-time assays were compared to assess the validity of using stopped-time assays to calculate kinetic constants for other, less rapidly hydrolysed substrates. The software package Hyper 1.01 (© 1991-1992, Dr J. S. Easterby, University of Liverpool, UK) was used to determine K_m, V_{max} and k_{cat} using the Hanes, Lineweaver-Burk, Michelis-Menten, Eadie-Hofstee and direct linear plots.

4.7 Hydrolysis of proteins

Since parasitic cysteine proteinases have been postulated to contribute to pathogenesis by hydrolysing various host proteins (Rautenberg *et al.*, 1982; Raimondi *et al.*, 1991; Mbawa *et al.*, 1992; Authié *et al.*, 1994), trypanopain hydrolysis of albumin, fibrinogen and IgG was investigated. Trypanopain-Tc has been shown to degrade IgG, albumin and fibrinogen *in vitro* (Mbawa *et al.*, 1992), while cruzipain degrades gelatin (Tomas and Kelly, 1994), oxidised insulin A and B chains (Raimondi *et al.*, 1991), casein, BSA, denatured haemoglobin (Bontempi *et al.*, 1984) and IgG (Bontempi *et al.*, 1990) *in vitro*.

4.7.1 Materials

Test proteins. Fibrinogen (100 μM, 34 mg), BSA (1 mM, 68 mg) and rabbit immunoglobulin (IgG, 100 μM, 16 mg) were dissolved in 0.1% (m/v) Brij-35 diluent (1 ml, Section 3.2.1).

Protein assay buffer. As per Section 4.8.6.

4.7.2 Method

Fibrinogen (8.8 µM final concentration) was incubated with trypanopain (88 nM or 8.8 nM final concentration, to give 1:100 and 1:1000 molar ratios of enzyme:fibrinogen respectively) in assay buffer (30 mM final cysteine concentration) at 37°C for 0, 15, 30 and 60 min. Aliquots were removed at the stated intervals and the reaction stopped by addition of E-64 (1 mM final concentration). Samples were mixed with an equal volume of reducing treatment buffer, boiled for 90 sec and electrophoresed as per Section 2.4.

BSA (100 µM final concentration) was similarly incubated with trypanopain (1 µM or 100 nM final concentration to give 1:500 and 1:1000 molar ratios of enzyme:BSA respectively). IgG (25 µM final concentration) was similarly incubated with trypanopain (250 nM or 25 nM to give 1:100 and 1:1000 molar ratios of enzyme:IgG respectively).

4.8 Results

4.8.1 Effect of pH on trypanopain activity against Z-Phe-Arg-AMC

Trypanopain activity against Z-Phe-Arg-AMC was optimal across a fairly wide plateau between pH 6.5 and 8.0 (Figure 20). Outside of this range, enzyme activity was substantially lower. All subsequent assays against Z-Phe-Arg-AMC were thus carried out at pH 7.0, in the optimal pH range, using 400 mM NaH_2PO_4 (see Section 4.8.6 for revised synthetic substrate assay buffer).

4.8.2 Effect of pH on activity against ^{14}C-gelatin

Trypanopain activity against ^{14}C-gelatin was optimal at pH 5.5, with activity dropping markedly on either side of this optimum (Figure 21). In contrast with activity against Z-Phe-Arg-AMC, trypanopain was thus active against ^{14}C-gelatin across a narrower pH range and at more acidic pH values. Minimal activity was observed at physiological pH values. All subsequent assays against ^{14}C-gelatin were thus carried out at pH 5.5 (see Section 4.8.6 for revised protein assay buffer).

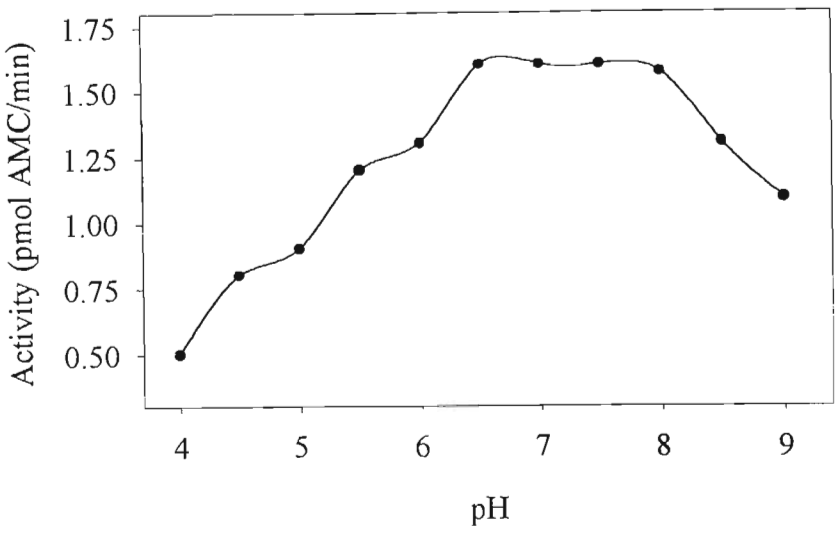


Figure 20: Effect of pH on trypanopain activity against Z-Phe-Arg-AMC.

Trypanopain (1.5 ng) was assayed against Z-Phe-Arg-AMC at 37°C in quintuplicate stopped-time assays as described in Section 3.2.3, except that the assay buffer was replaced by AMT buffers (ionic strength 0.1, 100 mM, pH 4.0 to 9.0) with DTT (6 mM) as reducing agent.

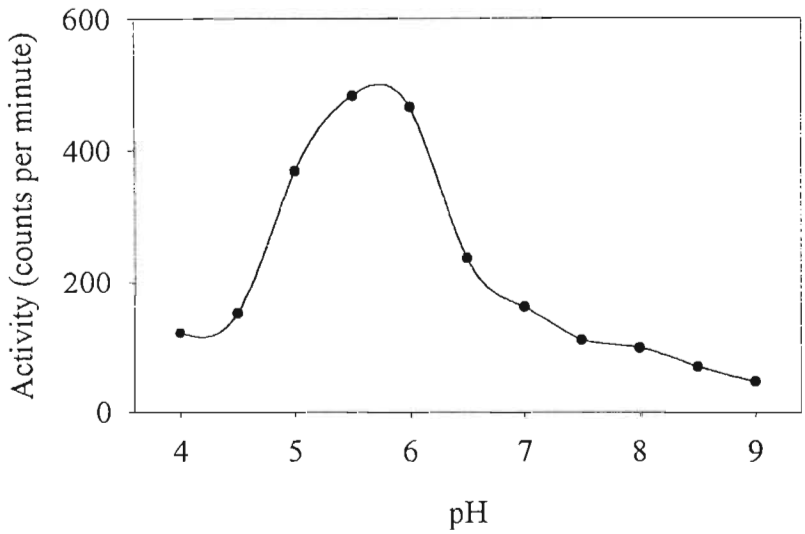


Figure 21: Effect of pH on trypanopain activity against ^{14}C-gelatin.

Trypanopain (6 ng) was incubated with ^{14}C-gelatin for 15 h at RT in quintuplicate stopped-time assays. Stop solution was added and the mixture maintained on ice for 30 min. Following centrifugation (4 800×*g*, 15 min, 25°C), the supernatant (500 µl) was mixed with scintillation cocktail (5 ml) and the counts per minute read in a Packard Tri-Carb Liquid Scintillation counter.

4.8.3 Effect of reducing agents on trypanopain activity against Z-Phe-Arg-AMC

Trypanopain was inactive against Z-Phe-Arg-AMC in the absence of reducing agents, while DTT, cysteine, β-mercaptoethanol and reduced glutathione all enhanced trypanopain activity (Figure 22). This indicates that trypanopain displays typical cysteine proteinase latency. At low concentrations (up to 50 mM), cysteine was the most effective reducing agent, with an optimal concentration of 30 mM. While DTT was also most effective at low concentrations (20 mM optimal), β-mercaptoethanol was more effective at higher concentrations (optimal at 250 mM, not shown). Reduced glutathione was a comparatively poor activator. Cysteine (30 mM final concentration) was thus used as reducing agent for all subsequent assays (see Section 4.8.6 for revised assay buffers).

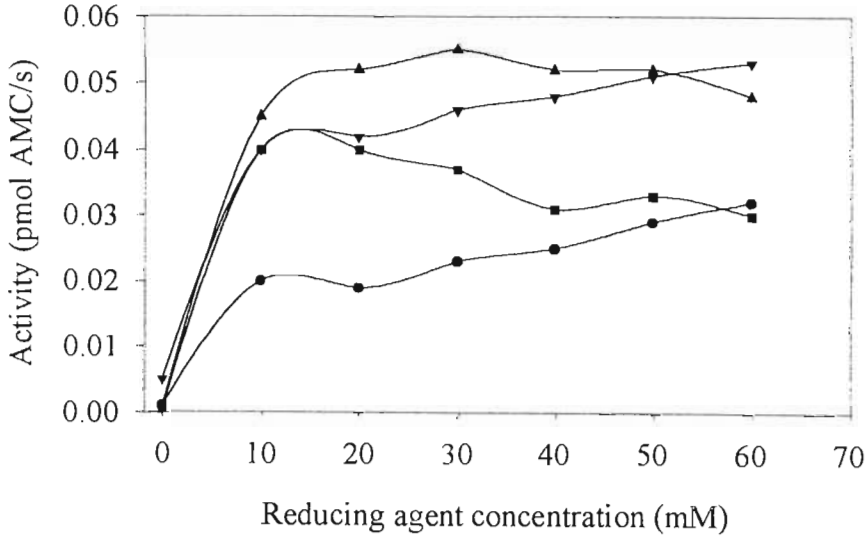


Figure 22: Effect of reducing agents on trypanopain activity against Z-Phe-Arg-AMC. Trypanopain (1.5 ng) was assayed against Z-Phe-Arg-AMC at 37°C in quintuplicate stopped-time assays as described in Section 3.2.2, except that various concentrations of DTT (■), cysteine (▲), β-mercaptoethanol (▼) or reduced glutathione (●) were used as reducing agents.

4.8.4 Optimal activation time with cysteine

Highest trypanopain activity was observed after 1 min pre-activation with cysteine (Figure 23). With increasing incubation times, reduced activity was observed.

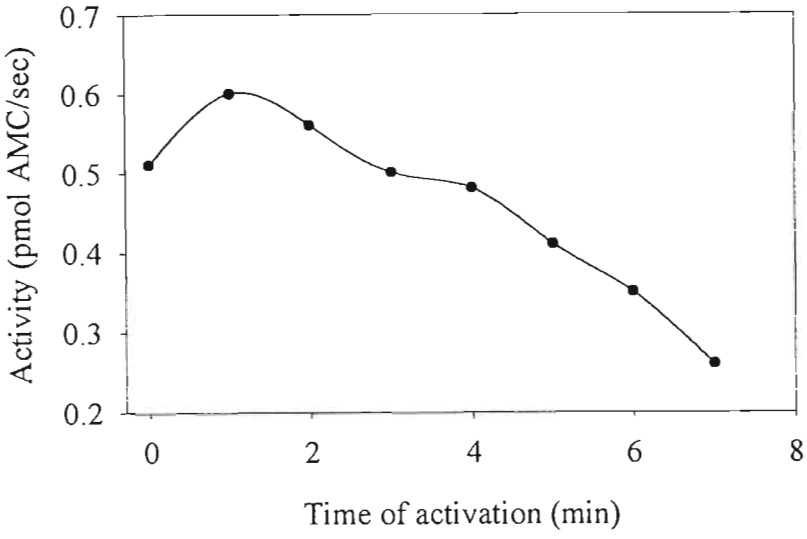


Figure 23: Optimal time of trypanopain activation with 30 mM cysteine.

Trypanopain (1.5 ng) hydrolysis of Z-Phe-Arg-AMC at 37°C was monitored using standard quintuplicate stopped-time assays (Section 3.2.2), except that the enzyme was activated with cysteine (30 mM) for 0-7 minutes before addition of Z-Phe-Arg-AMC.

4.8.5 Effect of pH on trypanopain stability

Below pH 7.5, trypanopain was slightly more stable in the absence of cysteine than in its presence (Figure 24). When pre-incubated without cysteine, trypanopain was relatively stable from pH 4.0 to 7.0, but above this pH the stability decreased sharply. In the presence of cysteine, trypanopain was moderately stable between pH 4.0 and 8.0, above which decreased stability was observed (Figure 24).

4.8.6 Revised trypanopain assay buffers

The results in Sections 4.8.1 to 4.8.4 necessitated modification of the cathepsin L assay buffer described in Section 3.2.1 for optimal assay of trypanopain activity. A Na-phosphate buffer of pH 7.0 was chosen as the synthetic substrate assay buffer. This buffer contained 400 mM $NaH_2PO_4.2H_2O$ (62.4 g/l), 4 mM $Na_2EDTA.2H_2O$ (1.5 g/l), 60 mM cysteine (10.51 g/l) and 0.02% (m/v) NaN_3 (0.2 g/l). Thus for standard assays against Z-Phe-Arg-AMC, trypanopain [diluted to 250 µl in 0.1% (m/v) Brij-35] was pre-activated for 1 min in synthetic substrate assay buffer (500 µl) before addition of Z-Phe-Arg-AMC (250 µl of 20 µM solution). After 10 min, stop reagent (1 ml) was added and the fluorescence read. For micro-assays, 1/10

volumes of the above reagents were used.

For subsequent experiments using ^{14}C-gelatin as a substrate, a "protein assay buffer" of 200 mM Na-acetate (11.45 ml acetic acid/l), 1 mM Na_2EDTA (0.37 g/l), 68 mM cysteine (11.91 g/l, 30 mM final concentration) and 0.02% (m/v) NaN_3 (0.2 g/l), pH 5.5 was used.

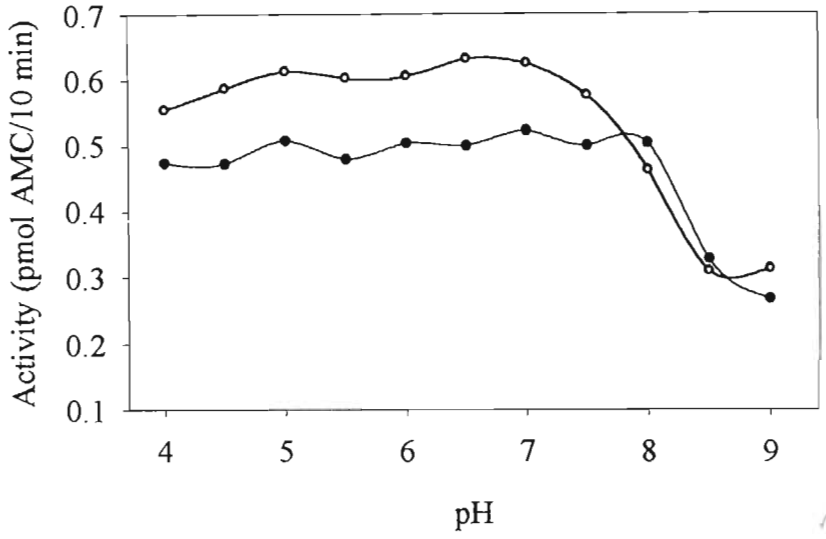


Figure 24: Effect of pH on the stability of trypanopain in the presence or absence of cysteine. Trypanopain (15 ng) was incubated for 1 h at 37°C in AMT buffers (100 mM, 0.1 ionic strength) over the pH range 4.0 to 9.0 (quintuplicate samples at each pH). Cysteine (30 mM) was either present (●) or absent (○) during pre-incubation. Residual enzyme activity against Z-Phe-Arg-AMC was determined using stopped-time assays as described in Section 3.2.3.

4.8.7 Hydrolysis of synthetic substrates

Of the synthetic substrates tested, Z-Phe-Arg-AMC was most readily cleaved by trypanopain, with a K_m of 1.2 µM and a k_{cat} of 13.4 s^{-1}, giving a k_{cat}/K_m of 11.3 $s^{-1}.\mu M^{-1}$ (Table 5). Pro-Phe-Arg-AMC was also readily hydrolysed with a K_m of 4.2 µM and a k_{cat} of 4.2 s^{-1}, (k_{cat}/K_m of 1 $s^{-1}.\mu M^{-1}$, Table 5). Compared with these two substrates, other tested substrates were poorly hydrolysed. Generally, only substrates with Phe in P_2 and Arg in P_1 were significantly cleaved (notation of Schechter and Berger, 1967). The cathepsin B substrate (Barrett and Kirschke, 1981) Z-Arg-Arg-AMC was comparatively poorly hydrolysed (k_{cat}/K_m of 0.01 $s^{-1}.\mu M^{-1}$ in 4 h assay).

As Z-Phe-Arg-AMC was by far the best of the substrates tested, a more accurate determination of its kinetic constants was undertaken, with v_o determined more accurately from continuous assays performed at a range of substrate concentrations (Figure 25). K_m (0.93 μM) and k_{cat} (9.9 s^{-1}) values calculated using this method were very similar to those calculated initially from 10 minute stopped time assays (1.2 μM and 13.4 s^{-1} respectively). This suggests that stopped time assays provide a reasonably accurate estimate of kinetic constants and that the values given below (Table 5) for the other substrates tested are thus reliable.

Table 5: Kinetic constants for trypanopain hydrolysis of various AMC substrates.

Values are calculated at 37°C from 10 minute or 4 h (*) quintuplicate stopped-time assays as described in Section 3.2.3, using various substrate concentrations (25-100 μM). Results below represent the average of K_m and k_{cat} values determined using the Hanes, Lineweaver-Burk, Michaelis-Menten, Eadie-Hofstee and direct linear plots.

Substrate	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.\mu M^{-1}$)
*Z-Arg-Arg-AMC	12	0.126	0.01
Z-Phe-Arg-AMC	1.2	13.4	11.3
Pro-Phe-Arg-AMC	4.2	4.2	1
Val-Leu-Lys-AMC	25	8.2	0.32
Ala-Leu-Lys-AMC	27	6.5	0.24
Suc-Ala-Phe-Lys-AMC	42	0.006	0.002
Ala-Phe-Lys-AMC	28	5.2	0.19
*Suc-Leu-Tyr-AMC	54	0.05	0.001
*MeOSuc-Asp-Tyr-Met-AMC	68	0.09	0.001
*MeOSuc-Gly-Trp-Met-AMC	4	0.04	0.0105

Note: Bz-Arg-AMC, Z-Arg-AMC, Z-Ala-Arg-Arg-AMC, Arg-AMC, Glu-Gly-Arg-AMC, Z-Gly-Gly-Arg-AMC, Boc-Val-Gly-Arg-AMC, Boc-Leu-Gly-Arg-AMC, Boc-Ile-Glu-Gly-Arg-AMC, Z-Pro-Arg-AMC, Ac-Ala-Ala-Pro-Ala-AMC, Ac-Ala-Ala-Tyr-AMC, Leu-AMC, Gly-AMC, Z-Gly-Pro-AMC, Suc-Ala-Ala-Phe-AMC and Glu-Gly-Gly-Phe-AMC were not detectably cleaved in 4 h.

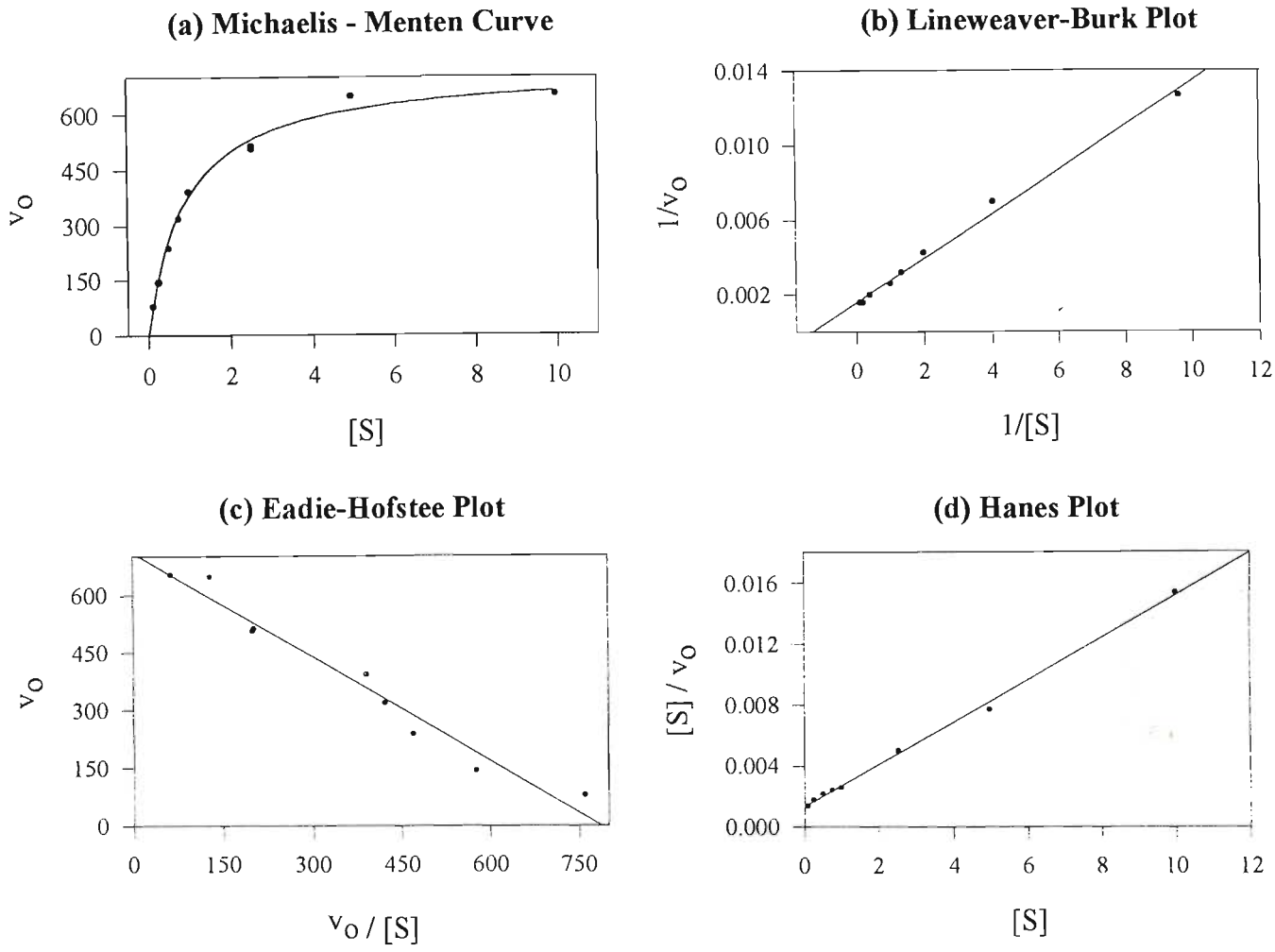


Figure 25: Calculation of K_m and k_{cat} values for trypanopain hydrolysis of Z-Phe-Arg-AMC. a) Michaelis-Menten curve, (b) Lineweaver-Burk plot, (c) Eadie-Hofstee plot and (d) Hanes plot for trypanopain (0.05 pmol) hydrolysis of Z-Phe-Arg-AMC (0.1-10 μM) at 37°C. Initial velocities (v_o in pmol AMC/s) were measured from quintuplicate continuous assays at different substrate concentrations, [S] in μM.

4.8.8 Hydrolysis of proteins

Trypanopain readily degraded the α- (67 kDa) and β-chains (56 kDa) of fibrinogen (Jakubke and Jeschkeit, 1983) within 15 min when incubated at a 1:100 molar ratio of enzyme:fibrinogen (Figure 26). The γ-chain (47 kDa), however, remained resistant for the full hour. By comparison, the entire fibrinogen molecule was resistant to degradation over 1 h at a 1:1 000 molar ratio of enzyme:fibrinogen. Trypanopain readily degraded BSA at 1:500 and 1:1 000 molar ratios of enzyme:BSA (Figure 27), with more degradation observed at the higher enzyme concentration. Increased degradation was also seen with increasing incubation times, although

substantial degradation was already evident after 15 min at both enzyme concentrations. Trypanopain readily degraded IgG at both a 1:100 and 1:1 000 molar ratio of enzyme:IgG (Figure 28). A similar degree of digestion was observed with both concentrations of enzyme, with only slightly greater degradation seen at the higher enzyme concentration.

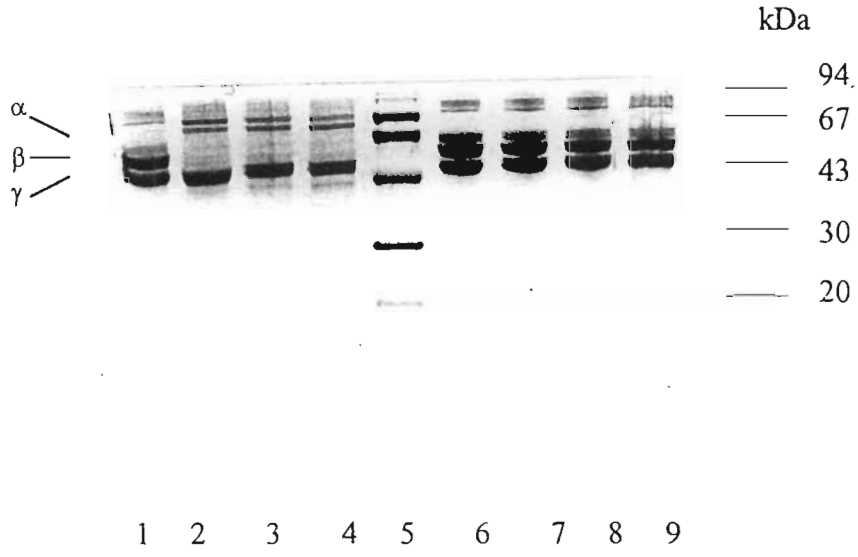


Figure 26: Trypanopain digestion of fibrinogen.

Fibrinogen was incubated with trypanopain in assay buffer at 37°C for 0, 15, 30 and 60 min. Aliquots were removed at the stated time intervals and the reaction stopped by addition of E-64 (1 mM final concentration). Samples were electrophoresed as per Section 2.4, using reducing treatment buffer. Trypanopain (88 nM) digestion of fibrinogen (8.8 μM, 1:100 molar ratio of enzyme:fibrinogen) after 0 min (lane 1), 15 min (lane 2), 30 min (lane 3) and 60 min (lane 4), Pharmacia molecular mass markers (Section 2.4), and trypanopain (8.8 nM) digestion of fibrinogen (8.8 μM, 1:1 000 molar ratio of enzyme:fibrinogen) after 0 min (lane 6), 15 min (lane 7), 30 min (lane 8) and 60 min (lane 9) are shown. The molecular mass of the fibrinogen α-chain is 67 kDa; the β-chain, 56 kDa and the γ-chain, 47 kDa.

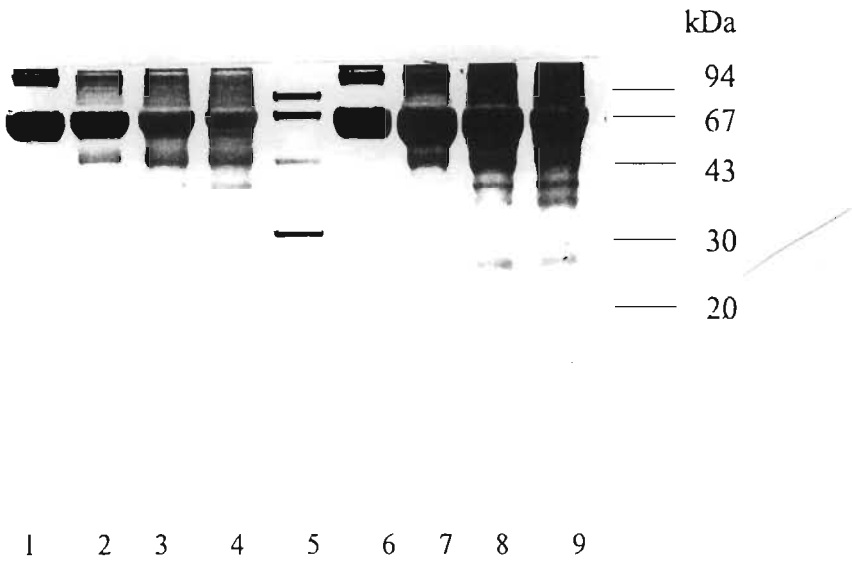


Figure 27: Trypanopain digestion of BSA.

BSA was incubated with trypanopain in assay buffer at 37°C for 0, 15, 30 and 60 min. Samples were prepared as in Figure 26. Trypanopain (100 nM) digestion of BSA (100 μM, 1: 1 000 molar ratio of enzyme:BSA) after 0 min (lane 1), 15 min (lane 2), 30 min (lane 3) and 60 min (lane 4), Pharmacia molecular mass markers (lane 5, Section 2.4), and trypanopain (200 nM) digestion of BSA (100 μM, 1:500 molar ratio of enzyme:BSA) after 0 min (lane 6), 15 min (lane 7), 30 min (lane 8) and 60 min (lane 9) are shown.

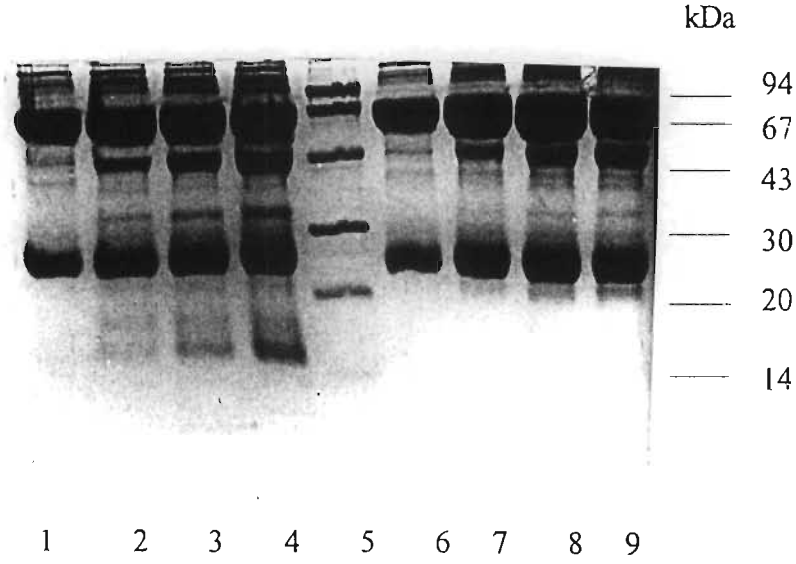


Figure 28: Trypanopain digestion of IgG.

Trypanopain (250 nM) digestion of IgG (8.8 μM, 1:100 molar ratio of enzyme:IgG) after 0 min (lane 1), 15 min (lane 2), 30 min (lane 3) and 60 min (lane 4), Pharmacia molecular mass markers (Section 2.4) and trypanopain (25 nM) digestion of IgG (8.8 μM. 1: 1 000 molar ratio of enzyme: IgG) after 0 min (lane 6), 15 min (lane 7), 30 min (lane 8) and 60 min (lane 9) are shown.

4.9 Discussion

Trypanopain was shown to be optimally active against Z-Phe-Arg-AMC between pH 6.5 and 8.0. This is in agreement with previous studies carried out with partially purified trypanopain (Huet *et al.*, 1992). By comparison, other trypanosomal cysteine proteinases are optimally active at slightly more acidic pH values, with trypanopain-Tc optimally active against Z-Phe-Arg-AMC between pH 5.5 and 6.0 (Lonsdale-Eccles and Grab, 1987a; Mbawa *et al.*, 1992) and cruzipain optimally active between pH 5.0 and 7.0 (Murta *et al.*, 1990; Stoka *et al.*, 1995). A cysteine proteinase from the nematode *Nippostrongylus brasiliensis* is also optimally active against Z-Phe-Arg-AMC at pH 5.5 (Kamata *et al.*, 1995). Mammalian cathepsins are generally also optimally active at slightly more acidic values than trypanopain, with cathepsin H optimally active against Arg-AMC at pH 6.8 (Barrett and Kirschke, 1981) and cathepsin L against Z-Phe-Arg-AMC at pH 6.5 (Dehrmann *et al.*, 1995). Cathepsin B, however, is optimally active against Z-Arg-Arg-AMC at pH 7.0-8.0 (Dehrmann *et al.*, 1996).

Trypanopain was optimally active against the proteinaceous substrate ^{14}C-gelatin at pH 5.5, which is more in line with the acidic pH optimum expected for a lysosomal cysteine proteinase. These results confirm previous zymogram studies using lysed *T. b. brucei*, which suggested that trypanopain is optimally active against fibrinogen at pH 5.0 and against collagen at pH 6.0 (Lonsdale-Eccles and Mpimbaza, 1986). Cruzipain is optimally active against IgG and gelatin between pH 5.0 and 7.5 (Murta et al., 1990). Since a pH optimum determined using a proteinaceous substrate is more likely to be physiologically relevant than a pH optimum determined using a synthetic substrate, it is considered likely that the pH optimum of trypanopain against its natural substrates is approximately pH 5.5.

Trypanopain displays typical cysteine proteinase latency (North *et al.*, 1990a), in that it is only active in the presence of reducing agents. Such latency has been reported for trypanopain-Tc, cruzipain and crude trypanopain-Tb (Rautenberg *et al.*, 1982; Lonsdale-Eccles and Grab, 1987a; Murta *et al.*, 1990). DTT (4-5 mM) and β-mercaptoethanol (10 mM) are commonly used for activation of trypanopain-Tc (Mbawa *et al.*, 1992) and cruzipain (Cazzulo *et al.*, 1989; Lima *et al.*, 1992). Compared with these enzymes, it appears that trypanopain requires slightly higher concentrations of reducing agents for maximal activation.

To ensure a constant maximum reaction rate during a stopped-time assay of a cysteine proteinase, it is necessary to fully reduce the active site cysteine residue before addition of the substrate. Trypanopain was shown to require 1 min pre-activation in assay buffer before maximum levels of activity were reached. Decreased levels of activity were seen if the enzyme was activated for longer than 1 min. For example, less than half the activity present after 1 min activation remained if trypanopain was activated for 7 min. This suggests that the active enzyme may undergo substantial autocatalysis in the absence of an added substrate. Continuous assays against Z-Phe-Arg-AMC indicated that the reaction velocity over 20 min was unchanged, suggesting that trypanopain does not undergo any appreciable autocatalysis when in the presence of substrates such as Z-Phe-Arg-AMC. Autocatalysis thus appears to occur only when active trypanopain has no potential substrate present other than neighbouring trypanopain molecules. For this reason, reducing agents were omitted from all buffers used during purification of trypanopain. However, sufficient endogenously activated enzyme and reducing agents are likely to have been present in the lysed parasites to cause some autocatalysis of trypanopain. Since the lysed parasites contain many potential trypanopain substrates, this effect is likely to have been minor initially, but would have increased with increasing enzyme purity.

Across the pH range tested, trypanopain was more stable in the absence of cysteine than in its presence, supporting the contention that the active enzyme undergoes autocatalysis. Approximately 17% less enzyme was present after the 1 h incubation period if cysteine was present. Compared with the activation experiment discussed above, the lower percentage of autocatalysis over a longer time period is thought to be a function of the higher concentration of enzyme (and thus potential proteinaceous substrate for remaining active enzyme molecules) used. In both the presence and absence of cysteine, sharp decreases in stability were only observed at alkaline pH values, suggesting that trypanopain undergoes denaturation in alkaline conditions but is fairly stable in acidic environments. Trypanopain is both stable and active at the pH chosen for the assay of synthetic peptide or protein substrates (pH 7.0 and pH 5.5 respectively). Cruzipain is very similar to trypanopain in this respect, being optimally stable between pH 4.5 and 9.5 (Stoka *et al.*, 1995).

As expected, trypanopain has cathepsin L-like specificity for synthetic substrates. Of the substrates tested, Z-Phe-Arg-AMC was most readily hydrolysed by trypanopain. Only

substrates with more than one amino acid residue were cleaved by trypanopain, suggesting that the S_2 site must be occupied by an amino acid for hydrolysis to proceed. Hydrophobic residues such as Phe, Leu, Tyr and Trp were accepted in the P_2 position while basic (Arg) and small, uncharged (Ala, Gly) residues were not accepted. Further information about trypanopain's P_2 preferences was gained from comparisons of the kinetic parameters determined for the hydrolysis of various substrates with Lys instead of Arg in P_1. Since these substrates were poorly cleaved, requiring 4 h incubation periods to generate kinetic constants, the differences observed between the rates of hydrolysis of these substrates should be viewed with caution. Nevertheless, comparison of Ala-Leu-Lys-AMC and Ala-Phe-Lys-AMC suggests that substrates with Leu in P_2 might be as good as, or slightly better than, those with Phe in P_2. Unfortunately, Z-Leu-Arg-AMC is not commercially available and this hypothesis was therefore not tested.

Comparison of Val-Leu-Lys-AMC and Ala-Leu-Lys-AMC suggests that hydrophobic residues (e.g. Val) in P_3 are slightly preferred over small, uncharged residues (e.g. Ala). Thus trypanopain is fairly specific in its catalysis of synthetic substrates. It acts most readily on substrates with basic residues (Arg, Lys in order of preference) in P_1 and hydrophobic residues in P_2 (e.g. Leu, Phe) and P_3 (e.g. Val) and non-acidic residues in P_4. Similar specificity has been reported for trypanopain-Tc (Mbawa *et al.*, 1992), cruzipain (Cazzulo *et al.*, 1990a), mammalian cathepsin L (Barrett and Kirschke, 1981; Dufour and Ribadeau-Dumas, 1988) and papain (Ménard, 1993) (Table 6). Since the determinations were made at different temperatures, direct comparison of the kinetic constants is not possible. Nevertheless, trypanopain-Tc and cathepsin L appear to catalyse the hydrolysis of Z-Phe-Arg-AMC most rapidly, as they have the highest k_{cat} values (17 s^{-1}) even though analysed at lower temperatures. Trypanopain-Tb has the lowest K_m for Z-Phe-Arg-AMC, indicating that trypanopain has comparatively high affinity for this substrate.

Table 6 : Kinetic constants for the hydrolysis of Z-Phe-Arg-AMC at various temperatures by human cathepsin L and the cathepsin L-like cysteine proteinases of various parasites.

Parasite	k_{cat} (s^{-1})	K_m (μM)	Temperature (°C)	Reference
F. hepatica	nd	15	37	Smith *et al.* (1993)
T. b. rhodesiense	nd	0.85	nd	Pamer *et al.* (1991)
T. congolense	17	4.4	25	Mbawa *et al.* (1992)
T. cruzi	3.1	10	37	Murta *et al.* (1990)
T. cruzi	0.88	2.4	37	Lima *et al.* (1992)
T. cruzi	nd	1.8	30	Serveau *et al.* (1996)
Human cathepsin L	17	2.4	30	Mason *et al.* (1985)
T. b. brucei	13.4	1.2	37	Present study.

Note: nd not determined

This study shows that trypanopain hydrolyses the cathepsin B substrate Z-Arg-Arg-AMC poorly. While trypanopain-Tc and trypanopain from *T. b. rhodesiense* do not hydrolyse Z-Arg-Arg-AMC (Pamer *et al.*, 1991; Mbawa *et al.*, 1992), cruzipain does so readily (Cazzulo *et al.*, 1990a; Lima *et al.*, 1992).

Trypanopain readily degrades BSA, fibrinogen and IgG. It is, however, very difficult to suggest a physiological substrate for trypanopain and the enzyme's ability to degrade these proteins *in vitro* does not imply that they are physiological substrates. It does, however, confirm that mammalian proteins are potential substrates.

Once the basic enzymatic properties of trypanopain had been elucidated, the interactions of the enzyme with various synthetic and naturally occurring host proteinase inhibitors were investigated. The results of these experiments are presented in the following chapter.

Chapter 5

Trypanopain interactions with inhibitors

5.1 Inhibitor profile

Proteinases can be divided into four classes (namely, cysteine, serine, aspartic and metalloproteinases) on the basis of the amino acid residues in their active sites that are responsible for substrate catalysis. Most generally used low molecular mass proteinase inhibitors are specific for one class of proteinase, with the result that a proteinase can be assigned to one of the four main classes of proteinases (Hartley, 1960) on the basis of its sensitivity to these diagnostic inhibitors.

Cysteine proteinases are specifically and irreversibly inhibited by epoxides (e.g. E-64), peptide diazomethylketones and alkylating agents [e.g. iodoacetate (IAA), iodoacetamide (IAN) and N-ethylmaleimide (NEM)]. Peptide diazomethylkettones will be discussed in more detail in Chapter 7. E-64 is the classical diagnostic inhibitor of cysteine proteinases that was first isolated from *Aspergillus japonicus* (Section 3.4). The alkylating agents react preferentially with active site cysteine residues, but also show some reactivity towards free thiols (Salvesen and Nagase, 1989). These inhibitors can be inactivated by an excess of reducing agent, so care must be taken when using them in cysteine proteinase assay buffers that contain reducing agents to activate the proteinase under study. The final concentration of reducing agent is minimised in such cases by pre-activating the enzyme in a small volume of concentrated reducing agent before addition of the inhibitor in a buffer free of reducing agents.

Serine proteinases are specifically and irreversibly inhibited by organophosphates [e.g. diisopropylphosphofluoridate (DFP)], sulfonyl fluorides [e.g. phenylmethylsulfonyl fluoride (PMSF) and 4-(2-aminoethyl)-benzenesulfonyl fluoride (AEBSF)] and coumarins [e.g. 3, 4-dichloroisocoumarin (DCI)] and reversibly by soybean trypsin inhibitor (SBTI, Salvesen and Nagase, 1989) and the arginine analogue, benzamidine (Powers and Harper, 1986).

Metalloproteinases are inhibited by zinc chelators such as 1, 10-phenanthroline and EDTA (Salvesen and Nagase, 1989). Aspartic proteinases are characteristically inhibited by isovaleryl-Val-Val-statine-Ala-statine (pepstatin A), a pentapeptide-like compound secreted by *Streptomyces* species, which has two residues of the unusual β-amino acid statine [(3S, 4S)-4-amino-3-hydroxy-6-methylheptanoic acid] (Salvesen and Nagase, 1989). The statine residues enable pepstatin A to act as a transition state analogue for aspartic proteinases (Kay *et al.*, 1983).

Some inhibitors react with more than one class of proteinase. For example, the peptide aldehydes leupeptin and antipain inhibit both cysteine and serine proteinases (Rich, 1986; Salvesen and Nagase, 1989). In place of the normal α-carboxylate present in the backbone of a peptide chain, these reversible inhibitors contain an aldehyde moiety that reacts with the active site residues of proteinases. These inhibitors mimic the tetrahedral intermediate formed during peptide bond hydrolysis and are the only transition-state analogue inhibitors identified for cysteine proteinases to date (Rich, 1986). Peptidyl chloromethylketones [e.g. N-tosyl-L-lysyl chloromethylketone (TLCK) and N-tosyl-phenylalanyl chloromethylketone (TPCK)] are irreversible inhibitors of cysteine and serine proteinases. They bind the target enzyme in a substrate-like manner, and then alkylate the active site histidine of serine proteinases or the active site cysteine of cysteine proteinases. These inhibitors will be discussed in more detail in Chapter 7.

Although trypanopain has not been previously isolated, preliminary inhibitor studies on crude lysed *T. b. brucei* suggest that the enzyme is a typical cysteine proteinase (Lonsdale-Eccles and Mpimbaza, 1986). Purified trypanopain-Tc and cruzipain are completely inhibited by E-64, mercurial compounds and cystatins and thus display typical cysteine proteinase inhibitor profiles.

5.1.1 Materials

As per Section 3.2.1, with the assay buffer of Section 4.8.6.

The following inhibitors were dissolved in H_2O (1 ml) on the day of use:

IAA (100 mM, 20 mg),

NEM (10 mM, 1.3 mg),

IAN (10 mM, 1.9 mg),

Leupeptin (1 mM, 0.5 mg),

Antipain (1 mM, 0.6 mg) and

EDTA (100 mM, 37 mg).

The following inhibitors were dissolved in methanol (1 ml) on the day of use:

PMSF (100 mM, 17 mg) and

TPCK (10 mM, 35 mg).

The following inhibitors were dissolved in DMSO (1 ml) on the day of use:

Pepstatin A (1 mM, 0.7 mg),

E-64 (0.2 mM, 1 mg/15 ml),

AEBSF (5 mM, 1 mg) and

Chymostatin (10 mM, 6 mg).

SBTI (10 μM; 1 mg/5 ml) was dissolved in assay buffer (Section 4.8.6) on the day of use.

TLCK (1 mM, 1.1 mg) was dissolved in 1 mM HCl (3 ml) on the day of use.

DFP (100 mM). Extreme care was taken in the preparation of this highly toxic inhibitor. Stock DFP (5.43 M) was diluted in assay buffer (Section 4.8.6) to 100 mM and used immediately. All residual material, including pipette tips and glassware, was soaked in 2 M NaOH to neutralise the inhibitor.

5.1.2 Method

The activity of trypanopain in the presence of various inhibitors was investigated by incubating the enzyme (2 ng, 150 μl) with each inhibitor (1-10 mM, 100 μl) in assay buffer (500 μl) for 15 min at 37°C before assaying residual activity against Z-Phe-Arg-AMC (250 μl) in stopped-time assays as described in Section 3.2.3. Activity was compared to the appropriate solvent-containing control in all cases. Since chloromethylketones and alkylating agents are inactivated by reducing agents, care was taken to minimise the relative amount of reducing agents used with these inhibitors. Concentrated enzymes were thus pre-activated (10 min at 37°C in 30 mM cysteine) and then diluted to the required working concentration in assay buffer minus reducing agent (final reducing agent

concentration of 3 mM cysteine). Activity was compared to controls assayed under the same conditions with the same concentration of reducing agent.

For pepstatin A, the reversibility of inhibition was investigated by pre-incubating trypanopain (2 ng, 5 µl) with assay buffer (250 µl) and pepstatin A [various concentrations, diluted to 245 µl in 0.1% (m/v) Brij-35] for 15 min at 37°C. Controls were incubated with 0.1% (m/v) Brij-35 alone. Aliquots of these inhibited and control enzyme mixtures were diluted 10-fold and 100-fold in 0.1% (m/v) Brij-35, giving three concentrations of inhibited and control enzyme. Samples (250 µl) of each of these mixtures were diluted into assay buffer (500 µl) and assayed against Z-Phe-Arg-AMC (250 µl) in continuous assays as described in Section 3.2.2. If the percentage of active enzyme at each dilution was the same, then inhibition was diagnosed as irreversible. If, however, the percentage of active enzyme increased with dilution, then inhibition was diagnosed as reversible.

For reversible inhibitors, control and inhibited K_m and V_{max} values (calculated as in Section 4.6) were then compared to further diagnose the class of inhibition. K_i was determined from the equation (Salvesen and Nagase, 1989)

$$K_i = \frac{[I]}{\frac{v_o}{v_i} - 1} \Big/ 1 + \frac{[S]}{K_m}$$

where K_i is the equilibrium constant for inhibition (M),

[S] is the substrate concentration (M),

[I] is the inhibitor concentration (M),

v_o is the initial velocity of the uninhibited reaction (arbitrary units),

v_i is the initial velocity of the inhibited reaction (arbitrary units), and

K_m is the Michaelis constant for the substrate used (M).

5.2 Association and inhibition rate constants of cystatins

Trypanopain has been postulated to contribute to pathogenesis by digestion of various host proteins (reviewed in Chapter 1). However in mammals, host and pathogen cysteine proteinases are usually kept under tight control by a variety of endogenous proteinase inhibitors. The most common of these belong to the cystatin superfamily. The interaction

between trypanopain and various cystatins was investigated to determine whether trypanopain is likely to be active *in vivo* or whether it is likely to be effectively controlled by these inhibitors. At the time this project was undertaken, no kinetic study of trypanosomal cysteine proteinase interaction with cystatins had been reported, although cruzipain interactions have been subsequently investigated (Stoka *et al.*, 1995).

Cystatins are tight-binding, reversible, competitive cysteine proteinases inhibitors, that are currently divided into three families on the basis of structure (Barrett *et al.*, 1986; Salvesen and Nagase, 1989; Abrahamson, 1993). New cystatins are, however, being discovered annually and await classification into families (Watanabe *et al.*, 1991; Misaka *et al.*, 1996; Yamashita and Konagaya, 1996; Gruden *et al.*, 1997). Family 1 cystatins (e.g. human cystatin A and B) are single chain, acidic polypeptides of 11-12 kDa with no intrachain disulfide bridges. The family is largely intracellular, with cystatin B being widespread in the cytoplasm of many cell types, and cystatin A found primarily in the skin and some white blood cells (Turk and Bode, 1993). Family 2 cystatins (e.g. cystatins C, D, S, SN and SA) are also of low molecular mass (13-14 kDa) but have two intrachain disulfide bridges and are found mostly in secretions like saliva and seminal plasma (Abrahamson, 1993). Family 3 cystatins, or kininogens, are complex high molecular mass (60-120 kDa) glycoproteins with various multi-functional domains (Barrett *et al.*, 1986). The two most common are high (H-) and low (L-) molecular mass kininogen, which are also parent molecules for the vasoactive kinins. Kallikreins cleave both L- and H-kininogen to liberate vasoactive kinins, thereby converting the parent kininogens into two-chain molecules joined by a single disulfide bond. L- and H-kininogen have identical heavy chains, but the light chain of H-kininogen is larger than that of L-kininogen (reviewed by Turk *et al.*, 1997). The heavy chains consist of three tandemly repeated cystatin-like domains (D1, D2 and D3), two of which (D2 and D3) are inhibitory for cysteine proteinases. Each L-kininogen molecule has two binding sites for cysteine proteinases, one tight-binding site on domain 3 and the other of 7 to 18-fold lower affinity on domain 2 (Turk *et al.*, 1995). Turk *et al.* (1995) stated, however, that the lower affinity site is likely to remain unoccupied under normal experimental conditions and need not be corrected for when calculating K_i for association with L-kininogen, provided that there is at least a 10-fold molar excess of inhibitor over enzyme. Since a 20-fold molar excess of L-kininogen was used in the present study, association with the weaker binding site was safely ignored.

Chicken egg white cystatin was the first cystatin to be crystallised (Bode *et al.*, 1988) and, since cystatins are well conserved, provided much of our understanding of the manner in which cystatins inhibit cysteine proteinases. Cystatins are folded into a five-stranded anti-parallel β-sheet surrounding a five-turn α-helix. The N-terminal section (Shibuya *et al.*, 1995) and two hairpin loops that connect adjacent strands of the β-sheet (Stubbs *et al.*, 1990) are thought to be crucial for hydrophobic interaction with the active site of the target enzyme (Figure 29).

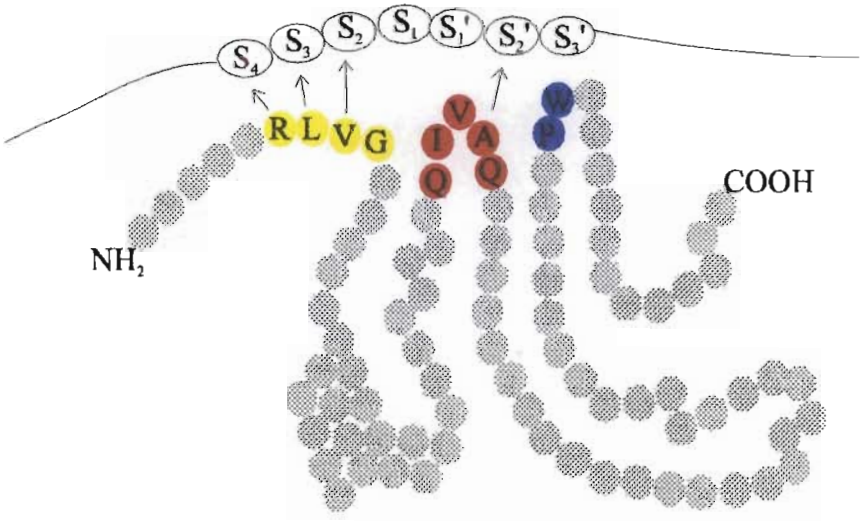


Figure 29: Schematic representation of chicken egg white cystatin binding to a cysteine proteinase.

Conserved residues of the cystatin bind to the active site of a target proteinase in a substrate-like manner, but the conformation of the cystatin prevents it being cleaved. The N-terminal (yellow) cystatin region interacts with proteinase subsites S_1, S_2 and S_3, while two cystatin hairpin loops (red and blue) connecting adjacent β-sheets interact with the S_2' and S_3' proteinase subsites. After Abrahamson (1993).

The interaction between an enzyme and a reversible inhibitor can be described (Bieth, 1980) by the simplified scheme

$$E + I \underset{k_{diss}}{\overset{k_{ass}}{\rightleftharpoons}} EI$$

where E is the initial enzyme concentration (M),

I is the initial inhibitor concentration (M),

EI is the concentration of the enzyme/inhibitor complex (M^2),

k_{ass} is the association rate constant ($M^{-1}.s^{-1}$) and

k_{diss} is the dissociation rate constant (s^{-1}).

K_i, the equilibrium constant for inhibition, is defined (Bieth, 1980) as

$$K_i = \frac{k_{ass}}{k_{diss}}$$

To investigate the possible physiological effects of cystatins on trypanopain activity, K_i and k_{ass} for trypanopain association with human recombinant cystatin A, human L-kininogen, human L-kininogen domain 3, sheep cystatin B and human cystatin C were determined. The interaction between an enzyme and a reversible competitive inhibitor such as a cystatin can be investigated under either first or second order conditions (Salvesen and Nagase, 1989). Under first order conditions, the inhibitor is in excess over the enzyme (at least a 20-fold molar excess) and so the rate of reaction is essentially independent of enzyme concentration. Conversely, under second order conditions enzyme and inhibitor are at similar concentrations and the rate of reaction is dependent on the concentrations of both reactants. First order conditions were employed in this study, with inhibitors at a 20-fold molar excess over enzyme.

5.2.1 Materials

Papain assay buffer [350 mM KH_2PO_4, 50 mM Na_2HPO_4, 4 mM Na_2EDTA, 40 mM cysteine, 0.02% (m/v) NaN_3, pH 6.0]. KH_2PO_4 (49.9 g), $Na_2HPO_4.2H_2O$ (8.90 g), $Na_2EDTA.2H_20$ (1.49 g) and NaN_3 (0.2 g) were dissolved in about 950 ml of H_20, adjusted to 6.0 with KOH and made up to 1 l with H_2O. Cysteine (200 µl of 1 M solution) was added to 5 ml of assay buffer just before use.

Cystatins were donated by the following researchers: Human recombinant cystatin A: Dr Ingemar Björk (Department of Veterinary Medical Chemistry, Swedish University of Agricultural Sciences, Uppsala Biomedical Centre, Uppsala, Sweden); Human L-kininogen: Dr Rob Pike (Department of Biochemistry and Molecular Biology, Monash University, Victoria, Australia); Sheep cystatin B: Dr Theresa Coetzer (Department of Biochemistry, UNP); Human L-kininogen domain 3: Dr Ennes Auerswald (Abteilung für Klinische Chemie und Klinische Biochemie in der Chirurgischen Klinik und Poliklinik,

Klinikum Innenstadt, Ludwig-Maximilians-Universität München, Germany); Human cystatin C: Dr Magnus Abrahamson (Department of Clinical Chemistry, University of Lund, Malmö General Hospital, Malmö, Sweden).

5.2.2 Methods

The active concentration of commercial papain was determined by titration against E-64 as described in Section 3.4, except that the pH 6.0 assay buffer described in Section 5.2.1 was used. Each inhibitor was then titrated against papain as described in Section 3.4 to determine the active concentration of the inhibitor.

Trypanopain reaction velocity in the presence (v_i) and absence (v_o) of inhibitor was determined using a modification of the standard continuous assay described in Section 4.8.6. Trypanopain (0.05 nM final active concentration, 10 µl) was incubated with Brij-35 diluent (240 µl) and trypanopain assay buffer (500 µl) for 1 min at 30°C. The assay was performed at 30°C because association at 37°C was too fast for accurate measurement. Z-Phe-Arg-AMC (250 µl, 5 µM final concentration) was added and the reaction allowed to proceed until a steady-state velocity (v_o) was achieved. Inhibitor (1-5 nM final concentration, giving first order conditions) was then added and the reaction further monitored to determine v_i (Salvesen and Nagase, 1989). To ensure accuracy, the assay was repeated at various inhibitor concentrations (1-5 nM).

For data generated in this manner to be valid, linearity of the assay over the time period used was confirmed, as it is essential that any decrease in reaction velocity is due only to the addition of inhibitor and not to other factors such as enzyme instability or substrate depletion (Salvesen and Nagase, 1989). As a general rule, to ensure that reaction velocity does not change over the period of an assay due to substrate depletion, no more than 5% of the substrate present must be hydrolysed. Additionally, to ensure that first order conditions are maintained and that the rate of inhibition is independent of enzyme concentration, there must be at least a 20-fold molar excess of inhibitor over enzyme (Salvesen and Nagase, 1989). All of these conditions were satisfied in the present study.

5.2.3 Calculation of K_i

For tight-binding inhibitors, it is inappropriate to use variations of the Lineweaver-Burk or Dixon plots to analyse inhibition data, since these assume that the concentration of enzyme-complexed inhibitor is negligible compared to the total inhibitor concentration. This assumption does not hold if a substantial degree of inhibition is seen at approximately equimolar concentrations of enzyme and inhibitor, which is the case with tight-binding inhibitors (Bieth, 1995) such as cystatins. K_i should then be calculated from the equation (Henderson, 1972; Salvesen and Nagase, 1989; Abrahamson, 1994):

$$K_i = \frac{[I]}{\frac{v_o}{v_i} - 1} \Big/ 1 + \frac{[S]}{K_m}$$

where: [I] is the inhibitor concentration (M),

v_o is the reaction velocity without inhibitor present (arbitrary units),

v_i is the reaction velocity with inhibitor present (arbitrary units),

[S] is the substrate concentration (M) and

K_m is the Michaelis constant for the substrate used (M).

This equation is valid when the enzyme concentration used is of the same magnitude as the calculated K_i and the inhibitor is sufficiently in excess so that it is not appreciably depleted during the assay (Abrahamson, 1994). For an inhibitor to be considered physiologically relevant, its *in vivo* concentration must be at least 10 times K_i (Bieth, 1980).

5.2.4 Calculation of k_{ass}

k_{ass}, which gives a measure of how fast an inhibitor associates with an enzyme, can be calculated from pre-steady state experiments, by measuring the rate at which enzyme activity decreases when inhibitor is added (Lenarcic *et al.*, 1996). This is done by plotting the natural log of residual enzyme activity against time. The gradient of the linear portion of this curve gives the observed (or pseudo-first order) association rate constant, $-k_{obs}$, and the true association rate constant, k_{ass}, can then be calculated from the equation (Salvesen and Nagase, 1989):

$$k_{ass} = \frac{k_{obs}}{[I]} . 1 + \frac{[S]}{K_m}$$

where: [S] is the substrate concentration (M),
[I] is the inhibitor concentration (M) and
K_m the Michaelis constant for the substrate used (M).

k_{ass} can be used to predict the half life of association *in vivo*, using the equation (Bieth, 1980):

$$t_{½} = \frac{1}{k_{ass}.[I]}$$

where: $t_{½}$ is the half life of association (s),
k_{ass} is the association rate constant ($M^{-1}.s^{-1}$) and
[I] is the *in vivo* inhibitor concentration (M^{-1}).

If the enzyme is considered to be effectively inhibited after 5 half lives, then the delay time of inhibition *in vivo* can be considered as 5 times the half life (Bieth, 1980).

k_{ass} can also be used to calculate both a predicted dissociation rate constant ($k_{diss} = K_i \times k_{ass}$; Bieth, 1980) and thus the half life for dissociation for the enzyme-inhibitor complex, using the equation (Bieth, 1980):

$$t_{1/2} = \frac{0.693}{k_{diss}}$$

where: $t_{½}$ is the half life of dissociation (s) and
k_{diss} is the dissociation rate constant (s^{-1}).

5.3 Association with α_2-macroglobulin (α_2-M)

α_2-M is a non-specific proteinase inhibitor, reacting with all four classes of proteinases in an equimolar manner (Barrett and Starkey, 1973). The inhibitor is one of the major human plasma proteins and is structurally homologous to some of the complement proteins that contain internal thioester bonds. Proteinase cleavage of the exposed "bait-region" of α_2-M induces a conformational change in the inhibitor that physically entraps the proteinase (Barrett and Starkey, 1973). The change in conformation of the α_2-M molecule causes the α_2-M and its entrapped proteinase to be rapidly cleared from the circulation through the multiligand α_2-M receptor on monocytes (Ashcom *et al.*, 1990; Strickland *et al.*, 1990; Moestrup *et al.*, 1992). Due to the physical constraints of the entrapment, the trapped

enzyme retains full activity against small peptides and inhibitors of less than 10 kDa in size (Barrett, 1981). Thus, while the enzyme is inactive against physiological protein substrates, it remains active against synthetic substrates commonly used to monitor enzyme activity (Harpel, 1976). Only small changes in K_m and V_{max} for synthetic substrates are observed when an enzyme is bound to α_2-M. To test whether a proteinase is inhibited by α_2-M, the purified enzyme is mixed with the inhibitor and subjected to gel filtration chromatography. If the enzyme is inhibited by α_2-M, it will elute from the column as a high molecular mass complex and the increase in apparent molecular mass can be visualised as a shift in the elution position of activity against the enzyme's synthetic substrate. To confirm that the α_2-M/proteinase complex was inactive against protein substrates, hydrolysis of fluorescein isothiocyanate (FITC)-albumin was investigated (De Lumen and Tappell, 1974; Twining, 1984).

5.3.1 Materials

Chromatography buffer [50 mM Tris-Cl, 1 mM DTT, 0.02% (m/v) NaN_3, pH 8.0]. Tris (6.055 g), DTT (0.15 g) and NaN_3 (0.2 g) were dissolved in 900 ml of H_2O, adjusted to pH 8.0 with HCl and made up to 1 l with H_2O.

FITC-albumin (5 mg/ml). FITC-albumin (5 mg) was dissolved in 1 ml of 0.5% (m/v) Brij-35.

Stop solution [5% (m/v) TCA]. TCA (5 g) was dissolved in 90 ml of H_2O and made up to 100 ml with H_2O.

FITC diluent (500 mM Tris-Cl, pH 8.5). Tris (6 g) was dissolved in 60 ml of H_2O, adjusted to pH 8.5 and made up to 100 ml with H_2O.

5.3.2 Method

The active concentration of trypanopain was determined by titration against E-64 (with Z-Phe-Arg-AMC as the substrate), while the active concentration of α_2-M was determined by titration against E-64-titrated papain [with azocasein as the substrate (Barrett and Kirschke, 1981)]. Trypanopain and α_2-M were individually applied to a Sephacryl S-100 column (10 cm/h, 15×300 mm) equilibrated in chromatography buffer and the elution

position of the individual components determined (by appearance of the A_{280} peak for α_2-M and Z-Phe-Arg-AMC hydrolysis for trypanopain). Trypanopain (10 pmol, 300 ng) was incubated with an equimolar amount of α_2-M in trypanopain protein assay buffer (Section 4.8.6) allowing complete association (1 h, 37°C). The mixture was then applied to the Sephacryl S-100 column and the A_{280} profile and Z-Phe-Arg-AMC activity of the eluate monitored.

To investigate whether the α_2-M/proteinase complex was active against proteins, digestion of FITC-albumin was investigated. Control and α_2-M-complexed samples (10 ng trypanopain, 20 µl) were activated in trypanopain protein assay buffer (Section 4.8.6, 20 µl) and FITC-albumin (20 µl) added (24 h at 37°C). Stop solution was added (120 µl, 1 h, RT) and the solution centrifuged (10 000×*g*, 10 min, RT). An aliquot (60 µl) was diluted with 1 ml of FITC diluent and the fluorescence read on a Hitachi F-2000 spectrofluorometer (excitation 490 nm, emmision 525 nm). Fluorescence of control, uninhibited samples was compared with that of α_2-M-complexed samples.

5.4 Effect of enzyme inhibitors on Z-Phe-Arg-AMC hydrolysis by live *T. b. brucei*

Zymogram analyses of the proteinases of *T. b. brucei* and *T. congolense* have shown that the molecular masses of the proteinases from different parasite preparations can be highly variable. This variation is thought to be due, in part, to the effects of unknown components of mammalian serum on the proteinases (Lonsdale-Eccles and Mpimbaza, 1986; Lonsdale-Eccles and Grab, 1987a). Preliminary work by colleagues in this laboratory showed that trypanopain can associate with a kininogen-like molecule (called rat trypanopain-modulator, rTM) to produce additional bands of higher molecular mass proteolytic activity on zymograms. The effects of L-kininogen on trypanopain hydrolysis of Z-Phe-Arg-AMC in live *T. b. brucei* were investigated as part of a collaborative study investigating the *in vivo* significance of these novel, active complexes more directly (Lonsdale-Eccles *et al.*, 1995). However, since both trypanopain and the trypanosomal oligopeptidase OP-Tb hydrolyse Z-Phe-Arg-AMC, E-64 (which inhibits trypanopain) and leupeptin (which inhibits both trypanopain and OP-Tb) were first added to live *T. b. brucei* to estimate what percentage of the total Z-Phe-Arg-AMC hydrolysis occurring in live

parasites was contributed by each enzyme. Once this was known, the effects of L-kininogen on trypanopain activity alone could be investigated.

5.4.1 Materials

As per Section 3.3 and Section 3.2.1.

Inhibitors. Leupeptin (1 mg), E-64 (1 mg) and L-kininogen (1 mg) were dissolved in a 50:50 mixture of H_20:DMSO (1 ml) on the day of use.

5.4.2 Method

Live *T. b. brucei* (1.8×10^6 parasites/ml, kept on ice until required) were equilibrated to 25°C or 37°C in pH 7.4 PSG (Section 3.3). Z-Phe-Arg-AMC was then added and the increase in fluorescence monitored continuously as in Section 3.2.2. E-64, leupeptin or L-kininogen (100 µl, 0.1 mg/ml final concentration) were added to replicates of the above experiment to assess their effects on *T. b. brucei* hydrolysis of Z-Phe-Arg-AMC.

5.5 Is trypanopain active in the bloodstream of infected animals?

Studies in Section 5.2 suggest that trypanopain is likely to be effectively controlled in the bloodstream of infected mammals by endogenous cystatins. To test this prediction directly, the blood of experimentally infected rats and naturally infected cattle was tested for trypanopain activity. As controls, samples were also assayed for the presence of OP-Tb and endogenous mammalian kallikrein. Since all three enzymes hydrolyse Z-Phe-Arg-AMC, combinations of inhibitors were used to discriminate between the two serine proteinases (OP-Tb and kallikrein) and the cysteine proteinase, trypanopain. OP-Tb is not inhibited by large proteinaceous serine proteinase inhibitors such as SBTI (Troeberg *et al.*, 1996). This fact was used to discriminate between OP-Tb and SBTI-sensitive kallikrein activity. Z-Phe-Arg-AMC hydrolysis at pH 8.0 in the presence of SBTI (inhibits kallikrein) and E-64 (inhibits trypanopain) thus indicates the presence of active OP-Tb in infected sera, while similar hydrolysis at pH 7.0 in the presence of SBTI (inhibits kallikrein) and AEBSF (inhibits kallikrein and OP-Tb) indicates trypanopain activity in the test sera.

5.5.1 Materials

Inhibitors (120 µM SBTI, 12 mM E-64 and 12 mM AEBSF). SBTI (3.6 mg/1.5 ml), E-64 (6.4 mg/1.5 ml) and AEBSF (4 mg/1.5 ml) were dissolved in DMSO on the day of use.

Experimentally infected rat serum. Two adult rats were infected with *T. b. brucei* as in Section 3.3, while a control rat was injected with PSG alone. Infected rats were sacrificed at peak parasitaemia and the control rat sacrificed at the same time. Blood was left to stand at RT to allow complete coagulation and centrifuged (1 000×*g*, 10 min, 4°C) to remove fibrin clots and parasites. The supernatant was microscopically confirmed to contain no parasites.

Naturally infected cattle serum. Serum from cattle infected with *T. b. brucei* was a gift from Dr Olivier Matthee (Department of Protozoology, Onderstepoort Veterinary School). Control, uninfected cattle serum was obtained from the local abattoir.

Trypanopain synthetic substrate assay buffer. As per Section 4.8.6.

OP-Tb assay buffer (150 mM Tris-Cl, 0.45 M NaCl, 30 mM DTT, pH 8.0). Tris (0.9 g) and NaCl (0.46 g) were dissolved in 45 ml of H_2O, adjusted to pH 8.0 with HCl and made up to 50 ml with H_2O. DTT (4.6 mg/ml) was added just before use.

Kallikrein assay buffer (150 mM Tris-Cl, 0.45 M NaCl, pH 8.0). Tris (0.9 g) and NaCl (0.46 g) were dissolved in 45 ml of H_2O, adjusted to 8.0 with HCl and made up to 50 ml with H_2O.

5.5.2 Method

Replicates of infected or control serum (25 µl) were incubated with each assay buffer for 10 min at 37°C. Inhibitors (25 µl) were then added and the mixture incubated for a further 10 min at 37°C. Following addition of Z-Phe-Arg-AMC, fluorescence was monitored in stopped-time assays as described in Section 3.2.3.

5.6 Detection of trypanopain/cystatin complexes in infected serum

Sandwich ELISAs were conducted to determine whether trypanopain is present in the serum of infected animals in the form of inactive trypanopain/cystatin complexes. Detection of such complexes implies that trypanopain released from dying trypanosomes becomes tightly bound to endogenous cystatins. Test sera from experimentally infected rats were applied to ELISA plates pre-coated with anti-cystatin B or anti-kininogen antibodies. Both free and trypanopain-complexed cystatins or kininogens were expected to bind to these antibodies. Anti-trypanopain antibodies were then applied to determine whether any of the inhibitors captured on the ELISA plate were also bound to trypanopain.

5.6.1 Materials

As per Section 2.11.1

Antibodies. Rabbit anti-cystatin B IgG was a gift from Nicola Scholfield (Department of Biochemistry, UNP) and rabbit anti-human H-kininogen IgG was from CalBiochem (La Jolla, CA, USA).

5.6.2 Method

A variation on the ELISA described by Takeda *et al.* (1992) to detect cathepsin/kininogen complexes was devised. Rabbit anti-human H-kininogen IgG or rabbit anti-cystatin B IgG (25 µg/ml in 100 µl PBS per well, 4°C, 16 h) was adsorbed to the well of Nunc Immunosorb microtitre plates and remaining sites blocked with 0.5% (m/v) BSA-PBS (200 µl/well, 1 h, 37°C). Infected rat or cattle serum was incubated (100 µl of various dilutions per well, 2 h, 37°C). Chicken anti-trypanopain IgY [75 µg/ml in 120 µl per well of 0.5% (m/v) BSA-PBS, 2 h, 37°C] was applied as the second antibody layer and rabbit anti-IgY-HRPO as the detection antibody (150 µl/well, 1 h, 37°C). ABTS/H_2O_2 (150 µl/well) was used as the substrate and the A_{405} monitored as described in Section 2.11.

5.7 Results

5.7.1 Inhibitor profile

In terms of inhibitor sensitivity, trypanopain was shown to be a fairly typical cysteine proteinase. Typical cysteine proteinase inhibitors (including E-64 and IAA, IAN and NEM) inhibited trypanopain completely, while typical serine and metálloproteinases inhibitors (such as DFP and EDTA, respectively) had no effect on enzyme activity. Of the alkylating agents, IAA was the most effective, inhibiting trypanopain activity by 100% at 0.1 mM. By comparison, IAN was only 36% effective at this concentration, while NEM was ineffective (Table 7). Leupeptin, antipain and chymostatin were all effective inhibitors of trypanopain (99-100% inhibition). Data from previous studies for the related enzymes trypanopain-Tc and cruzipain are given for comparison.

Interestingly, the aspartic proteinase inhibitor pepstatin A also inhibited trypanopain (by 22%). Pepstatin A inhibition of trypanopain was further investigated by calculating the apparent K_m and V_{max} in the presence of the inhibitor. K_m was substantially increased (by approximately 50%) while V_{max} remained unchanged, indicating that pepstatin A is a competitive trypanopain inhibitor (Price and Stevens, 1989) with a K_i of 207±51 μM.

5.7.2 Kinetics of association between trypanopain and various cystatins

k_{ass} and predicted k_{off} values for trypanopain association with various cystatins were in the 10^7-10^8 $M^{-1}.s^{-1}$ and 10^{-4} s^{-1} range respectively, while K_i values were in the 10^{-11} M range (Table 8). Of the inhibitors tested, L-kininogen domain 3 and cystatin C associated fastest with trypanopain (10^8 $M^{-1}.s^{-1}$). The slowest dissociation was observed for L-kininogen (8.8×10^5 s^{-1}).

Table 7: Effects of various proteinase inhibitors on the activity of trypanosomal enzymes against Z-Phe-Arg-AMC.

Trypanopain from *T. b. brucei* (2 ng) was incubated with each of the inhibitors below (15 min, 37°C) and residual activity against Z-Phe-Arg-AMC measured in quintuplicate stopped-time assays as described in Section 3.2.3. Activity was compared to the appropriate solvent-containing controls in all cases. Data from previous studies for trypanopain-Tc (from *T. congolense*) and cruzipain (from *T. cruzi*) are given for comparison.

Inhibitor	**Proteinases inhibited** [1]	**Concentration** (mM)	**Trypanopain activity** (% of control)	**Trypanopain-Tc**[2]	**Cruzipain**[2]
E-64	C	0.001	0	+	+
IAN	C	10	0	+	+
IAA	C	0.1	0	+	+
NEM	C	10	0	nd	+
TLCK	S & C	1	0	nd	+
TPCK	S & C	1	0	nd	+
Leupeptin	S & C	0.1	0	+	+
Chymostatin	S & C	0.1	1	+	+
Antipain	S & C	0.1	0	+	+
AEBSF	S	0.5	100	nd	nd
DFP	S	10	100	nd	nd
PMSF	S	10	100	+	0
SBTI	S	0.01	100	+	0
EDTA	M	10	100	nd	nd
Pepstatin A	A	0.1	78	+	0

Note 1. C cysteine proteinase; S serine proteinase
A aspartic proteinase; M metalloproteinase
2. + inhibited
0 unaffected
nd not done

Data from Steiger *et al.* (1979); Rautenberg *et al.* (1982); Lonsdale-Eccles and Mpimbaza (1986); Lonsdale-Eccles and Grab (1987a); Cazzulo *et al.* (1989); Murta *et al.* (1990); Healy *et al.* (1992); Mbawa *et al.* (1992).

Table 8: Association, dissociation and inhibition rate constants for cystatin inhibition of trypanopain (0.05 nM) at 30 °C.

Inhibitor	**K_i** (nM)	**k_{ass}** ($M^{-1}.s^{-1}$)	**Predicted k_{off}** (s^{-1})
Cystatin A	0.045	2.13×10^{7}	9.6×10^{-4}
Sheep cystatin B	0.004	6.9×10^{7}	2.8×10^{-4}
Cystatin C	0.001	1.7×10^{8}	1.7×10^{-4}
L-kininogen	0.0035	2.5×10^{7}	8.8×10^{-5}
L-kininogen domain 3	0.0044	1.1×10^{8}	4.8×10^{-4}

Note: 1. Cystatin C and L-kininogen domain 3 association with trypanopain was too rapid for accurate measurement without stopped-flow apparatus and the value given is thus only an estimate.

2. Results represent the average of quintuplicate at each of four different inhibitor concentrations assays.

As L-kininogen and cystatin C are the most abundant cystatins in blood (Abrahamson, 1993), the half-lives of association and dissociation were calculated for these inhibitors. These calculations, using physiological concentrations of inhibitors cited by Abrahamson *et al.* (1986), showed that trypanopain associates with both L-kininogen and cystatin C within fractions of a second and that the inhibitor-enzyme complex dissociates fairly slowly (over a period of approximately 1-2 hours, as shown in Table 9). Thus trypanopain is likely to be inhibited effectively by cystatin C and (especially) L-kininogen *in vivo*.

Table 9: Half lives for association and dissociation of trypanopain and selected cystatins at physiological concentrations.

Inhibitor	**Physiological concentration**	**Half life of association** (s)	**Half life of dissociation** (min)
L-kininogen	7.5 μM	0.006	131
Cystatin C	80 nM	0.08	68

5.7.3 Association with α_2-M

α_2-M alone eluted from Sephacryl S-100 in the void volume, while trypanopain alone eluted in the included volume. Following incubation with α_2-M, trypanopain eluted in the void volume, indicating that the enzyme had associated with α_2-M (Figure 30). The α_2-M-complexed enzyme was inactive against FITC-albumin, showing that inhibition of proteinase activity had occurred.

5.7.4 Effects of inhibitors on Z-Phe-Arg-AMC hydrolysis by live *T. b. brucei*

Leupeptin (which inhibits both trypanopain and OP-Tb) consistently inhibited Z-Phe-Arg-AMC hydrolysis by nearly 100% at both 25°C and 37°C, while E-64 (inhibits only trypanopain) inhibited hydrolysis by approx. 50%. This indicates that approximately half of the Z-Phe-Arg-AMC hydrolysis by live *T. b. brucei* can be ascribed to trypanopain. In the presence of L-kininogen, a 10-20% increase in the rate of hydrolysis of Z-Phe-Arg-AMC was observed. This suggests that trypanopain activity was increased by 20-40%, and that *in vitro* L-kininogen interacts with trypanopain in live *T. b. brucei* in an unusual, activity-enhancing manner.

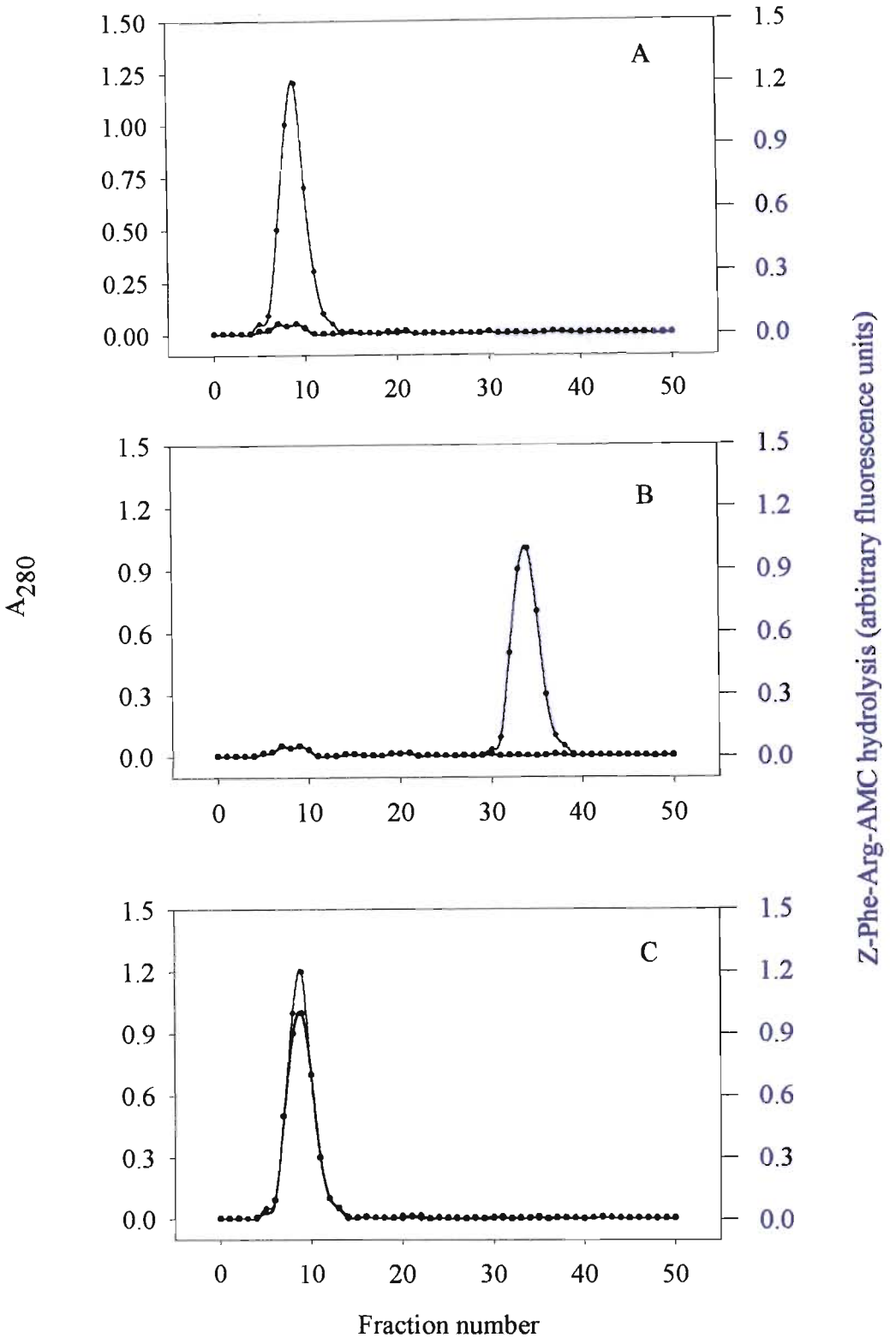


Figure 30: Association between trypanopain and α_2-M.

α_2-M alone (A) eluted in the void volume of Sephacryl S-100, while trypanopain alone (B) eluted in the included volume. After pre-incubation with α_2-M, trypanopain activity against Z-Phe-Arg-AMC eluted in the void volume (C), indicating that it had associated with α_2-M.

5.7.5 Is trypanopain active in the blood of infected rats?

Very low levels of Z-Phe-Arg-AMC hydrolysis were detected in the serum of control, uninfected animals, while high levels of activity against Z-Phe-Arg-AMC were detected in infected rats (Figure 31) and cattle. This activity was insensitive to E-64 and SBTI, but entirely eliminated by AEBSF. This indicates, firstly, that OP-Tb was predominantly responsible for the hydrolysis observed and that it is present in an active form in the bloodstream of infected animals. Secondly, this study suggests that trypanopain is effectively controlled in the mammalian bloodstream, supporting the conclusion arrived at from *in vitro* studies with the purified enzyme and purified serum cystatins. Zymogram analyses of infected samples supported the results of this experiment, with no proteolytic activity detectable in infected sera (results not shown).

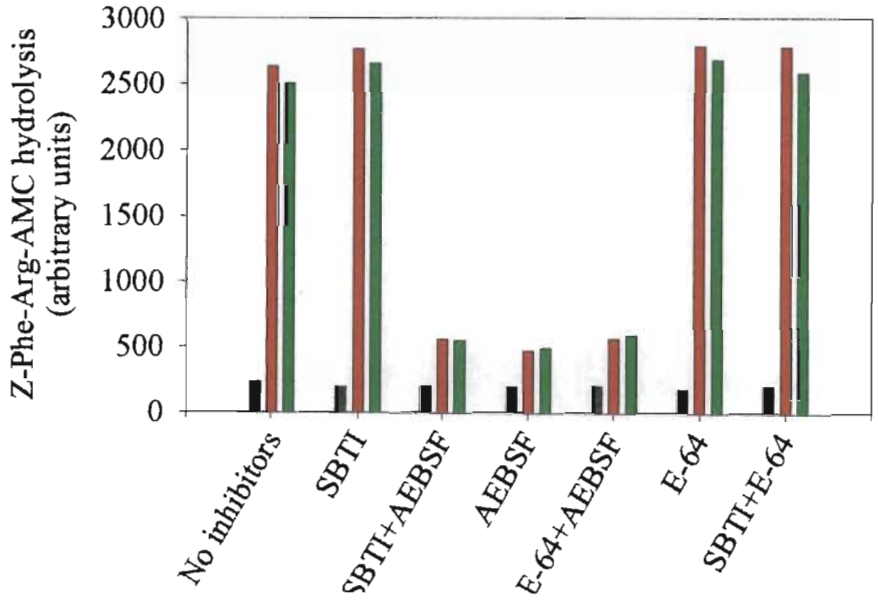


Figure 31: Z-Phe-Arg-AMC activity in the sera of control and infected animals.
Infected rat (■) and cattle (■) serum or control, uninfected serum (■) (25 µl) was incubated with assay buffer for 10 min at 37°C. Inhibitors (25 µl) were then added and the mixture incubated for a further 10 min at 37°C. Following addition of Z-Phe-Arg-AMC, fluorescence was monitored in a microplate fluorimeter. Results represent the average of quintuplicate assays.

5.7.6 Detection of trypanopain/cystatin complexes in infected serum

Sandwich ELISAs showed that trypanopain/cystatin B and trypanopain/H-kininogen complexes are present in the serum of infected rats (Figure 32) and cattle. This implies that trypanopain released from dying trypanosomes is tightly bound to cystatins in the bloodstream. Slightly higher levels of H-kininogen complexes were detected.

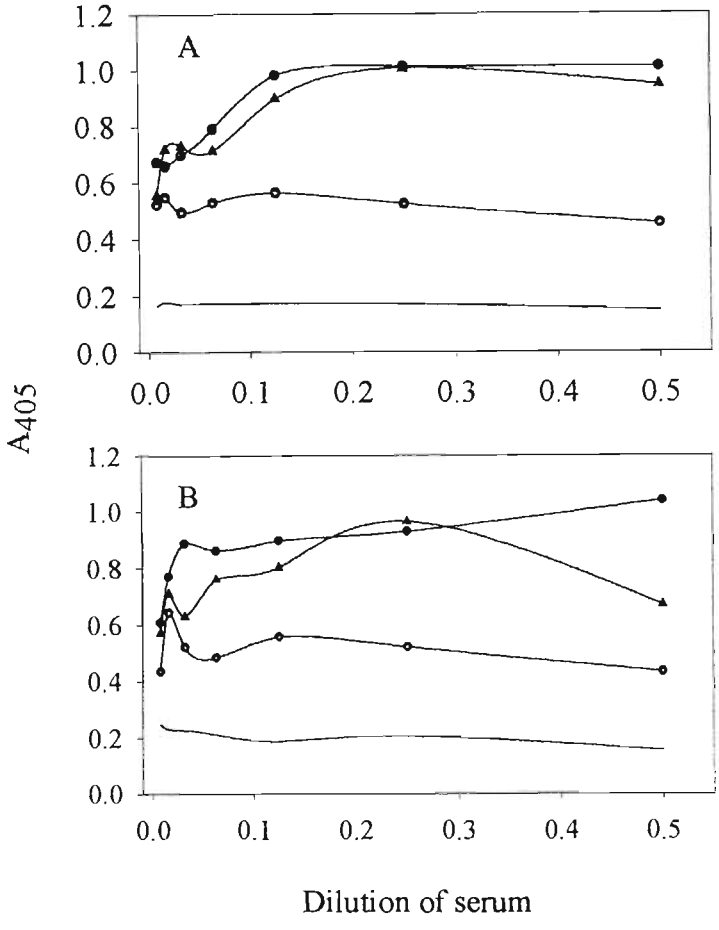


Figure 32: Detection of trypanopain/cystatin complexes in the serum of *T. b. brucei* infected rats.

Rabbit anti-human H-kininogen IgG (A) and rabbit anti-cystatin B IgG (B) (25 µg/ml) were coated as the first antibody layer and infected rat serum (various dilutions) applied as the antigen layer, followed by IgY anti-trypanopain (75 µg/ml) as the second antibody layer. Rabbit anti-IgY-HRPO and ABTS/H_2O_2 were used as the detection system. Absorbance readings at 405 nm of serum from infected rats (● and ▲), uninfected control animals (—) and non-immune antibodies (○) represent the average of duplicate determinations.

5.8 Discussion

Trypanopain has a fairly typical cysteine proteinase inhibitor profile, in agreement with the inhibitor profiles of trypanopain-Tc and cruzipain. Trypanopain is sensitive to E-64, cystatins and alkylating agents, but insensitive to typical serine and metalloproteinase inhibitors (such DFP and EDTA respectively). Interestingly, the aspartic proteinase inhibitor pepstatin inhibited trypanopain to some extent, as has been previously reported for partially purified trypanopain from *T. b rhodesiense* (Pamer *et al.*, 1989) and trypanopain-Tc (Mbawa *et al.*, 1992). Pepstatin A does not, however, inhibit cruzipain (Cazzulo *et al.*, 1989). The inhibitor was found to increase trypanopain's apparent K_m for Z-Phe-Arg-AMC (by approx. 50%) while V_{max} remained unchanged, indicating that pepstatin A is a competitive inhibitor of trypanopain (K_i calculated to be 207±51 μM). The competitive nature of its inhibition suggests that this pentapeptide-like compound (Salvesen and Nagase, 1989; Kay *et al.*, 1983) acts as a pseudo-substrate for trypanopain. It may bind into the trypanopain active site differently from its mode of binding with aspartic proteinases.

Trypanopain was shown to associate with α_2-M, suggesting that this inhibitor would inhibit any trypanopain present in the mammalian bloodstream. At the time this study was undertaken, this was the first report of α_2-M association with a trypanosomal proteinase (Troeberg *et al.*, 1996). Recently however, Morrot *et al.* (1997) showed that cruzipain is also effectively inhibited by α_2-M. They further showed that cruzipain/α_2-M complexes are effectively taken up by monocytes through the multiligand α_2-M receptor, resulting in enhanced presentation of cruzipain-derived peptides to $CD4^+$ T-cells. This indicates that binding of α_2-M to cruzipain enhances immune responses against the enzyme. It is likely that the same is true in African trypanosomiasis, where trypanopain-Tc is reportedly one of the major antigens recognised in cattle (Authié *et al.*, 1992).

This study also showed that trypanopain is similar to cathepsin L (Barrett *et al.*, 1986) and papain (Abrahamson, 1993) in its interactions with cystatins *in vitro* (Table 10). Later studies showed that cruzipain is also similar to these enzymes in its association with cystatins (Stoka *et al.*, 1995). All four enzymes have very similar K_i and k_{ass} values for the various cystatins. In all three cases, association is very fast and the resultant complexes dissociate very slowly.

The time required for association and dissociation of a tight-binding, reversible inhibitor with an enzyme is partially dependent on the concentration of the inhibitor. When assessing the possible physiological effects of cystatins on an enzyme, the physiological concentrations of the cystatins must thus be considered. Cystatin C and L-kininogen are the most abundant cystatins in blood, with *in vivo* concentrations of 80 nM and 7.5 μM respectively (Abrahamson *et al.*, 1986). For an inhibitor to be considered physiologically relevant, its *in vivo* concentration must be at least 10 times K_i (Bieth, 1980). Clearly, this holds for cystatin C and L-kininogen and so these inhibitors are likely to effectively inhibit trypanopain in the bloodstream of *T. b. brucei*-infected animals. Since cystatins are relatively conserved between species (Barrett *et al.*, 1986) and the trypanosomal cysteine proteinases sequenced to date are highly homologous (Mottram *et al.*, 1989; Pamer et al., 1990; Eakin *et al.*, 1992; Fish *et al.*, 1995), the present study suggests that trypanopains from various trypanosome species are likely to be effectively inhibited by cystatins in a variety of mammalian hosts.

Table 10: Comparison of the inhibition of trypanopain, cathepsin L, cruzipain and papain by various cystatins.

	Cystatin A	Cystatin B	Cystatin C	L-kininogen	L-kininogen domain 3
K_i (nM)					
Cathepsin L	1.3	0.23	0.005	0.0017	0.005
Papain	0.019	0.12	0.005	0.0015	0.0030
Trypanopain	0.045	0.004	0.001	0.0035	0.0044
k_{ass} ($M^{-1}.s^{-1}$)					
Cathepsin L	3.8×10^7	2.2×10^7	nd	1×10^7	1×10^8
Papain	4.31×10^6	2.2×10^7	1.1×10^7	2×10^7	3×10^7
Cruzipain	3.4×10^6	3×10^7	7.9×10^7	1.8×10^7	nd
Trypanopain	2.13×10^7	6.9×10^7	1.7×10^8	2.5×10^7	1.1×10^8

Note: 1. nd not determined

2. Data from Barrett *et al.* (1986), Abrahamson (1993), Turk *et al.* (1995), Stoka *et al.* (1995), Lenarcic *et al.* (1996) and the present study.

The blood of experimentally infected rats was assayed for trypanopain activity to test this prediction. High levels of OP-Tb activity were detectable, as had been predicted from *in vitro* studies with serpins (Troeberg *et al.*, 1996). However, as expected, no trypanopain-like activity was detectable in any of the tested samples. Furthermore, sandwich ELISAs carried out on infected rat serum showed that detectable levels of trypanopain/cystatin and trypanopain/kininogen complexes are present in infected serum, but that these were inactive against Z-Phe-Arg-AMC. This suggests that trypanopain is effectively controlled in the mammalian bloodstream by association with cystatins. A previous study of Nwagwu *et al.* (1988) possibly supports this conclusion. In their study, isolated live parasites were shown to release two proteinases active against BSA: one which is active at pH 5.4 (thought to be trypanopain) and a second which is active at pH 8.0. However, when rat plasma was added to the isolated parasites, the trypanopain-like activity at pH 5.4 was not observed, suggesting that host plasma cysteine proteinase inhibitors, possible cystatins, effectively inhibited the released trypanopain.

However, a kininogen-like molecule from rat serum reportedly enhances trypanopain activity in fibrinogen zymograms of lysed *T. b. brucei* (Lonsdale-Eccles *et al.*, 1995). This molecule, rTM, cross-reacts to some extent with anti-human kininogen antibodies. rTM has many other features in common with kininogens, such as its size (68 kDa), its acid and heat stability, and the presence of 15 kDa proteinase-sensitive domains. Additionally, L-kininogen was shown to enhance trypanopain activity on fibrinogen zymograms in a similar manner to rTM and so Lonsdale-Eccles *et al.* (1995) conclude that kininogen-like molecules have the potential to enhance trypanopain activity. The present study showed that leupeptin inhibited Z-Phe-Arg-AMC hydrolysis by live *T. brucei* by 100%, while E-64 inhibited activity by 50%. This suggests that about half of the Z-Phe-Arg-AMC hydrolysis by live *T. brucei* is contributed by trypanopain and the other half by OP-Tb (Kornblatt *et al.*, 1992). L-kininogen was shown in the present study to mediate a 10 to 20% increase in Z-Phe-Arg-AMC hydrolysis, which suggests that it increased trypanopain activity by 20-40%. The present study, however, clearly indicates that purified human L-kininogen is an effective inhibitor of purified trypanopain with a K_i of 0.0035 nM. Pike *et al.* (1992) reported a similar apparent contradiction in the interaction between mammalian cathepsin L and cystatin B. While purified cystatin B inhibited purified cathepsin L as expected,

abnormally active covalent complexes of the enzyme and inhibitor were shown to form *in vivo*. An unidentified factor, which may be absent in *in vitro* studies using purified components, may contribute to the formation of these active complexes *in vivo* (Dr Robert Pike, Department of Biochemistry and Molecular Biology, Monash University and Dr Theresa Coetzer, Department of Biochemistry, UNP, personal communication). It is possible that this putative additional factor modifies the interaction between trypanopain within live trypanosomes and L-kininogen *in vivo*, resulting in the formation of active complexes as reported by Lonsdale-Eccles *et al.* (1995).

There are thus two apparently contradictory bodies of evidence concerning the effects of cystatins on trypanopain in the mammalian bloodstream. Firstly, trypanopain is effectively inhibited *in vitro* by all tested cystatins, no trypanopain-like activity is detectable in the blood of infected animals and *in vivo*-formed cystatin/trypanopain complexes are inactive against Z-Phe-Arg-AMC. On the other hand, L-kininogen enhances trypanopain-like Z-Phe-Arg-AMC hydrolysis by live *T. b. brucei* and also enhances trypanopain-like hydrolysis of fibrinogen by lysed *T. b. brucei* on zymograms. The most convincing and apparently irrefutable piece of data is that no trypanopain activity is detectable in the bloodstream of infected animals. The assay used was shown to be sensitive enough to detect the presence in the bloodstream of another trypanosomal proteinase, OP-Tb, which contributes 50% of the Z-Phe-Arg-AMC hydrolysis by live parasites, so it is unlikely that the assay is not sensitive enough to detect trypanopain.

If L-kininogen does activate the enzyme, why is no activity detectable in the bloodstream? It was considered possible that L-kininogen does enhance trypanopain activity *in vivo*, but that the enzyme is effectively inhibited by another proteinase inhibitor, e.g. α_2-M. However, sandwich ELISAs showed that cystatin B/trypanopain and kininogen/trypanopain complexes formed *in vivo* are not active against Z-Phe-Arg-AMC. Alternatively, L-kininogen may activate trypanopain in live *T. b. brucei*, but not once the enzyme is outside of the trypanosome, in the bloodstream. A trypanosomal component may alter the interaction between trypanopain and L-kininogen in the parasite, but be ineffective in the bloodstream. It is considered most likely that the enhancement of trypanopain activity seen in zymograms is an artifact of the method used. Michaud *et al.* (1996) reported that cystatin/cysteine proteinase complexes with K_i values above 10 nM

are likely to dissociate during electrophoresis, while those with K_i values below 10 nM remain intact. However, the authors reported that papain/ chicken egg white cystatin complexes (K_i of 5 pM) dissociate during electrophoresis, enabling the enzyme to regain its activity on zymograms. This suggests that the active trypanopain/kininogen complexes reported by Lonsdale-Eccles *et al.* (1995) and in the present study may be the result of dissociation of inhibitory complexes during electrophoresis. The high sensitivity of zymogram assays may have additionally inflated the apparent amount of activity seen at higher molecular weights. It is interesting that Michaud *et al.* (1996) only report activity at the molecular mass of the studied proteinase, while Lonsdale-Eccles *et al.* (1995) and the present study report additional activity at increased molecular masses. Whether this occurs only with *Trypanosoma* and *Leishmania* remains to be investigated.

In summary, the majority of available evidence suggests that trypanopain is inactive in the mammalian bloodstream. Most convincingly, all *in vivo* data shows no enzyme activity. It is thus considered most likely that trypanopain is indeed effectively inhibited in the mammalian bloodstream by cystatins and α_2-M. However, the possibility that active trypanopain/cystatin complexes form under certain as yet undefined conditions cannot be excluded.

Following investigation of the effects of mammalian proteinase inhibitors on trypanopain activity, the effects of anti-trypanopain antibodies on enzyme activity were investigated. The following chapter describes the production of antibodies against a peptide from the active site of trypanopain and evaluation of their effects on trypanopain activity against a variety of substrates.

Chapter 6

Production and evaluation of anti-peptide antibodies against a peptide from the trypanopain active site

6.1 Introduction

Trypanopain-Tc and cruzipain have been identified as major antigens recognised by cattle and humans in *T. congolense* and *T. cruzi* infections respectively (de Souza *et al.*, 1990; Murta *et al.*, 1990; Authié *et al.*, 1993; González *et al.*, 1996), while cysteine proteinases of the helminth *Spirometra mansoni* (Kong *et al.*, 1997) and the nematode *Nippostrongylus brasiliensis* (Kamata *et al.*, 1995) are also major antigens recognised by infected hosts. It is thus likely that trypanopain from *T. b. brucei* is also highly immunogenic in natural infections. Authié *et al.* (1993) report that production of anti-trypanopain-Tc antibodies correlates with resistance to trypanosomiasis in cattle, suggesting that immuno-targeting of trypanopain is beneficial to the infected host. Various authors have suggested that parasite cysteine proteinases may contribute to disease pathogenesis by degrading host proteins (McKerrow, 1993; McKerrow *et al.*, 1993; Robertson *et al.*, 1996), so it is tempting to speculate that immuno-targeting of trypanopain plays a role in disease resistance, possibly by antibody inhibition of trypanopain activity. Binding of anti-enzyme antibodies to enzymes reportedly has varied effects, with inhibition or enhancement of activity, as well as no alteration in enzyme activity being observed (Richmond, 1977). The possible inhibitory effects of anti-trypanopain antibodies were investigated in the present study by producing and evaluating antibodies directed against a peptide corresponding to a portion of the trypanopain active site. The active site was targeted because of the increased likelihood that anti-active site antibodies would modulate enzyme activity compared with antibodies directed against other regions of the enzyme.

Production of antibodies against a protein of interest can be accomplished in various ways, to produce antibodies with different properties. The method chosen is thus dependent on experimental requirements. For the purposes of this study, a versatile yet specific antibody preparation was required to recognise the enzyme in its native form (to assess the effects of the antibodies on enzyme activity) and preferably also in its denatured form (for use in ELISAs, Western Blots and immunocytochemical labelling). The most common method of raising antibodies is immunisation of an experimental animal with a whole protein (to produce

polyclonal anti-protein antibodies), but production of anti-peptide (to produce polyclonal anti-peptide antibodies) or monoclonal antibodies is also possible. Each of these methods was evaluated in the context of the experimental requirements of the present study.

Since a whole protein contains many epitopes, immunisation of an experimental animal with such an antigen stimulates several B cell clones and results in the production of antibodies of many different specificities. The heterogeneity of such polyclonal antisera makes them unsuitable for applications that require antibodies of a single specificity, as only a fraction of the antibodies elicited are directed against any one part of the protein. Immunisation with the whole enzyme was not considered suitable for this study since antibodies exclusively recognising the active site were required.

A homogenous antibody population of single and known specificity can be produced from a single B-cell clone and these are thus termed monoclonal antibodies (mAbs) (Köhler and Milstein, 1975). Such clones are generated by fusing a single primed B-cell with an immortal cancerous cell to produce an immortal B-cell hybridoma. While the absolute specificity of mAbs is attractive, they are expensive and time-consuming to produce. Their specificity can also limit the applications of these antibodies, because they only recognise a single conformation of the antigen and are thus not sufficiently versatile for some of the applications required for this study.

An animal immunised with a single peptide from a target protein produces what are known as anti-peptide antibodies. In addition to recognising the free peptide, these antibodies cross-react with the target protein, provided that some of the conformations adopted by the peptide in solution mimic those adopted in the corresponding region in the target protein. Since several B-cell clones are stimulated by the peptide, the antibodies are polyclonal, but because they are all directed against the selected peptide, they have specificity more similar to mAbs than to anti-protein polyclonal antibodies (Lerner, 1984). The specificity of such anti-peptide antibodies for the target protein arises from the fact that a sequence of ten or more residues is likely to be unique to a particular protein. In addition to their specificity, anti-peptide antibodies are versatile because they recognise the target sequence in many different conformations and all have different "reading frames" in that they bind to different, overlapping regions of the peptide. This versatility presented an advantage for this study, since it increased the likelihood that the

antiserum would recognise whole trypanopain in both its native and denatured forms. This versatility of anti-peptide antibodies is their greatest advantage over mAbs. The combined versatility and specificity of anti-peptide antibodies thus resulted in the decision to produce anti-trypanopain antibodies by immunising experimental animals with a peptide from the active site of trypanopain.

6.2 Choosing an immunogenic peptide

The choice of an immunogenic peptide was based on a number of factors. A peptide from the active site region was chosen because antibodies against this region were considered more likely to modulate trypanopain activity than antibodies directed against other regions of the enzyme. In addition, the peptide had to be immunogenic, i.e. able to stimulate B-cells in the experimental animal to produce antibodies. Most methods of predicting antibody-eliciting B-cell epitopes are based on the premise that such epitopes are usually located on the surfaces of proteins, where they can interact with antibodies. The three-dimensional structure of the protein can be studied to identify surface regions or, where such structural data is unavailable, there are various methods for predicting stretches of surface-located amino acids from the primary sequence of the protein. Most of these methods assign each amino acid a numerical value on a scale, which is then applied to the primary sequence of a protein whose three-dimensional structure is unknown. Some of the methods are listed below:

- Hopp (1985) constructed an acrophilicity scale based on the frequency with which each amino acid appeared on the surface of proteins of known three-dimensional structure.
- Surface residues are usually more mobile and flexible than internal residues and crystallographic measurement of this mobility has been used to assign atomic temperature factors (also known as B-factors, or Debye-Waller factors) to the various amino acids. Karplus and Schulz (1985) devised an epitope prediction method based on B-factors, which Van Regenmortel (1986) suggests is the most reliable of the presently available prediction methods.
- Hydrophobic amino acid residues are typically buried within the protein core to minimise interactions with the polar environment, while hydrophilic side chains are usually located on the surface to maximise interactions with water. Stretches of hydrophilic residues are thus more likely to be surface-located and accessible to antibodies (Hopp and Woods, 1981; Kyte and Doolittle, 1982). In some cases, hydrophilicity peaks of stretches of polypeptide

chains have been shown to correspond with known protein epitopes (Hopp, 1986), although Hopp and Woods (1981) caution that "not all antigenic determinants are associated with high points of hydrophilicity and not all high points are associated with antigenic determinants". Nevertheless, they maintain that one determinant is always located at the highest point of hydrophilicity.

The three-dimensional structure of trypanopain is not yet known, which precluded a direct search for surface-located regions. Trypanopain is, however, 38% similar to papain (Mottram *et al.*, 1989), the three-dimensional structure of which is known (Drenth *et al.*, 1971; Figure 33), thus allowing consideration of the papain structure for the prediction of trypanopain epitopes. To increase the likelihood of success, epitope prediction algorithms, such as those listed above, were also used. Such a combination of methods has been successful for choosing an immunogenic peptide from the homologous enzyme, cathepsin L, where antibodies produced against the chosen active site peptide cross-reacted well with the native enzyme (Coetzer *et al.*, 1991). In light of the success of this previous study, a peptide analogous to that chosen from cathepsin L by Coetzer *et al.* (1991) was used in the present study. The 16-residue peptide chosen corresponds with residues 274 to 289 in the primary sequence of trypanopain (Mottram *et al.*, 1989) and includes the active site histidine residue, increasing the likelihood that antibodies would inhibit enzyme activity if this histidine residue is exposed in the native enzyme. The sequence of the chosen peptide (residues 274-289) in trypanopain is

ILTSCTSKQLDHGVL

where H represents the active site histidine. Some modifications to the original sequence were made in the synthesis of the peptide, namely

- an N-terminal cysteine was added to allow thioether coupling to a carrier molecule,
- the internal cysteine residue was synthesised as an α-amino butyric acid residue to prevent dimerisation of peptides via disulfide bonds (Muller, 1988), and
- the C-terminal end was synthesised as an amide, mimicking an uncharged peptide bond and hopefully increasing cross-reactivity with whole trypanopain.

The peptide (1461.93 Da) was synthesised by the Peptide Synthesis Core Facility at the University of Georgia, Athens, USA. The chosen peptide was expected to be immunogenic as it is greater than ten residues in length; is exposed in the parent protein and contains a local peak

of flexibility, mobility, accessibility and hydrophilicity (Figure 34). The trypanopain peptide chosen shows high sequence homology with corresponding regions of other trypanosomal cysteine proteinases, papain and mammalian cathepsin L. This is particularly marked immediately adjacent to the active site histidine residue (shown in bold in Table 11).

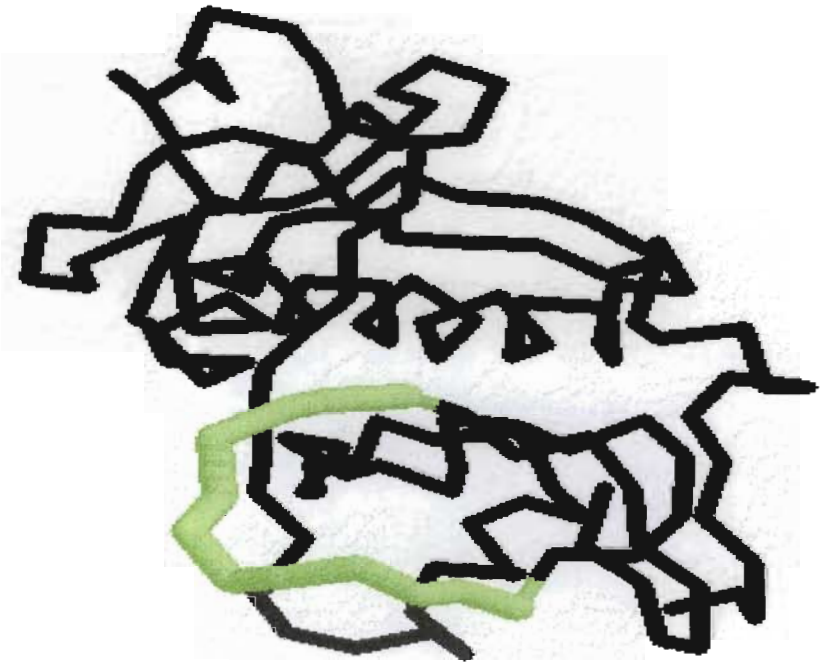


Figure 33: Schematic representation of the three-dimensional structure of papain.
The region corresponding to the chosen trypanopain peptide is shown in green. After Drenth *et al.* (1971).

Table 11: Amino acid sequence homology between the chosen trypanopain peptide and corresponding regions of other trypanosomal cysteine proteinases, human cathepsin L and papain.

Enzyme	**Sequence**	**Reference**
Trypanopain	IL-TSCTSKQLD**H**GVL	Mottram *et al.* (1989)
Trypanopain-Tc	VL-TSCISKGLD**H**DVL	Fish *et al.* (1995)
Cruzipain	VM-TSCVSEQLD**H**GVL	Cazzulo *et al.* (1989)
Human cathepsin L	IYFEPDCSSEDMD**H**GVL	Gal and Gottesman (1988)
Papain	I-FVGPC-GNKVD**H**AVA	Cohen *et al.* (1986)

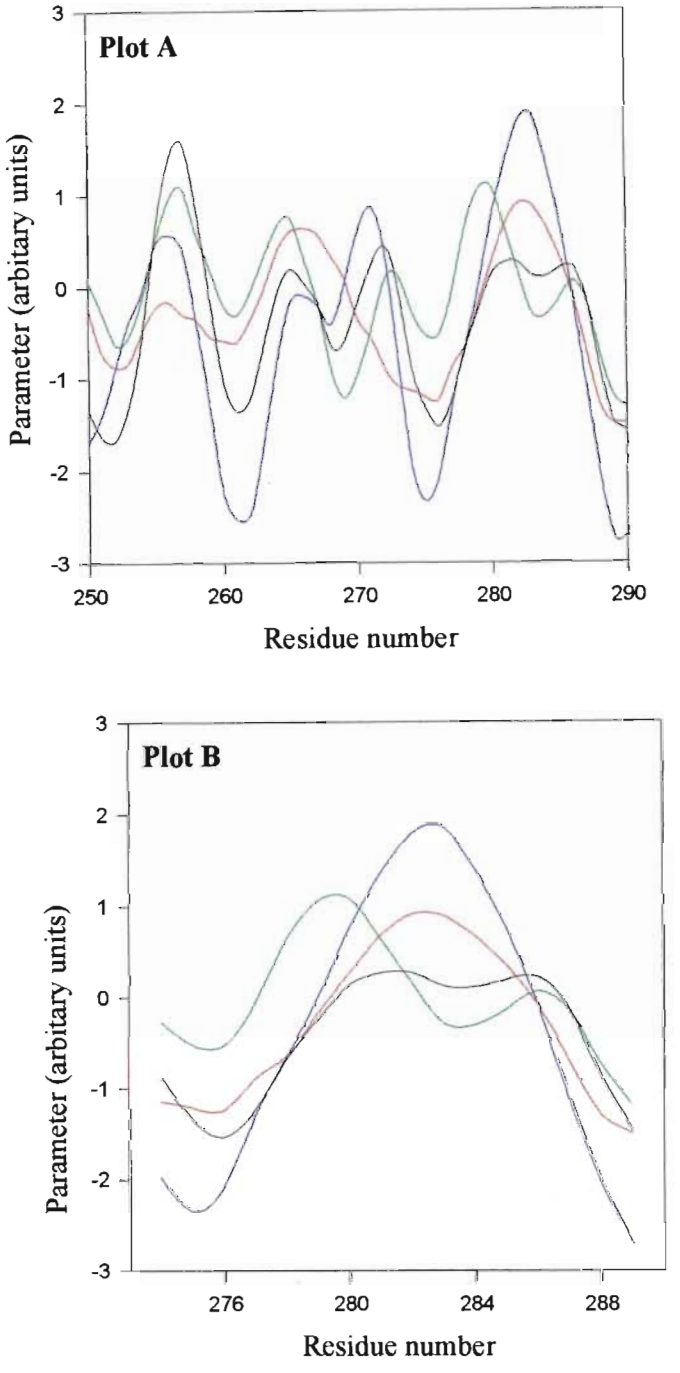


Figure 34: Epitope prediction plots for regions of trypanopain.

Plot **A** shows epitope prediction plots for residues 250 to 290 of trypanopain and Plot B shows epitope prediction plots for the peptide sequence (trypanopain residues 274-289) selected for anti-peptide antibody production. The Y-axis has arbitrary units of hydrophilicity (—), accessibility (–), flexibility (—) and antigenicity (—). A software package, Predict7 (Cármenes *et al.*, 1989) was used to generate the data.

6.3 Peptide conjugation

Although peptides of 10 residues or longer are considered immunogenic by some authors (Harlow and Lane, 1988), peptides are usually coupled to carrier proteins before immunisation, increasing their immunogenicity up to 1000-fold (Mariani *et al.*, 1987). The selected peptide was conjugated to carrier proteins using the coupling reagent meta-maleimidobenzoyl N-hydroxysuccinimide ester (MBS) as described by Kitagawa and Aikawa (1976), using the modifications of Liu *et al.* (1979). This heterobifunctional reagent acylates amino groups via its active ester and also forms thioether bonds with sulfhydryl groups (Kitagawa and Aikawa, 1976). In this study, MBS was used to link peptides to carrier proteins by the acylation of amino groups on the carrier protein and subsequent formation of thioether bonds with the N-terminal cysteine of the peptide. Due to the heterobifunctional nature of MBS, conjugation must be carried out in two distinct steps, which serendipitously prevents the formation of carrier or peptide dimers. A 30-50% yield of conjugate is usually obtained using MBS (Muller, 1988). Glutaraldehyde conjugation was unsuitable for the peptide used in this study because exclusive conjugation of the peptide to the carrier by the N-terminus would be prevented by the presence of the ε-amino group on the lysine residue of the peptide (Avrameas and Ternynck, 1969).

Ovalbumin was used as a carrier protein for immunisation into rabbits and rabbit albumin for chickens. These carriers were used instead of keyhole limpet haemocyanin (KLH), which is extremely immunogenic in experimental animals (Polson *et al.*, 1980) and elicits antibodies that have been found to react non-specifically in Western blots (Coetzer *et al.*, 1991).

6.3.1 Materials

50 mM sodium phosphate buffer, pH 7.0. NaH_2PO_4. $2H_2O$ (0.69 g) was dissolved in 90 ml of H_2O, titrated to pH 7.0 with NaOH and made up to 100 ml with H_2O.

Ellman's reagent [10 mM 5,5'-dithiobis(2-nitrobenzoic acid), (DTNB) in 50 mM sodium phosphate buffer, pH 7.0, 10% (v/v) methanol]. DTNB (40 mg) was dissolved in methanol (100 µl) and diluted to 10 ml with the sodium phosphate buffer described above.

MBS (16 mM). MBS (5 mg) was dissolved in dimethylformamide (DMF, 1 ml). DMF must be amine-free and "dry" to prevent undesired side reactions.

Reducing buffer [100 mM Tris-Cl, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, pH 8.0]. Tris (1.21 g), $Na_2EDTA.2H_2O$ (0.037 g) and NaN_3 (0.02 g) were dissolved in 90 ml of H_2O, titrated to pH 8.0 with HCl and made up to 100 ml with H_2O.

10 mM DTT in reducing buffer. DTT (7.71 mg) was dissolved in 5 ml of reducing buffer just before use.

Chromatography buffer [100 mM sodium phosphate buffer, 0.02% NaN_3, pH 7.0]. $NaH_2PO_4.2H_2O$ (6.9 g) and NaN_3 (0.1 g) were dissolved in 450 ml of H_2O, titrated to pH 7.0 with NaOH and made up to 500 ml with H_2O.

6.3.2 Method

Carrier protein (0.089 µmol, equivalent to 4.16 mg ovalbumin or 6.20 mg rabbit albumin) was dissolved in phosphate buffer (2 ml). MBS (279 µl, 3.56 µmol, giving a 1:40 molar ratio of carrier:MBS) was added and stirred slowly into the carrier protein solution and acylation of the carrier allowed to proceed at RT for 30 min. Unreacted MBS was removed by chromatography on Sephadex G-25 (1.5×11.5 cm) pre-equilibrated in chromatography buffer at a flow rate of 10 cm/h. The A_{280} of the eluate was monitored and the activated carrier collected as the first peak. The subsequent peak, representing unreacted MBS was discarded.

Peptide (5 mg, 3.4 µmol, giving a molar ratio of peptide to activated carrier of 40:1) was dissolved in reducing buffer (1 ml). DTT (1 ml) was added and reduction of the peptide allowed to proceed for 1½ h at 37°C. Reduced peptide was separated from excess DTT by chromatography on Sephadex G-10 (1×12 cm, pre-equilibrated at RT in chromatography buffer at 10 cm/h). Fractions (500 µl) were collected manually in Eppendorf tubes and 10 µl of each fraction mixed with an equal volume of Ellman's reagent to construct the elution profile. This reagent produces a yellow product after reaction with reducing agents, with a light yellow colour marking the elution of the reduced peptide and an intensely yellow colour marking the elution of excess DTT.

The reduced peptide was immediately mixed with activated carrier and incubated for 3 h at RT. Carrier activation and peptide reduction should ideally proceed at the same time so that they are mixed immediately after preparation but, in practice, the time of elution of each is difficult to control exactly. Where necessary, the activated carrier, rather than the reduced peptide, was allowed to stand before the final reaction commenced, as Muller (1988) states that coupling yield is strongly dependent on the availability of sulfhydryl groups. Unbound peptide was removed by dialysis against 4 changes of sodium phosphate buffer over 16 h at 4°C.

Estimating the efficiency of coupling proved difficult. Kitagawa and Aikawa (1976) titrated the maleimide groups on the carrier using a sulfydryl reagent (such as β-mercaptoethanol, which binds to MBS) and Ellman's reagent. By determining the maleimide content of the carrier before and after conjugation, the efficiency of both carrier activation and coupling were estimated. In the present study, irreproducible results were obtained using this method, so three alternative methods of estimating coupling efficiency were investigated. The carrier-conjugated peptide (called the conjugated peptide throughout) and the free carrier were scanned between 185 and 900 nm, but no marked differences in their spectra were noted. However, both the free, unmodified carrier and MBS-modified carrier gave sharp single bands on a silver stained SDS-PAGE gel, while the conjugates gave a large number of bands of greater size (Figure 35). The increased molecular mass indicates that successful conjugation to the carrier had occurred.

Finally, electrospray mass spectroscopy (ES-MS), kindly performed by Ron Berry and Andrew Howes from this department while at the University of Stellenbosch, showed that numerous higher molecular weight species were present in conjugated peptide preparations but not in unmodified albumin (results not shown), suggesting that coupling was successful. However, the complexity of the ES-MS profile did not allow determination of the percentage of peptide moieties coupled to the carrier.

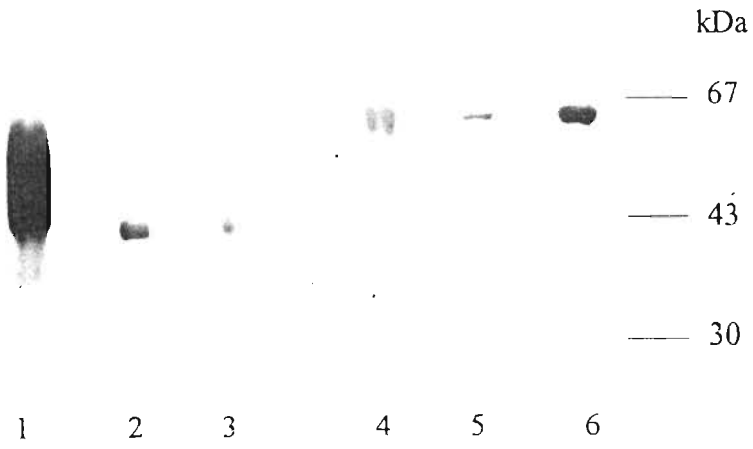


Figure 35: SDS-PAGE gel showing coupling of the trypanopain peptide to rabbit albumin and ovalbumin.

Ovalbumin-MBS-peptide (lane 1), ovalbumin-MBS (lane 2) and unmodified ovalbumin (45 kDa, lane 3), rabbit albumin-MBS-peptide (lane 4), rabbit albumin-MBS (lane 5) and unmodified rabbit albumin (66 kDa, lane 6) are shown. Pharmacia molecular mass markers (Section 2.4) were used.

6.4 Immunisation of experimental animals

Because different species may respond differently to an immunogen (Harlow and Lane, 1988), both rabbits and chickens were immunised with the trypanopain peptide. Parallel experiments were conducted with the free and conjugated peptide to assess their relative immunogenicity.

6.4.1 Rabbits

Two young rabbits were each immunised with free peptide (200 μg each, dissolved in heat-sterilised 100 mM sodium phosphate buffer, pH 6.0) and two with peptide-ovalbumin conjugate (about 200 μg each of peptide). Since the exact efficiency of peptide-carrier coupling could not be calculated (see Section 6.3.2), 40% coupling efficiency was assumed (Muller, 1988) to calculate the required dose of conjugated peptide. Immunogens were triturated with an equal volume of Freund's adjuvant until a thick emulsion formed (Freund and McDermott, 1942). Freund's complete adjuvant was used for all immunisations with free peptide and for initial immunisations with conjugated peptide. Freund's incomplete adjuvant was used for subsequent immunisations with the conjugated peptide. Rabbits were injected subcutaneously at 4-6 sites on either side of the spine (Chase, 1977) at weeks 0, 2, 6 and 10.

Blood was collected 3, 8 and 12 weeks after the first immunisation. The area surrounding the central ear vein was shaved and cleaned with alcohol. A needle was inserted into the ear vein and blood collected into dry test tubes. Clots were carefully loosened from the walls of the collection tubes with a sealed Pasteur pipette and blood was stored for 16 h at 4°C to allow complete clotting. The serum was aspirated and centrifuged (1500×*g*, 10 min, RT) to remove any remaining erythrocytes.

6.4.2 Chickens

Chickens are excellent animals for experimental antibody production, since large quantities of antibody are concentrated in the egg yolk to provide passive immunity for the developing chick (Polson *et al.*, 1980). These antibodies are termed IgY, where the Y refers to their location in the egg yolk. Since antibodies produced throughout the immunisation protocol can be obtained by collecting the eggs, no bleeding of the chicken is necessary. Two chickens were immunised with free peptide (200 μg each, prepared as in Section 6.4.1) and two with peptide-rabbit albumin conjugate (about 200 μg peptide each, calculated as in Section 6.4.1). The immunogen was triturated with adjuvant until a thick emulsion formed. Because chickens respond unfavourably to repeated doses of Freund's complete adjuvant (Dr Theresa Coetzer, Department of Biochemistry, UNP, personal communication), the free peptide was alternately administered in Freund's complete and incomplete adjuvant. Conjugated peptide was administered in complete adjuvant for the first immunisation and subsequently in incomplete adjuvant. Birds were injected intramuscularly at 4 to 6 sites on either side of the sternum at weeks 0, 1, 2, 6 and 10 and eggs collected from the start of the immunisation protocol.

6.5 Assessment of antibody production and cross-reactivity with whole enzymes

Following isolation of chicken (Section 2.8) and rabbit (Section 2.9) antibodies, ELISAs were used to monitor antibody production in experimental animals by titrating test antibodies against the free peptide, coated as antigen (Section 2.10). The titre of each antibody is defined as the lowest antibody concentration that gives an absorbance value higher than the controls. Once the highest titre anti-peptide antibodies produced by each animal were identified, the ability of these antibodies to recognise whole trypanopain and cathepsin L was investigated. Only some of the anti-peptide antibodies were expected to cross-react with whole trypanopain (or

cathepsin L) since the free peptide used as antigen can adopt many conformations, only some of which mimic the naturally occurring conformations in the corresponding constrained region of the target protein. Hence only some of the elicited antibodies are directed against peptide conformations normally occurring in the parent enzyme.

6.5.1 Materials

As per Section 2.10.1.

6.5.2 Method for ELISAs monitoring progression of antibody titre

For ELISAs monitoring the production of anti-peptide antibodies in chickens and rabbits, the free peptide was coated as antigen (5 µg/ml in PBS, 150 µl per well, 16 h, 4°C) and remaining sites blocked with BSA-PBS (200 µl, 1 h, 37°C). Test antibodies were then titrated (between 250 and 2 µg/ml in BSA-PBS, 100 µl per well, 2 h, 37°C) and the HRPO-linked secondary antibody added (120 µl per well in BSA-PBS, 1 h, 37°C). ABTS/H_2O_2 (150 µl per well) was used as the substrate and absorbance monitored at 405 nm.

6.5.3 Method for ELISAs to determine recognition of whole trypanopain and cathepsin L by anti-peptide antibodies

To investigate the cross-reactivity between anti-peptide antibodies and whole trypanopain, various steps were taken to increase the sensitivity over that achieved in Section 2.10. Whole trypanopain was coated directly onto Nunc "PolySorb" ELISA plates (serial two-fold dilutions from 1 µg/ml in pH 6.0 sodium carbonate buffer, 150 µl per well, 16 h, 4°C), which have superior antigen coating ability and lower background than the routinely used "MaxiSorb" plates. Coating was done in carbonate buffer, since this has been shown to improve recognition of whole cathepsin-like enzymes by anti-peptide antibodies (Coetzer, 1992). Remaining sites were blocked with BSA-PBS and primary antibody (fixed concentration of 50 µg/ml in BSA-PBS) applied. Tween-20 [0.1% (v/v)] was added to this BSA-PBS to reduce non-specific protein-protein interactions and so reduce background. The secondary antibody was applied as in Section 6.5.2, but a more sensitive HRPO substrate, namely TMB (150 µl per well) was used to increase the signal generated by the HRPO.

ELISAs to determine whether the anti-peptide trypanopain antibodies recognise whole cathepsin L were conducted as for the trypanopain ELISA above, with sheep cathepsin L coated as antigen [serial doubling dilutions from 1 µg/ml in pH 6.0 sodium carbonate buffer (Coetzer, 1992), 16 h, 4°C].

6.6 Evaluation of antibodies with Western blots

Following evaluation of anti-peptide antibodies by ELISA, the antibodies were evaluated by Western blots. Electroblotting denatures the antigen to a larger extent than occurs in an ELISA, so this method evaluates antibody recognition of fully denatured antigen. When initial experiments showed no recognition of fully denatured trypanopain by these antibodies, the renaturing buffer system of Dunn (1986) was used, in the hope that the partially renatured trypanopain would be successfully recognised.

6.6.1 Materials

As per Section 2.11.1.

Renaturing solution [50 mM Tris-Cl, 20% (m/v) glycerol, pH 7.4]. Tris (6.055 g) was dissolved in 700 ml of H_2O and glycerol (200 g) added. The solution was adjusted to pH 7.4 with HCl and made up to 1 l with H_2O.

Dunn carbonate transfer buffer [10 mM $NaHCO_3$, 3 mM Na_2CO_3, 20% (v/v) methanol, pH 9.9]. $NaHCO_3$ (1.6802 g) and Na_2CO_3 (0.63594 g) were dissolved in 1.4 l, methanol (400 ml) added and the solution made up to 2 l. The pH of this buffer should not be adjusted (see Section 2.11.1).

6.6.2 Method

For initial Western blots evaluating anti-peptide antibodies, the gel was electroblotted at 200 V for 1½ h and blocked with block II (Section 2.11.1). In separate experiments, various IgY and rabbit anti-peptide antibody preparations were applied at 500 µg/ml and 250 µg/ml respectively (3 h), followed by rabbit anti-IgY-AP or goat anti-rabbit-AP secondary antibodies (manufacturer's recommended dilutions, 1 h) and BCIP/NBT substrate.

When these blots were unsuccessful, various steps were taken to improve on the initial method. Following tricine SDS-PAGE, gels were soaked in renaturing solution to promote renaturation of antigens (Dunn, 1986) and gels then blotted using the high pH transfer buffer. Primary antibodies were incubated with these blots for 3½ h.

6.7 Effect of anti-peptide antibodies on trypanopain activity against Z-Phe-Arg-AMC, ^{14}C-gelatin and FITC-albumin

Antibodies can activate, inhibit or have no effect at all on an enzyme's activity, depending on factors such as the epitopes targeted by the antibodies, antibody concentration and the substrate used (Richmond, 1977). Antibody-mediated inhibition is thought to be due either to occlusion of the active site or induction of an inactive conformation (Oppenheim and Nachbar, 1977). Since activity against large substrates is often completely or largely inhibited, while hydrolysis of small substrates is affected less, the former mechanism is thought to predominate. On the other hand, antibody-mediated enhancement of enzyme activity is thought to result from stabilising of an active enzyme conformation.

Since the effect of an anti-enzyme antibody on the enzyme's activity is in part dependent on the substrate used to assess the effect (Richmond, 1977), three trypanopain substrates were used in these studies. Because the size of the substrate is often an important factor, large protein substrates [^{14}C-gelatin and fluorescein isothiocyanate (FITC)-albumin (Lumen and Tappell, 1974; Twining, 1984)] and a small synthetic substrate (Z-Phe-Arg-AMC) were chosen.

6.7.1 Materials

As per Sections 4.2 and 4.8.6.

Antibody diluting buffer [100 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3, 0.1% (v/v) Tween-20, pH 8.0]. Glacial acetic acid (5.7 ml), $Na_2EDTA.2H_2O$ (0.37 g) and NaN_3 (0.2 g) were dissolved in 900 ml of H_2O and Tween-20 (1 ml) added. The solution was adjusted to pH 8.0 with NaOH and made up to 1 l with H_2O.

FITC-albumin (5 mg/ml). FITC-albumin (5 mg) was dissolved in 1 ml of 0.5% (m/v) Brij-35.

Stop solution [5% (m/v) TCA]. TCA (5 g) was dissolved in 90 ml of H_2O and made up to 100 ml with H_2O.

FITC diluent (500 mM Tris-Cl, pH 8.5). Tris (6 g) was dissolved in 60 ml of H_2O, adjusted to pH 8.5 with HCl and made up to 100 ml with H_2O.

6.7.2 Method

Trypanopain (1.5 ng for hydrolysis of Z-Phe-Arg-AMC, 5.5 ng for hydrolysis of ^{14}C-gelatin and FITC-albumin) was diluted to 250 µl with 0.1% (m/v) Brij-35. The antibody under study was diluted as required (final concentration of 31.25-1000 µg/ml) with antibody dilution buffer (diluted volume 250 µl) and the mixture incubated for 15 min at 30°C. An aliquot (250 µl for Z-Phe-Arg-AMC hydrolysis; 150 µl for ^{14}C-gelatin hydrolysis) was assayed as usual against Z-Phe-Arg-AMC (Section 3.2.2) or ^{14}C-gelatin (Section 4.2 and 4.8.6) at 37°C. Aliquots (50 µl) to be assayed against FITC-albumin were added to this substrate (20 µl) and incubated for 24 h at 37°C. Stop solution was added (120 µl, 1 h, RT) and the solution centrifuged (10 000×*g*, 10 min, RT). An aliquot (60 µl) was diluted with 1 ml of FITC diluent and the fluorescence read on a Hitachi F-2000 spectrofluorometer (excitation 490 nm, emmision 525 nm). Since the addition of non-immune antibodies slightly reduced enzyme activity against all three substrates, enhancement or inhibition of enzyme activity is expressed as a percentage of the activity detected after incubation with the same concentration of non-immune antibody.

6.8 Inhibition of antibody effect on enzyme activity by addition of free peptide

Interactions between trypanopain and anti-trypanopain antibodies were measured relative to interactions with non-immune antibodies so the results obtained from experiments described in Section 6.7 point to specific recognition of the enzyme by anti-trypanopain antibodies. To further confirm the specificity of the interaction, experiments in Section 6.7 were repeated in the presence of the free trypanopain peptide. Specific anti-peptide antibodies are expected to bind to the free peptide, leaving less free antibody to modulate trypanopain activity. If the antibody effect arises from specific recognition of the peptide region in trypanopain, then addition of free peptide should reduce the effect of antibodies on enzyme activity.

6.8.1 Materials

As in Section 6.7.1.

6.8.2 Method

The trypanopain peptide was dissolved in 0.1% (m/v) Brij 35 to give 10 000:1 to 1:1 molar ratios of peptide:enzyme. The peptide solution (100 µl) was incubated with antibody (500 µl, final concentration 500 µg/ml) for 15 min at 30°C to allow specific anti-peptide antibodies to bind to the peptide. The antibodies were then incubated with trypanopain as in Section 6.7.2.

6.9 Effects of antibodies on trypanopain activity against Z-Phe-Arg-AMC and ^{14}C-gelatin in *T. b. brucei* lysates

6.9.1 Materials

As per Section 6.7.1.

6.9.2 Method

As per Section 6.7.2, except that *T. b. brucei* lysates were used instead of purified trypanopain. The concentration of trypanopain in these lysates was estimated by E-64 titration (Section 3.4) to be approximately 350 nM.

6.10 Effect of antibodies on cathepsin L activity against Z-Phe-Arg-AMC and ^{14}C-gelatin

Since trypanopain and sheep cathepsin L are 9% homologous (Mottram *et al.*, 1989; Ritonja *et al.*, 1996), some degree of cross-reactivity between anti-trypanopain antibodies and the mammalian enzyme was expected. Section 6.5.3 showed that anti-trypanopain anti-peptide antibodies recognised denatured cathepsin L in an ELISA. To determine whether these antibodies also recognise native cathepsin L, the effect of these antibodies on cathepsin L activity were determined.

6.10.1 Materials

As per Section 6.7.1, except with the following changes:

Cathepsin L assay buffer [340 mM sodium acetate, 60 mM acetic acid, 4 mM Na_2EDTA, 0.02% NaN_3, 4 mM DTT, pH 5.5]. Sodium acetate.$3H_2O$ (23.13 g), glacial acetic acid (1.72 ml), $Na_2EDTA.2H_2O$ (0.75 g) and NaN_3 (0.1 g) were dissolved in 450 ml of H_2O, adjusted to pH 5.5 with NaOH and made up to 500 ml with H_2O. DTT (6.2 mg) was added to 5 ml of buffer just before use.

6.10.2 Method

As per Section 6.7.2.

6.11 Results

6.11.1 Determination of antibody titre in rabbits and chickens

Rabbits immunised with the conjugated peptide produced antibodies that recognised the free peptide effectively. Only minimal differences were observed between the responses of individual rabbits immunised with identical antigens, so the response of a single animal is shown (Figure 36A). Antibodies produced 3, 8 and 12 weeks post-immunisation all gave high signals compared to those generated by non-immune antibodies, with the antibody titre below 10 µg antibody/ml for all weeks tested.

Rabbits immunised with the free peptide also produced high titre antibodies, showing that the free peptide is immunogenic in rabbits. Only minimal differences were observed between the responses of individual rabbits immunised with identical antigens. Compared with the conjugated peptide, the free peptide appeared to be slightly less immunogenic (Figure 36B). While the titre of anti-(conjugated peptide) antibodies was high (below 10 µg/ml) by 3 weeks post-immunisation, the titre of anti-(free peptide) antibodies was still low (below 100 µg/ml) at this time. By weeks 8 and 12 post-immunisation, however, the titre of anti-(free peptide) and anti-(conjugated peptide) antibodies was comparable (below 10 µg/ml), showing that the peptide ultimately elicited antibodies of similar strength irrespective of whether it was conjugated or not.

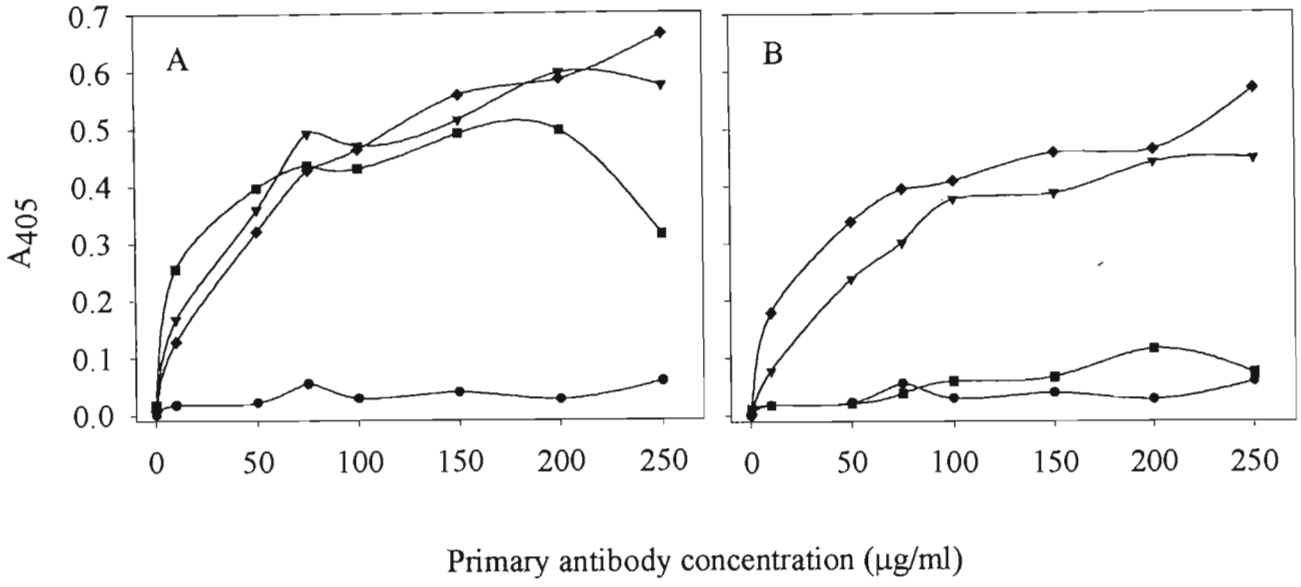


Figure 36: Rabbit antibodies produced against the conjugated and free trypanopain peptide at various weeks post-immunisation.

Peptide (5 μg/ml) was coated for 16 h at 4°C in PBS. Antibodies produced against the conjugated (A) and free peptide (B) are shown. Absorbance values at 405 nm represent the average of duplicate experiments. Non-immune antibodies (●) and antibodies from week 3 (■) week 8 (▼) and week 12 (◆) post-immunisation were titrated between 250 μg and 0.1 μg antibody/ml. Sheep anti-rabbit-HRPO was used as secondary antibody with ABTS/H_2O_2 as substrate.

Only one of the chickens immunised with the conjugated peptide laid eggs, so comparison between individuals was not possible. Antibodies from weeks 3, 6, 8, 10 and 12 post-immunisation all recognised the peptide well, with week 8 giving the highest signal (Figure 37A). Chickens immunised with the free peptide also produced high titre antibodies, showing that the free peptide was also immunogenic in chickens (Figure 37B). Antibodies from 6, 8 and 12 weeks post-immunisation recognised the peptide well (titre below 10 μg antibody/ml), with week 8 giving the highest signal. Week 3 antibodies gave poor signals compared to the corresponding anti-(conjugated peptide) antibodies, showing that was the case with rabbits, chicken responses to the free peptide were slower than against the conjugate. Antibodies against the conjugated peptide gave slightly higher A_{405} readings than anti-(free peptide) antibodies.

Production of anti-peptide antibodies was thus similar in rabbits and chickens, with respect to titre and timing of the immune response. Individuals immunised with identical immunogens responded similarly. The two species tested, namely rabbits and chickens, also responded comparably. High titre antibodies were produced faster against the conjugated peptide than against the free peptide, but by 8 weeks post-immunisation, titres of antibodies against the two forms of immunogen were comparable.

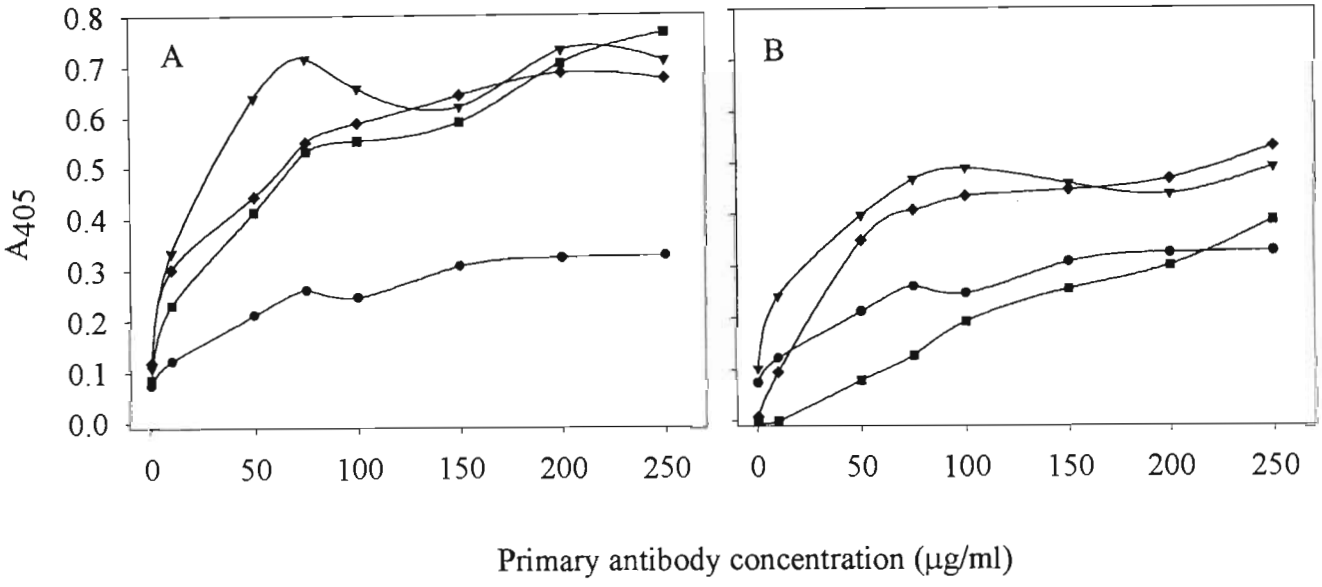


Figure 37: Chicken antibodies produced against the conjugated and free trypanopain peptide at various weeks post-immunisation.

Peptide (5 µg/ml) was coated for 16 h at 4°C. Antibodies directed against the conjugated (A) and free (B) peptide are shown. Absorbance values at 405 nm represent the average of duplicate experiments. Non-immune antibodies (●) and antibodies from week 3 (■) week 8 (▼) and week 12 (◆) post-immunisation were titrated between 250 and 0.1 µg antibody/ml. Rabbit anti-chicken-HRPO was used as secondary antibody with ABTS/H_2O_2 as substrate.

6.11.2 Recognition of whole trypanopain

In addition to recognising the free trypanopain peptide, the anti-peptide antibodies recognised whole trypanopain in an ELISA. Rabbit responses are shown in Figure 38. Antibodies against both forms of the antigen (free and conjugated peptide) and from both species (rabbit and chicken) recognised the whole enzyme comparably, with titres below 31 µg/ml antibody.

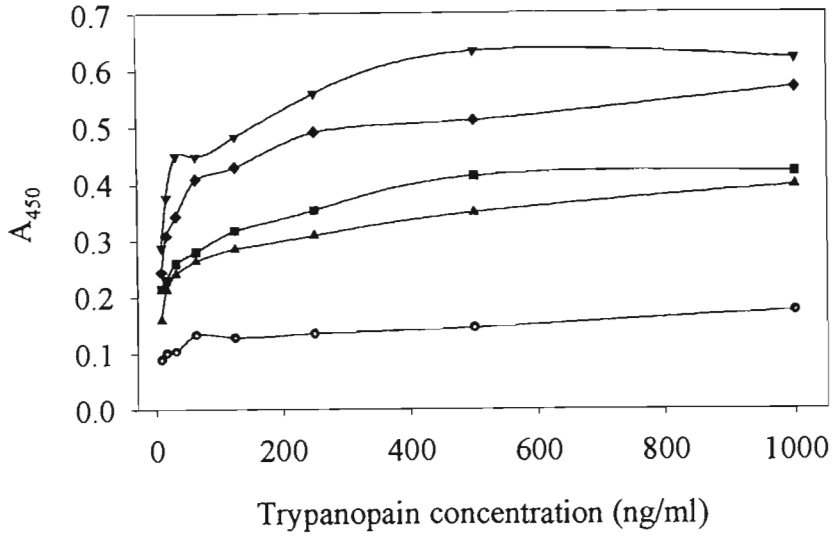


Figure 38: Recognition of whole trypanopain by rabbit anti-(free peptide) and anti-(conjugated peptide) antibodies in an ELISA.

Trypanopain (serial two-fold dilutions from 1 µg/ml) was coated for 16 h at 4°C in pH 6.0 sodium carbonate buffer, after which a fixed concentration of test primary antibody (50 µg antibody/ml) was applied. Absorbance values at 450 nm of non-immune antibodies (●), anti-(free peptide) antibodies from rabbits I (▲) and II (◆) and anti-(conjugated peptide) antibodies from rabbits III (■) and IV (▼) represent the average of duplicate experiments. Sheep anti-rabbit-HRPO was used as secondary antibody with TMB/H_2O_2 as substrate.

6.11.3 Cross-reactivity with sheep cathepsin L

Since the trypanopain peptide sequence targeted is similar to the corresponding sequence in mammalian cathepsin L (Ritonja *et al.*, 1988), cross-reactivity between anti-trypanopain antibodies and sheep cathepsin L was investigated in ELISAs performed in parallel with the trypanopain ELISA in Section 6.11.2. Both chicken and rabbit anti-(free peptide) and anti-(conjugated peptide) antibodies recognised cathepsin L in an ELISA (Figure 39). Generally weaker signals were generated by cathepsin L than by trypanopain, showing that the antibodies recognise trypanopain more readily than they do cathepsin L.

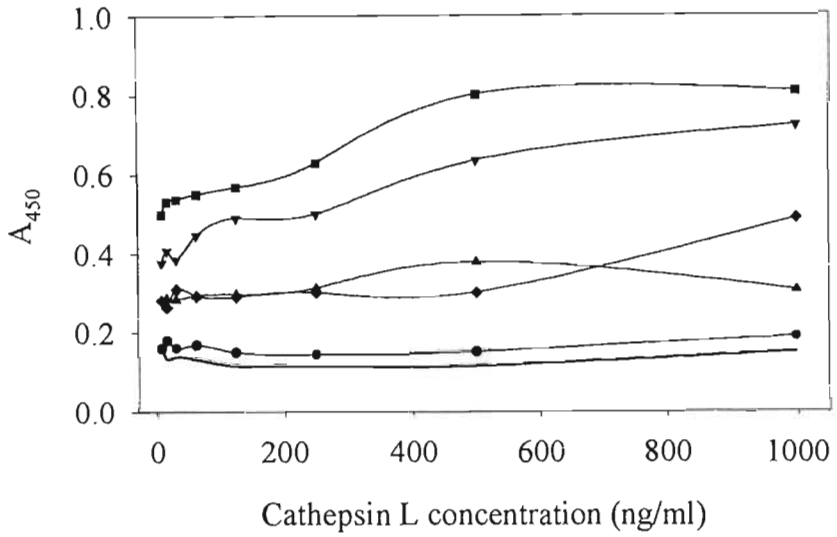


Figure 39: Cross reactivity between chicken and rabbit anti-trypanopain peptide antibodies and sheep cathepsin L in an ELISA.

Sheep cathepsin L (serial two-fold dilutions from 1 µg/ml) was coated for 16 h at 4°C in pH 6.0 sodium carbonate buffer. Absorbance values at 450 nm for rabbit non-immune antibodies (●), rabbit anti-(free peptide) (◆) and anti-(conjugated peptide) antibodies (▲), chicken non-immune antibodies (–), chicken anti-(free peptide) (▼) and anti-(conjugated peptide) antibodies (■) represent the average of duplicate experiments. Sheep anti-rabbit-HRPO or rabbit anti-chicken-HRPO secondary antibodies were used for detection and TMB/H_2O_2 was used as the substrate.

6.11.4 Western blots with anti-peptide antibodies

The anti-peptide antibodies did not recognise trypanopain on Western blots, whether purified enzyme or lysed *T. b. brucei* were used as antigen. Methods reported to increase recognition of antigens (Dunn, 1986) were unsuccessful.

6.11.5 Effect of antibodies on trypanopain activity against Z-Phe-Arg-AMC

Rabbit antibodies directed against both the free and conjugated peptide enhanced trypanopain hydrolysis of Z-Phe-Arg-AMC relative to non-immune antibodies (Figure 40). Antibodies directed against the free peptide activated the enzyme slightly more (by 64% at 500 µg antibody/ml) than those directed against the conjugated peptide (by 49% at 500 µg antibody/ml). The effects of antibodies from individual rabbits immunised with identical

antigens were comparable. Slightly greater enhancement was mediated by week 12 antibodies irrespective of whether raised against the free or conjugated peptide.

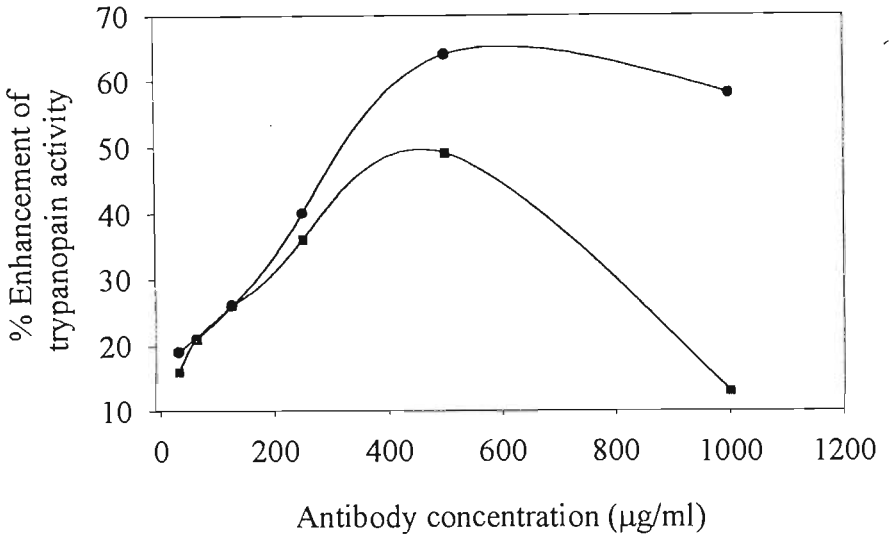


Figure 40: Enhancement of trypanopain hydrolysis of Z-Phe-Arg-AMC by rabbit anti-trypanopain peptide antibodies.

Trypanopain (1.5 ng) was incubated in triplicate with rabbit anti-peptide antibodies (31.25 to 1000 µg antibody/ml) at pH 8.0 (15 min, 37°C). Residual enzyme activity against Z-Phe-Arg-AMC was then determined as described in Section 4.8.6. Effects of antibodies raised against the free peptide (●) and the conjugated peptide (■) are shown.

In contrast with rabbit antibodies, the effects of chicken antibodies on trypanopain hydrolysis of Z-Phe-Arg-AMC were more complex. Individual chickens produced both activity-enhancing and activity-inhibiting antibodies at different stages in their immune response. This effect was observed both in chickens immunised with the free peptide and those immunised with the conjugated peptide. In the case of the free peptide, antibodies produced 3, 6, 8 and 10 weeks after the first immunisation all enhanced trypanopain activity against Z-Phe-Arg-AMC, with enhancement increasing to an optimum at week 8 (Figure 41). Week 10 antibodies inhibited activity by 80%, while week 11 antibodies enhanced activity by 75% and week 12 inhibited by 64% (Figure 41). Consideration of the immunisation schedule sheds some light on the results obtained.

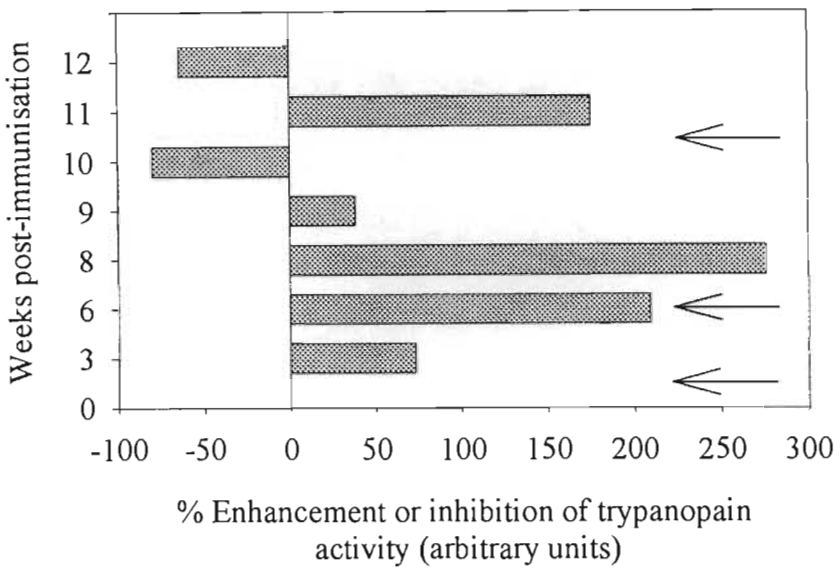


Figure 41: Modulation of trypanopain hydrolysis of Z-Phe-Arg-AMC by chicken anti-(free peptide) antibodies.

Trypanopain (1.5 ng) was incubated in triplicate with chicken anti-(free peptide) antibodies (500 μg/ml) at pH 8.0 (15 min, 37 C). Residual enzyme activity against Z-Phe-Arg-AMC was then determined as described in Section 4.8.6. Activity is expressed relative to that observed after incubation with non-immune antibodies (500 μg/ml) with % enhancement of activity expressed as positive values and % inhibition of activity expressed as negative values. Chickens were immunised at weeks 0, 1, 2, 6 and 10 (arrows).

Antibodies produced in chickens soon after immunisation/boosting with the free peptide appear to enhance activity while those produced a few weeks later appear inhibitory. Chicken responses to the conjugated peptide were similar to those against the free peptide. For example, antibodies produced 3, 6 and 8 weeks post-immunisation enhanced enzyme activity (by up to 46% at 500 μg/ml) while those from weeks 10 and 12 inhibited activity (by up to 91% at 500 μg/ml) (results not shown).

Chicken anti-whole trypanopain antibodies (Chapter 3) inhibited trypanopain hydrolysis of Z-Phe-Arg-AMC by up to 60% at 1 mg/ml (results not shown).

6.11.6 Inhibition of antibody effect on Z-Phe-Arg-AMC hydrolysis by incubation with free peptide

If the effects of the anti-peptide antibodies on trypanopain activity are specific, then pre-incubation of the antibodies with the free peptide should substantially reduce the antibody effects on trypanopain activity. This was shown to be the case. For example, week 8 chicken antibodies raised against the free peptide enhanced trypanopain activity by 276% (Section 6.11.5) but after pre-incubation with the free peptide, this enhancement was greatly reduced. At a 10 000:1 molar ratio of peptide:enzyme, only 46% enhancement of trypanopain activity was observed (Table 12). The effects of the peptide on activation can thus be expressed as follows:

$$\text{Peptide} - \text{induced \% reduction of enzyme activition} = \frac{276 - x}{x}\%$$

where x represents enhancement of trypanopain activity by antibodies pre-incubated with the free peptide. Using this formula, the reduction in activity enhancement from 276% to 46% represents an 83% reduction of the antibody effect by pre-incubation with the peptide.

Table 12: Effect of free peptide on subsequent modulation of trypanopain hydrolysis of Z-Phe-Arg-AMC by activity enhancing chicken anti-peptide antibodies.

Peptide:enzyme molar ratio	% Enhancement	% Reduction of enhancement
0 : 1	276	0
1 : 1	113	59
1 00 : 1	99	64
1 000 : 1	73	74
10 000 : 1	46	83

In contrast with week 8 chicken anti-(free peptide) antibodies, week 10 chicken anti-(free peptide) antibodies inhibited trypanopain activity by 80% (Section 6.11.5). Pre-incubation of these antibodies with the free trypanopain peptide reduced the inhibition observed to the extent

that at 10 000:1 molar ratio of peptide:enzyme, no inhibition was seen (Table 13). This represents a 100% reduction in the antibody effect by addition of free peptide.

Table 13: Effect of free peptide on subsequent modulation of trypanopain hydrolysis of Z-Phe-Arg-AMC by activity inhibiting chicken anti-peptide antibodies.

Peptide:enzyme molar ratio	**% Inhibition**	**% Reduction of inhibition**
0 : 1	80	0
1 : 1	59	26
10 : 1	58	28
1 00 : 1	54	33
1 000 : 1	56	31
10 000 : 1	0	100

In contrast with chicken anti-peptide antibodies, rabbit anti-peptide antibodies (all weeks) enhanced trypanopain activity against Z-Phe-Arg-AMC by up to 64% (for week 12 anti-(free peptide) antibodies at 500 µg antibody/ml, Section 6.11.5). Pre-incubation of the antibodies with the free peptide reduced the enhancement of trypanopain activity substantially, to the extent that at a 10 000:1 molar ratio of peptide:enzyme only 15% enhancement of activity was seen (Table 14) This decrease from 64% to 15% enhancement represents a 77% reduction in the antibody effect by addition of the free peptide.

Table 14: Effect of free peptide on subsequent enhancement of trypanopain activity against Z-Phe-Arg-AMC by activity enhancing rabbit anti-peptide antibodies.

Peptide:enzyme molar ratio	**% Enhancement**	**% reduction of enhancement**
0 : 1	64	0
1 : 1	57	11
10 : 1	50	22
1 00 : 1	48	25
1 000 : 1	28	56
10 000 : 1	15	77

Addition of a non-related peptide should have no effect on binding of anti-trypanopain antibody to the enzyme. Poly-L-glutamic acid was used to confirm that this occurred in the system under study. As expected, different molar excesses of poly-L-glutamate had no effect on anti-peptide antibody binding to trypanopain (results not shown) indicating that the peptide effect is specific.

6.11.7 Effect of antibodies on trypanopain activity against ^{14}C-gelatin

All the tested anti-peptide antibodies activated trypanopain digestion of ^{14}C-gelatin (Figure 42). The greatest level of enhancement was seen with chicken anti-(free peptide) antibodies (349% of non-immune activity at 250 μg/ml). Anti-whole trypanopain antibodies (Chapter 3) had no effect on trypanopain hydrolysis of ^{14}C-gelatin.

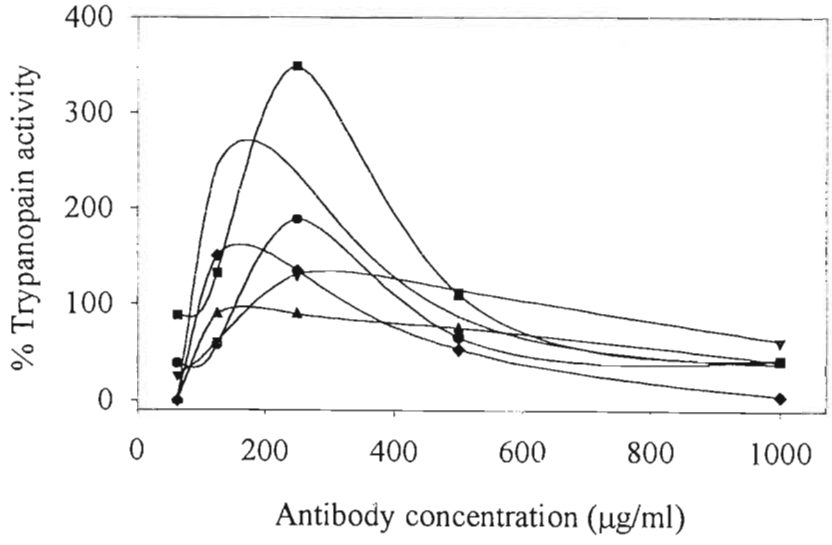


Figure 42: Effect of anti-peptide antibodies on trypanopain digestion of ^{14}C-gelatin.
Trypanopain (5.5 ng) was activated with pH 5.5 assay buffer and incubated in quintuplicate with various concentrations (62.5 to 1000 μg/ml) of the antibody under study (30 min, 37°C). ^{14}C-gelatin was then added and digestion allowed to proceed at 37°C for 3½ h. Chicken anti-(free peptide) antibodies (■ and ●), chicken anti-(conjugated peptide) antibodies (▼), rabbit anti-(free peptide) antibodies (◆) and rabbit anti-(conjugated peptide) antibodies (▲ and —) from various weeks all enhanced trypanopain activity against ^{14}C-gelatin relative to non-immune antibodies.

6.11.8 Reduction of antibody effect on ^{14}C-gelatin hydrolysis by pre-incubation with free peptide

To test the specificity of anti-peptide antibody enhancement ^{14}C-gelatin hydrolysis by trypanopain, the antibodies were pre-incubated with the free trypanopain peptide before incubation with trypanopain, as discussed in Section 6.11.6.

Pre-incubation of anti-peptide antibodies with the free trypanopain peptide reduced their subsequent enhancement of trypanopain activity against ^{14}C-gelatin. Rabbit antibodies showed a complete abolition of enhancing activity when pre-incubated with the free peptide, while chicken antibodies showed up to 57% reduction in their enhancing activity (results not shown).

6.11.9 Effect of anti-trypanopain antibodies on trypanopain digestion of FITC-albumin

All tested anti-peptide and anti-whole enzyme (Chapter 3) antibodies inhibited trypanopain digestion of FITC-albumin. Rabbit antibodies inhibited trypanopain activity poorly (14-18%), but chicken antibodies inhibited trypanopain activity by up to 40% (Figure 43).

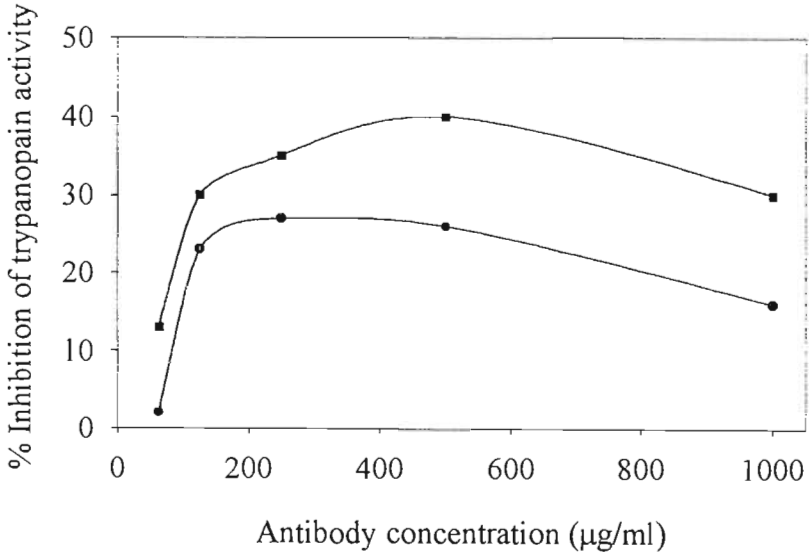


Figure 43: Effect of anti-trypanopain peptide antibodies on trypanopain digestion of FITC-albumin.

Trypanopain (5.5 ng) was incubated with various concentrations of anti-trypanopain peptide antibodies and hydrolysis of FITC-albumin measured in quintiplicate assays. Inhibition of trypanopain digestion of FITC-albumin by chicken anti-(free peptide) (●) and anti-(conjugated peptide) (■) antibodies is shown.

6.11.10 Effect of anti-trypanopain peptide antibodies on trypanopain hydrolysis of Z-Phe-Arg-AMC and ^{14}C-gelatin in *T. b. brucei* lysates

The chicken antibodies [anti-(free peptide) week 8] that enhanced activity of purified trypanopain against Z-Phe-Arg-AMC (Section 6.11.5) also enhanced hydrolysis of this substrate in crude lysates of *T. b. brucei* (Figure 44). Comparable enhancement of pure and crude lysate enzyme was observed at 1 and 0.5 mg antibody/ml. Below these concentrations, lower enhancement was seen in lysates than with the free enzyme at equal antibody concentrations.

Chicken anti-(conjugated peptide) antibodies (week 10) that inhibited pure trypanopain also inhibited Z-Phe-Arg-AMC hydrolysis in lysed *T. b. brucei*. For example, up to 82% inhibition was observed with the pure enzyme at 500 µg antibody/ml, while up to 70% inhibition was seen in lysates at the same antibody concentration (results not shown).

Chicken anti-peptide antibodies thus modulated trypanopain activity in lysates at levels comparable to those seen for the purified enzyme. However, rabbit anti-(free peptide) and anti-(conjugated peptide) antibodies that enhanced the activity of pure trypanopain, had no effect on trypanopain in lysates. Even at high concentrations of antibody (1 mg/ml) comparable activity was observed in trypanosome lysate samples incubated with non-immune and test antibody.

Chicken and rabbit anti-peptide antibodies all enhanced digestion of ^{14}C-gelatin by purified trypanopain (Section 6.11.7). However, in lysed *T. b. brucei*, this effect was markedly reduced, so that little or no enhancement of activity was seen.

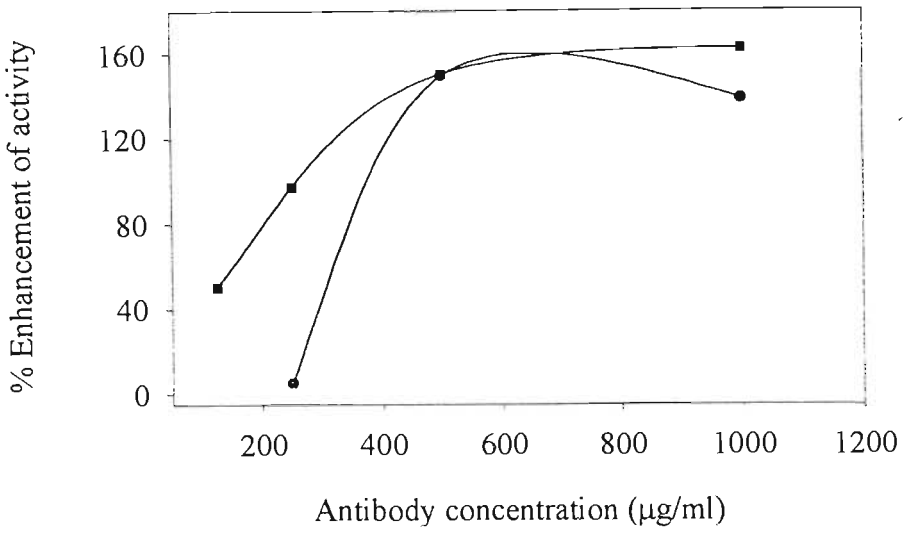


Figure 441: Effect of activity-enhancing chicken antibodies on trypanopain activity against Z-Phe-Arg-AMC in lysed *T. b. brucei*.

The effects of week 8 chicken anti-(free peptide) antibodies (1 000 to 31.25 µg antibody/ml) on Z-Phe-Arg-AMC hydrolysis in lysed *T. b. brucei* (●) and by pure trypanopain (■) were investigated in triplicate assays.

6.11.11 Effect of anti-trypanopain antibodies on cathepsin L activity against Z-Phe-Arg-AMC and ^{14}C-gelatin

The tested anti-peptide trypanopain antibodies modulated cathepsin L activity against Z-Phe-Arg-AMC and ^{14}C-gelatin (Table 15). Most of the tested antibodies inhibited cathepsin L activity against Z-Phe-Arg-AMC. For example, anti-(free peptide) antibodies from chicken 1 (week 20) enhanced trypanopain activity against this substrate by up to 82%, but inhibited cathepsin L activity by 68%.

Similarly, anti-(free peptide) antibodies from rabbit I (week 12) enhanced trypanopain activity against Z-Phe-Arg-AMC by 14%, but inhibited cathepsin L activity by 24% (Table 15). Thus, while anti-trypanopain antibodies do modulate cathepsin L activity against Z-Phe-Arg-AMC, the nature of the effect differs from that seen with trypanopain. Compared with their effects on trypanopain activity, the antibodies enhanced cathepsin L activity to a lesser extent, or inhibited

activity. No difference was seen between rabbit and chicken antibodies or between antibodies directed against the free or conjugated peptide.

Table 15: Maximal effect of anti-trypanopain antibodies on trypanopain and cathepsin L activity against Z-Phe-Arg-AMC.

Antibody	Maximum effect on enzyme activity against Z-Phe-Arg-AMC	
	Trypanopain	Cathepsin L
IgY anti-(free peptide) (chicken I, week 20)	82% enhancement	68% inhibition
IgY anti-(conjugated peptide) (chicken II, week 10)	81% inhibition	72% inhibition
IgY anti-(conjugated peptide) (chicken III, week 6)	124% enhancement	55% enhancement
IgG anti-(free peptide) (rabbit I, week 12)	14% enhancement	24% inhibition
IgG anti-(free peptide) (rabbit II, week 12)	64% enhancement	16% enhancement
IgG anti-(conjugated peptide) (rabbit III, week 12)	27% enhancement	44% inhibition
IgG (anti-conjugated peptide) (rabbit IV, week 12)	49% enhancement	53% inhibition

The anti-trypanopain antibodies also modulated cathepsin L hydrolysis of ^{14}C-gelatin. While these antibodies all activated trypanopain hydrolysis of this substrate, some enhanced cathepsin L activity, while others inhibited activity (results not shown). Generally, the effects on cathepsin L activity were substantially lower than was seen against trypanopain.

6.11.12 Summary of results

Table 16 gives a summary of the results obtained with anti-peptide and anti-whole enzyme antibodies.

Table 16: Effects of various antibodies on trypanopain digestion of synthetic and proteinaceous substrates.

Substrate	Rabbit anti-peptide IgG	Chicken anti-peptide IgY	Chicken anti-trypanopain IgY
Z-Phe-Arg-AMC	-	+/-	-
^{14}C-gelatin	0	+	+
FITC-albumin	-	-	-

Note: +, activity enhanced; -, activity inhibited; 0, activity unaffected

6.12 Discussion

High titre antibodies were produced in rabbits and chickens against all the immunogens tested. Chickens proved to be far more convenient experimental animals than rabbits, both in terms of the higher total amount of antibody obtained and in obviating the need to bleed animals to obtain these antibodies. The major limitation of chicken antibodies is that they do not bind protein A (Larsson *et al.*, 1993), so an additional mammalian linker antibody must be used in applications such as immuno-electron microscopy where gold probes are attached to protein A. This procedure was, however, beyond the scope of the present study, so this did not present a significant disadvantage.

The trypanopain peptide was found to be immunogenic in rabbits and chickens, with antibody responses being of similar magnitude and timing in both species. Antibodies of comparable titre were produced against the conjugated and free peptides by 8 weeks post-immunisation. The only observed effect of conjugation of the peptide to a carrier molecule was to increase the titre of antibodies produced in the first 3 weeks after immunisation. Thus, while the conjugated peptide elicited high titre antibodies 3 weeks after the initial immunisation, the titre of anti-(free peptide) antibodies remained low at this time. While the free peptide is less immunogenic than the conjugate, the free peptide is still immunogenic in its own right. Similar immunogenicity has been reported for other small, unconjugated peptides. For example, Coetzer (1992) found that a similarly sized peptide corresponding to a region of the cathepsin L active site is immunogenic in its own right.

The free peptide was used as antigen instead of the conjugated peptide in all ELISAs monitoring antibody titre, because coupling chemistry creates epitopes unique to the conjugate, termed CAMOR (carrier modified residues) regions (Briand *et al.*, 1985). If the conjugated peptide (containing carrier and CAMOR regions) is used as an ELISA antigen, anti-carrier and anti-CAMOR antibodies cause false positive results. Use of the free peptide as the ELISA antigen obviates this problem, without having to remove anti-carrier and CAMOR antibodies from antisera. Some authors (Muller, 1988) maintain that peptides of 6-15 residues bind poorly to plastics, but successful binding of 10 to 20-residue peptides has been reported by others (Tanaka *et al.*, 1985; Davies *et al.*, 1987; Coetzer *et al.*, 1991; Moroder *et al.*, 1992) and gave good results in the present study.

In addition to recognising the free peptide, all anti-peptide antibodies tested recognised whole trypanopain in an ELISA. This shows that at least a proportion of the conformations adopted by the highly mobile peptide in solution mimic those that occur in the corresponding constrained region of the parent protein. A decreased signal was observed in ELISAs where trypanopain was used as antigen, compared with ELISAs using the free peptide as antigen. This is thought to be due to the fact that some of the anti-peptide antibodies bind only to conformations of the free peptide that have no equivalent in the parent protein and these antibodies are thus unable to recognise the whole protein. The concentration of antibodies effectively recognising the coated antigen is thus lower when trypanopain is used as the antigen. Conjugation of the peptide to a carrier protein had no discernible effect on the degree of cross-reactivity of the corresponding antibodies with trypanopain. Similar cross-reactivity between anti-peptide antibodies and their corresponding whole protein antigen has been previously reported for cathepsin L and D (Coetzer *et al.*, 1991) and cytochrome P-450IA2 (Edwards *et al.*, 1990).

Anti-trypanopain peptide antibodies not only recognised whole trypanopain in an ELISA, but were also shown to cross-react with whole sheep cathepsin L in an ELISA. This is thought to be a reflection of the homology between the peptide used in this study and the corresponding region of the cathepsin L active site (Ritonja *et al.*, 1988).

None of the anti-peptide antibodies recognised trypanopain on a Western blot, despite attempts made to improve recognition. This indicates that the antibodies are unable to recognise their target sequence in fully denatured trypanopain.

Once the anti-peptide antibodies raised had been shown to recognise the intact enzyme, their effect on trypanopain activity was analysed. While the ELISAs discussed above show that the anti-peptide antibodies bind to whole, partially denatured trypanopain, the ability of these antibodies to modulate trypanopain substrate hydrolysis shows that they recognise and bind to the native enzyme in the form it is likely to be encountered *in vivo*. Various other enzymes, such as cathepsin D (Dingle *et al.*, 1971), papain (Arnon and Shapira, 1967), human acetylcholinesterase (Olson *et al.*, 1990), cathepsin L (Dennison and Pike, 1991) and cytochrome P-450IA2 (Edwards *et al.*, 1990) are inhibited by their respective antibodies. In this study, the different anti-trypanopain peptide antibodies modulated trypanopain activity against Z-Phe-Arg-AMC, ^{14}C-gelatin and FITC-albumin in different ways.

All of the rabbit anti-(free peptide) and anti-(conjugated peptide) antibodies studied enhanced trypanopain hydrolysis of Z-Phe-Arg-AMC. Rabbit antibodies directed against the free peptide enhanced enzyme activity slightly more (64%) than those directed against conjugated peptide (49%), suggesting that the titre of antibodies recognising the native form of trypanopain is slightly higher in anti-(free peptide) antibody preparations than in those directed against the conjugated peptide. Immuno-enhancement of enzyme activity is thought to occur by antibody stabilisation of an active enzyme conformation (Richmond,1977). Z-Phe-Arg-AMC is a small synthetic substrate, and is thought to be small enough to gain access to the antibody-occluded trypanopain active site.

The effects of chicken antibodies on trypanopain were more complex, with individual chickens producing activity-enhancing and activity-inhibiting antibodies at different stages in their immune response. Antibodies produced shortly after each immunisation appeared to enhance trypanopain activity, while those produced a few weeks later appeared inhibitory. A polyclonal immune response, by definition, consists of a heterogeneous antibody population produced by numerous B-cell clones and the properties of the antiserum are a balance of the individual properties of the various clones. Depending on the mode of binding, individual antibodies can either enhance or inhibit enzyme activity. The varied effect of antibodies on enzyme activity observed in this study could thus be a function of different B-cell clones being dominant at different stages in the immune response. A peptide can adopt numerous conformations in solution, some of which may elicit activity-enhancing antibodies and some of which elicit

inhibitory antibodies. Variation in the balance between these antibody populations may explain the result obtained in this study. Thus the B-cell clones that produce activity-enhancing antibodies may have been dominant at all tested stages of the rabbit immune response, while in chickens, B cell clones producing activity-enhancing antibodies may have been dominant soon after immunisation, followed later by B cell clones producing activity-inhibiting antibodies.

Binding of antibodies to an enzyme active site is thought to have two primary effects on the enzyme. Firstly, the active site is occluded to some extent by the antibody, and , secondly, a conformational change is induced in the active site. The practical consequences of occlusion of the active site vary mainly with the size of a substrate used to assess enzyme activity (Richmond, 1977). Small substrates (e.g. Z-Phe-Arg-AMC) can often still gain access to the partially occluded active site, and occlusion will thus not appreciably inhibit activity against small substrates. Occlusion is more likely to lead to inhibition of larger substrates. Conformational changes in the enzyme active site may induce either a more or less active conformation. The nett effects of antibody binding on enzyme activity are thus a balance between the effects of occlusion and the effects of conformational changes. Since Z-Phe-Arg-AMC is a small substrate, its hydrolysis is more likely to have been affected by antibody-induced conformational changes than by occlusion effects. For larger protein substrates, occlusion effects are likely to predominate.

The region of the trypanopain active site corresponding to the peptide immunogen used may be partially buried in the native trypanopain molecule. If this is the case, only portions of anti-peptide antibodies may bind to this region. For example, antibody-induced conformational change and occlusion of the active site will differ depending upon whether all or some of the 6 hypervariable regions of an antigen binding site of the antibody interact with the enzyme (Roitt, 1991). Not all of these interactions are necessarily with the target peptide.

The specificity of the antibody effects on trypanopain activity was investigated by pre-incubating antibodies with the free trypanopain peptide and with an unrelated peptide. Antibody modulation of trypanopain activity was substantially reduced when the antibodies were pre-incubated with the trypanopain peptide, whereas pre-incubation with an unrelated peptide (poly-L-glutamic acid) had no such effect. This implies that the specific peptide competes with trypanopain for binding to the antibodies and antibody binding to trypanopain is

thus thought to occur via the peptide sequence. The effects of the antibodies on trypanopain activity are thus unlikely to be due to non-specific interactions between the enzyme and the antibody. Absolute abolition of antibody binding to trypanopain was not always achieved by pre-incubation with the trypanopain peptide, but this is thought to be an unavoidable consequence of the assay design rather than a suggestion of non-specificity on the part of the antibody. The high sensitivity of the fluorometric assay used in this study necessitated the use of highly dilute enzyme, which, in turn, promotes dissociation of enzyme-antibody complexes. High concentrations of antibody are thus required for the formation of trypanopain-antibody complexes and to neutralise such high concentrations of antibody, vast molar excesses of peptide are required. It is thought that further increases in peptide concentration would reduce antibody binding to trypanopain almost entirely. This hypothesis was not tested experimentally because of the high cost of the synthetic peptide.

The effects of anti-peptide antibodies on Z-Phe-Arg-AMC hydrolysis by trypanopain were thus complex. Richmond (1977) stated that the effect of antibodies on enzyme activity can vary with the substrate used, and since Z-Phe-Arg-AMC is not a potential physiological substrate, the results obtained using this substrate were not considered representative of the *in vivo* situation. Thus, to investigate the effect of these antibodies on trypanopain activity *in vivo* more directly, their effects on digestion of protein substrates were investigated. Since only small amounts of purified trypanopain were available, highly sensitive assays for trypanopain hydrolysis of proteins were developed, using ^{14}C-gelatin and FITC-albumin.

It was expected that binding of the anti-peptide antibodies to trypanopain would occlude the active site and thus inhibit digestion of protein substrates. However, all tested anti-peptide antibodies enhanced trypanopain hydrolysis of ^{14}C-gelatin, while all antibodies inhibited hydrolysis of FITC-albumin. While such variation in antibody activity with differing substrates is poorly understood, it has been well documented (Richmond, 1977). Pre-incubation of the anti-peptide antibodies with the free peptide reduced both these effects, showing that both are specific. In both cases, the antibodies are thought to bind specifically to the trypanopain active site, protecting the enzyme from autocatalysis during the 30 min pre-incubation period. While non-specific antibodies are also likely to prevent such autocatalysis by acting as alternative substrates, they are likely to do so less effectively than antibodies that specifically bind the active site. Interestingly, such a concentration-dependent enhancement of trypanopain activity against

^{14}C-gelatin was seen in the presence of non-immune antibodies, presumably due to enzyme stabilisation. Following such postulated stabilisation of trypanopain during the pre-incubation phase of the experiment, either ^{14}C-gelatin or FITC-albumin was added. Compared with non-immune antibodies, activity against ^{14}C-gelatin was enhanced, while activity against FITC-albumin was inhibited.

A possible explanation for this result might be that the two substrates have different K_m values for trypanopain. If ^{14}C-gelatin has a higher affinity for trypanopain than do the anti-peptide antibodies, then it would displace these antibodies from the trypanopain active site when added, and the enhanced activity seen would then be a result of the increased stabilising ability of specific antibodies compared with non-immune antibodies. If on the other hand, FITC-albumin has a lower affinity for the trypanopain active site than do the anti-peptide antibodies, then it would be unable to displace these antibodies from the trypanopain active site and the inhibition of activity seen reflects continued antibody binding to the active site. To test this hypothesis, the K_m values for trypanopain hydrolysis of ^{14}C-gelatin and FITC-albumin would have to be determined, as well as the binding affinity of the various antibodies for trypanopain. However, the large amounts of trypanopain required for a statistically valid calculation of these constants (especially the binding affinity of the antibodies) precluded such a study. Additionally, the heterogeneous nature of the polyclonal antibodies raised in the present study is likely to complicate interpretation of such kinetic constants and may necessitate further purification of the antibodies or raising of monoclonal antibodies against the target peptide. It is, however, clear from the present results that the effects of anti-trypanopain antibodies on enzyme activity are largely dependent on the substrate in use. Without knowledge of the true *in vivo* substrates of trypanopain, it is not possible to undertake a physiologically relevant study.

The anti-peptide antibodies also modulated cathepsin L activity against ^{14}C-gelatin. Compared with their effects on trypanopain activity against this substrate, the antibodies enhanced cathepsin L activity less or inhibited activity. This suggests that the antibodies bind differently to the cathepsin L and trypanopain active sites Since cathepsin L is unlikely to be remain active in the mammalian bloodstream, these antibodies are not likely to have a physiological effect.

Anti-peptide antibodies were added to lysed *T. b. brucei* to assess the potential effects of other trypanosomal factors on such antibodies. Chicken antibodies had the same effect in lysed cells

as was previously observed with the pure enzyme, that is, some antibodies mediated inhibition and others enhancement of trypanopain hydrolysis of Z-Phe-Arg-AMC. The enhancement/inhibition observed with the pure enzyme and in lysates was comparable, suggesting that trypanosome-derived factors do not interfere with the effects of these antibodies on trypanopain activity. In contrast, rabbit antibodies that substantially activated the pure enzyme, had no effect in lysed trypanosomes even at 1 mg antibody/ml. This suggests that the interaction between these antibodies and trypanopain is altered by some factor in trypanosome lysates that does not affect chicken antibodies. Nevertheless, it appears that anti-trypanopain antibodies may have the potential to modulate trypanopain activity in a specific manner.

Trypanopain is likely to be effectively controlled in the mammalian bloodstream by cystatins, so the physiological relevance of the studies reported in this chapter are unclear. If trypanopain is not active in the bloodstream, then why does the production of anti-trypanopain antibodies correlate with disease resistance in cattle (Authié *et al.*, 1993)? If, as Lonsdale-Eccles *et al.* (1995) suggest, trypanopain interacts with kininogens in an unusual manner to produce an active complex, then it is feasible that trypanopain may contribute to pathogenesis in such cases and thus that inhibitory anti-trypanopain antibodies would be protective for the infected host.

However, it is also possible that anti-trypanopain antibodies are not protective for the infected host, and that the correlation between anti-trypanopain antibody production and disease resistance is not meaningful. If this is true, then there would be no therapeutic advantage to eliciting anti-trypanopain antibodies. Since an evaluation of trypanopain as a potential target for novel anti-trypanosomal agents was the aim of this study, the anti-peptide antibody study was abandoned at this stage in favour of an investigation of proteinase inhibitors as trypanocidal agents.

Chapter 7

Effect of proteinase inhibitors on cultured *T. b. brucei*

7.1 Introduction

Proteinases play important roles in the normal functioning of living systems as well as in various pathologies and disease processes. Proteinase inhibitors are thus being investigated as treatments for a wide variety of diseases, such as cancer (reviewed in De Clerk and Imren, 1994), arthritis (Esser *et al.*, 1994), bone resorption (Delaissé *et al.*, 1980) emphysema, atherosclerosis, glomerulonephritis, septicaemia, periodontitis, coaggulation and complement cascade disorders (reviewed in Schnebli and Braun, 1986). In some cases, proteinase inhibitors have already proved to be effective drugs and are in clinical use. For example, inhibitors of angiotensin converting enzyme (ACE) are used in the treatment of hypertension, and inhibitors of the human immunodeficiency virus (HIV) aspartic proteinase are commonly used in the treatment of acquired immunodeficiency syndrome (AIDS) (reviewed by Scharpe *et al.*, 1991). The cysteine proteinases of various pathogens have also received attention as potential targets for chemotherapeutic intervention. Cysteine proteinase inhibitors kill *T. cruzi* (Ashall *et al.*, 1990; Harth *et al.*, 1993; Franke de Cazzulo *et al.*, 1994), *T. congolense* (Mbawa *et al.*, 1992) and *Trichomonas vaginalis* (Irvine *et al.*, 1997), and halt the development of *S. mansoni* (Wasilewski *et al.*, 1996), *P. vinckei* (Rosenthal *et al.*, 1993), *P. falciparum* (Dluzewski *et al.*, 1986; Rosenthal *et al.*, 1988; Rockett *et al.*, 1990; Rosenthal *et al.*, 1993; Bailly *et al.*, 1992; Gluzman *et al.*, 1994) and *Tritrichomonas foetus* (Irvine *et al.*, 1997). Additionally, inhibitors of the major cysteine proteinases of *P. vinckei* and *S. mansoni* have been shown to be effective against murine malaria and schistosomiasis *in vivo* (Rosenthal *et al.*, 1993; Wasilewski *et al.*, 1996).

Since trypanopain is the major cysteine proteinase of *T. b. brucei*, the effects of various cysteine proteinase inhibitors on the activity of purified trypanopain and on the growth of cultured bloodstream forms of *T. b. brucei* were investigated.

7.2 *T. b. brucei* cell culture

While culture of procyclic forms of trypanosomes is a well established technique (Tobie, 1958; Cross and Manning, 1973; Brun and Schonenberger, 1973), the culture of mammalian bloodstream forms of trypanosomes has been more difficult to achieve. Hirumi *et al.* (1977) made the initial breakthrough in this field, culturing bloodstream trypanosomes using fibroblasts as a feeder cell layer. This method of culture has various drawbacks for metabolic studies, the greatest of which is the difficulty in discriminating between fibroblast and trypanosome metabolism. The discovery that reducing agents such as cysteine (Duszenko *et al.*, 1985) or β-mercaptoethanol (Baltz *et al.*, 1985) are essential growth factors for trypanosomes paved the way for successful culturing of trypanosomes without feeder cells. However, copper ions present in the medium readily oxidise cysteine to cystine, which does not stimulate growth. This problem can be circumvented by addition of the copper-specific chelator bathocuproine sulfonate (BCDS) (Zak, 1958) to the culture medium (Duszenko *et al.*, 1992; Hesse *et al.*, 1995).

Hesse *et al.* (1995) reported that trypanosomes cultured according to the protocol described below mimic a natural trypanosome infection in various ways. Most importantly, cultured trypanosomes undergo the oscillations in population size characteristic of natural populations during an infection.

7.2.1 Materials

Antibiotic solution. Lyophilised penicillin/streptomycin mix was reconstituted in 10 ml of autoclave-sterilised deionised H_2O according to the manufacturer's instructions, giving 10 000 units of penicillin and 10 mg of streptomycin per ml.

BCDS/cysteine stock (1 mM BCDS, 25 mM cysteine). BCDS (0.028 g) and cysteine (0.2195 g) were dissolved in 50 ml of deionised H_2O and filter-sterilised through a 0.22 μm syringe filter.

Foetal calf serum (FCS). Complement was heat-inactivated by incubation of the serum at 56°C for 1 h.

Minimal essential medium (MEM) with Earle's salts, 0.25 mM cysteine and 0.01 mM BCDS, 15% (v/v) FCS. MEM was prepared as described by Hesse *et al.* (1995). MEM powder with Earle's salts (4.8 g) and $NaHCO_3$ (1.1 g) were dissolved in 405 ml of deionised water and the medium filter-sterilised through a 0.45 μm pre-filter and a 0.22 μm filter unit into an autoclave-sterilised glass bottle. FCS (75 ml) was added aseptically. For each 100 ml of medium, 1 ml each of BCDS/cysteine stock and antibiotic solution were added aseptically.

7.2.2 Method

Aseptic technique was maintained throughout (Adams, 1990). All work was carried out in a laminar flow hood equipped with 0.22 μm air filters and an ultraviolet light, using autoclaved glassware and pipette tips, and commercially sterilised plastic pipettes (10 ml and 1 ml), culture flasks (with vented cap containing 0.2 μm filters) and petri dishes (50 mm diameter). All items were liberally sprayed with 70% (v/v) isopropanol before introduction into the hood. Culture flasks were used for initial population growth experiments and petri dishes for subsequent proteinase inhibitor experiments.

Medium was warmed to 37°C and aliquoted into flasks (5 ml of medium) or petri dishes (2.5 ml of medium) using sterile plastic pipettes. Any test-specific reagents were added to the flask or dish, followed by the addition of approximately 7.5×10^5 trypanosomes, thawed from stabilates in liquid nitrogen. Flasks or petri dishes were placed in a CO_2 incubator at 37°C and 5% (v/v) CO_2. Trypanosome growth was assessed using a haemocytometer to count the number of visibly motile trypanosomes.

7.3 Effects of proteinase inhibitors on *T. b. brucei* growth

Inhibitors of various classes of proteinases were added to cultured *T. b. brucei* to determine which classes might be necessary for parasite viability. Experiments with similar inhibitors have been conducted on cultured bone tissue (Delaissé *et al.*, 1980), various mammalian cell lines (Wilcox and Mason, 1992) and *P. falciparum* (Dluzewski *et al.*, 1986; Rosenthal *et al.*, 1988; Bailly *et al.*, 1992).

7.3.1 Materials

EDTA (100 mM). $Na_2EDTA.2H_2O$ (37 mg) was dissolved in 1 ml of DMSO.

IAN (100 mM). IAN (18 mg) was dissolved in 1 ml of DMSO.

Pepstatin A (100 mM). Pepstatin A (3.4 mg) was dissolved in 50 µl of DMSO.

PMSF (100 mM). PMSF (17.4 mg) was dissolved in 1 ml of DMSO.

E-64 (100 mM). E-64 (4 mg) was dissolved in 111 µl of DMSO.

TLCK (100 mM). TLCK (36 mg) was dissolved in 1 ml of DMSO.

SBTI (4 mM). SBTI (40 mg) was dissolved in 500 µl of DMSO.

Leupeptin (100 mM). Leupeptin (2.9 mg) was dissolved in 61 µl of DMSO.

Aprotinin (6.8 mM). Aprotinin (4.4 mg) was dissolved in 100 µl of DMSO.

NEM (100 mM). NEM (6 mg) was dissolved in 500 µl of DMSO.

IAA (100 mM). IAA (10 mg) was dissolved in 500 µl of DMSO.

7.3.2 Method

Petri dishes were set up as described in Section 7.2.2. Inhibitors (25 µl, 100 mM) were added to MEM (2.5 ml) to give a final concentration of 1 mM. SBTI and aprotinin were tested at 40 µM and 68 µM respectively because of limitations on the amount of inhibitor available. DMSO [25 µl, giving 1% (v/v) DMSO] was added to solvent control experiments containing no inhibitors. The number of visibly motile trypanosomes present after 24 h was counted using a haemocytometer.

7.4 k_{ass} of CMK, FMK and DMK

Peptidyl chloromethylketones (CMK) are synthetic, irreversible inhibitors of cysteine and serine proteinases. The peptidyl portion of the inhibitor is designed with the substrate specificity of the target enzyme in mind, so that the resulting inhibitors are able to discriminate between proteinases to some extent (Kurachi *et al.*, 1973; Coggins *et al.*, 1974; Kettner and Shaw, 1981). Following targeting of the inhibitor to a proteinase active site via the peptide sequence, the reactive CMK moiety binds covalently to the active site cysteine of cysteine proteinases (Whitaker and Perez-Villaseñor, 1968) or the active site histidine of serine proteinases (Schoellmann and Shaw, 1963; Ong *et al.*, 1965; Glover and Shaw, 1971), thereby irreversibly inactivating the respective enzymes.

Several other classes of peptidyl inhibitors have been designed based on the CMK theme. These newer inhibitors [e.g. peptidyl diazomethylketones (DMK) and fluoromethylketones (FMK)] are generally less reactive than the CMK, making them more specific for the target proteinase. FMK bind covalently with the active site histidine of serine proteinases and the active site cysteine of cysteine proteinases (Rauber *et al.*, 1986; Angliker *et al.*, 1987). Given the same peptide moeities, FMK are less reactive than CMK, due to the fact that chlorine is a stronger nucleophile than fluorine (Rasnick, 1985). While FMK are only 2% as reactive as CMK against serine proteinases (Rauber *et al.*, 1986), they are 35-40% as reactive as CMK against cysteine proteinases (Rasnick, 1985). Thus FMKs react more readily with cysteine than serine proteinases but, as they are irreversible inhibitors, this does not imply that they are specific for cysteine proteinases. Of the ketone family of inhibitors, FMK have been used most extensively *in vivo*, with much success. For example, FMK are active against murine malaria (Rosenthal *et al.*, 1993) and *S. mansoni* (Wasilewski *et al.*, 1996) *in vivo*.

DMK are considered specific for cysteine proteinases (Green and Shaw, 1981; Shaw, 1994; Rasnick, 1996), although isolated cases of inhibition of serine proteinases have been reported (Rasnick, 1996). The DMK moiety has low reactivity with thiols (Brocklehurst and Malthouse, 1978) and reacts only with the active site cysteine residue following positioning of the inhibitor by the peptide portion of the DMK (Leary *et al.*, 1977). The positioning role of the peptide moiety enables the synthesis of DMK with some degree of selectivity for various cysteine proteinases (Crawford *et al.*, 1988).

Since these inhibitors have low reactivity with thiols, they are stable in the presence of reducing agents in cysteine proteinase assay buffers (Leary *et al.*, 1977). The active site cysteine residue of the target enzyme is thought to protonate the diazomethyl carbon atom of DMK, forming a diazonium salt. The C_1 carbon of the diazonium salt undergoes a nucleophilic attack by the sulfhydryl anion of the enzyme, releasing N_2 and alkylating the active site cysteine residue (Leary *et al.*, 1977; Figure 45). More complicated reaction mechanisms, involving the formation of thiohemiketal intermediates, have also been proposed (Brocklehurst and Malthouse, 1978).

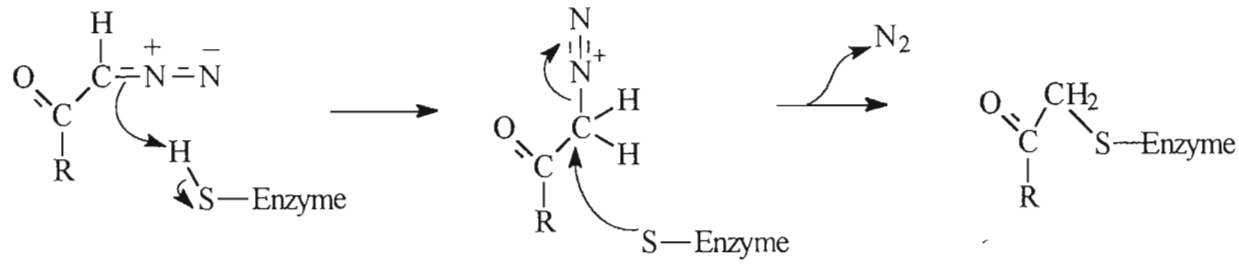


Figure 45: Postulated scheme for DMK inactivation of cysteine proteinases.
The active site cysteine residue is thought to protonate the diazomethyl carbon atom, forming a diazonium salt. The C_1 carbon of the diazonium salt then undergoes a nucleophilic attack by the sulfhydryl anion of the enzyme, releasing N_2 and alkylating the active site cysteine residue. After French *et al.* (1963) and Leary *et al.* (1977).

The rate of association (k_{ass}) between an irreversible inhibitor and its target enzyme may be determined using either first- or second-order conditions, depending on the expected rate of association (Salvesen and Nagase, 1989). Since, based on preliminary studies, k_{ass} for trypanopain inactivation by DMK, FMK and CMK was suspected to be greater than 10^5 $M^{-1}.s^{-1}$, second order conditions were employed in the present study. For enzyme and inhibitor to be combined in equimolar amounts, the active concentrations of both reactants are determined and the two then combined in assay buffer (Kirschke and Shaw, 1981). Aliquots are removed at timed intervals and residual enzyme activity determined. A ten-fold dilution of enzyme and inhibitor in the assay mixture is sufficient to prevent further association of enzyme and inhibitor (Salvesen and Nagase, 1989). k_{ass} can be determined from the equation (Salvesen and Nagase, 1989)

$$\frac{1}{[E]} = \frac{1}{[E]_0} + k_{ass}.\ t$$

where [E] is the enzyme concentration (M) at time, t,
$[E]_0$ is the initial enzyme concentration (M),
k_{ass} is the association rate constant ($M^{-1}.s^{-1}$), and
t is the time of incubation (s).

A plot of time of incubation versus 1/[E] has a slope of k_{ass} and a Y-intercept of $1/[E]_0$.

7.4.1 Materials

Sheep cathepsin L was a gift from Dr Theresa Coetzer, Department of Biochemistry, UNP.

Inhibitor stock solutions (100 mM) These were prepared by dissolving the following mass of each inhibitor in 100 µl of DMSO:

Z-Phe-Phe-DMK	4.7 mg
Z-Phe-Phe-CMK	4.8 mg
Z-Phe-CMK	3.3 mg
MeOSuc-Ala-Ala-Pro-Val-CMK	5.0 mg
Z-Phe-Ala-DMK	3.9 mg
Z-Phe-Ala-CMK	4.0 mg
Z-Phe-Ala-FMK	3.9 mg
Z-Lys-CMK	3.5 mg
Phe-Pro-Arg-CMK	4.5 mg
Z-Gly-Leu-Phe-CMK	5.0 mg
Z-Gly-Gly-Phe-CMK	4.4 mg
TLCK	3.3 mg
TPCK	3.5 mg

7.4.2 Method

The active concentrations of sheep cathepsin L and trypanopain were determined by E-64 titration (Section 3.4). CMK, FMK and DMK were titrated against cathepsin L to determine the active concentration of each inhibitor. All inhibitors were found to be 100% active (results not shown). Trypanopain (10 nM, 250 µl) was activated in 500 µl of pH 7.0 assay buffer (5 min, 37°C) and inhibitor (10 nM, 250 µl) added. Aliquots (40 µl) were removed from the mixture at timed intervals and assayed against Z-Phe-Arg-AMC for 10 min at 37°C as described previously (Section 3.2.2). Values for k_{ass} and $[E]_0$ were determined from plots of time of incubation versus 1/[E].

7.5 IC_{50} of CMK, DMK and FMK

Initial experiments in this laboratory showed that CMK, DMK and FMK kill cultured *T. b. brucei*. To quantify this trypanocidal effect, the IC_{50} of each inhibitor was calculated,

where the 50% inhibitory concentration (IC_{50}) is defined as the concentration of inhibitor required to inhibit trypanosome population growth by 50% compared to control populations after 24 h.

7.5.1 Materials

As per Section 7.3.1.

7.5.2 Method

Various concentrations of each inhibitor (2.5 µl in DMSO) were added to trypanosomes (7.5×10^5 parasites) in MEM (2.5 ml) in petri dishes and the dishes incubated as described in Section 7.2.2. The number of motile trypanosomes was counted after 24 h using a haemocytometer. Control flasks were incubated with 2.5 µl DMSO (0.001% DMSO). Plots of inhibitor concentration versus the number of live trypanosomes were prepared and IC_{50} values determined.

7.6 Detection of biotinylated FMK and DMK

Killing of trypanosomes by cysteine-proteinase specific DMK suggests that a cysteine proteinase is essential for *T. b. brucei* viability. To identify the targeted proteinase(s) more directly, biotinylated inhibitors were added to live and lysed *T. b. brucei*. After electrophoresis and electroblotting, a streptavidin-alkaline phosphatase conjugate was used to detect the biotinylated inhibitors and thereby determine the molecular mass of the targeted proteinase(s). Such biotinylated versions of CMK and DMK have been used to detect cathepsin B (Cullen *et al.*, 1992; Walker *et al.*, 1992) and chymotrypsin- and trypsin-like serine proteinases (Kay *et al.*, 1992).

7.6.1 Materials

As per Section 2.11.

Biotinylated inhibitors. Biotin-Phe-Ala-DMK was purchased from BioSyn (The Queen's University of Belfast, UK) and biotin-Phe-Ala-FMK was a gift from Dr James McKerrow (University of California, San Francisco, USA).

7.6.2 Method

Live and lysed *T. b. brucei* (containing 21 pmol trypanopain, as estimated by E-64 titration) were incubated with biotin-Phe-Ala-FMK (420 pmol, 24 h, 37°C) and biotin-Phe-Ala-DMK (420 pmol, 1 h, 37°C) respectively. Samples were electrophoresed on reducing tricine SDS-PAGE gels (Section 2.4) and electroblotted onto nitrocellulose (30 V, 16 h, Section 2.11). After blocking with BSA-TBS, the blot was incubated with ExtrAvidin®-alkaline phosphatase in BSA-TBS (3 h) and results visualised using BCIP/NBT substrate.

7.7 Design of chalcones

Most enzyme inhibitors and antiparasitic drugs have been discovered in large, random screens (Kuntz, 1992) and their specificity and reactivity subsequently modified by chemical manipulation. While this method has worked well, it is time consuming and labour intensive. International funding for anti-parasite research is limited and major pharmaceutical companies have little interest in the "small profit and high cost of development" of antiparasite drugs (McKerrow *et al.*, 1995). Any means of reducing the total cost and time required for the development of a new antiparasite drug would thus be welcomed. Once the mechanism of action and three-dimensional shape of an enzyme are known, it becomes possible to design molecules with high affinity for the enzyme active site. Such a method, known as rational drug design, is potentially a rapid method of drug development. Rational design of enzyme inhibitors has gained momentum in recent years, with the two most successful and well-known examples being the design of inhibitors of ACE and the HIV proteinase.

ACE acts on a number of physiologically important substrates (angiotensin I, neurotensin, enkephalins, bradykinin, substance P, cholecystokinin), producing a variety of physiological effector molecules (reviewed by Scharpe *et al.*, 1991). While ACE had not been crystallised, primary sequence data shows that it is very similar to pancreatic carboxypeptidase A, the three-dimensional structure of which is known. Specific and potent ACE inhibitors were thus designed based on the structure of carboxypeptidase A, taking the known differences in specificity of the two enzymes into account in designing a model of the ACE active site (Ondetti *et al.*, 1977). Orally active, peptide-based ACE

inhibitors designed in this manner are now clinically used to control hypertension (Scharpe *et al.*, 1991).

The *gag* and *pol* genes of HIV are expressed as polyproteins ($Pr55^{gag}$ and $Pr160^{gag\text{-}pol}$) that are proteolytically processed to generate the virion core structural proteins (p17, p24 and p7) and various essential enzymes (reverse transcriptase, ribonuclease H and endonuclease) (reviewed by Dreyer *et al.*, 1989). This processing is done by a viral aspartic proteinase that cleaves at a consensus sequence occurring at various positions along the polyproteins. Since HIV proteinase mutants are non-infectious and show altered morphology (Kohl *et al.*, 1988), the proteinase was considered a potential target for drug therapy. The known consensus sequence for enzyme cleavage of its natural polypeptide substrate was used as a basis for rationally designed inhibitors using various peptidyl mimics of aspartic proteinase transition state analogues (Dreyer *et al.*, 1989; Roberts *et al.*, 1990). These inhibitors were found to competitively inhibit the HIV-1 and HIV-2 proteinases (Dreyer *et al.*, 1989, Roberts *et al.*, 1990) and also to have anti-retroviral activity (McQuade *et al.*, 1990; Meek *et al.*, 1990) and now form part of the drug cocktail commonly used to treat AIDS patients.

Rational design of novel drugs is increasingly benefiting from advances in computer technology. For example, potential enzyme inhibitors can be predicted using the computer programme DOCK, which assesses the complementarity and energy interactions between two molecules (Kuntz, 1992). The programme can be used to scan a database of available small molecules to predict those that will bind strongly to a target enzyme. The starting point for such a search is a three-dimensional structure of the target enzyme, from which the programme identifies grooves and invaginations on the enzyme surface, amongst which will be the enzyme active site. The three-dimensional structures of molecules in a database are evaluated in various orientations and conformations for their complementarity to the target enzyme. Once the programme has identified suitable lead compounds, the top 100 to 200 can be individually examined using graphics programmes. The best 10-50 of these are tested *in vitro* for inhibition of the enzyme. Databases of 100 000 compounds can be evaluated in less than a week using DOCK, with about 2-20% of identified compounds inhibiting the target enzyme at micromolar concentrations (Kuntz, 1992).

While it is optimal to have a high resolution X-ray crystallographic structure of the target enzyme for DOCK searches, models can be used in cases where the three-dimensional structure is not known. Considering that X-ray crystallography remains a slow process, application of DOCK to model structures has obvious advantages. Such models can be constructed using the primary sequence of the target enzyme and the three-dimensional structures of homologous enzymes. The quality of the model generated in this manner is directly dependent on the degree of homology between the target enzyme and the homologous enzymes (Ring *et al.*, 1993). DOCK has identified successful inhibitors using models constructed from enzymes showing 20-30% homology with the target enzyme (Ring *et al.*, 1993).

Li *et al.* (1995) used DOCK to search for inhibitors of the *P. falciparum* cysteine proteinase (falcipain). While the three-dimensional structure of falcipain is not yet known, the primary structure of the enzyme is approximately 33% homologous to cruzipain, the three-dimensional structure of which is known (McGrath *et al.*, 1995). A falcipain model, built using the primary sequence of falcipain and the three-dimensional structure of cruzipain (Li *et al.*, 1994), was used in a DOCK search of a chemical database. Chalcones (1, 3-diphenyl-2-propen-1-one) were identified by this search as potential lead compounds. Interestingly, an independent research group published their finding that a chalcone-related molecule (lipochalcone A) isolated from licorice roots has anti-malarial activity (Chen *et al.*, 1994). Li *et al.* (1995) synthesised a variety of chalcone derivatives, and showed that the molecules do indeed reversibly inhibit falcipain and also kill cultured *P. falciparum*. Chalcones were also shown to inhibit cruzipain (Li *et al.*, 1995).

Since trypanopain is 6-9% homologous to falcipain (Rosenthal *et al.*, 1992; Rosenthal, 1993; Rosenthal *et al.*, 1994) and 60% homologous to cruzipain (Cazzulo *et al.*, 1989), it was considered likely that the chalcones designed and synthesised by Li *et al.* (1995) would inhibit trypanopain and may have trypanocidal activity. In collaboration with Cohen and co-workers, the effects of chalcones on trypanopain and cultured *T. b. brucei* were investigated. To evaluate the potential toxicity of these compounds in mammals, the effects of chalcones on mammalian cathepsin L were also investigated.

7.8 K_i for chalcone interaction with trypanopain and cathepsin L

Preliminary studies in our laboratory showed that chalcones are effective inhibitors of both trypanopain and cathepsin L. The interaction between chalcones and trypanopain or cathepsin L was quantitatively investigated by calculating K_i values for the interaction of the inhibitors with the enzymes. Inhibition was shown to be reversible by dilution of enzyme/inhibitor complexes (as described in Section 5.1.2). K_i values were calculated from the ratio between steady-state inhibited (v_i) and uninhibited (v_o) reaction velocities, as described in Section 5.2.3.

7.8.1 Materials

As per Section 4.8.6, 6.10.1 and 7.4.

Chalcones. Chalcones were designed and synthesised by the research group of Professor Fred Cohen at the Departments of Pharmaceutical Chemistry and Medicine of the University of San Francisco, California, USA.

7.8.2 Method

Dilution of chalcone/enzyme mixtures reduced the percentage of inhibition, showing that chalcones are reversible inhibitors of trypanopain and cathepsin L (Section 5.1.2). K_i was calculated from continuous assays (Section 3.2.2) by comparing the rates of Z-Phe-Arg-AMC hydrolysis in the absence and presence of chalcones (Section 5.2.3). Enzyme (3 ng, 0.1 pmol, 240 µl) was activated in pre-warmed assay buffer (25°C, 5 min, 500 µl), Z-Phe-Arg-AMC (250 µl, 5 µM) added and the initial rate of substrate hydrolysis (v_o) determined. Inhibitor (0.1-10 nmol in 10 µl DMSO, 0.1-100 µM final concentration) was added at several thousand-fold molar excess over the enzyme (first order conditions, Salvesen and Nagase, 1989) and the inhibited reaction rate (v_i) determined. K_i was calculated from the equation (Section 5.1.2)

$$K_i = \frac{[I]}{\frac{v_o}{v_i} - 1} \Big/ 1 + \frac{[S]}{K_m}$$

Since chalcones are competitive inhibitors, both [S] and [I] were considered. The affinity between substrate and enzyme was accounted for by inclusion of the Michaelis constant for hydrolysis of Z-Phe-Arg-AMC (K_m), which is 1.2 μM for trypanopain-Tb (Troeberg *et al.*, 1996) and 6.81 μM for sheep cathepsin L (Dehrmann *et al.*, 1995).

7.9 IC_{50} for chalcones against cultured *T. b. brucei*

Once chalcones were shown to be effective inhibitors of purified trypanopain, their effect on cultured *T. b. brucei* was investigated as described in Section 7.5.

7.9.1 Materials

As per Section 7.5.1.

7.9.2 Method

As described in Section 7.5.2, various concentrations of each tested chalcone (2.5 μl in DMSO, usually 0.8-20 μM final concentration) were added to *T. b. brucei* in MEM (2.5 ml) in duplicate petri dishes and the number of motile trypanosomes counted (twice from each petri dish) after 24 h using a haemocytometer. Plots of inhibitor concentration versus the number of live trypanosomes were prepared and IC_{50} values calculated.

7.10 *In vivo* testing of chalcones

Chalcones were shown to be highly trypanocidal in *in vitro* cell culture assays, suggesting that they might be effective anti-trypanosomal agents *in vivo*. The effects of the most trypanocidal chalcones on experimental *T. b. brucei* infection in mice were thus tested.

7.10.1 Materials

As per Sections 3.3.1 and 7.5.

7.10.2 Method

The chalcone most effective against purified trypanopain (ZLIII43A) and two of the most trypanocidal chalcones (ZLIII44A and MC161) *in vitro* were chosen for testing in mice.

The toxicity of chalcones was evaluated by injecting adult Balb/c mice intraperitoneally with 100 µl of phosphate/saline/glucose (PSG) and 25 µl of DMSO containing 2 mg of chalcone. The effect of chalcones on experimental *T. b. brucei* infection was examined first by injecting mice intraperitoneally with 100 µl of trypanosomes (5 000 trypanosomes total) in PSG and immediately afterwards with 25 µl of chalcone (0.5 mg) in DMSO. Control mice were injected with 100 µl trypanosomes (5 000 trypanosomes) and 25 µl of DMSO alone. The effects of a delayed injection of chalcones on *T. b. brucei* infection were investigated by injecting mice intraperitoneally with 100 µl of trypanosomes (5 000 trypanosomes total) in PSG and 3 h later with one of the three tested chalcones (0.5 mg in 25 µl DMSO). Control mice were injected with 100 µl trypanosomes (5 000 trypanosomes) and 3 h later with 25 µl of DMSO alone. Parasitaemia was monitored in both cases by microscopic examination of blood taken from the tail vein (Section 3.3).

7.11 Results

7.11.1 Growth curve

Assessment of trypanosome population growth was done by counting the numbers of visibly motile trypanosomes using a haemocytometer. Usually only a few non-motile trypanosomes were visible. These were assumed to be dead and were thus not counted. It appears that dead trypanosomes rapidly succumb to osmotic stress and burst. This serendipitously simplifies counting of live trypanosomes. Ashall *et al.* (1990) reported that such counting methods give the same results as dye (e.g. eosin) exclusion methods. Growth characteristics similar to those described by Hesse *et al.* (1995) were seen in this laboratory. Following inoculation into the culture medium, about 20% of the population died. After about 2 h, the population size began increasing as the trypanosomes entered the logarithmic phase of their growth. After about 20 h, the stationary phase of growth was entered, followed by the decline phase. This occurred even if the medium was changed every 6 h. No active trypanopain was detectable in the supernatant of culture flasks. This was not unexpected, since FCS contains cystatins, which were shown in Chapter 6 to effectively inhibit trypanopain.

7.11.2 Effect of proteinase inhibitors on growth of cultured *T. b. brucei*

The alkylating agents IAN, IAA and NEM were highly effective trypanocidal agents, killing all trypanosomes within 24 h (Figure 46). TLCK and SBTI were effective to a lesser extent, killing 54% and 40% of trypanosomes respectively in 24 h. All other inhibitors tested did not alter trypanosome population numbers relative to the controls.

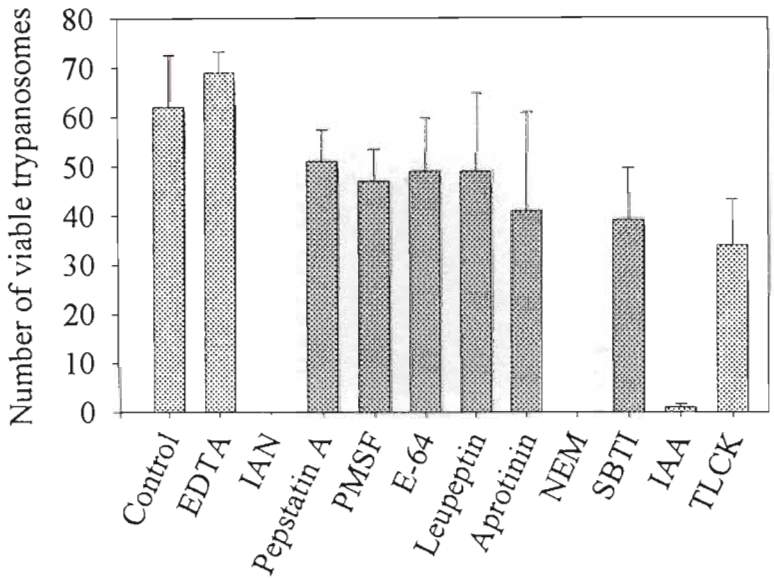


Figure 46: Effect of proteinase inhibitors on growth of cultured *T. b. brucei.*

Trypanosomes (approximately 7.5×10^5 cells) were placed in duplicate petri dishes in MEM (2.5 ml) with various proteinase inhibitors [in 25 µl DMSO, 100 mM, except for SBTI (40 µM) and aprotinin (68 µM)]. DMSO [25 µl, 1% (v/v) DMSO] was added to duplicate control experiments. Petri dishes were placed in a CO_2 incubator at 37°C and the number of visibly motile trypanosomes in the 0.1 µl haemocytometer chamber counted (twice from each dish) after 24 h using a haemocytometer.

7.11.3 k_{ass} of CMK, DMK and FMK

Most of the tested inhibitors effectively inhibited purified trypanopain (Table 17, Figure 47). Z-Phe-Phe-CMK had the highest k_{ass} of the tested inhibitors (2.16×10^6 $M^{-1}.s^{-1}$), followed by Z-Phe-Ala-FMK (1.53×10^6 $M^{-1}.s^{-1}$) and Z-Phe-Ala-CMK (1.1×10^6 $M^{-1}.s^{-1}$). MeOSuc-Ala-Ala-Pro-Val-CMK had the lowest k_{ass} (5.6×10^4 $M^{-1}.s^{-1}$). The other inhibitors tested all had fairly high k_{ass} values, in the 10^5 $M^{-1}.s^{-1}$ range (e.g. Z-Gly-Leu-Phe-CMK).

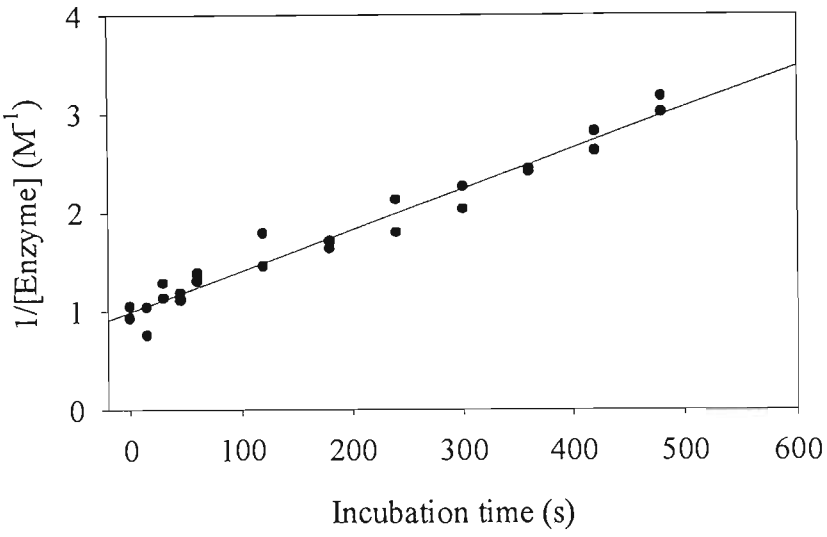


Figure 47: Determination of k_{ass} for Z-Lys-CMK inhibition of trypanopain.

Trypanopain (10 nM, 250 µl) was activated in pH 5.5 assay buffer (500 µl, 5 min, 37°C) and Z-Lys-CMK (10 nM, 250 µl) added. Duplicate aliquots (40 µl) were removed from the mixture at timed intervals and assayed against Z-Phe-Arg-AMC in stopped-time assays for 10 min at 37°C. Data was plotted as time of incubation versus 1/[Enzyme], with the gradient of the line equal to k_{ass} ($3.4 \times 10^5 M^{-1}. s^{-1}$).

Table 17: Effects of CMK, FMK and DMK on purified trypanopain and cultures of live *T. b. brucei*.

Inhibitor	**$k_{ass} \times 10^5$ ($M^{-1}.s^{-1}$)**	**IC_{50} (µM)**
Z-Phe-Phe-CMK	21.6 ± 1.6	3.6
Z-Phe-Ala-FMK	15.3 ± 0.6	31
Z-Phe-Ala-CMK	11.3 ± 1.4	21
Z-Phe-Ala-DMK	6.5 ± 0.4	6.1
Phe-Pro-Arg-CMK	5.0 ± 0.5	>75
Z-Phe-CMK	3.7 ± 0.4	20
Z-Lys-CMK	3.4 ± 0.2	65
TLCK	2.7 ± 0.2	48
Z-Phe-Phe-DMK	2.4 ± 0.2	4.8
TPCK	1.7 ± 0.2	14
Z-Gly-Leu-Phe-CMK	1.3 ± 0.1	33
MeOSuc-Ala-Ala-Pro-Val-CMK	0.6 ± 0.1	>75

7.11.4 IC_{50} of CMK, DMK and FMK

Trypanosomes treated with micromolar concentrations of CMK, DMK and FMK inhibitors either died, or grew more slowly than controls. The morphology of some of the trypanosomes remaining in inhibitor treated dishes was noticeably altered, with the normally long and slender trypanosomes becoming short and round. The inhibitors can be divided into three groups on the basis of their effectiveness, namely the highly effective inhibitors (Z-Phe-Phe-CMK; Z-Phe-Phe-DMK and Z-Phe-Ala-DMK) with IC_{50} values of approx. 5 μM; the least effective inhibitors (Phe-Pro-Arg-CMK and MeOSuc-Ala-Ala-Pro-Val-CMK) with IC_{50} values greater than 75 μM; and an intermediate group (remainder) (Table 17). Figure 48 shows an example of one such IC_{50} calculation.

7.11.5 Detection of biotinylated inhibitors

Biotin-Phe-Ala-FMK and biotin-Phe-Ala-DMK strongly labelled a single band of approximately 30 kDa in live and lysed *T. b. brucei* respectively (Figure 49). This corresponds to the reported molecular mass of trypanopain (Lonsdale-Eccles and Grab, 1987a; Pamer *et al.*, 1989; Boutignon *et al.*, 1990; Troeberg *et al.*, 1996).

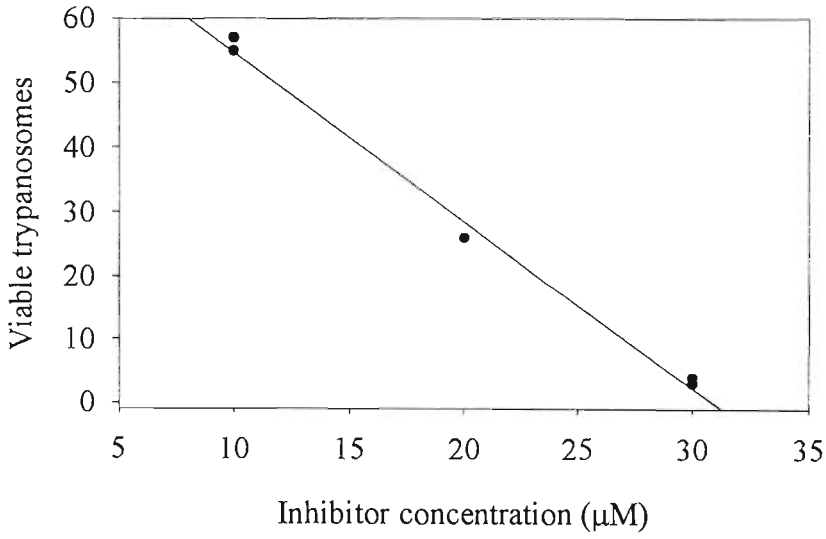


Figure 48: Determination of IC_{50} for TPCK.

Trypanosomes (7.5×10^5 cells) were grown in duplicate petri dishes in MEM with various concentrations (10 -75 μM) of TPCK. The number of viable trypanosomes was counted (twice from each dish) after 24 h using a haemacytometer. The IC_{50} was determined to be 14 μM.

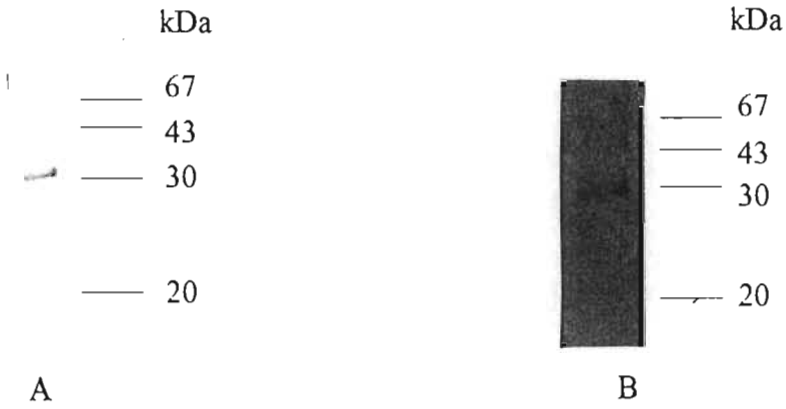


Figure 49: Biotin blots of live and lysed *T. b. brucei*.

A: Live *T. b. brucei* (containing 21 pmol trypanopain) were incubated with biotin-Phe-Ala-FMK (0.42 nmol, 24 h). B: Lysed *T. b. brucei* (21 pmol trypanopain) were incubated with with biotin-Phe-Ala-DMK (0.42 nmol, 24 h). Both samples were electrophoresed as in Section 2.4 and electroblotted as in Section 2.11 (30 V, 16 h). After blocking with BSA-TBS, the nitrocellulose blot was incubated with ExtrAvidin®-alkaline phosphatase BSA-TBS (3 h) and results visualised using BCIP/NBT substrate.

7.11.6 K_i for chalcone interaction with trypanopain and cathepsin L

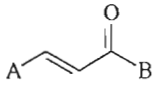
Chalcones inhibited trypanopain and sheep cathepsin L, with low micromolar or high nanomolar K_i values (Tables 18-21). The rate of association (k_{ass}) between the enzymes and chalcones was too fast for accurate determination without a stopped-flow apparatus. Against trypanopain, ZLIII43A had 10-fold lower K_i (27 nM) than any of the other tested inhibitors. MC23A24, HH27A13, HH27A44 and TF-1-56 all had Ki values of approx. 0.20 μM. MC153, MC135, MC159, MC161, BG21A11, HH29A43 and HH25A43 did not inhibit trypanopain at all at 100 μM. Against cathepsin L, ZLIII115A was most effective (K_i = 0.90 μM), with ZLIII115A, MC361A, MC96A4I and TF-1-60 also having low K_i values. HH29A13, HH29A14, HH26A13, BG21A11 and BG31A02 were least effective against cathepsin L.

7.11.7 IC_{50} for chalcone activity against cultured *T. b. brucei*

The tested chalcones inhibited growth of cultured *T. b. brucei* substantially, with IC_{50} values in the low micromolar and high nanomolar range (Table 18-21). MC161 had the lowest IC_{50} value (0.24 μM), with ZLIII44A, MC357 and TF-1-52 also having IC_{50} values

below 1 μM. MC24A24 had the highest IC_{50} value (28.56 μM), while MC96A4I and HH29A13 also had IC_{50} values above 23 μM.

Table 18: Effects of chalcones on cultured *T. b. brucei*, trypanopain & cathepsin L.



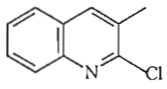
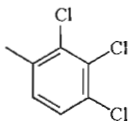
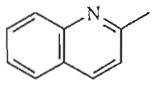
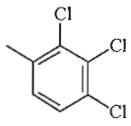
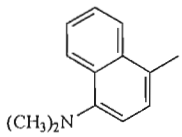
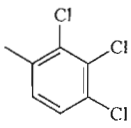
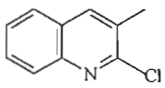
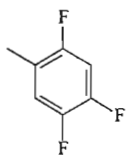
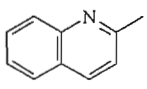
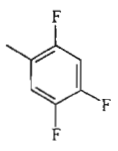
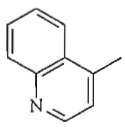
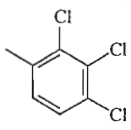
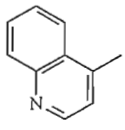
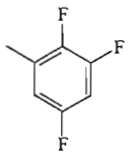
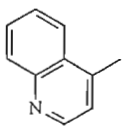
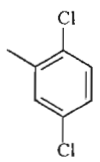
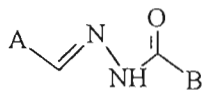
Chalcone	A	B	*T. b. brucei* IC_{50} (μM)	Trypanopain K_i (μM)	Cathepsin L K_i (μM)
MC131b			13.80	0.37±0.06	7.16±0.62
MC135			5.49	No inhibition	9.55±1.42
MC143			3.50	2.25±0.29	2.66±0.62
MC151			9.07	9.65±0.91	9.48±1.59
MC153			4.79	No inhibition	12.11±1.41
MC159			3.52	No inhibition	4.57±0.55
MC161			0.24	No inhibition	40.72±4.81
JD159			1.88	17.14±1.76	6.95±0.80

Table 19: Effects of hydrazide derivatives on cultured *T. b. brucei* and purified trypanopain and cathepsin L.



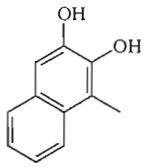
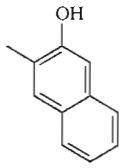
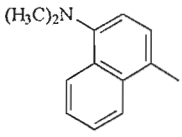
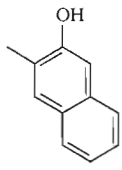
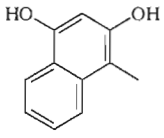
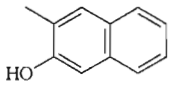
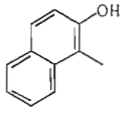
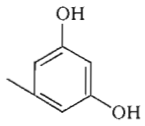
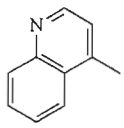
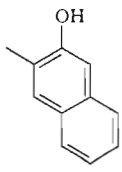
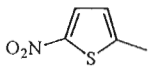
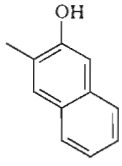
Chalcone	**A**	**B**	***T. b. brucei*** **IC_{50}** (μM)	**Trypanopain** **K_i** (μM)	**Cathepsin L** **K_i** (μM)
MC357			0.42	0.64±0.08	1.07±0.14
ZLIII115A			1.01	0.34±0.04	0.90±0.14
ZLIII43A			1.53	0.027±0.002	1.49±0.17
ZLIV44A			0.72	1.31±0.20	9.48±0.52
TF-1-51			1.55	4.47±0.64	1.52±0.28
TF-1-52			0.58	1.12±0.24	1.59±0.31

Table 19 continued…..

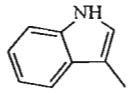
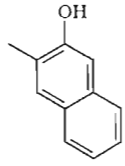
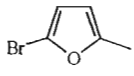
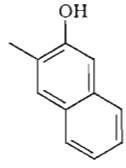
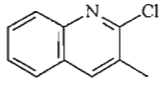
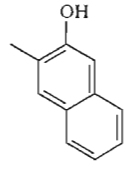
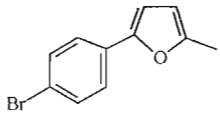
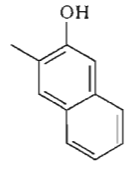
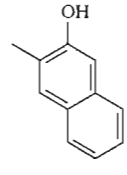
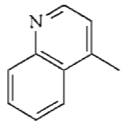
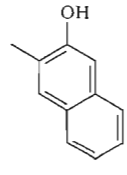
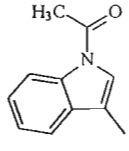
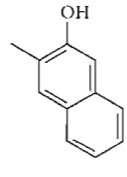
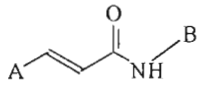
Chalcone	A	B	*T. b. brucei* IC_{50} (μM)	Trypanopain K_i (μM)	Cathepsin L K_i (μM)
TF-1-53			2.70	2.74±0.44	3.39±0.31
TF-1-54			2.34	3.45±0.27	3.18±0.48
TF-1-55			1.91	4.06±0.66	2.32±0.16
TF-1-56			1.66	0.22±0.06	1.90±0.10
TF-1-58			1.44	1.20±0.01	6.47±1.18
TF-1-59			2.14	1.38±0.14	3.81±0.59
TF-1-60			1.02	0.39±0.12	1.35±0.21

Table 20: Effects of amide derivatives on cultured *T. b. brucei* and purified trypanopain and cathepsin L.



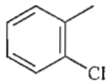
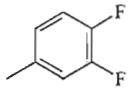
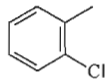
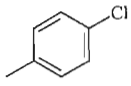
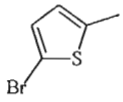
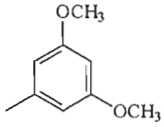
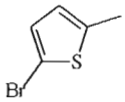
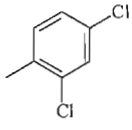
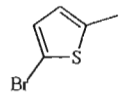
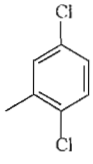
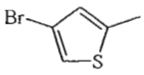
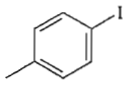
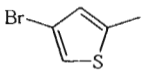
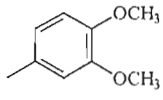
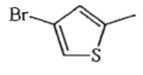
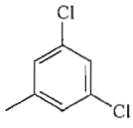
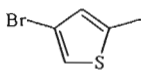
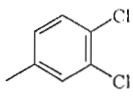
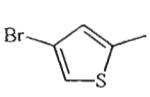
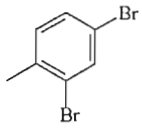
Name	A	B	*T. b. brucei* LD_{50} (μM)	Trypanopain K_i (μM)	Cathepsin L K_i (μM)
MC08A02			6.86	6.29±0.93	20.55±2.73
MC08A27			13.50	0.66±0.11	13.01±1.49
MC23A13			12.39	2.95±0.39	11.21±1.00
MC23A24			10.00	0.26±0.04	1.42±0.14
MC23A25			25.00	2.09±0.27	8.34±0.55
MC24A4I			11.82	1.06±0.15	3.25±0.62
MC24A12			18.28	6.20±0.77	21.69±3.04
MC24A22			18.88	2.47±0.33	1.45±0.14
MC24A24			28.56	0.61±0.09	11.52±1.38
MC24A31			12.94	9.69±1.33	5.33±1.25

Table 20 continued...

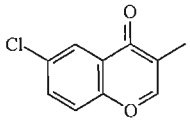
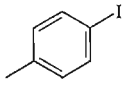
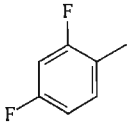
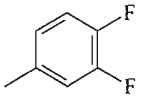
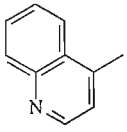
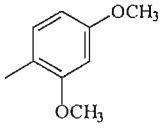
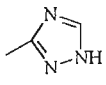
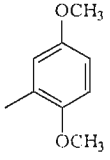
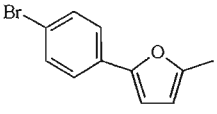
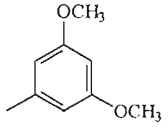
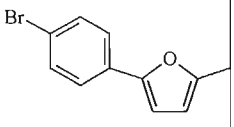
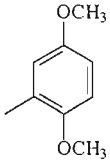
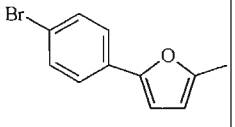
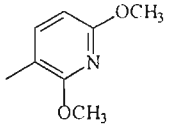
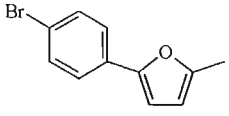
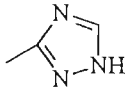
Chalcone	A	B	*T. b. brucei* IC_{50} (μM)	Trypanopain K_i (μM)	Cathepsin L K_i (μM)
MC96A4I			24.78	0.63±0.06	2.94±0.90
BG31A02			1.82	0.50±0.08	52.17±3.43
BG21A11			4.44	No inhibition	61.07±7.54
HH25A43			4.72	No inhibition	49.70±11.32
HH26A13			10.21	41.49±2.15	53.16±5.19
HH27A13			7.46	0.26±0.04	4.77±1.00
HH27A14			10.23	1.51±0.24	2.35±1.00
HH27A36			9.68	0.83±0.14	6.64±1.28
HH27A43			4.40	1.49±0.24	16.09±3.94

Table 20 continued…

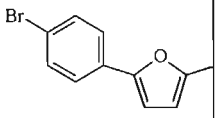
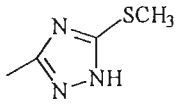
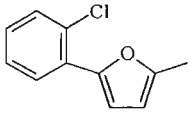
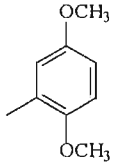
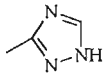
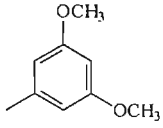
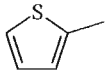
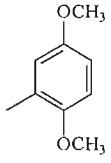
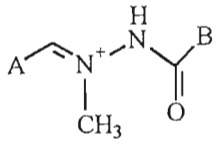
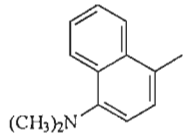
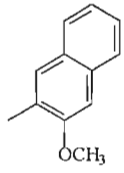
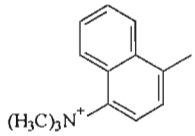
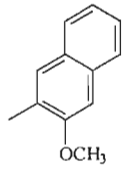
Chalcone	A	B	*T. b. brucei* IC_{50} (μM)	Trypanopain K_i (μM)	Cathepsin L K_i (μM)
HH27A44			17.14	0.22±0.02	12.46±0.86
HH28A14			7.16	2.20±0.21	3.08±0.42
HH29A43			4.40	No inhibition	20.49±1.87
HH29A13			23.77	9.08±2.80	60.05±9.62
HH29A14			18.19	13.06±1.87	81.86±3.15

Table 21: Effects of acyl hydrazide derivatives on cultured *T. b. brucei* and purified trypanopain and cathepsin L.



Name	A	B	*T. b. brucei* IC_{50} (μM)	Trypanopain K_i (μM)	Cathepsin L K_i (μM)
MC361A			10.52	0.70±0.06	0.97±0.10
MC361			13.55	25.56±2.82	No inhibition

7.11.8 *In vivo* testing of chalcones against *T. b. brucei*

An intraperitoneal dose of 2 mg of each of ZLIII43A, ZLIV44A and MC161 in DMSO was found to be non-lethal to adult Balb/c mice. Tested mice experienced slight side effects (appeared slightly bloated and photo-sensitive) only during the week following administration. Additionally, a dose of 5 000 trypanosomes (in 100 μl PSG, 25 μl DMSO) was shown to be lethal to adult Balb/c mice in 8-10 days.

Control mice injected with *T. b. brucei* (5 000 trypanosomes) alone were compared with mice simultaneously injected with the same amount of parasite and 0.5 mg of chalcone. Control mice (n=8) died on day 7.2±1.3. However, all mice (n=8) injected with ZLIII43A and MC161 survived, with no parasites evident in the bloodstream 1 month after infection. Of the 8 mice injected with ZLIV44A, 3 died on day 5.3±0.6, and the remainder (n=5) survived, with no parasites evident in the bloodstream 1 month after infection. Control mice injected with *T. b. brucei* (5 000 trypanosomes) alone were compared to mice injected with 0.5 mg of chalcone 3 h after *T. b. brucei* infection. MC161 was very

effective, prolonging the life of infected mice by 48%, while ZLIV44A prolonged life by 18% and ZLIII43A was ineffective (Table 22).

Table 22: Effects of chalcones on murine model of *T. b. brucei* infection.

Mice (n=8 in each group) were injected intraperitoneally with 100 µl of trypanosomes (5 000 trypanosomes total) in PSG and chalcone (0.5 mg in 25 µl) in DMSO injected 3 h later. Control mice (n=8) were injected with 100 µl trypanosomes (5 000 trypanosomes) and 3 h later with 25 µl of DMSO alone.

Drug	Day of death	% Increase in lifespan
Control	4.4±1.1	-
ZLIV44A	5.2±0.8	18
ZLIII43A	4.4±0.7	0
MC161	6.5±1.1	48

7.12 Discussion

Of the proteinase inhibitors tested, the alkylating agents IAN, IAA and NEM were the most trypanocidal, killing all trypanosomes within 24 h. Since *T. b. brucei* growth is known to be dependent on the presence of free cysteine in the medium (Duszenko *et al.*, 1985; 1992), it is likely that these inhibitors are trypanocidal in part because they starve the parasite of this essential nutrient. TLCK was less effective, killing 54% of cultured trypanosomes within 24 h. TLCK inhibits serine and cysteine proteinases, so its trypanocidal action implies that such a proteinase is likely to be essential for *T. b. brucei* viability. SBTI killed 40% of trypanosomes in 24 h, suggesting that a serine proteinase may be essential for viability. All other inhibitors tested did not alter trypanosome population numbers relative to the controls. Similar experiments have shown that leupeptin, chymostatin, pepstatin A and E-64 inhibit *P. falciparum* invasion of erythrocytes and haemoglobin digestion (Dluzewski *et al.*, 1986; Rosenthal *et al.*, 1988; Bailly *et al.*, 1992) and that serine and metallo-proteinase inhibitors reduce basement membrane degradation by cancer cells (Stonelake *et al.*, 1997). Some of the inhibitors are toxic to cells for reasons unrelated to proteinase inhibition (as is thought to be the case with the alkylating agents) and others do not readily enter cells [e.g E-64, Wilcox and Mason

(1992)]. Therefore, such tests with proteinase inhibitors can only be used as an initial guide to further experiments. The potential role of cysteine proteinases (including trypanopain) in *T. b. brucei* viability was thus further examined using DMK, CMK and FMK inhibitors.

Most of the tested inhibitors inhibited purified trypanopain with k_{ass} values in the 10^5 $M^{-1}.s^{-1}$ range (Table 17). Reports of k_{ass} values for inhibition of other parasite and mammalian cysteine proteinases by CMK, DMK and FMK unfortunately show very little overlap, limiting the comparisons that may be made between the present and previous studies. Additionally, considerable differences are evident between the small number of repeat experiments reported. For example, Franke de Cazzulo *et al.* (1994) reported a k_{ass} of 3×10^3 $M^{-1}.s^{-1}$ for the interaction of Z-Phe-Phe-DMK with cruzipain (the major cysteine proteinase of *T. cruzi*), while Mierelles *et al.* (1992) reported a 7-fold higher k_{ass} for the same inhibition (2×10^4 $M^{-1}.s^{-1}$). The value determined in the present study for inhibition of trypanopain by this inhibitor is 2.44×10^5 $M^{-1}.s^{-1}$, which is 80-fold higher than the value reported by Franke de Cazzulo (1994) and 12-fold higher than the value reported by Mierelles *et al.* (1992), both for cruzipain. In light of the discrepancy between the reported cruzipain values, it is difficult to postulate whether the difference between cruzipain and trypanopain values represents a true difference between the inhibition of cruzipain and trypanopain or rather differences in the methods used to calculate k_{ass}.

The majority of previous studies (Mbawa *et al.*, 1992; Mierelles *et al.*, 1992; Franke de Cazzulo *et al.*, 1994; Wasilewski *et al.*, 1996) on the inhibition of parasite cysteine proteinases by CMK, DMK and FMK have been conducted under first-order conditions, with the inhibitor in excess over the enzyme under study. However, this is only appropriate in cases where k_{ass} is suspected to be less than 10^5 $M^{-1}.s^{-1}$ (Salvesen and Nagase, 1989). Most studies on the inhibition of mammalian cysteine proteinases by CMK, DMK and FMK have been done on cathepsin L, with k_{ass} values above 10^5 $M^{-1}.s^{-1}$ for all tested inhibitors (Shaw, 1994). With this in mind, second-order conditions were employed in the present study, with the resultant k_{ass} values determined to be above 10^5 $M^{-1}.s^{-1}$ (Table 17). These values are more in agreement with Shaw's values for cathepsin L than with those reported for cruzipain (Mierelles *et al.*, 1992; Franke de

Cazzulo *et al.*, 1994). Reported k_{ass} values for ketone inhibition of the *S. mansoni* cysteine proteinase are in the 10^6 $M^{-1}.s^{-1}$ range (Wasilewski *et al.*, 1996), which are also in agreement with those obtained by Shaw (1994) and in the present study. However, irrespective of the actual k_{ass} values, most studies agree that derivatives of Phe-Phe and Phe-Ala are highly effective against these parasitic cysteine proteinases, reflecting their similarity to mammalian cathepsin L in terms of specificity. In light of trypanopain's substrate specificity, it would have been optimal to test inhibitors with Arg in the P1 position, but these were not commercially available at the time of the study.

In addition to inhibiting purified trypanopain, micromolar concentrations of the tested DMK, CMK and FMK inhibitors were also trypanocidal for cultured bloodstream forms of *T. b. brucei*. IC_{50} values were mostly in the 10-50 μM range (Table 17). Previous studies have shown that cysteine proteinase inhibitors kill *T. cruzi* (Ashall *et al.*, 1990; Harth *et al.*, 1993; Franke de Cazzulo *et al.*, 1994), *T. congolense* (Mbawa *et al.*, 1992) and *T. vaginalis* (Irvine *et al.*, 1997), and halt the development of *S. mansoni* (Wasilewski *et al.*, 1996), *T. foetus* (Irvine *et al.*, 1997), *P. vinckei* (Rosenthal *et al.*, 1993) and *P. falciparum* (Rockett *et al.*, 1990; Rosenthal *et al.*, 1990). No IC_{50} values were reported in these studies, so the relative effectiveness of the inhibitors cannot be assessed.

An absolute correlation between trypanocidal activity and inhibition of trypanopain was not observed. This can, however, be ascribed to the differing properties of the inhibitors in a cell culture system. For example, Z-Phe-Ala-FMK was a highly effective inhibitor of the enzyme but only moderately effective against the parasite, possibly because it penetrates living cells less readily than CMK and DMK. On the other hand, TPCK was very effective against the parasite but only moderately effective against the enzyme. TPCK is, however, a broad spectrum inhibitor and its trypanocidal effect could be due to inhibition of enzymes other than trypanopain-Tb. To the author's knowledge, no evaluation of the relative toxicities of CMK, DMK and FMK for mammalian cells or trypanosomes has been published, but the two DMK inhibitors tested had two of the three lowest IC_{50} values, suggesting that DMK may be more toxic to parasite cells than CMK or FMK. Since factors (e.g. membrane permeability, general toxicity) other than inhibition of the target enzyme are likely to affect the relative trypanocidal efficacy of inhibitors in a cell culture system, it is not unexpected that there is not an absolute correlation between IC_{50} and k_{ass}

values. However, Z-Phe-Phe-CMK was the most effective inhibitor against both the purified enzyme and the cultured parasite, and MeOSuc-Ala-Ala-Pro-Val-CMK was least effective in both cases.

The *in vitro* trypanocidal activity of the cysteine proteinase-specific DMK inhibitors suggests that a cysteine proteinase is required for *T. b. brucei* viability. Biotinylated derivatives of two inhibitors (biotin-Phe-Ala-FMK and biotin-Phe-Ala-DMK) were thus incubated with live and lysed *T. b. brucei* respectively to visualise the population of enzyme(s) binding to these inhibitors. A single band of 30 kDa (corresponding to the molecular mass of trypanopain) was strongly labelled in both experiments, suggesting that trypanopain is a major intracellular target for these inhibitors. Since FMK react with cysteine and serine proteinases, the specificity of labelling in live trypanosomes by biotin-Phe-Ala-FMK strengthens the likelihood that trypanopain (and no other serine or cysteine proteinase with this specificity) is essential for parasite viability. Studies previously conducted on other parasites using FMK, CMK and DMK showed that cysteine proteinases are essential for the viability of *T. cruzi* (Ashall *et al.*, 1990; Mierelles *et al.*, 1992; Franke de Cazzulo *et al.*, 1994), *T. congolense* (Mbawa *et al.*, 1992), *P. falciparum* (Rosenthal *et al.*, 1988; Rockett *et al.*, 1990), *P. vinckei* (Rosenthal *et al.*, 1993) and *S. mansoni* (Wasilewski *et al.*, 1996). Ketone derivatives of Phe-Ala and Phe-Phe were also highly effective in many of these studies, suggesting that a cathepsin L-like proteinase is targeted by these inhibitors.

While FMK, CMK and cysteine proteinase-specific DMK inhibitors are active against various parasites *in vitro*, it is necessary to demonstrate their action *in vivo*. CMK, however, have not been extensively tested *in vivo*, since they react with various cellular components other than serine or cysteine proteinases (Rossman *et al.*, 1974; Pong *et al.*, 1975). DMK are unstable at low pH and thus unsuitable for oral administration, and are reportedly also mutagenic (Rasnick, 1996). DMK and CMK have thus been used primarily as research tools. On the other hand, while various toxic FMK metabolites are produced *in vivo*, making high concentrations of FMK toxic and unsuitable for human use (Rasnick, 1996), FMK have been extensively tested in animals. The LD_{50} for FMK in rats is 300-400 mg/kg (Smith *et al.*, 1988). Mice are cured of murine malaria (caused by *P. vinckei*) by morpholine urea-Phe-homophenylalanine-FMK (Rosenthal *et al.*, 1993),

which strongly inhibits the haemoglobin-degrading cysteine proteinase of *P. vinckei in vitro* and is active against murine malaria *in vivo*. Only moderate adverse side effects were seen in the treated mice, possibly due to cross-reactivity with host proteinases (e.g. cathepsins) which could be reduced by careful inhibitor selection (Rosenthal *et al.*, 1993). Additionally, this inhibitor was effective in treating mice infected with *S. mansoni*, reducing the number of adult worms (by approx. 50%), egg production by adult worms, and hepatomegaly when administered at 1 mg twice daily intraperitonealy (Wasilewski *et al.*, 1996). The effects of morpholine urea-Phe-homophenylalanine-FMK and related inhibitors on experimental trypanosome infection should be determined. Insufficient FMK was available for *in vivo* testing against *T. b. brucei*, so the *in vivo* trypanocidal activity of FMK could not be evaluated.

In addition to their toxicity, peptide-based compounds such as DMK, CMK and FMK have various disadvantages as potential drugs, the greatest of which are their short half lives *in vivo* and their limited oral bioavailability (Kuntz, 1992). Various steps have been taken to counteract these problems. *In vivo* hydrolysis can be reduced by inclusion of unusual or D-amino acids, backbone modifications and peptide cyclisation. Non-invasive alternatives to oral delivery (e.g. inhalation, nasal absorption, electroporation) are also being investigated (Kuntz, 1992). Such investigations were beyond the scope of the present study, so more bio-stable, non-peptide inhibitors of cysteine proteinases were sought for *in vivo* testing. Reports by Li *et al.* (1995) suggested that chalcones might be appropriate and, in collaboration with Fred Cohen and his co-workers (Departments of Pharmaceutical Chemistry and Medicine of the University of San Francisco, California, USA), the trypanocidal effects of this new group of reversible cysteine proteinase inhibitors were evaluated. Most the tested chalcones and chalcone derivatives effectively inhibited purified trypanopain as well as mammalian cathepsin L. DOCK modelling suggests that chalcones bind into the S_2, S_1 and S_1' sites of falcipain (Li *et al.*, 1995), and the same is likely to be true of trypanopain.

The chalcones were fairly effective cathepsin L inhibitors. The chalcones series, which contains quinolinyl A rings, were almost all poor inhibitors of trypanopain (Table 18). Only those with substituents [Cl or $(CH_3)_2N$) on their quinolinyl A rings (MC131b, MC143 and MC151) were effective against trypanopain at less than 10 μM. All of these

compounds were, however, effective against cathepsin L at approx 10 μM or less. This suggests that the trypanopain active site is less able to tolerate substituted and unsubstituted quinolinyl A rings than is cathepsin L. However, the hydrazide derivative series contains three effective inhibitors with quinolinyl rings (TF-1-59, TF-1-55 and TF-1-51) suggesting that it is the combination of the chalcone linker with the quinolinyl A ring that is unfavourable for trypanopain. Of the various substitutions made, it appears that substitution of the B ring with F atoms is least favourable.

The hydrazide derivatives were highly effective trypanopain inhibitors, with all K_i values falling below 5 μM (Table 19). In addition, this group contained the most effective trypanopain inhibitor, ZLIII43A. Hydrazides with bulky non-quinolinyl A groups were generally more effective against trypanopain. This group was also effective cathepsin L inhibitors, with all K_i values below 2 μM. While no direct comparison was made, it appears that acylation of the hydrazide linker had no effect on the efficacy of these inhibitors (Table 21). For example, MC361A is approx. as effective as ZLIII115A as an inhibitor of both trypanopain and cathepsin L. The effects of substitution on the A and B groups was most extensively studied in the group of amide derivatives (Table 20). Addition of Br to the A (5-membered S-ring) of HH29A13 (i.e. MC2A13) lowers the trypanopain K_i from 9.08 μM to 2.95 μM and the cathepsin L K_i from 17.35 μM to 3.24 μM. Comparison of MC24A24 and MC24A22 suggests that trypanopain favours Cl groups in positions 3 and 4 on the B phenyl ring rather than in positions 3 and 5, while the opposite is true for cathepsin L. Methyl groups are favoured in positions 3 and 5 (HH29A13, K_i = 9.08 μM) on the B ring rather than 2 and 5 (HH29A14, K_i = 13.06 μM) by both trypanopain and cathepsin L. Additionally, both enzymes favour Cl substitutents on the B group over methyl groups (compare MC24A24 and MC24A12, Table 20). Methyl substitution on the B ring seems to be particularly unfavourable for cathepsin L.

All of the tested chalcones were trypanocidal against cultured *T. b. brucei*. The chalcone MC161 had the lowest LD_{50} value (0.24 μM), with the hydrazides ZLIII44A, MC357 and TF-1-52 also having LD_{50} values below 1 μM. It thus seems that the hydrazide linker is best suited to crossing membranes. The amides MC24A24, MC96A4I and HH29A13 had the highest LD_{50} value (above 23 μM), suggesting that this linker crosses *T. b. brucei* membranes poorly.

There was not an absolute correlation between inhibition of purified trypanopain and trypanocidal activity. This is not unexpected since trypanocidal activity requires additional properties (e.g. membrane solubility) not relevant to inhibition of trypanopain directly. Thus, some poor trypanopain inhibitors were highly trypanocidal (MC161 and MC159), and some excellent enzyme inhibitors fared less favourably against the parasite (MC96A4I, MC24A24 and HH27A44).

The most effective trypanopain inhibitor ZLIII43A and two of the most trypanocidal chalcones (ZLIII44A and MC161) were tested in mice. When chalcones were injected simultaneously with *T. b. brucei*, all mice injected with ZLIII43A and MC161 and 63% of mice injected with ZLIV44A survived. When chalcones were administered 3 h after *T. b. brucei* infection, ZLIV44A prolonged life by 18%, MC161 prolonged life by 48% and ZLIII43A was ineffective. The decreased effectiveness observed in the second study is thought to indicate that the effective concentration of chalcone trypanosomes were exposed to was higher in the first experiment. Chalcones are poorly water soluble, and it is likely that once injected into the body they will precipitate, so that only a small fraction of the administered dose remains available. When chalcones are co-administered with parasites, their concentration in the peritoneum appears to have been sufficient to kill enough parasites to prevent the establishment of an infection. However, when chalcones were injected 3 h after infection, the majority of parasites are likely to have migrated from the peritoneum, and the concentration of chalcone outside the peritoneum does not appear to have been sufficient to prevent completely the establishment of an infection.

MC161 was the most effective of the chalcones in *in vitro* cell culture experiments and *in vivo* mouse experiments. This chalcone is, however, a poor trypanopain inhibitor, with no inhibition evident at 100 μM. The low water solubility of the compound prevents its analysis at higher concentrations. The highly trypanocidal nature of this inhibitor may suggest that it is more able to cross parasite cell membranes than ZLIII43A or ZLIV44A. Alternatively, its intracellular target may not be trypanopain. ZLIII43A and ZLIV44A were effective trypanopain inhibitors and also highly effective trypanocidal agents in *in vitro* cell culture experiments. While these chalcones were less effective than MC161 *in vivo*, the current results suggest that increased doses of these inhibitors (especially

ZLIV44A) are likely to give better results. Experiments are currently under way in our laboratory to assess the effects of repeated administration of chalcones on *T. b. brucei* infection. The low water solubility of the agents is likely to be a problem, with DMSO toxicity predicted to occur with multiple administrations. Potential ways to improve the water solubility of chalcones are being considered.

The three chalcones were shown to be non-lethal to adult Balb/c mice, with only slight side effects seen in the first week after administration. Since all of these compounds inhibit mammalian cathepsin L, inhibition of the murine enzyme may be the cause of these side effects. While cysteine proteinases show many similarities, advances in the understanding of these enzymes is making it possible to exploit their small differences to create more specific inhibitors (Storer and Ménard, 1996). ZLIII43A is 16 times more effective against trypanopain than against cathepsin L. Salvesen and Nagase (1989), however, suggest that in order to suppress the activity of a single proteinase *in vivo*, the selective inhibitor should have a K_i for the enzyme 1000-fold less than for any other enzyme in the mix, or a k_{ass} 1000-fold greater than for any other proteinase in the mix. ZLIII43A clearly does not fit these criteria, but is nevertheless effective *in vivo*. Possibly chalcones are more able to enter trypanosomes than mammalian cells, so that the effective concentration of ZLIII43A is too low inside mammalian cells for cathepsin L inhibition to be toxic. *T. cruzi* selectively accumulates FMK-based inhibitors, leading McKerrow *et al.* (1995) to suggest that further study of the mechanisms used by parasites to endocytose small molecules from their surrounding medium may have benefits for drug design. Ideally, wholly specific inhibitors of cysteine proteinases should be used *in vivo* to prevent any side effects. To develop such inhibitors, the interactions between enzymes and their substrates/inhibitors that occur outside of the major subsites should be studied (Storer and Ménard, 1996). For example, the S' subsites of trypanopain have not been characterised.

Chalcones would be inexpensive to synthesise, and could thus potentially be of great use in the developing world. The major potential disadvantage of chalcones is their low water solubility, although attempts to improve this are being made. The *in vivo* half-life and oral bioavailability of chalcones remains to be tested. Various other classes of cysteine proteinase inhibitors have been recently described, and their effects on trypanopain and

T. b. brucei should be investigated. For example, α-keto amides reportedly inhibit calpains and cathepsin B (Li *et al.*, 1996), peptidyl acyloxymethanes (Krantz, 1994) and N, O-diacyl hydroxamates (Brömme and Demuth, 1994) inhibit various cathepsins, while vinyl sulfones inhibit falcipain (Rosenthal *et al.*, 1996) and papain (Hanzlik and Thompson, 1984). Vinyl sulfones have been shown to inhibit metabolism, growth and development of cultured *P. falciparum* (Rosenthal *et al.*, 1996). Studies are presently under way to assess the effects of vinyl sulfones on trypanopain and cultured *T. b. brucei*.

Chapter 8

General discussion

8.1 Introduction

Trypanosomiasis has historically been of substantial economic and social importance in many parts of Africa, and continues to exert a significant effect on many parts of the continent today. Gutteridge (1985) remarked that none of the currently available anti-trypanosomal drugs is entirely satisfactory, since they generally lack oral activity, have undesirable side-effects and are unable to treat all stages of the disease. Additionally, while there are few reports of drug resistant human trypanosomes, resistant cattle strains limit the veterinary efficacy of the current drugs. This, together with the time and cost involved in controlling tsetse fly populations, has led to a search for novel means of disease therapy and prevention. However, while trypanosomes have been studied for many decades, there remains a general lack of understanding of the parasite metabolites responsible for the pathology of trypanosomiasis. This is illustrated by the fact that most of the anti-trypanosomal drugs currently in use were developed many decades ago and additionally, that their mode of action and target molecules remain speculative (Gutteridge, 1985). DFMO is the only drug developed in the last decade, and is also the best understood of the currently available treatments (McCann *et al.*, 1981; Bacchi *et al.*, 1983; Fairlamb *et al.*, 1987).

The major lysosomal cysteine proteinases (trypanopains) of trypanosomes have been suggested to contribute to the pathogenesis of trypanosomiasis by degrading host proteins in the bloodstream. At the time this study was undertaken, such theories were purely speculative, since the interactions between trypanopains and endogenous host cysteine proteinase inhibitors (cystatins) had not been reported. Additionally, while trypanopains were known to be major antigens in infected cattle (Authié *et al.*, 1993), the effects of anti-trypanopain antibodies on trypanopain had not been investigated. Also, while the trypanopain-Tc and cruzipain had been purified from *T. congolense* (Mbawa *et al.*, 1992) and *T. cruzi* (Cazzulo *et al.*, 1989; Murta *et al.*, 1990; Lima *et al.*, 1992) respectively and their kinetic properties extensively characterised, trypanopain-Tb from *T. b. brucei* had not been purified to electrophoretic homogeneity or

similarly characterised. *T. b. brucei* is of considerable economic importance in Africa as a cattle parasite, and also serves as an excellent model for the trypanosome species that infect humans. The aims of this study were thus initially to isolate trypanopain from *T. b. brucei* and subsequently to characterise the enzyme's kinetic properties and its interactions with host proteinase inhibitors and antibodies.

8.2 Enzymatic characterisation

This study reports the first isolation to electrophoretic homogeneity of trypanopain from mammalian bloodstream forms of *T. b. brucei,* using a combination of three-phase partitioning between ammonium sulfate and *t*-butanol, anion exchange chromatography on Q-resins and pepstatin A-Sepharose affinity chromatography. These methods had not been previously utilised for the purification of trypanopains. The purification was particularly challenging because of the extremely small amounts of starting material available and the low stability of the enzyme. A rapid, efficient means of purification was thus essential. As discussed in Chapter 3, purification of trypanopain relied heavily on the fact that this enzyme binds to Q-Sepharose at pH 5.5, while most other trypanosomal proteins do not. This indicates either that trypanopain has an unusually low pI or that regions of the enzyme remain strongly negatively charged below the enzyme's pI.

Separation of trypanopain into three active peaks on Resource Q may indicate that the enzyme undergoes variable post-translational modifications. Alternatively, the different enzyme populations may consist of different gene products encoded by the different tandemly arranged genes (Mottram *et al.*, 1989). This appears to be the situation with the major cysteine proteinases of *T. cruzi* (Campetella *et al.*, 1992; Eakin *et al.*, 1992) and *Leishmania* (Souza *et al.*, 1992; Mottram *et al.*, 1996). In the case of cruzipain, the different gene products vary slightly in their substrate specificity (Lima *et al.*, 1994), possibly indicating that the gene products fulfil slightly different roles in the organism. Preliminary kinetic analysis of the three Resource Q peaks revealed no such variation in trypanopain specificity, but it remains possible that such variation would be revealed by a wider investigation involving more substrates. The separation of trypanopain into three peaks must reflect some underlying variability in trypanopain and further analysis of the translation of trypanopain genes is required to unravel

the cause and significance of this chromatographic separation and to better understand the reasons why trypanopain is encoded by multiple genes.

Since Resource Q columns were not cost-effective, pepstatin A-Sepharose was used as the final purification step in later purifications. While it is unusual that an aspartic proteinase inhibitor should inhibit a cysteine proteinase, such inhibition has been previously reported for trypanopain from *T. b. rhodesiense* (Pamer *et al.*, 1989) and trypanopain-Tc (Mbawa *et al.*, 1992). Pepstatin A was shown to be a competitive inhibitor of trypanopain, suggesting that it acts as a substrate analogue.

Only 30 μg of the purified trypanopain over 4 injections was required to elicit high titre antibodies in a chicken. This is much less than is normally considered necessary to evoke an effective immune response (Thorpe, 1994), suggesting that trypanopain is highly immunogenic in chickens. This is of particular significance where the immunogen is unstable in its purified form or starting material is limited, as is commonly the case with parasite enzymes. Western blots using these antibodies confirmed a previous report by Pamer *et al.* (1991) that anti-trypanopain antibodies recognise three bands in lysed *T. b. brucei*. Pamer *et al.* (1991) suggested from analysis of the trypanopain gene coding region that these are likely to be processing intermediates of the enzyme, but pulse-chase studies are obviously needed to confirm this suggestion.

Trypanopain was shown to be a fairly typical cysteine proteinase; active only in the presence of reducing agents and inhibited by E-64, cystatins and alkylating agents. Previous characterisations of trypanopain's kinetic properties were all carried out on partially purified enzyme or lysed *T. b. brucei* (Boutignon *et al.*, 1990; Robertson *et al.*, 1990; Pamer *et al.*, 1991; Huet *et al.*, 1992), so this study represents the most reliable reported characterisations. The enzyme was found to be optimally active against Z-Phe-Arg-AMC between pH 6.5 and 8.0, and against the proteinaceous substrate ^{14}C-gelatin at pH 5.5. Since a pH optimum determined using a proteinaceous substrate is more likely to be physiologically relevant than that optimum determined using a synthetic substrate, it is considered likely that the *in vivo* pH optimum of trypanopain is approximately pH 5.5. Across the pH range, trypanopain was more stable in the absence of cysteine than in its presence, suggesting that the active enzyme

undergoes autocatalysis. Trypanopain has cathepsin L-like specificity for synthetic substrates, with Z-Phe-Arg-AMC being by far the most favoured of the tested synthetic substrates. Substrates with basic residues (Arg or Lys) in P_1 and hydrophobic residues (Phe and Leu) in P_2 were most rapidly hydrolysed. Hydrophobic residues (e.g. Val) were preferred over small, uncharged residues (e.g. Ala) in P_3. Additionally, acidic residues were unacceptable in P_4. Trypanopain was thus shown to be kinetically similar to trypanopain-Tc (Mbawa et al., 1992) and cruzipain (Cazzulo *et al.*, 1989; Murta *et al.*, 1990; Lima *et al.*, 1992).

Trypanopain was shown to be able to hydrolyse a number of mammalian proteins (e.g. BSA, IgG, fibrinogen, albumin and gelatin) *in vitro*. Thus, if trypanopain remains active when it is released into the mammalian bloodstream (either actively or from dying parasites), then it is possible that the enzyme may contribute to disease pathogenesis by degrading host proteins. However, endogenous proteinase inhibitors (e.g. cystatins or kininogens) or anti-trypanopain antibodies may inhibit the enzyme *in vivo*. Consequently, the effects of both natural cysteine proteinase inhibitors (cystatins) and anti-trypanopain antibodies on enzyme activity were investigated in the present study.

8.3 The control of trypanopain by inhibitors in the bloodstream

Cystatin C and L-kininogen were both shown to strongly inhibit purified trypanopain *in vitro*. The binding constants for cystatin C and L-kininogen suggest that trypanopain is likely to be effectively inhibited in the host bloodstream and therefore unlikely to contribute to pathogenesis by the generalised digestion of host proteins. Indeed, no trypanopain-like activity was detectable in the blood of naturally-infected cattle or experimentally-infected rats, and *in vivo*-formed trypanopain/cystatin complexes were shown to be inactive against Z-Phe-Arg-AMC. This study thus argues against the widely held contention amongst researchers in this field that trypanopains contribute to pathogenesis by degrading host proteins in the bloodstream.

However, reports of the formation of active complexes between trypanopain and kininogen-like molecules (rTM) cast doubt on the conclusion that trypanopain is inhibited in all situations (Lonsdale-Eccles *et al.*, 1995). The present study showed that L-kininogen enhances hydrolysis of Z-Phe-Arg-AMC by live *T. b. brucei* by 20-40% *in vitro*. Thus, the possibility that active

trypanopain/cystatin complexes form under certain undefined conditions *in vivo* cannot be excluded.

This study has shown that anti-trypanopain antibodies can inhibit trypanopain digestion of Z-Phe-Arg-AMC and FITC-albumin. While trypanopain-Tc and cruzipain have been reported as major antigens in infected cattle (de Souza *et al.*, 1990; Murta *et al.*, 1990; Authié *et al.*, 1993; González *et al.*, 1996), this is the first investigation of the effects of anti-trypanopain antibodies on enzyme activity. If similar antibody inhibition of trypanopain occurs *in vivo* in infected hosts, then degradation of host proteins could be limited and pathology reduced. It is also possible that inhibitory anti-trypanopain antibodies could exert a protective effect by opsonising trypanopain and thus mediating more rapid clearance of the enzyme from the blood. Either possibility is supported by the observed correlation between the production of anti-trypanopain IgG by certain breeds of cattle and their tolerance to trypanosome infection (Authié *et al.*, 1993).

However, the present study has shown that some anti-trypanopain antibodies can also enhance trypanopain activity against Z-Phe-Arg-AMC and ^{14}C-gelatin. The presence of such activity-enhancing antibodies *in vivo* may contribute to a complex interplay between enhanced host protein degradation and enhanced enzyme clearance by opsonisation and inhibition of enzyme activity. Since antibody modulation of trypanopain activity varies depending on the substrate used, and since the *in vivo* substrates of trypanopain remain unknown, it is difficult to predict the effects of these antibodies on *in vivo* trypanopain activity. However, it should be possible to investigate the effects of these antibodies on disease parameters (e.g. parasitaemia, anaemia) in a natural infection directly by passive immunisation of infected animals (e.g. mice) with these antibodies.

Since it appears that trypanopain is effectively controlled by cystatins in the mammalian bloodstream under normal circumstances, the correlation between disease resistance and anti-trypanopain antibody production is puzzling. While the correlation does not necessarily imply a causal relationship, anti-trypanopain antibodies may be protective for reasons other than direct inhibition of the enzyme. As shown in this study, trypanopain is highly immunogenic in chickens. Trypanopain peptides are also immunogenic in chickens and rabbits. Trypanopain

may thus elicit high titre antibodies that protect the host by alerting the immune system to the presence of the parasites in the blood. To investigate the protective effects of trypanopain directly, it would be necessary to immunise a susceptible host with trypanopain and then challenge the immunised animal with a trypanosome infection. Such an experiment was beyond the scope of the present study, since it requires large amounts of trypanopain, and would thus require expression of recombinant enzyme.

8.4 Trypanocidal activity of trypanopain inhibitors

Numerous parasites, including trypanosomes, have been shown to be sensitive to cysteine proteinase inhibitors, eliciting interest in these inhibitors as potential chemotherapeutic agents (Rosenthal *et al.*, 1988; Ashall *et al.*, 1990; Mbawa *et al.*, 1992; Wasilewski *et al.*, 1996; Irvine *et al.*, 1997). With this in mind, the effects of cysteine proteinase inhibitors on *T. b. brucei* were investigated. Various DMK, FMK and CMK were shown to be effective inhibitors of the purified enzyme and additionally to be trypanocidal for cultured bloodstream forms of *T. b. brucei*. This study also showed for the first time that these inhibitors can associate with trypanopains at rates similar to those reported for mammalian enzymes (Shaw, 1994), and not only at the slower rates previously reported for cruzipain (Mierelles *et al.*, 1992; Franke de Cazzulo *et al.*, 1994). The higher rates of association observed in this study are attributed to the fact that second order conditions were used in the present study, while first order conditions were used in previous cruzipain studies. Labelling of lysed and live *T. b. brucei* with biotin-labelled derivatives of these inhibitors showed that trypanopain is the principal intracellular target of these inhibitors.

Salvesen and Nagase (1989) suggested that, in general, it is preferable to use a reversible inhibitor such as a chalcone *in vivo* rather than an irreversible one such as a CMK, DMK or FMK. An irreversible inhibitor will eventually inhibit proteinases it reacts with slowly, whereas a reversible inhibitor will never decrease the activity of an untargeted proteinase when that inhibitor is at a concentration above the K_i for the reaction (Salvesen and Nagase, 1989). In an attempt to develop less toxic cysteine proteinase inhibitors with greater bio-stability, the trypanocidal effects of chalcones were investigated. These reversible cysteine proteinase inhibitors were shown to inhibit purified trypanopain and to be trypanocidal for cultured *T. b. brucei*. Most significantly, these inhibitors protected mice from a lethal dose of

T. b. brucei when given at the time of infection and a single dose given 3 hours after infection prolonged the lives of infected mice. This suggests that a new class of anti-trypanosomal agents could be developed based on trypanopain inhibitors.

Chalcones do effectively inhibit mammalian cathepsin L, so their limited toxicity in the murine model is surprising. Predicted cross-reactivity with host cathepsins has been one of the predicted major stumbling blocks to the therapeutic use of cysteine proteinase inhibitors. The limited toxicity observed in the present study may suggest that trypanosomes accumulate the drug more actively or selectively than mammalian cells. Alternatively, the redundancy of mammalian cysteine proteinases may be such that inhibition of cathepsin L is not lethal to mammalian cells.

Various other reports have suggested that cysteine proteinase inhibitors are potential treatments for parasitic diseases (Rosenthal *et al.*, 1993; Wasilewski *et al.*, 1996). While this study presents much circumstantial evidence that trypanopain is essential for *T. b. brucei* viability, none of the evidence is conclusive. DMK and FMK react with some serine proteinases (Rasnick, 1996) and while the biotinylated inhibitors used in the present study bind predominantly to trypanopain, it is possible that their trypanocidal activity is a result of binding to a minor (but crucial) enzyme present at too low a level to be detected on such blots. Additionally, while chalcones were designed to inhibit cysteine proteinases, their actual specificity has not been directly investigated. The highly trypanocidal effect of the chalcone MC161, which is a poor trypanopain inhibitor, may indicate that chalcones have trypanosomal targets other than trypanopain. It is thus possible that the trypanocidal effects of these inhibitors are not a function of trypanopain inhibition, but rather of interaction with some other molecule. To confirm that trypanopain is essential for the viability of *T. b. brucei*, a gene knockout trypanosome should be produced. Gene knockout of the cysteine proteinase of *Streptococcus pyogenes* (Lukomski *et al.*, 1997) and *L. mexicana* (Mottram *et al.*, 1996) confirmed that these enzymes are important virulence factors, thus validating them as drug targets. Thus, similar studies on trypanopain are appropriate.

8.5 Future directions

Most of the currently available cysteine proteinase inhibitors all bind only to the S sites of the susceptible enzyme rather than to the S' sites. Inhibitors binding to the S' as well as the S sub-sites are likely to be effective at much lower concentrations than the currently available inhibitors. Additionally, their increased specificity should reduce cross-reaction with host proteinases, making them less toxic to mammalian cells. A better understanding of the S' subsite specificity of trypanopain would enable the rational design of more specific, less toxic inhibitors by exploiting the differences between parasite and host cysteine proteinases (Robertson *et al.*, 1996). With this is mind, the S′ subsites of trypanopain were investigated using the method of Schellenberger *et al.* (1993). Nucleophilic peptides of the general structure X-Ala-Ala-Ala (where X represents each of the 20 natural amino acids) act as replacements for H_2O in the final step of enzyme catalysis, with those peptides that bind best to the S' sites acting as better nucleophiles. HPLC is used to assess the rate of disappearance of each peptide from a mixture of such peptides, with this rate reflecting each peptide's success as a nucleophile and thus its success at binding the enzyme active site.

However, initial experiments suggested that none of the peptides were consumed over test periods of up to 16 h. A similar lack of utilisation was observed using the standard enzymes papain and cathepsin L. Variation of HPLC conditions, substrates for binding to the S subsites, assay buffers, enzyme and peptide concentrations was unsuccessful. More recently, considerable success has been reported using internally quenched substrates (Le Bonniec *et al.*, 1996), and it is likely that these would also prove successful for trypanopain.

Various new cysteine proteinase inhibitors have recently been reported, including α-keto amides (Li *et al.*, 1996), peptidyl acyloxymethanes (Krantz, 1994), N, O-diacyl hydroxamates (Brömme and Demuth, 1994) and vinyl sulfones (Hanzlik and Thompson, 1984; Rosenthal *et al.*, 1996). These new families of inhibitors should be evaluated against *T. b. brucei* in the same manner as chalcones have been evaluated in this study. Of these, vinyl sulfones seem to be the most promising, as they have shown *in vitro* antimalarial activity (Rosenthal *et al.*, 1996).

People living in areas where trypanosomiasis is common traditionally use various plants to treat infected people and animals. Studies have shown that many of these plants do contain anti-trypanosomal agents (Frieburghaus *et al.*, 1996; Sepúlveda-Boza and Cassels, 1996) and preliminary studies in this laboratory suggest that the indigenous plants *Ehretia rigida* and *Albizza adanthafolia* have anti-trypanosomal activity *in vitro*. Further investigation of these plants could uncover novel trypanosomal targets and may lead to the development of new anti-trypanosomal drugs.

In conclusion, this study has shown that trypanopain is unlikely to remain active in the mammalian bloodstream, and so is unlikely to contribute to pathogenesis by degrading host proteins. However, the enzyme does appear to be essential for parasite viability. In addition to killing parasites *in vitro*, trypanopain inhibitors seem to be active *in vivo*, since they prolong the lives of infected mice. Thus trypanopain appears to be a potentially suitable target for new anti-trypanosomal agents. Further investigation of trypanopain substrate specificity will enable the design of less toxic trypanocidal cysteine proteinase inhibitors.

Chapter 9

References

Abrahamson, M. (1993). Cystatins-protein inhibitors of papain-like cysteine proteinases. J. Braz. Assoc. for Adv. Sci. 45, 299-304.

Abrahamson, M. (1994). Cystatins. Meth. Enzymol. 244, 685-700.

Abrahamson, M., Barrett, A. J., Salvesen, G. and Grubb, A. (1986). Isolation of six cysteine proteinase inhibitors from human urine. J. Biol. Chem. 261, 11282-11289.

Adams, R. L. P. (1990). Cell culture for biochemists 2nd edition. (Elsevier, Amsterdam), pp 35-56, 71-94, 151-184.

Alafiatayo, R. A., Crawley, B., Oppenheim, B. A. and Penreath, V. W. (1993). Endotoxins and the pathogenesis of *Trypanosoma brucei brucei* infection in mice. Parasitology 107, 49-53.

Angliker, H., Wikstron, P., Rauber, P. and Shaw, E. (1987). The synthesis of lysylfluoromethanes and their properties as inhibitors of trypsin, plasmin and cathepsin B. Biochem. J. 241, 871-875.

Arnon, R. and Shapira, E. (1967). Antibodies to papain. A selective fractionation according to inhibitory capacity. Biochemistry 6, 3942-3950.

Ashall, F., Angliker, H. & Shaw, E. (1990). Lysis of trypanosomes by peptidyl fluoromethyl ketones. Biochem. Biophys. Res. Comm. 170, 923-929.

Ashcom, J. D., Tiller, S. E., Dickerson, K., Cravens, J. L., Argraves, W. S. and Strickland, D. K. (1990). The human α_2-macroglobulin receptor: identification of a 420 kDa cell surface glycoprotein specific for the activated conformation of α_2-macroglobulin. J. Cell Biol. 110, 1041-1046.

Åslund, L., Hendriksson, J., Campetella, O., Frasch, A. C. C., Petterson, U. and Cazzulo, J. J. (1991). The C-terminal extension of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 45, 345-348.

Authié, E. (1994). Trypanosomiasis and trypanotolerance in cattle: a role for congopain? Parasitol. Today 10, 360-364.

Authié, E., Muteti, D. K., Mbawa, Z. R., Lonsdale-Eccles, J. D., Webster, P. and Wells, C. W. (1992). Identification of a 33-kilodalton antigen of *Trypanosoma congolense* as a cysteine protease. Mol. Biochem. Parasitol. 56, 103-116.

Authié, E., Duvallet, G., Robertson, C. and Williams, D. (1993). Antibody response to a 33 kDa cysteine protease of *Trypanosoma congolense*: relationship to "trypanotolerance" in cattle. Parasite Immunology 15, 465-474.

Avila, E. E. and Calderon, J. (1993). *Entamoeba histolytica* trophozoites: a surface-associated cysteine protease. Exp. Parasitol. 76, 232-241.

Avrameas, S. and Ternynck, T. (1969). The cross-linking of proteins and its use for the preparation of immunosorbents. Immunochemistry 6, 53-66.

Bacchi, C. J., McCann, P. P., Nathan, H. C., Hutner, S, H. and Sjoerdsma, A. (1983). Antagonism of polyamine metabolism -a critical factor in chemotherapy of African trypanosomiasis. Adv. Polyamine Res. 4, 221-231.

Bailly, E., Jambou, R., Savel, J. and Jaureguiberry, G. (1992). *Plasmodium falciparum*: differential sensitivity *in vitro* to E-64 (cysteine protease inhibitor) and pepstatin A (aspartyl protease inhibitor). J. Protozool. 39, 593-599.

Bairoch, A. and Boeckmann, B. (1994). The SwissProt protein sequence databank: current status. Nucl. Acids Res. 22, 3578-3580.

Baltz, T., Baltz, D., Giroud, C. and Crockett, J. (1985). Cultivation in a semi-defined medium of animal infective forms of *Trypanosoma brucei*, *T. equiperdum*, *T. evansi*, *T. rhodesiense* and *T. gambiense*. EMBO J. 4, 1273-1277.

Barrett, A. J. (1981). α_2-Macroglobulin. Meth. Enzymol. 90, 737-754.

Barrett, A. J. and Kirschke, H. (1981). Cathepsin B, cathepsin H and cathepsin L. Meth. Enzymol. 80, 535-561.

Barrett, A. J. and Rawlings, N. D. (1996). Families and clans of cysteine peptidases. Persp. Drug Disc. Des. 6, 1-11.

Barrett, A. J. and Starkey, P. M. (1973). The interaction of α_2-macroglobulin with proteinases. Biochem. J. 133, 709-724.

Barrett, A. J., Kembhavi, A. A., Brown, M. A., Kirschke, H., Knight, C. G., Tamai, M. and Hanada, K. (1982). L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) and its analogues as inhibitors of cysteine proteinases including cathepsins B, H and L. Biochem. J. 201, 189-198.

Barrett, A. J., Rawlings, N. D., Davies, M. E., Machleidt, W., Salvesen, G. and Turk, V. (1986). Cysteine proteinase inhibitors of the cystatin superfamily. In: Proteinase inhibitors. A. J. Barrett and G. Salvesen, eds. (Elsevier, Amsterdam), pp. 515-569.

Becker, M. M., Harrop, S. A., Dalton, J. P., Kalinna, B. H., McManus, D. P. and Brindley, P. J. (1995). Cloning and characterisation of the *Schistosoma japonicum* aspartic proteinase involved in haemoglobin degradation. J. Biol. Chem. 270, 24496-24501.

Bieth, J. G. (1980). Pathophysiological interpretation of kinetic constants of protease inhibitors. Bull. Europ. Physiopath. Resp. 16, 183-195.

Bieth, J. G. (1995). Theoretical and practical aspects of proteinase inhibition kinetics. Meth. Enzymol. 248, 59-84.

Bitonti, A. J., Bacchi, C. J., McCann, P. P. and Sjoerdsma, A. (1985). Catalytic irreversible inhibition of *Trypanosoma brucei brucei* ornithine decarboxylase by substrate and product analogues and their effects on murine trypanosomiasis. Biochem. Pharm. 34, 1773-1777.

Blum, H., Beier, H. and Gross, H. J. (1987). Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. Electrophoresis 8, 93-99.

Bode, W., Engh, R., Musil, D, J., Thiele, U., Huber, R., Karshikov, A., Brzin, J., Kos, J. and Turk, V. (1988). The 2.0 Å X-ray crystal structure of chicken egg white cystatin and its possible mode of interaction with cysteine proteinases. EMBO J. 7, 2593-2599.

Bonaldo, M. C., D'Escoffier, L. N., Salles, J. M. and Goldberg, S. (1991). Characterisation and expression of proteases during *Trypanosoma cruzi* metocyclogenesis. Exp. Parasitol. 73, 44-51.

Bontempi, E. and Cazzulo, J. J. (1990). Digestion of human immunoglobulin G by the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. FEMS Microbiol Letters 70, 337-342.

Bontempi, E., Franke de Cazzulo, B. M., Ruiz, A. M. and Cazzulo, J. J. (1984). Purification and some properties of an acidic protease from epimastigotes of *Trypanosoma cruzi*. Comp. Biochem. Physiol. 77B, 599-604.

Bontempi, E., Martínez, J. and Cazzulo, J. J. (1989). Subcellular localization of a cysteine proteinase from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 33, 43-48.

Bordier, C. (1987). The promastigote surface protease of *Leishmania*. Parasitol. Today 3, 151-153.

Boreham, P. F. L. (1974). The pathogenesis of African and American trypanosomiasis. In: Biochemistry and physiology of protozoa volume 2. M. Levandowsky and S. H. Hutner. eds. (Academic Press, New York), pp. 429-457.

Boutignon, F., Huet-Duvillier, G., Demeyer, D., Richet, C. and Degand, P. (1990). Study of the proteolytic activities released by incubation of trypanosomes (*Trypanosoma brucei brucei*) in pH 5.5 and pH 7.0 phosphate/glucose buffers. Biochim. Biophys. Acta 1035, 369-377.

Bouvier, J., Etges, R. J. and Bordier, C. (1985). Identification and purification of membrane and soluble forms of the major surface protein of *Leishmania* promastigotes. J. Biol. Chem. 260, 15504-15509.

Briand, J. P., Muller, S. and Van Regenmortel, M. V. H. (1985). Synthetic antigens as antigens: pitfalls of conjugation methods. J. Immunol. Meth. 78, 59-69.

Briggs, G. E. and Haldane, J. B. S. (1925). A note on the kinetics of enzyme action. Biochem. J. 19, 338-339.

Brocklehurst, K. (1987). Acyl group transfer -cysteine proteinases. In: Enzyme mechanisms. M. I. Page and A. Williams, eds. (Royal Soc. Chem., London), pp 140-158.

Brocklehurst, K. and Malthouse, J. P. G. (1978). Mechanism of the reaction of papain with substrate-derived diazomethyl ketones. Biochem. J. 175, 761-764.

Brömme, D. and Demuth, H.-U. (1994). N, O-diacyl hydroxamates as selective and irreversible inhibitors of cysteine proteinases. Meth. Enzymol. 244, 671-685.

Brown, A., Burleigh, J. M., Billett, E. E. and Pritchard, D. I. (1995). An initial characterisation of the proteolytic enzymes secreted by the adult stage of the human hookworm *Necator americanus*. Parasitology 110, 555-563.

Brun, R. and Schonenberger, M. (1973). Cultivation of *Trypanosoma brucei* sspp. in semi-defined and defined media. Parasitology 67, 315-331.

Butler, R., Michel, A., Kunz, W. and Klinkert, M.-Q. (1995). Sequence of *Schistosoma mansoni* cathepsin C and its structural comparison with papain and cathepsins B and L of the parasite. Prot. Pept. Lett. 2, 313-320.

Campetella, O., Martínez, J. and Cazzulo, J. J. (1990). A major cysteine proteinase is developmentally regulated in *Trypanosoma cruzi*. FEMS Microbiol. Lett. 67, 145-150.

Campetella, O., Hendriksson, J., Åshlund, L., Frasch, A. C. C., Pettersson, U. and Cazzulo, J.J. (1992). The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is encoded by multiple polymorphic tandemly organised genes located on different chromosomes. Mol. Biochem. Parasitol. 50, 225-234.

Cármenes, R. S., Freije, J. P., Moline, M. M. and Martin, J. M. (1989). Predict7, a program for protein structure prediction. Biochem. Biophys. Res. Commun. 159, 687-693.

Cawston, T. E. and Barrett, A. J. (1979). A rapid and reproducible assay for collagenase using [1-^{14}C]acetylated collagen. Anal. Biochem. 99, 340-345.

Cazzulo, J. J and Fransch, A. C. A. (1992). SAPA/trans-sialidase and cruzipain: two antigens from *Trypanosoma cruzi* contain immunodominant but enzymatically inactive domains. FASEB J. 6, 3259-3263.

Cazzulo, J. J., Couso, R., Raimondi, A., Wernstedt, C. and Hellman, U. (1989). Further characterisation and partial amino acid sequence of a cysteine proteinase from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 33, 33-42.

Cazzulo, J. J., Hellman, U., Couso, R. and Parodi, A. J. A. (1990a). Amino acid and carbohydrate composition of a lysosomal cysteine proteinase from *Trypanosoma cruzi*. Absence of phosphorylated mannose residues. Mol. Biochem. Parasitol. 38, 41-48.

Cazzulo, J. J., Cazzulo Franke, M. C., Martínez, J. and Franke de Cazzulo, B. M. (1990b). Some kinetic properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. Biochim. Biophs. Acta 1037, 186-191.

Cazzulo, J. J., Stoka, V. and Turk, V. (1997). Cruzipain, the major cysteine proteinase from the protozoan parasite *Trypanosoma cruzi*. Biol. Chem. 378, 1-10.

CDR News (1994). Sleeping sickness on the boil in Zaire. TDR News 46, 6.

Chandler, A. C. and Read, C. P. (1961). Introduction to parasitology: with special reference to the parasites of man. (Wiley, London), pp. 131-160.

Chase, M. W. (1977). Chapter 2: Production of antiserum. In: Methods in Immunology and Immunochemistry. Volume 1: Preparation of antigens and antibodies. C. A. Williams and M. W. Chase, eds. (Academic Press, New York), pp 197-306.

Chen, M., Theander, T. G., Christensen, S. B., Hviid, L., Zhai, L., Kharazmi, A. (1994). Licochalcone A, a new antimalarial agent, inhibits *in vitro* growth of the human malarial parasite *Plasmodium falciparum* and protects mice from *P. yoelii* infection. Antimicrob. Agents Chemother. 38, 1470-1475.

Coetzer, T. H. T. (1985). Preparation and characterisation of antibodies against mouse Ig (all classes). Internal Report, Bioclones, (Pty.) Ltd. Immunology group, Stellenbosch.

Coetzer, T. H. T. (1992). Type IV collagenase and cathepsins L and H: proteinases involved in tumour invasion. PhD thesis, University of Natal, Pietermaritzburg.

Coetzer, T. H. T., Elliott, E., Fortgens, P. H., Pike, R. N. and Dennison, C. (1991). Anti-peptide antibodies to cathepsins B, L and D and type IV collagenase. J. Immunol. Methods 136, 199-210.

Coggins, J. R., Kray, W. and Shaw, E. (1974). Affinity labelling of proteinases with tryptic specificity by peptides with C-terminal lysine chloromethyl ketone. Biochem. J. 138, 579-585.

Cohen, L. W., Coghlan, V. M. and Dihel, L. C. (1986). Cloning and sequencing of papain-encoding DNA. Gene 48, 219-227. SwissProt (http://expasy.hcuge.ch) accession number P00784.

Coombs, G. and Baxter, J. (1984). Inhibition of *Leishmania* amastigote growth by antipain and leupeptin. Ann. Trop. Med. Parasitol. 78, 21-24.

Coombs, G. H., Hart, D. T. and Capaldo, J. (1982). Proteinase inhibitors as anti-leishmanial agents. Trans Royal Soc. Trop. Med. Hyg. 76, 660-663.

Cornish-Bowden, A. (1995). Fundamentals of enzyme kinetics. (Portland, London), pp 24-37.

Cornish-Bowden, A. and Eisenthal, R. (1974). Statistical considerations in the estimation of enzyme kinetic parameters by the direct linear plot and other methods. Biochem. J. 139, 721-730.

Cox, G. N., Pratt, D., Hageman, R. and Boisvenue, R. J. (1990). Molecular cloning and primary sequence of a cysteine protease expressed by *Haemonchus contortus* adult worms. Mol. Biochem. Parasitol. 41, 25-34.

Crawford, C., Mason, R. W., Wikstrom, P. and Shaw, E. (1988). The design of peptidyldiazomethane inhibitors to distinguish between the cysteine proteinases calpain II, cathepsin L and cathepsin B. Biochem. J. 253, 751-758.

Cross, G. A. M. and Manning, J. C. (1973). Cultivation of *Trypanosoma brucei* spp. in semi-defined and defined media. Parasitology 67, 315.

Cullen, B. M., Halliday, I. M., Kay, G., Nelson, J. and Walker, B. (1992). The application of a novel biotinylated affinity label for the detection of a cathepsin B-like precursor produced by breast-tumour cells in culture. Biochem. J. 283, 461-465.

Dalton, J. P., Smith, A. M., Clough, K. A. and Brindley, P. J. (1995). Digestion of haemoglobin by schistosomes: 35 years on. Parasitol. Today 11, 299-303.

Dalton, J. P., Clough, K. A., Jones, M. K. and Brindley, P. J. (1996). Characterisation of cathepsin-like cysteine proteases of *Schistosoma mansoni*. Infect. Immun. 64, 1328-1334.

Davies, A., Meeran, K. Cairns, M. T. and Baldwin, S. A. (1987). Peptide-specific antibodies as probes for the orientation of the glucose transporter in the human erythrocyte membrane. J. Biol Chem. 262, 9347-9352.

Davis, A. H., Nanduro, J. and Watson, D. C. (1987). Cloning and gene expression of *Schistosoma mansoni* protease. J. Biol. Chem. 262, 12851-12855.

Dawson, R. M. C., Elliot, D. C., Elliot, W. H. and Jones, K. M. (1968). Data for biochemical research. (Clarendon Press, Oxford), p 628.

De Clerk, Y. A. and Imren, S. (1994). Protease inhibitors: role and potential therapeutic use in human cancer. Eur. J. Cancer 30A, 2170-2180.

De Lumen, B. O. and Tappell, A. L. (1970). Fluorescein-hemoglobin as a substrate for cathepsin D and other proteases. Anal. Biochem. 36, 22-29.

De Raadt, P. (1989). Report and recommendations. Twentieth Meeting of the International Scientific Council for Trypanosomiasis Research and Control. O. A. U./S. T. R. C., Mombasa, Kenya.

de Souza, A. C. M., Persechini, P. M., de Souto Padron, T., de Souza, W., Guimarães, J. A.´and Scharfstein, J. (1990). Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase. Mol. Biochem. Parasitol. 43, 27-38.

Dehrmann, F. M., Coetzer, T. H. T., Pike, R. N. and Dennison, C. (1995). Mature cathepsin L is substantially active in the ionic milieu of the extracellular medium. Arch. Biochem. Biophys. 324, 93-98.

Dehrmann, F. M., Elliott, E. and Dennison, C. (1996). Reductive activation markedly increases the stability of cathepsins B and L to extracellular ionic conditions. Biol. Chem. Hoppe-Seyler 377, 391-394.

Delaissé, J.-M., Eeckhout, Y. and Vaes, G. (1980). Inhibition of bone resorption in culture by inhibitors of thiol proteinases. Biochem. J. 192, 365-368.

Dennison, C. and Pike, R. N. (1991). A peptide antibody that specifically inhibits cathepsin L. In: Immunobiology of proteins and peptides VI. M. Z. Atassi, ed. (Plenum Press, New York), pp 285-288.

Dingle, J. T., Barrett, A. J. and Weston, P. D. (1971). Cathepsin D. Characteristics of immunoinhibition and the confirmation of a role in cartilage breakdown. Biochem. J. 123, 1-13.

Dluzewski, A. R., Rangechari, K., Wilson, R. M. J. and Gratzer, W. B. (1986). *Plasmodium falciparum*: protease inhibitors and inhibition of erythrocyte invasion. Exp. Parasitol. 62, 416-422.

Dowd, A. J., McGonigle, S. and Dalton, J. P. (1995). *Fasciola hepatica* cathepsin L proteinase cleaves fibrinogen and produces a novel type of fibrin clot. Eur. J. Biochem. 232, 241-246.

Drenth, J., Janonius, J. N., Koekoek, R. and Wolthers, B. G. (1971). The structure of papain. Advan. Prot. Chem. 25, 79.

Dresden, M. H. and Deelder, A. M. (1979). *Schistosoma mansoni*: thiol proteinase properties of adult worm "haemoglobinase". Exp. Parasitol. 48, 190-197.

Dreyer, G. B., Metcalf, B. W., Tomaszek, T. A., Carr, T. J., Chandler, A. C., Hyland, L., Fakhoury, S. A., Magaard, V. W., Moore, M. L., Strickler, J. E., Debouck, C. and Meek, T. D. (1989). Inhibition of human immunodeficiency virus 1 protease *in vitro*: rational design of substrate analogue inhibitors. Proc. Natl Acad. Sci. (USA). 86, 9752-9756.

Duboise, S. M., Vannier-Santos, M. A., Costa-Pinto, D., Rivas, L., Pan, A. A., Traub-Cseko, Y., De Souza, W. and McMahon-Pratt, D. (1994). The biosynthesis, processing, and immunolocalisation of *Leishmania pifanoi* amastigote cysteine proteinases. Mol. Biochem. Parasitol. 68, 119-132.

Dufour, E. and Ribadeau-Dumas, B. (1988). Proteolytic specificity of chicken cathepsin L on bovine β-casein. Bioscience Reports 8, 185-191.

Dunn, B. M. (1989a). Determination of protease mechanism. In: Proteolytic enzymes, a practical approach. R. J. Benyon and J. S. Bond, eds. (IRL Press, Oxford), pp. 57-60.

Dunn, M. J. (1989b). Electrophoretic analysis methods. In: Protein purification methods: a practical approach. E. L. V. Harris and S. Angal, eds. (IRL Press, Oxford), pp 18-40.

Dunn, S. D. (1986). Effects of the modification of transfer buffer composition and the renaturation of proteins in gels on the recognition of proteins on Western blots by monoclonal antibodies. Anal Biochem. 157, 144-153.

Duszenko, M., Ferguson, M. A. J., Lamont, G. S., Rifkin, M. R. and Cross, G. A. M. (1985). Cysteine eliminates the feeder cell requirement for cultivation of *Trypanosoma brucei* bloodstream forms *in vitro*. J. Exp. Med. 162, 1256-1263.

Duszenko, M., Mühlstädt, K. and Broder, A. (1992). Cysteine is an essential growth factor for *Trypanosoma brucei* bloodstream forms. Mol. Biochem. Parasitol. 50, 269-274.

Eadie, G. S. (1942). The inhibition of cholinesterase by physostigmine and prostigmine. J. Biol Chem. 146, 85-93.

Eakin, A. E., Mills, A. A., Harth, G., McKerrow, J. H. and Craik, C. S. (1992). The sequence, organisation and expression of the major cysteine protease (cruzipain) from *Trypanosoma cruzi*. J. Biol. Chem. 267, 7411-7420. SwissProt (http://expasy.hcuge.ch) accession number P25779.

Eakin, A. E., McGrath, M. E., McKerrow, J. H., Fletterick, R. J. and Craik, C. S. (1993). Production of crystallizable cruzain, the major cysteine proteinase from *Trypanosoma cruzi*. J. Biol. Chem. 268, 6115-6118.

Edwards, R. J., Singleton, A. M., Murray, B. P., Sesardic, D., Rich, K. J., Davies, D. S. and Boobis, A. R. (1990). An anti-peptide antibody targeted to a specific region of rat cytochrome P-450IA2 inhibits enzyme activity. Biochem. J. 266, 497-504.

Eisenthal, R. and Cornish-Bowden, A. (1974). The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters. Biochem. J. 139, 715-720.

Ellis, K. J. and Morrison, J. F. (1982). Buffers of constant ionic strength for studying pH-dependent processes. Meth. Enzymol. 87, 405-426.

Engvall, E. and Perlmann, P. (1971). Enzyme-linked immunosorbent assay (ELISA): quantitative assay of immunoglobulin G. Immunochemistry 8, 871-874.

Esser, R. E., Angelo, R. A., Murphey, M. D., Watts, L. M., Thornburg, L. P., Palmer, J. T., Talhouk, J. W. and Smith, R. E. (1994). Cysteine proteinase inhibitors decrease articular cartilage and bone destruction in chronic inflammatory arthritis. Arthritis and Rheumatism 37, 236-247.

Fairlamb, A. H., Henderson, G. B., Bacchi, C. J. and Cerami, A. (1987). *In vivo* effects of difluoromethylornithine on trypanothione and polyamine levels in bloodstream forms of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 24, 185-191.

Fish, W. R., Nkhungulu, Z. M., Muriuki, C. W., Ndegwa, D. M., Lonsdale-Eccles, J. D. and Steyaert, J. (1995). The cDNA and deduced amino acid sequence of a cysteine protease from *Trypanosoma* (*Nannomonas*) *congolense* metacyclic forms. Gene, 161, 125-128.

Flynn, J. N. and Sileghem, M. (1993). Immunosuppression in trypanotolerant N'Dama cattle following *Trypanosoma congolense* infection. Parasite Immunology. 15, 547-552.

Franke de Cazzulo, B. M., Martínez, J., North, M., Coombs, G. H. and Cazzulo, J. J. (1994). Effects of proteinase inhibitors on the growth and differentiation of *Trypanosoma cruzi*. FEMS Microbiol. Lett. 124, 81-86.

French, T. C., Dawid, I. B. and Buchanan, J. M. (1963). Azaserine-reactive sulfhydryl group of 2-formamido-N-ribosylacetamide 5'-phosphate:L-glutamine amido ligase (adenosine diphosphate). J. Biol. Chem. 238, 2186-2193.

Freund, J. and McDermott, K. (1942). Sensitisation to horse serum by means of adjuvants. Proc. Soc. Exp. Biol. Med. 49, 548-553.

Frieburghaus, F., Kaminsky, R., Nkunya, M. H. H. and Brun, R. (1996). Evaluation of African medicinal plants for their *in vitro* trypanocidal activity. J. Ethnopharm. 35, 1-11.

Fripp, P. J. (1983). Blood and tissue flaggelates In: An introduction to human parasitology, with reference to southern Africa. (Macmillan, Johannesburg), pp 23-29.

Gal, S. and Gottesman, M. M. (1988). Isolation and sequence of a cDNA for human pro-(cathepsin L). Biochem. J. 253, 303-307. SwissProt (http://expasy.hcuge.ch) accession number P07711.

Ghendler, Y., Parizade, M., Arnon, R., McKerrow, J. H. and Fishelson, Z. (1996). *Schistosmoma mansoni*: evidence for a 23-kDa membrane-anchored protease on schistosomula. Exp. Parasitol. 83, 73-82.

Ghoneim, H. and Klinkert, M.-Q. (1995). Biochemical properties of purified cathepsin B from *Schistosoma mansoni*. Int. J. Parasitol. 25, 1515-1519.

Glover, G. and Shaw, E. (1971). The purification of thrombin and isolation of a peptide containing the active centre histidine. J. Biol. Chem. 246, 4594-4601.

Gluzman, I. Y., Francis, S. E., Oksmann, A., Smith, C. E., Duffin, K. L. and Goldberg, D. E. (1994). Order and specificity of the *Plasmodium falciparum* haemoglobin degradation pathway. J. Clin. Invest. 93, 1602-1608.

Goldberg, D. E., Slater, A. F. G., Beavis, R., Chait, B., Cerami, A. and Henderson, G. B. (1991). Haemoglobin degradation in the human malaria pathogen *Plasmodium falciparum*: a catabolic pathway initiated by a specific aspartic protease. J. Exp. Med. 173, 961-969.

González, G., Sunnemark, D., Örn, A. and Grönvik, K.-O. (1996). Detection of cruzipain, the major cysteine proteinase from *Trypanosoma cruzi* and its C-terminal extension in biological fluids during experimental infection in mice. Scand. J. Immunol. 44, 122-128.

Grab, D. J. and Bwayo, J. J. (1982). Isopycnic isolation of African trypanosomes on Percoll gradients formed in situ. Acta Tropica 39, 363-366.

Green, G. D. J. and Shaw, E. (1981). Peptidyl diazomethyl ketones are specific inactivators of thiol proteinases. J. Biol Chem 256, 1923-1928.

Grosvenor, G. H. (1986). Fly of the deadly sleep: Tsetse. National Geographic 170, 814-833.

Gruden, K., Štrukelj, B., Ravnikar, M., Poljšak-Prijatelj, M., Mavric, I., Brzin, J., Pungercar, J. and Kregar, I. (1997). Potato cysteine proteinase inhibitor gene family: molecular cloning, characterisation and immunocytochemical localisation studies. Plant Mol. Biol. 34, 317-323.

Gutteridge, W. E. (1985). Trypanosomiasis: existing chemotherapy and its limitations. Brit. Med. Bull. 41, 162-168.

Hadjuk, S. L. (1984). Antigenic variation during the development cycle of *Trypanosoma brucei*. J. Protozool. 31, 41-47.

Hall, H. T. B. (1977). Disease and parasites of livestock in the tropics. (Harlow, Longman), pp. 163-171.

Hanada, K., Tamai, M., Yamagishi, M., Omura, S., Sawada, J. and Tanaka, I. (1978a). Studies on thiol protease inhibitors. Part I. Isolation and characterisation of E-64, a new thiol proteinase inhibitor. Agric. Biol. Chem. 42, 523-528.

Hananda, K., Tamai, M., Ohmura, S., Sawada, J., Seki, T. and Tanaka, I. (1978b). Studies on thiol protease inhibitors. Part II. Structure and synthesis of E-64, a new thiol proteinase inhibitor. Agric. Biol. Chem. 42, 529-536.

Hanada, K., Tamai, M., Adachi, T., Oguma, K., Kashiwagi, K., Ohmura, S., Kominami, E., Towatari, T. and Katanuma, N. (1983). Characterisation of the three new analogues of E-64 and their therapeutic application. In: Proteinase inhibitors: medical and biological aspects. N. Katanuma, H. Umezawa. and H. Holzer, eds. (Springer Verlag, Berlin), pp 25-36.

Handmann, E., Button, L. L. and McMaster, T. W. (1990). *Leishmania major*: production of recombinant gp63, its antigenicity and immunogenicity in mice. Exp. Parasitol. 70, 427-435.

Hanes, C. S. (1932). Studies on plant amylases. I. The effect of starch concentration upon the velocity of hydrolysis by amylases of germinated barley. Biochem. J. 26, 1406-1421.

Hanzlik, R. P. and Thompson, S. A. (1984). Vinylogous amino acid esters: a new class of inactivators of thiol proteases. J. Med. Chem. 27, 711-712.

Harlow, E. and Lane, D. (1988). Antibodies, a laboratory manual. (Cold Spring Harbour Laboratories, Cold Spring Harbour), pp 55-114, 555-612.

Harpel, P. C. (1976). Human α_2-macroglobulin. Meth. Enzymol. 45, 639-647.

Harrop, S. A., Sawangjaroen, N., Prociv, P. and Brindley, P. J. (1995). Characterisation and localisation of cathepsin B proteinases secreted by adult *Ancylostoma caninum* hookworms. Mol. Biochem. Parasitol. 71, 163-171.

Harth, G., Andrews, N., Mills, A. A., Engel, J. C., Smith, R. and McKerrow, J. H. (1993). Peptide-fluoromethyl ketones arrest intracellular replication and intercellular transmission of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 58, 17-24.

Hartley, B. S. (1960). Proteolytic enzymes. Annu. Rev. Biochem. 29, 45-72.

Hartree, E. E. (1972). Determination of protein; a modification of the Lowry method that gives a linear photometric response. Anal Biochem. 48, 422-427.

Healy, N., Greig, S., Enahoro, H., Roberts, H., Drake, L., Shaw, E. and Ashall, F. (1992). Detection of peptidases in *Trypanosoma cruzi* epimastigotes using chromogenic and fluorogenic substrates. Parasitology 104, 315-322.

Henderson, P. J. F. (1972). A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors. Biochem. J. 127, 321-333.

Hesse, F., Selzer, P. M., Mühlstädt, K. and Duszenko, M. (1995). A novel cultivation technique for long-term maintenance of bloodstream form trypanosomes *in vitro*. Mol. Biochem. Parasitol. *70*, 157-166.

Heumann, D., Burger, D., Vischer, T., de Colmenares, M., Bouvier, J. and Bordier, C. (1989). Molecular interactions of *Leishmania* promastigote protease with human α_2-macroglobulin. Mol. Biochem. Parasitol. 33, 67-72.

Heussen, C. and Dowdle, E. B. (1980). Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and co-polymerised substrates. Anal. Biochem. 102, 192-202.

Hirumi, H., Doyle, J. J. and Hirumi, K. (1977). African trypanosomes: cultivation of animal infective *Trypanosoma brucei in vitro*. Science 196, 992-994.

Hoare, C. A. (1972). The trypanosomes of mammals, a zoological monograph. (Blackwell, Oxford), pp 401-429. Cited by Tait et al. (1984).

Hofstee, B. H. J. (1952). Specificity of esterases. I. Identification of two pancreatic aliesterases. J. Biol Chem. 199, 357-365.

Hopp, T. P. (1985). In: Synthetic peptides in biology and medicine. K. Alitalo, P. Partanen, and A. Vaheri, eds. (Elsevier, Amsterdam), pp 3-11. Cited by Van Regenmortel and Daney de Marcillac (1988).

Hopp, T. P. (1986) J. Immunol. Meth. 88, 1-18. Cited by Van Regenmortel and Daney de Marcillac (1988).

Hopp, T. P. and Woods, K. R. (1981). Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl Acad. Sci. (USA). 78, 3824-3828.

Hornby, H. E. (1950). Animal trypanosomiasis in Eastern Africa, 1949. (Colonial Office, London). Cited by Chandler and Read (1961).

Hudson, L. and Hay, F. C. (1980). Molecular weights and special properties of immunoglobulins and antigens of immunological interest. In: Practical Immunology, 2nd edition. (Blackwell Scientific, London), p 347.

Huet, G., Richet, C., Demeyer, D., Bisiau, H., Soudan, B., Tetaert, D., Han, K. K. and Degand, P. (1992). Characterisation of different proteolytic activities in *Trypanosoma brucei brucei*. Biochim. Biophys. Acta 1138, 213-221.

Hummel, K. M., Penheiter, A. R., Gathman, A. C. and Lilly, W. W. (1996). Anomalous estimation of protease molecular weights using gelatin-containing SDS-PAGE. Anal Biochem. 233, 140-142.

Hunter, C. A., Lockwood, B. C. and Coombs, G. H. (1992). Hydrolysis of L-leucine methyl ester by *Leishmania mexicana* amastigote cysteine proteinases. Int. J. Parasitol. 22, 711-717.

Ilg, T., Fuchs, M., Gnau, V., Wolfram, M., Harbecke, D. and Overath, P. (1994). Distribution of parasite cysteine proteinases in lesions of mice infected with *Leishmania mexicana* amastigotes. Mol. Biochem. Parasitol. 67, 193-203.

ILRAD (1992). International Laboratory for Research on Animal Diseases 1991: Annual report of the International Laboratory for Research on Animal Diseases (Nairobi: International Laboratory for Research on Animal Diseases), pp 31-47.

ILRAD (1993). International Laboratory for Research on Animal Diseases 1992: Annual report of the International Laboratory for Research on Animal Diseases (Nairobi: International Laboratory for Research on Animal Diseases), pp 19-27.

ILRAD (1994). International Laboratory for Research on Animal Diseases.1993/4: Annual report of the International Laboratory for Research on Animal Diseases (Nairobi: International Laboratory for Research on Animal Diseases), pp 21-29.

Irvine, J. W., North, M. J. and Coombs, G. H. (1997). Use of inhibitors to identify essential cysteine proteinases of *Trichomonas vaginalis*. FEMS Microbiol. Lett. 149, 45-50.

Ishidoh, K., Towatari, T., Imajoh, S., Kawasaki, H., Kominami, I., Katanuma, N. and Suzuki, K. (1987). Molecular cloning and sequencing of cDNA for rat cathepsin L. FEBS Lett. 223, 69-73. SwissProt (http://expasy.hcuge.ch) accession number P07154.

Ishii, S.-I. (1994). Legumain: asparaginyl endopeptidase. Meth. Enz. 244, 604-615.

Jaffe, C. L., Shor, R., Trau, H. and Passwell, J. H. (1990). Parasite antigens recognised by patients with cutaneous leishmaniasis. Clin. Exp. Immunol. 80, 77-82.

Jakubke, H.-D. and Jeschkeit, H. (1983). Concise encyclopaedia of biochemistry. (Walter de Gruyter, Berlin), p 163.

Josephy, P. D., Elings, T. and Mason, R. P. (1982). The horseradish peroxidase-catalysed oxidation of 3, 3', 5, 5'-tetramethylbenzidine. J. Biol. Chem. 257, 3669-3675.

Kamata, I., Yamada, M., Uchikawa, R., Matsuda, S. and Arizono, N. (1995). Cysteine protease of nematode *Nippostrongylus brasiliensis* preferentially evokes an IgE/IgG1 antibody response in rats. Clin. Exp. Immunol. 102, 71-77.

Karplus, P. A and Schulz, G. E. (1985). Naturwissenschaften 72, 212-213. Cited by Van Regenmortel and Daney de Marcillac (1988).

Kaufmann, J. (1996). Parasitic infections of domestic animals. (Birkhäuser Verlag, Basel), pp 59, 134, 167, 224, 272.

Kay, G., Bailie, J. R., Halliday, I. M., Nelson, J. and Walker, B. (1992). The synthesis, kinetic characterisation and application of biotinylated aminoacylchloromethanes for the detection of chymotrypsin and trypsin-like enzymes. Biochem. J. 283, 455-459.

Kay, J., Valler, M. J. and Dunn, B. M. (1983). Naturally-occurring inhibitors of aspartic proteinases. In: Proteinase inhibitors: Medical and biological aspects. N. Katunama, ed. (Springer-Verlag, Berlin), pp 201-210.

Keene, W. E., Petitt, M. G., Allen, S. and McKerrow, J. H. (1986). The major neutral proteinase of *Entamoeba histolytica*. J. Exp. Med. 163, 536-549.

Keene, W. E., Hidalgo, M. E., Orozco, E. and McKerrow, J. H. (1990). *Entamoeba histolytica*: correlation of the cytopathic effect of virulent trophozoites with secretion of cysteine proteinase. Exp. Parasitol. 71, 199-206.

Kemeny, D. M. and Chantler, S. (1988). An introduction to ELISA. In: ELISA and other solid phase immunoassays. Theoretical and practical aspects. D. M. Kemeny and S. J. Challacombe, eds. (Wiley, Chichester), pp 14-15.

Kettner, C. and Shaw, E. (1981). Inactivation of trypsin-like enzymes with peptides of arginine chloromethyl ketone. Meth. Enzymol. 80, 826-841.

Kinabo, L. D. B. (1993). Pharmacology of existing drugs for animal trypanosomiasis. Acta Trop. 54, 69-183.

Kirschke, H. and Shaw, E. (1981). Rapid inactivation of cathepsin L by Z-Phe-Phe-CHN_2 and Z-Phe-Ala-CHN_2. Biochem. Biophys. Res. Commun. 101, 454-458.

Kitagawa, T. and Aikawa, T. (1976). Enzyme coupled immunoassay of insulin using a novel coupling reagent. J. Biochem. 79, 233-236.

Klinkert, M.-Q., Felleisen, R., Link, G., Ruppel, A. and Beck, E. (1989). Primary structures of Sm31/32 diagnostic proteins of *Schistosoma mansoni* and their identification as proteases. Mol. Biochem. Parasitol. 33, 113-122.

Knight, C. G. (1995). Fluorometric assays of proteolytic enzymes. Meth. Enzymol. 248, 18-34

Kohl, N. E., Emini, E. A., Schleif, W. A., Davis, L. J., Heimbach, J. C., Dixon, R. A. F., Scolnick, E. M. and Sigal, I. S. (1988). Active human immunodeficiency virus protease is required for viral infectivity. Proc. Natl Acad. Sci. (USA) 85, 4846-4890.

Köhler, G. and Milstein, C. (1975). Continuous cultures of fused cells secreting antibody of predefined specificity. Nature. 256, 495-497.

Kong, Y., Kang, S.-Y., Kim, S. H., Chung, Y.-B. and Cho, S.-Y. (1997). A neutral cysteine protease of *Spirometra mansoni* pleroceroid invoking an IgE response. Parasitology 114, 263-271.

Koritas, V. M. and Atkinson, H. J. (1995). An assay for nanogram levels of proteolytic enzymes. Anal. Biochem. 227, 22-26.

Kornblatt, M. J., Mpimbaza, G. W. N. and Lonsdale-Eccles, J. D. (1992). Characterisation of an endopeptidase of *Trypanosoma brucei brucei*. Arch. Biochem. Biophy. 293, 25-31.

Krantz, A. (1994). Peptidyl (acyloxy)methanes as quiescent affinity labels for cysteine proteinases. Meth. Enzymol. 244, 656-671.

Kreier, J. P. and Baker, J. R. (1987). Parasitic protozoa. (Allen and Unwin, Boston), pp 1-89.

Kuntz, I. D. (1992). Structure-based strategies for drug design and discovery. Science 257, 1078-1082.

Kurachi, K., Powers, J. C. and Wilcox, P. E. (1973). Kinetics of the reaction of chymotrypsin A with peptide chloromethyl ketones in relation to its subsite specificity. Biochemistry 12, 771-777.

Kuzoe, F. A. S. (1993). Current situation of African trypanosomiasis. Acta Trop. 54, 153-162.

Kyte, J. and Doolittle, R. F. (1982). A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105-132.

Labriola, C., Sousa, M. and Cazzulo, J. J. (1993). Purification of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* by affinity chromatography. Biological Res. 26, 101-107.

Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of the bacteriophage T. Nature 277, 680-685.

Lanham, S. M. and Godfrey, D. G. (1970). Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. 28, 521-534.

Larsson, A., Balow, R. M., Lindahl, T. L. and Forsberg, P. O. (1993). Chicken antibodies-taking advantage of evolution-a review. Poultry Science 72, 1807-1812.

Le Bonniec, B. F., Myles, T., Johnson, T., Knight, C. G., Tapparelli, C. and Stone, S. R. (1996). Characterisation of the P_2' and P_3' specificities of thrombin using fluorescence-quenched substrates and mapping of the subsites by mutagenesis. Biochemistry 35, 7114-7122.

Leary, R., Larsen, D., Watanabe, H. and Shaw, E. (1977). Diazomethyl ketone substrate derivatives as active-site-directed inhibitors of thiol proteases. Papain. Biochemistry 16, 5857-5861.

Lenarcic, B., Krizaj, I., Zunec, P. and Turk, V. (1996). Differences in specificity for the interactions of stefins A, B and D with cysteine proteinases. FEBS Lett. 395, 113-118.

Lerner, R. A. (1984). Antibodies of predetermined specificity in biology and medicine. Adv. Immunol. 36, 1-44.

Li, R., Kenyon, G. L., Cohen, F. E., Chen, X., Gong, B., Dominguez, J. N., Davidson, E., Kurzban, G., Miller, R. E., Nuzum, E. O., Rosenthal, P. J. and McKerrow, J. H. (1995). *In vitro* antimalarial activity of chalcones and their derivatives. J. Med. Chem. 38, 5031-5037.

Li, Z., Chen, X., Davidson, E., Zwang, O., Mendis, C., Ring, C. S., Rousch, W. R., Fegley, G., Li, R., Rosenthal, P. J., Lee, G. K., Kenyon, G. L., Kuntz, I. D. and Cohen, F. E. (1994). Anti-malarial drug development using models of enzyme structure. Chem. Biol. 1, 31-37.

Li, Z., Ortega-Vilain, A.-C., Patil, G. S., Chu, D.-L., Foreman, J. E., Eveleth, D. D. and Powers, J. C. (1996). Novel peptidyl α-keto amide inhibitors of calpains and other cysteine proteases. J. Med. Chem. 39, 4089-4096.

Lima, A. P. C. A., Scharfstein, J., Storer, A. C. and Ménard, R. (1992). Temperature-dependent substrate inhibition of the cysteine proteinase (GP57/51) from *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 56, 335-338.

Lima, A. P. C. A., Tessier, D. C., Thomas, D. Y., Scharfstein, J., Storer, A. C. and Vernet, T. (1994). Identification of new cysteine protease gene isoforms in *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 67, 333-338.

Lineweaver, H. and Burk, D. (1934). The determination of enzyme dissociation constants. J. Amer. Chem. Soc. 56, 658-666.

Liu, F.-T., Zinnecker, M., Hamaoka, T. and Katz, D. H. (1979). New procedure for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterisation of such conjugates. Biochemistry 18, 690-697.

Longstaffe, J. A. and Terry, R. J. (1982). Phytohaemagglutinin reactivity in circulating peripheral blood lymphocytes during a *Trypanosoma brucei* infection: sequential studies in individual guinea-pigs. Parasite Immunology 4, 187-196. Cited by Flynn and Sileghem (1993).

Lonsdale-Eccles, J. D. and Grab, D. J. (1987a). Lysosomal and non-lysosomal peptidyl hydrolases of the bloodstream forms of *Trypanosoma brucei brucei*. Eur. J. Biochem. 169, 467-475.

Lonsdale-Eccles, J. D. and Grab, D. J. (1987b). Purification of African trypanosomes can cause biochemical changes in the parasites. J. Protozool. 34, 405-408.

Lonsdale-Eccles, J. D. and Mpimbaza, W. N. (1986). Thiol-dependent proteases of African trypanosomes. Analysis by electrophoresis in sodium dodecyl sulfate/polyacrylamide gels co-polymerised with fibrinogen. Eur. J. Biochem. 155: 469-473.

Lonsdale-Eccles, J. D., Mpimbaza, G. W. N., Nkhungulu, Z. R. M., Olobo, J., Smith, L., Tosomba, O. M. and Grab, D. J. (1995). Trypanosomatid cysteine protease activity may be enhanced by a kininogen-like moiety from host serum. Biochem. J. 305, 549-556.

Lopez, J. A., Reims, H.-A., Etges, R. J., Button, L. L. and McMaster, W. R. (1991). Genetic control of the immune response in mice to *Leishmania mexicana* surface protease. J. Immunol. 146, 1328-1324.

Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193, 265-275.

Luaces, A. L. and Barrett, A. J. (1988). Affinity purification and biochemical characterisation of histolysin, the major cysteine protease of *Entamoeba histolytica*. Biochem. J. 250, 903-909.

Lukomski, S., Sreetvatsan, S., Amberg, C., Reichardt, W., Woischnik, M., Podbielski, A. and Musser, J. M. (1997). Inactivation of *Streptococcus pyogenes* extracellular cysteine protease significantly decreases mouse lethality of serotype M3 and M49 strains. J. Clin. Invest. 99, 2574-2580.

Malchiodi, E. L., Chiaramonte, M. G., Martinez, J. A., Zwirner, N. W., Margni, R. A. and Cazzulo, J. J. (1993). Identity of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* and an antigen (Ag163B6) isolated with a monoclonal antibody. Immunol. Letters 35, 59-62.

Mariani, M., Bracci, L., Presentini, R., Nucci, D., Neri, P and Antoni, G. (1987). Immunogenicity of free synthetic peptide: carrier-conjugation enhances antibody affinity for the native protein. Mol. Immunol. 24, 297-303.

Markell, E. K., Voge, M. and John, D. T. (1992). Other blood- and tissue-dwelling protozoa. In: Medical Parasitology 7th edition. (Saunders, Philadelphia), pp 126-147.

Martìnez, J. and Cazzulo, J. J. (1992a). Anomalous electrophoretic behaviour of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* in relation to its apparent molecular mass. FEMS Microbiol. Lett. 95, 225-230.

Martìnez, J. and Cazzulo, J. J. (1992b). Variations in electrophoretic mobility, staining with silver nitrate and immunoreactivity of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* run under reducing or non-reducing conditions. Asoc. Quém. Argent. 80, 227-233.

Martìnez, J., Campetella, O., Fransch, A. C. C. and Cazzulo, J. J. (1991). The major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* is antigenic in human infections. Infect. Imm. 59, 4275-4277.

Martìnez, J., Campetella, O., Fransch, A. C. C. and Cazzulo, J. J. (1993). The reactivity of sera from chagasic patients against different fragments of cruzipain, the major cysteine proteinase from *Trypanosoma cruzi*, suggests the presence of defined antigenic and catalytic domains. Immunol. Letters 35, 191-196.

Mason, D. and Wilcox, R. W. (1992). Inhibition of cysteine proteinases in lysosomes and whole cells. Biochem. J. 285, 495-502.

Mason, R. W., Green, G. D. J. and Barrett, A. J. (1985). Human liver cathepsin L. Biochem. J. 226, 233-241.

Mbawa, Z. R., Webster, P. and Lonsdale-Eccles, J. D. (1991a). Immunolocalisation of a cysteine protease within the lysosomal system of *Trypanosoma congolense*. Eur. J. Cell Biol. 56, 243-250.

Mbawa, Z. R., Gumm, I. D., Fish, W. D. and Lonsdale-Eccles, J. D. (1991b). Endopeptidase variations among different life-cycle stages of African trypanosomes. Eur. J. Biochem. 195, 183-190.

Mbawa, Z. R., Gumm, I. D., Shaw, E. and Lonsdale-Eccles, J. D. (1992). Characterisation of a cysteine protease from bloodstream forms of *Trypanosoma congolense*. Eur. J. Biochem. 204, 371-379.

McCann, P. P., Bacchi, P. P., Hanson, W. L., Cain, G. D., Nathan, H. C., Hutner, S. H. and Sjoerdsma, A. (1981). Effect on parasitic protozoa of α-difluoromethylornothine -an inhibitor of ornithine decarboxylase. Adv. Polyamine Res. 3, 97-110.

McGrath, M. E., Eakin, A. E., Engel, J. C., McKerrow, J. H., Craik, C. S. and Fletterick, R. J. (1995). The crystal structure of cruzipain: a therapeutic target for Chaga's disease. J. Mol. Biol. 247, 251-259.

McKerrow, J. H. (1989). Parasite proteases. Exp. Parasitol. 68, 111-115.

McKerrow, J. H. (1991). New insights into the structure of a *Trypanosoma cruzi* protease. Parasitology Today 7, 132-133.

McKerrow, J. H. (1993). The diversity of protease function in parasitic infections. J. Braz. Assoc. Adv. Sci. 45, 319-321.

McKerrow, J. H., Sun, E., Rosenthal, P. J. and Bouvier, J. (1993). The proteases and pathogenicity of parasitic protozoa. Annu. Rev. Microbiol. 47, 821-853.

McKerrow, J. H., McGrath, M. E. and Engel, J. C. (1995). The cysteine protease of *Trypanosoma cruzi* as a model for antiparasitic drug design. Parasitol. Today 11, 279-282.

McQuade, T. J., Tomasselli, A. G., Liu, L., Karacostas, V., Moss, B., Sawyer, T. K., Heinrikson, R. L. and Tarpley, W. G. (1990). A synthetic HIV-1 protease inhibitor with antiviral activity arrests HIV-like particle maturation. Science 247, 454-456.

Meara, J. P. and Rich, D. H. (1996). Mechanistic studies on the inactivation of papain by epoxysuccinyl inhibitors. J. Med. Chem. 39, 3357-3366.

Medawar, P. (1996). Darwin's Illness. In: The Strange Case of the Spotted Mice. (Oxford University Press, Oxford), pp 52-58.

Meek, T. D., Lambert, D. M., Dreyer, G. B., Carr, T. J., Tomaszek, T. A., Moore, M. L., Strickler, J. E., Debouck, C., Hyland, L. J., Matthews, T. J., Metcalf, B. W. and Petteway, S. R. (1990). Inhibition of HIV-1 protease in infected T-lymphocytes by synthetic peptide analogues. Nature 343, 90-92.

Ménard, R. (1993). Structure-function studies in the papain family of cysteine proteases. J. Braz. Assoc. for Adv. Sci. 45, 292-298.

Michaud, D., Cantin, L., Raworth, D. A. and Vrain, T. C. (1996). Assessing the stability of cystatin/cysteine proteinase complexes using mildly-denaturing gelatin polyacrylamide gel electrophoresis. Electrophoresis 17, 74-79.

Michel, A., Ghoneim, H., Resto, M., Klinkert, M.-Q. and Kunz, W. (1995). Sequence, characterisation and localisation of a cysteine proteinase cathepsin L in *Schistosoma mansoni*. Mol. Biochem. Parasitol. 73, 7-18.

Mierelles, M. N. L., Juliano, L., Carmona, E., Silva, S. G., Costa, E. M., Murta, A. C. M. & Scharfstein, J. (1992). Inhibitors of the major cysteinyl proteinase (GP57/51) impair host cell invasion and arrest the intracellular development of *Trypanosoma cruzi* in vitro. Mol. Biochem. Parasitol. 52, 175-184.

Mirelman, D., Nuchamowitz, Y., Böhm-Gloning, B. and Walderich, B. (1996). A homologue of the cysteine proteinase gene (*ACP_1* or *Eh-CPp3*) of pathogenic *Entamoeba histolytica* is present in non-pathogenic *E. dispar* strains. Mol. Biochem. Parasitol. 78, 47-54.

Misaka, T., Kuroda, M., Iwabuchi, K., Abe, K. and Arai, S. (1996). Soyacystatin, a novel cysteine proteinase inhibitor from soybean, is distinct in protein structure and gene organisation from other cystatins of animal and plant origin. Eur. J. Biochem. 240, 609-614.

Moestrup, S. K., Gleimann, J. and Pallensen, G. (1992). Distribution of the α_2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. Cell Tissue Res. 269, 375.

Moroder, L., Papini, A., Sigmüller, G., Köcher, K., Dörrer, K. and Schneider, C. H. (1992). Induction and detection of anti-peptide antibody specificity is critically affected by the mode of hapten presentation. Biol. Chem. Hoppe-Seyler 372, 315-321.

Morrot, A., Strickland, D. K., de Lourdes Higuchi, M., Reis, M., Pedrosa, R. and Scharfstein, J. (1997). Human T cell responses against the major cysteine proteinase (cruzipain) of *Trypanosoma cruzi*: role of the multifunctional α_2-macroglobulin receptor in antigen presentation by monocytes. Internat. Immunol. 9, 825-834.

Mottram, J. C., North, M. J., Barry, M. J. and Coombs, G. H. (1989). A cysteine protease cDNA from *Trypanosoma brucei* predicts an enzyme with an unusual C-terminal extension. FEBS Letters 258, 211-215.

Mottram, J. C., Robertson, C. D., Coombs, G. H. and Barry, J. D. (1992). A developmentally regulated cysteine proteinase gene of *Leishmania mexicana*. Mol. Biochem. Parasitol. 6, 1925-1932. SwissProt (http://expasy.hcuge.ch) accession number P25775.

Mottram, J. C., Souza, A. E., Hutchison, J. E., Carter, R., Frame, M. J. and Coombs, G. H. (1996). Evidence from disruption of the *lmcpb* gene array of *Leishmania mexicana* that cysteine proteinases are virulence factors. Proc. Natl Acad. Sci. (USA). 93, 6008-6013.

Mottram, J. C., Frame, M. J., Brooks, D. R., Tetley, L., Hutchinson, J. E., Souza, A. E. and Coombs, G. H. (1997). The multiple *cpb* cysteine proteinase genes of *Leishmania mexicana* encode isoenzymes that differ in their stage regulation and substrate preferences. J. Biol. Chem. 272, 14285-14293.

Müller, N., Imboden, M., Detmer, E., Mansfield, J. M. and Seebeck, T. (1993). Cytoskeleton-associated antigens from African trypanosomes are recognised by self-reactive antibodies of uninfected mice. Parasitology 107, 411-417.

Muller, S. (1988). Peptide-carrier conjugation. In: Laboratory techniques in biochemistry and molecular biology Volume 19. Synthetic peptides as antigens. R. H., Burdon and P. H. Van Knippenberg, eds. (Elsevier, Amsterdam), pp 131-141.

Muñoz, M. L., Lamoyi, E., Leön, G., Tovar, R. and Pérez-García, J. (1990). Antigens in electron-dense granules from *Entamoeba histolytica* as possible markers for pathogenicity. J. Clin. Microbiol. 28, 2418-2424.

Murray, M. and Dexter, T. M. (1988). Anaemia in bovine African trypanosomiasis. Acta Tropica 45, 389-432.

Murta, A. C. M., Persechini, P. M., de Souto Padron, T., de Souza, W., Guimaraes, J. A. and Scharfstein, J. (1990). Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase. Mol. Biochem. Parasitol. 43, 27-38.

Nakane, P. K., Sri Ram, J. and Pierce, G. B. (1966). Enzyme-labelled antibodies for light and electron microscopic localisation of antigens. J. Histochem. Cytochem. 14, 789-791.

Nene, V., Gobright, E., Musoke, A. J. and Lonsdale-Eccles, J. D. (1990). A single exon codes for the enzyme domain of a protozoan cysteine protease. J. Biol. Chem. 265, 18047-18050.

Nesterenko, M. V., Tilley, M. and Upton. S. J. (1995). A metallo-dependent cysteine proteinase of *Cryptosporidium parvum* associated with the surface of sporozoites. Microbios 83, 77-88.

North, M. J. (1991). Trypanosomes and tomatoes. Parasitology Today 7, 249.

North, M. J., Mottram, J. C. and Coombs, G. H. (1990a). Cysteine proteinases of parasitic protozoa. Parasitology Today. 6, 270-275.

North, M. J., Robertson, C. J. and Coombs, G. H. (1990b). The specificity of trichomonad cysteine proteinases analysed using fluorogenic substrates and specific inhibitors. Mol. Biochem. Parasitol. 39, 183-194.

Nwagwu, M., Okenu, D. M. N., Olusi, T. A. and Molokwu, R. I. (1988). *Trypanosoma brucei* releases proteases extracellularly. Trans. Royal Soc. of Trop. Med. Hyg. 82, 577.

Ochs, D. (1983). Protein contaminants of sodium dodecyl sulfate-polyacrylamide gels. Anal. Biochem. 135, 470-474.

Olson, C. E., Chhajlani, V., August, J. T. and Schmell, E. D. (1990). Novel allosteric sites on human erythrocyte acetylcholinesterase identified by two monoclonal antibodies. Arch. Biochem. Biophys. 277, 361-367.

Ondetti, M. A., Rubin, B. and Cushman, D. W. (1977). Design of specific inhibitors of angiotensin-converting enzyme: new class of orally active antihypertensive agents. Science 196, 441-444.

Ong, E. B., Shaw, E. and Schoellmann, G. (1965). The identification of the histidine residue at the active centre of chymotrypsin. J. Biol. Chem. 240, 694-698.

Oppenheim, J. D. and Nachbar, M. S. (1977). Immunochemistry of bacterial ATPases. In: Immunochemistry of enzymes and their antibodies. M. R. J. Salton, ed. (Wiley, New York), pp 104-109.

Pamer, E. G., So, M. and Davis, C. E. (1989). Identification of a developmentally regulated cysteine protease of *Trypanosoma brucei*. Mol. Biochem. Parasitol. 33, 27-32.

Pamer, E. G., Davis, C. E., Eakin, A. and So, M. (1990). Cloning and sequencing of the cysteine protease cDNA from *Trypanosoma brucei rhodesiense*. Nucl. Acids Res. 18, 6164.

Pamer, E. G., Davis, C. E. and So, M. (1991). Expression and deletion analysis of the *Trypanosoma brucei rhodesiense* cysteine protease in *Escherichia coli*. Infect. Imm. 59, 1074-1078.

Pamer, T. (1981). Understanding enzymes. (Ellis Horwood, Chichester), pp 119-169.

Paulnock, D. M., Smith, C. and Mansfield, J. M. (1988). Antigen presenting cell function in African trypanosomiasis. In: Antigen presenting cells: diversity, differentiation and regulation. L. B. Schook and J. G. Tew, eds. (Alan R. Liss, New York), pp 135-143. Cited by Pamer *et al.* (1991).

Pépin, J. (1994). The treatment of human African trypanosomiasis. Adv. Parasitol. 33, 1-47.

Pike, R. N. and Dennison, C. (1989). Protein fractionation by three-phase partitioning (TPP) in aqueous/t-butanol mixtures. Biotech. Bioeng. 33, 221-228.

Pike, R. N., Coetzer, T. H. T. and Dennison, C. (1992). Proteolytically active complexes of cathepsin L and a cysteine proteinase inhibitor; purification and demonstration of their formation *in vitro*. Arch. Biochem. Biophy. 294, 623-629.

Piras, M. M., Henríquez, D. and Piras, R. (1985). The effect of proteolytic enzymes and protease inhibitors on the interaction *Trypanosoma cruzi*-fibroblasts. Mol. Biochem. Parasitol. 14, 151-163.

Polson, A., Potgieter, G. M., Largier, J. F., Mears, G. E. F. and Joubert, F. J. (1964). The fractionation of protein mixtures by linear polymers of high molecular weight. Biochim. Biophy. Acta 82, 463-475.

Polson, A., von Wechmar, M. B. and Fazakerley, G. (1980). Antibodies to proteins from the yolk of eggs. Immunol. Comm. 9, 495-514.

Pong, S.-S., Nuss, D. L. and Kock, G. (1975). Inhibition of initiation of protein synthesis in mammalian tissue culture cells by L-1-tosylamido-2-phenylethyl chloromethyl ketone. J. Biol. Chem. 250, 240-245.

Powers, J. C. and Harper, J. W. (1986). Inhibitors of serine proteinases. In: Proteinase inhibitors. A. J. Barrett and G. Salvesen, eds. (Elsevier, Amsterdam), pp 55-152.

Price, N. C. and Stevens, L. (1989). Fundamentals of enzymology 2nd edition. (Oxford University Press, Oxford), pp 136-180.

Raimondi, A., Wernstedt, C., Hellman, U. and Cazzulo, J. J. (1991). Degradation of oxidised insulin A and B chains by the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi* epimastigotes. Mol. Biochem. Parisitol. 49, 341-344.

Rangel, H. A., Araújo, P. M. F., Camargo, I. J. B., Bonfitto, M., Repka, D., Sakurada, J. K. and Atta, A. M. (1981). Detection of a protease common to epimastigote, trypomastigote and amastigote of different strains of *Trypanosoma cruzi*. Tropenmed. Parasitol. 32, 87-92.

Rasnick, D. (1985). Synthesis of peptide fluoromethyl ketones and the inhibition of human cathepsin B. Anal. Biochem. 149, 461-465.

Rasnick, D. (1996). Small synthetic inhibitors of cysteine proteinases. Persp. Drug Disc. Des. 6, 47-63.

Rauber, P., Angliker, H., Walker, B. and Shaw, E. (1986). The synthesis of peptidyl fluoromethanes and their properties as inhibitors of serine proteinases and cysteine proteinases. Biochem. J. *239*, 633-640.

Rautenberg, P., Schädler, R., Reinwald, E. and Risse, H.-J. (1982). Study on a proteolytic enzyme from *Trypanosoma congolense*. Purification and some biochemical properties. Mol. Cell. Biochem. 47, 151-159.

Ray, C. and McKerrow, J. H. (1992). Gut-specific and developmental expression of a *Caenorhabditis elegans* cysteine protease gene. Mol. Biochem. Parasitol. 51, 239-249.

Reed, S., Keene, W. E. and McKerrow, J. H. (1989). Thiol proteinase expression correlates with pathogenicity of *Entamoeba histolytica*. J. Clin. Microbiol. 27, 2772-2777.

Rich, D. H. (1986). Inhibitors of cysteine proteinases. In: Proteinase inhibitors. A. J. Barrett and G. Salvesen, eds. (Elsevier, Amsterdam), pp 153-178.

Richmond, M. H. (1977). β-Lactamase/anti-β-lactamase interactions. In: Immunochemistry of enzymes and their antibodies. M. R. J. Salton, ed. (Wiley, New York), pp 39-55.

Ring, C. S., Sun, E., McKerrow, J. H., Lee, G. K., Rosenthal, P. J., Kuntz, I. D. and Cohen, F. E. (1993). Structure-based inhibitor design by using protein models for the development of antiparasitic agents. Proc. Natl Acad. Sci. (USA). 90, 3583-3587.

Ritonja, A., Popovic, T., Kotnik, M., Machleidt, W. and Turk, V. (1988). Amino acid sequence of human kidney cathepsins H and L. FEBS Lett. 228, 341-345.

Ritonja, A., Coetzer, T. H. T., Pike, R. N. and Dennison, C. (1996). The amino acid sequences, structure comparisons and inhibition kinetics of sheep cathepsin L and sheep stefin B. Comp. Biochem. Physiol. 1148, 193-198. SwissProt (http://expasy.hcuge.ch) accession number Q10991.

Roberts, N. A., Martin, J. A., Kinchington, D., Broadhurst, A. V., Craig, J. C., Duncan, I. B., Galpin, S. A., Handa, B. K., Kay, J., Kröhn, A., Lambert, R. W., Merret, J. H., Mills, J. S., Parkes, K. E. B., Redshaw, S., Ritchie, A. J., Taylor, D. L., Thomas, G. J. and Machin, P. J. (1990). Rational design of peptide-based HIV proteinase inhibitors. Science 248, 358-361.

Robertson, C. D. and Coombs, G. H. (1990). Characterisation of three groups of cysteine proteinases in the amastigotes of *Leishmania mexicana mexicana*. Mol. Biochem. Parasitol. 42, 269-276.

Robertson, C. D. and Coombs, G. H. (1992). Stage-specific proteinases of *Leishmania mexicana mexicana* promastigotes. FEMS Microbiol. Lett. 94, 127-132.

Robertson, C. D., North, M. J., Lockwood, B. C. and Coombs, G. H. (1990). Analysis of the proteinases of *Trypanosoma brucei*. J. Gen. Microbiol. 136, 921-925.

Robertson, C. D., Coombs, G. H., North, M. J. and Mottram, J. C. (1996). Parasite cysteine proteinases. Persp. Drug Disc. Des. 6, 99-118.

Rockett, K. A., Playfair, J. H. L., Ashall, F., Targett, G. A. T., Angliker, H. & Shaw, E. (1990). Inhibition of intraerythrocytic development of *Plasmodium falciparum* by proteinase inhibitors. FEBS Lett. 259, 257-259.

Roitt, I. M. (1991). Essential Immunology 7th Edition. (Blackwell Scientific; Oxford), p 43.

Rosenthal, P. J. (1993). A *Plasmodium vinckei* cysteine proteinase shares unique features with its *Plasmodium falciparum* analogue. Biochim. Biophys. Acta. 1173, 91-93. SwissProt (http://expasy.hcuge.ch) accession number P46102.

Rosenthal, P. J. and Nelson, R. G. (1992). Isolation and characterisation of a cysteine proteinase gene of *Plasmodium falciparum*. Mol. Biochem. Parasitol. 51, 143-152. SwissProt (http://expasy.hcuge.ch) accession number P25805.

Rosenthal, P. J., McKerrow, J. H., Aikawa, M., Nagasawa, H. and Leech, J. H. (1988). A malarial cysteine proteinase is necessary for haemoglobin degradation by *Plasmodium falciparum*. J. Clin. Invest. 82, 1560-1566.

Rosenthal, P. J., Wolllish, W. S., Palmer, J. T. and Rasnick, D. (1990). Antimalarial effects of peptide inhibitors of a *Plasmodium falciparum* cysteine proteinase. J. Clin. Invest. 88, 1467-1472.

Rosenthal, P. J., Lee, G. K. and Smith, R. E. (1993). Inhibition of a *Plasmodium vinckei* cysteine proteinase cures murine malaria. J. Clin. Invest. 91, 1052-1056.

Rosenthal, P. J., Ring, C. S., Chen, X. and Cohen, F. E. (1994). Characterisation of a *Plasmodium vivax* cysteine proteinase gene identifies uniquely conserved amino acids that may mediate the substrate specificity of malarial hemoglobinases. J. Mol. Biol. 241, 312-316. Cited as SwissProt (http://expasy.hcuge.ch) sequence P42666.

Rosenthal, P. J., Olson, J. E., Lee, G. K., Palmer, J. T., Klaus, J. L. and Rasnick, D. (1996). Antimalarial effects of vinyl sulfone cysteine proteinase inhibitors. Antimicrob. Agents Chemother. 40, 1600-1603.

Rossman, T., Norris, T. and Troll, W. (1974). Inhibition of macromolecular synthesis in *Escherichia coli* by protease inhibitors. J. Biol. Chem. 249, 3412-3417.

Russel, D. G. and Alexander, J. (1988). Effective immunisation against cutaneous leishmaniasis with defined membrane antigens reconstituted into liposomes. J. Immunol. 140, 1274-1279.

Russo, D. C. W., Grab, D. J., Lonsdale-Eccles, J. D., Shaw, M. K. and Williams, D. J. L. (1994). Directional movement of variable surface glycoprotein-antibody complexes in *Trypanosoma brucei*. Cell Biology. 62, 432-441.

Salvesen, G. and Nagase, H. (1989). Inhibition of proteolytic enzymes. In: Proteolytic enzymes: a practical approach. R. J. Beynon and J. S. Bond, eds. (IRL Press, Oxford), pp 83-104.

Schaffer, M. A. and Fischer, R. L. (1988). Analysis of mRNAs that accumulate in response to low temperature identifies a thiol protease gene in tomato. Plant Physiology 87, 431-436.

Schägger, H. and von Jagow, G. (1987). Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Anal. Biochem. 166, 368-379.

Scharpe, S., De Meester, I., Hendriks, D., Vanhoof, G., van Sande, M. and Vriend, G. (1991). Proteases and their inhibitors: today and tomorrow. Biochimie 73, 121-126.

Schechter, I. and Berger, A. (1967). On the size of the active site in proteases. I. Papain. Biochem. Biophys. Res. Commun. 27, 157-162.

Schellenberger, V., Turck, C. W., Hedstrom, L. and Rutter, W. J. (1993). Mapping the S' subsites of serine proteases using acyl transfer to mixtures of peptide nucleophiles. Biochemistry 32, 4349-4353.

Schnebli, H. P. and Braun, N. J. (1986). Proteinase inhibitors as drugs. In: Proteinase inhibitors. A. J. Barrett and G. Salvesen, eds. (Elsevier, Amsterdam), pp 613-627.

Schoellmann, G. and Shaw, E. (1963). Direct evidence for the presence of histidine in the active centre of chymotrypsin. Biochemistry 2, 252-255.

Scholze, H. and Schulte, W. (1988). On the specificity of a cysteine proteinase from *Entamoeba histolytica*. Biomed. Biochim. Acta 47, 115-123.

Scholze, H. and Schulte, W. (1990). Purification and partial characterisation of the major cysteine protease from *Entamoeba histolytica*. Biomed. Biochim. Acta 49, 455-463.

Seed, J. R. and Sechelski, J. B. (1989). Mechanism of long slender (LS) to short stumpy (SS) transformation in the African trypanosomes. J. Protozool. 36, 572-577.

Sepúlveda-Boza, S. and Cassels, B. (1996). Plant metabolites active against *Trypanosoma cruzi*. Planta Medica 62, 98-105.

Serveau, C., Lalmanach, G., Juliano, M. A., Scharfstein, J., Julianao, L. and Gauthier, F. (1996). Investigation of the substrate specificity of cruzipain, the major cysteine proteinase of *Trypanosoma cruzi*, through the use of cystatin-derived substrates and inhibitors. Biochem. J. 313, 951-956.

Shapiro, S. Z. (1987). Elimination of the detection of an artifactual 65 kDa keratin band from immunoblots. J. Immunol. Methods 102, 143-146.

Sharma, M., Hirata, K., Herdman, S. and Reed, S. (1996). *Entamoeba invadens*: characterisation of cysteine proteinases. Exp. Parasitol. 84, 84-91.

Shaw, E. (1994). Peptidyl diazomethanes as inhibitors of cysteine and serine proteinases. Meth. Enzymol. 244, 659-656.

Shibuya, K., Kaji, H., Itoh, T., Ohyama, Y., Tsujikami, A., Tate, S., Tadeka, A., Kumagai, Í., Hirao, I., Miura, K., Inagaki, F. and Samejima, T. (1995). Human cystatin A is inactivated by engineered truncation. The NH_2-terminal region of the cysteine proteinase inhibitor is essential for expression of its inhibitory activity. Biochemistry 34, 12185-12192.

Smith, A. M., Dowd, A. J., McGonigle, S., Keegan, P. S., Brennan, G., Trudget., A. and Dalton, J. P. (1993). Purification of a cathepsin L-like proteinase secreted by adult *Fasciola hepatica*. Mol. Biochem. Parasitol. 62, 1-8.

Smith, A. M., Dalton, J. P., Clough, K. A., Kilbane, C. L., Harrop, S. A., Hole, N. and Brindley, P. J. (1994). Adult *Schistosoma mansoni* express cathepsin L proteinase activity. Mol. Biochem. Parasitol. 67, 11-19.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. and Klenk, D. C. (1985). Measurement of protein using bicinchoninic acid. Anal. Biochem. 150, 76-85.

Smith, R. E., Rasnick, D., Burdick, C. O., Cho, K., Rose, J. C. and Vahratian, A. (1988). Visualisation of time-dependent inactivation of human tumour cathepsin B isozymes by a peptidyl fluoromethyl ketone using a fluorescent print technique. Anticancer Res. 8, 525-530.

Soulsby, E. J. L. (1982). Phylum Sarcomastogophora. In: Helminths, arthropods and protozoa of domesticated animals. (Baillière Tindall, London), pp. 514-543.

Souto-Padròn, T., Campetella, O. E., Cazzulo, J. J. and de Souza, W. (1990). Cysteine proteinase in *Trypanosoma cruzi*: immunocytochemical localisation and involvement in parasite-host cell interaction. J. Cell Science 96, 485-490.

Souza, A. E., Waugh, S., Coombs, G. H. and Mottram, J. C. (1992). Characterization of a multi-copy gene for a major stage-specific cysteine proteinase of *Leishmania mexicana*. FEBS Lett. 311, 124-127. SwissProt (http://expasy.hcuge.ch) accession number P36400.

Spinola, S. M. and Gannon, J. G. (1985). Different blocking agents cause variation in the immunologic detection of proteins transferred to nitrocellulose membranes. J. Immunol. Meth. 81, 161-165.

Steiger, R. F., Van Hoof, F., Bontemps, J., Nyssens-Jadin, M. and Druetz, J.-E. (1979). Acid hydrolases of trypanosomid flagellates. Acta Tropica 36, 335-341.

Stoka, V., Nycander, M., Lenarcic, B., Labriola, C., Cazzulo, J. J., Björk, I. and Turk, V. (1995). Inhibition of cruzipain, the major cysteine proteinase of the protozoan parasite, *Trypanosoma cruzi*, by proteinase inhibitors of the cystatin superfamily. FEBS Lett. 370, 101-104.

Stonelake, P. S., Jones, C. E. and Baker, P. R. (1997). Proteinase inhibitors reduce basement membrane degradation by human breast cancer cell lines. Brit. J. Canc. 75, 951-959.

Storer, A. C. and Ménard, R. (1994). Catalytic mechanism in papain family of cysteine peptidases. Meth. Enzymol. 244, 486-500.

Storer, A. C. and Ménard, R. (1996). Recent insights into cysteine proteinase specificity: lessons for drug design. Persp. Drug Disc. Des. 6, 33-46.

Strickland, D. K., Ashcom, J. D., Williams, S., Burgess, W. H., Migliorini, M. and Argraves, W. S. (1990). Sequence identity between the α_2-macroglobulin receptor and Low Density Lipoprotein Receptor-related Protein suggests that this molecule is a multifunctional receptor. J. Biol. Chem. 265, 17401-17404.

Stubbs, M. T., Laber, B., Bode, W., Huber, R., Jerala, R., Lenarcic, B. and Turk, V. (1990). The refined 2.4 Å X-ray crystal structure of recombinant human stefin B in complex with the cysteine proteinase papain: a novel type of proteinase inhibitor interaction. EMBO J. 9, 1939-1947.

Swellengrebel, N. H. and Sterman, M. M. (1961). Hemoflaggelates. In: Animal parasites in man. (Van Nostrand, Princeton), pp 49-71.

Tabel, H., Rurangirwa, F. R. and Losos, G. J. (1978). Is the anaemia in bovine trypanosomiasis caused by immunologic mechanisms? In: Pathogenicity of trypanosomes. Proceedings of a workshop held at Nairobi, Kenya, 20-23 November 1978. G. Losos and A. Chouinard, eds. (International Development Research Centre, Ottawa), pp 91-93.

Tait, A., Eldirdiri, A., Babiker, E. A. and Le Ray, D. (1984). Enzyme variation in *Trypanosoma brucei* species. I. Evidence for the sub-speciation of *Trypanosoma brucei gambiense*. Parasitol. 89, 311-326.

Takeda, A., Nakamura, Y. and Aoki, Y. (1992). Enzyme-linked immunosorbent assay for the detection of cathepsin-kininogen complexes in human plasma. J. Immunol. Meth. 147, 217-223.

Tanaka, T., Slamon, D. J. and Cline, M. J. (1985). Efficient generation of antibodies to oncoproteins by using synthetic peptides as antigens. Proc. Natl Acad. Sci. (USA) 82, 3400-3404.

Thorpe, R. (1994). Producing antibodies. In: Immunochemistry LabFax. M A. Kerr and R. Thorpe, eds. (Bios Scientific, Oxford), pp 63-81.

Tizard, I., Nielsen, K. H., Seed, J. R., and Hall, J. E. (1978). Biologically active products from African trypanosomes. Microbiological Reviews 42, 661-681.

Tobie, E. J. (1958). The cultivation of *Trypanosoma congolense in vitro*. J. Parasitol. 44, 241-242.

Todorova, V. K., Knox, D. P. and Kennedy, M. W. (1995). Proteinases in the excretory/secretory products (ES) of adult *Trichinella spiralis*. Parasitology 111, 201-208.

Tomás, A. M. and Kelly, J. M. (1994). Transformation as an approach to functional analysis of the major cysteine protease of *Trypanosoma cruzi*. Biochem. Soc. Trans 22, 90S.

Towbin, J., Staehelin, T. and Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proc. Natl Acad. Sci. (USA) 76, 4350-4354.

Traub-Cseko, Y. M., Duboise, M., Boukai, L. K. and McMahon-Pratt, D. (1993). Identification of two distinct cysteine proteinase genes of *Leishmania pifanoi* axenic amastigotes using the polymerase chain reaction. Mol. Biochem. Parasitol. 57, 101-115. SwissProt (http://expasy.hcuge.ch) accession number Q05094.

Troeberg, L., Pike, R. N., Morty, R. E., Berry, R. K., Coetzer, T. H. T. and Lonsdale-Eccles, J. D. (1996). Proteases from *Trypanosoma brucei brucei*: Purification, characterisation and interaction with host regulatory molecules. Eur. J. Biochem. 238, 728-736.

Troeberg, L., Pike, R. N., Lonsdale-Eccles, J. D. and Coetzer, T. H. T. (1997). Production of anti-peptide antibodies against trypanopain-Tb from *Trypanosoma brucei brucei*: effects of antibodies on enzyme activity against Z-Phe-Arg-AMC. Immunopharm. 36, 295-303.

Troen, B. R., Gal, S. and Gottesmann, M. M. (1987). Sequence and expression of the cDNA from MEP (major excreted protein), a transformation-regulated secreted cathepsin. Biochem. J. 246, 731-735. SwissProt (http://expasy.hcuge.ch) accession number P06797.

Turk, B., Stoka, V., Björk, I., Boudier, C., Johansson, G., Dolenc, I., Colic, A., Bieth, J. G. and Turk, V. (1995). High-affinity binding of two molecules of cysteine proteinases to low-molecular-weight kininogen. Protein Science 4, 1874-1880.

Turk, B., Turk, V. and Turk, D. (1997). Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. Biol. Chem. 378, 141-150.

Turk, V. and Bode, W. (1993). Lysosomal cysteine proteinases and their inhibitors cystatins. In: Innovations in proteases and their inhibitors. F. C. Avilas, ed. (Walter de Gruyter, Berlin), pp 161-178.

Twining, S. S. (1984). Fluorescein isothiocyanate-labelled casein assay for proteolytic enzymes. Anal. Biochem. 143, 30-34.

Ukoli, F. M. A. (1984). Introduction to parasitology in tropical Africa. (Wiley, Chichester), pp 364-389.

Urquhart, G. M., Armour, J., Duncan, J. L., Dunn, A. M. and Jennings, F. W. (1987). Veterinary parasitology. (Longman, London), pp 206-212.

Van Regenmortel, M. V. H. (1986). Which structural features determine protein antigenicity? TIBS 11, 36-39.

Van Regenmortel, M. H. V. and Daney de Marcillac, G. (1988). An assessment of prediction methods for locating continuous epitopes in proteins. Immunol. Lett. 17, 95-108.

Vickerman, K. (1970). Ultrastructure of trypanosoma in relation to function. In: the African trypanosomiases. H. W. Mulligan, ed. (Allen and Unwin, London), pp 60-66.

Walker, B., Cullen, B. M., Kay, G., Halliday, I. M., McGinty, A. and Nelson, J. (1992). The synthesis. kinetic characterisation and application of a novel biotinylated affinity label for cathepsin B. Biochem. J. 283, 449-453.

Walsh, J. A. (1989). Disease problems in the Third World. Ann. N. Y. Acad. Sci. 569, 1-16. Cited in McKerrow *et al.*(1993).

Wasilewski, M. M., Lim, K. C., Phillips, J. and McKerrow, J. H. (1996). Cysteine proteinase inhibitors block schistosome haemoglobin degradation *in vitro* and decrease worm burden and egg production *in vivo*. Mol. Biochem. Parasitol. 81, 179-189.

Watanabe, H., Abe, K., Emori, Y., Hosoyama, H. and Arai, S. (1991). Molecular cloning and gibberellin induced expression of multiple cysteine proteinases of rice seed (oryzains). J. Biol. Chem. 266, 16897-16902.

Whitaker, J. R. and Perez-Villasenor, J. (1968). Chemical modification of papain. I. Reaction with the chloromethyl ketones of phenylalanine and lysine and with phenylmethyl sulfonylfluoride. Arch. Biochem. Biophys. 124, 70-78.

Wilcox, D. and Mason, R. W. (1992). Inhibition of cysteine proteinases in lysosomes and whole cells. Biochem. J. 285, 495-502.

Wong, J. Y. M., Harrop, S. A., Ray, S. R. and Brindley, P. J. (1997). Schistosomes express two forms of cathepsin D. Biochim. Biophys. Acta 1338, 156-160.

Wooff, R. and Phillemon-Motsu, T. (1993). Tsetse control -the how, why, where and when. Okavango Observer, p 6.

Yamashita, M. and Konagaya, S. (1996). A novel cysteine protease inhibitor from the egg of chum salmon, containing a cysteine-rich thyroglobulin-like motif. J. Biol. Chem. 271, 1282-1284.

Yong Song, C. and Chappell, C. L. (1993). Purification and partial characterisation of a cysteine proteinase from *Spirometra mansoni* plerocercoids. J. Parasitol. 79, 517-524.

Zak, B. (1958). Simple procedure for the single sample determination of serum copper and iron. Clinica Chemica Acta 3, 328-334.

Zimmerman, M., Yurewicz, E. and Patel, G. (1976). A new fluorogenic substrate for chymotrypsin. Anal. Biochem. 70, 258-262.

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Production of anti-peptide antibodies against trypanopain-Tb from *Trypanosoma brucei brucei*: effects of antibodies on enzyme activity against Z-Phe-Arg-AMC

Linda Troeberg, Robert N. Pike [1], John D. Lonsdale-Eccles [2], Theresa H.T. Coetzer *

Department of Biochemistry, University of Natal (Pietermaritzburg), Private Bag X01, Scottsville, Pietermaritzburg, 3209, South Africa

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Production of anti-peptide antibodies against trypanopain-Tb from *Trypanosoma brucei brucei*: effects of antibodies on enzyme activity against Z-Phe-Arg-AMC

Linda Troeberg, Robert N. Pike [1], John D. Lonsdale-Eccles [2], Theresa H.T. Coetzer *

Department of Biochemistry, University of Natal (Pietermaritzburg), Private Bag X01, Scottsville, Pietermaritzburg, 3209, South Africa

Abstract

Anti-peptide antibodies were produced against the cysteine proteinase trypanopain-Tb from *Trypanosoma brucei brucei* and the effects of these antibodies on enzyme activity against carboxybenzoyl (Z)-Phe-Arg-aminomethylcoumarin (AMC) investigated. A peptide was synthesised corresponding to a region of the trypanopain-Tb active site around the active site histidine so that the resulting anti-peptide antibodies specifically targeted the active site of the enzyme. Such antibodies were considered more likely to modulate enzyme activity compared with antibodies directed against other regions of the enzyme. Trypanopain-Tb activity was modulated by rabbit and chicken antibodies produced against both the free and conjugated peptide. Rabbit anti-peptide antibodies enhanced trypanopain-Tb activity by up to 64% at 500 μg/ml relative to non-immune antibodies. Chicken antibodies on the other hand, both enhanced (by up to 176% at 500 mg/ml) and inhibited (by up to 85% at 250 mg/ml) trypanopain-Tb activity against Z-Phe-Arg-AMC. The nature of the antibody effect depended on the stage during the immunisation protocol at which the antibodies were produced. Chicken antibodies also modulated trypanopain-Tb activity in lysates of *T.b. brucei*, while rabbit antibodies were only effective against the purified enzyme. Anti-trypanopain-Tb peptide antibodies were thus shown to have the potential to affect trypanopain-Tb activity.

Keywords: Anti-peptide antibodies; *Trypanosoma brucei brucei*; Trypanopain

1. Introduction

Trypanosoma brucei brucei is the causative agent of bovine and human trypanosomiasis, called nagana and sleeping sickness, respectively. Both of these diseases are of great economic importance in regions of Africa where the disease vector (the tsetse fly) occurs. Currently available treatments are limited in their efficacy by their toxicity as well as emerging parasite resistance. Various aspects of the parasite's

Abbreviations: ABTS, 2, 2-azino-di-(3-ethylbenthiazoline sulfonate); AMC, aminomethylcoumarin; BSA, bovine serum albumin; ELISAs, enzyme-linked immunosorbent assays; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; HRPO, horseradish peroxidase; PBS, phosphate buffered saline; TMB, 3,3',5,5'-tetramethyl benzidine; Z, carboxybenzoyl

* Corresponding author.

[1] Present address: Department of Haematology, University of Cambridge, MRC Centre, Hills Road, Cambridge CB2 2QH, UK.

[2] Present address: 1309 Panorama Drive, Birmingham, AL 35216, USA.

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metabolism are thus under study as potential future points for chemotherapeutic intervention. The major lysosomal cysteine proteinases of *T. congolense* and *T. cruzi* (called trypanopain-Tc and cruzipain, respectively) have been identified as major antigens recognised in cattle and human infections (de Souza et al., 1990; Authiè et al., 1993) and it is thus considered likely that trypanopain-Tb from *T.b. brucei* is also highly immunogenic in natural infections. While previous studies have indicated that trypanopain-Tb is unlikely to remain active in the host bloodstream (Troeberg et al., 1996), cattle breeds resistant to trypanosomiasis produce anti-trypanopain IgG1, while susceptible breeds do not. This correlation between trypanotolerance and the production of anti-trypanopain-Tb antibodies suggests that anti-trypanopain antibodies may help protect the infected host (Authiè et al., 1993). We thus sought to investigate the effects of anti-trypanopain-Tb antibodies on enzyme activity. We elected to produce antibodies against the trypanopain-Tb active site because of the increased possibility that antibodies against this region would modulate enzyme activity compared to antibodies directed against other regions of the enzyme.

Production of antibodies against a protein of interest can be accomplished in various ways, to produce antibodies with differing specificities. For the purposes of this study, antibodies able to recognise the enzyme active site in its native form (to assess the effects of the antibodies on activity) and also in its partially denatured form (for use in ELISAs) were required. The broad specificity of polyclonal anti-protein antibodies excluded them as potential tools for specifically targeting the trypanopain-Tb active site. Conversely, the absolute specificity of monoclonal antibodies can limit their versatility. Anti-peptide antibodies have advantages over both monoclonal and traditional polyclonal antibodies which made them especially suited to the requirements of this study. Firstly, since anti-peptide antibodies are all directed against a single peptide, they have specificity more similar to monoclonal antibodies than to anti-protein polyclonal antibodies (Lerner, 1984). The specificity of such anti-peptide antibodies for the target protein arises from the fact that a sequence of ten or more residues is likely to be unique to a particular protein. Secondly, since several B-cell clones are stimulated by the peptide, the individual antibodies all have different specificities in that they bind to different, overlapping regions of the peptide. These antibodies are thus highly versatile in that they are able to specifically recognise the target sequence in many different conformations (e.g. both the native and denatured forms). This combined versatility and specificity of anti-peptide antibodies led us to produce anti-trypanopain-Tb antibodies by immunising rabbits and chickens with a peptide immunogen.

Here we describe the selection of a peptide corresponding to a sequence in the active site of trypanopain-Tb and the subsequent production of anti-peptide antibodies in chickens and rabbits. The effect of the anti-peptide antibodies on trypanopain-Tb activity was investigated using the fluorometric peptide substrate Z-Phe-Arg-AMC, previously found to be an appropriate synthetic substrate for trypanopain-Tb (Troeberg et al., 1996).

2. Materials and methods

2.1. Choosing a peptide from the active site

The choice of an immunogenic peptide from the trypanopain-Tb active site was based on identification of surface-located regions. In the absence of a three dimensional structure for trypanopain-Tb, the known three dimensional structure of papain (Drenth et al., 1971), which is 38% similar to that of trypanopain-Tb (Mottram et al., 1989) was used for the prediction of trypanopain B-cell epitopes. To increase the likelihood of immunogenicity, epitope prediction algorithms, such as hydrophilicity, hydrophobicity, accessibility and antigenicity plots (Hopp and Woods, 1981, Kyte and Doolittle, 1982; Van Regenmortel, 1986) were also used. Such a combination of methods has successfully identified an immunogenic peptide from the active site of the homologous mammalian proteinase, cathepsin L (Coetzer et al., 1991).

In light of the success of the previous study by Coetzer et al. (1991), a peptide analogous to that chosen from cathepsin L was chosen for the current study. The 16-residue peptide chosen corresponds with residues 274 to 289 in the primary sequence of trypanopain-Tb (Mottram et al., 1989) and includes

the active site histidine residue, increasing the possibility that antibodies would inhibit enzyme activity. The sequence of the chosen peptide (residues 274–289, Mr = 1461.93) in trypanopain-Tb is ILTSCTSKQLD*H*GVL, where *H* represents the active site histidine. The corresponding peptide is surface-located in papain and contains local peaks of hydrophilicity, accessibility, antigenicity and flexibility in trypanopain-Tb, as determined using the software programme Predict7 (Cármenes et al., 1989). Modifications were made to the original sequence for the synthesis of the peptide by the Peptide Synthesis Core Facility at the University of Georgia, GA, USA, namely: (a) an N-terminal cysteine was added to allow coupling of the peptide to a carrier molecule via a thioether bond; (b) the internal cysteine residue was synthesised as an α-amino butyric acid residue to prevent dimerisation of peptides via disulphide bonds (Muller, 1988) and (c) the C-terminal end was synthesised as an amide, mimicking an uncharged peptide bond, thereby potentially increasing cross-reactivity with whole trypanopain-Tb.

2.2. Peptide conjugation and immunisations

The selected peptide was conjugated to carrier proteins by means of meta-maleimidobenzoyl N-hydroxysuccinimide ester as described by Kitagawa and Aikawa (1976), using the modifications of Liu et al. (1979).

Two rabbits and two chickens were immunised with free peptide (200 μg each, dissolved in heat-sterilised 100 mM sodium phosphate buffer, pH 6.0) and two rabbits and two chickens with peptide–ovalbumin conjugate (200 μg each of conjugated peptide, assuming a 40% coupling efficiency (Muller, 1988)). Freund's complete adjuvant (FCA) was used for all immunisations with the free peptide in rabbits and for the first immunisation with the conjugated peptide in rabbits and chickens (Muller, 1988). Freund's incomplete adjuvant (FIA) was used for subsequent immunisations of rabbits and chickens with the conjugated peptide. In chickens, the free peptide was administered alternately in FCA and FIA. Rabbits were injected subcutaneously, at 4–6 sites on either side of the spine at weeks 0, 2, 6 and 10. Blood was collected from the ear vein 3, 8 and 12 weeks after the initial immunisation. Chickens were injected intramuscularly at 4 to 6 sites on either side of the sternum at weeks 0, 1, 2, 6 and 10 and eggs collected from the start of the immunisation protocol.

2.3. Isolation of antibodies

IgY was isolated from egg yolk by polyethylene glycol precipitation, as described by Polson et al. (1985). Rabbit IgG was similarly isolated from rabbit serum.

2.4. ELISAs

The production of anti-peptide antibodies by experimental animals was assessed using an enzyme-linked immunosorbent assay (ELISA) as described by Coetzer et al. (1991).

To investigate the cross-reactivity between anti-peptide antibodies and whole trypanopain-Tb, trypanopain-Tb (purified as described by Troeberg et al., 1996) was coated (Nunc PolySorb, 16 h at 4°C, titrated between 28 and 0.2 ng per well in 40 mM carbonate buffer, pH 6.0). The remainder of the ELISA was carried out essentially as described by Coetzer et al. (1991) except that a fixed concentration of primary antibody was applied (50 μg/ml, in 0.5% (m/v) BSA–PBS, 0.1% (v/v) Tween-20, 2 h at 37°C), 3,3′,5,5′-tetramethyl benzidine (TMB)/H_2O_2 was used as HRPO-substrate and 1 M H_2SO_4 added to stop the HRPO reaction.

The recognition of cathepsin L by anti-trypanopain-Tb peptide antibodies was investigated by coating sheep cathepsin L (Nunc MaxiSorb, 16 h at 4°C, 1 μg/ml in 40 mM carbonate buffer, pH 6.0). Test antibodies were titrated between 250 and 0.1 μg/ml in 0.5% BSA–PBS (2 h at 37°C). The remainder of the ELISA was carried out as described above for the trypanopain-cross reactivity ELISA.

2.5. Effect of anti-peptide antibodies on trypanopain-Tb activity against Z-Phe-Arg-AMC

The effects of anti-peptide antibodies on enzyme activity against Z-Phe-Arg-AMC were investigated as described by Coetzer et al. (1991). Trypanopain-Tb (1.5 ng, purified or present in lysates of *T.b. brucei*) was incubated with the antibody under study before assaying trypanopain-Tb activity (Troeberg et al.,

1996). Enhancement or inhibition of enzyme activity was expressed as a percentage of the activity detected after incubation with the same concentration of non-immune antibody.

2.6. Inhibition of anti-peptide antibody effect by addition of free peptide

To confirm the specificity of the anti-peptide antibody effects on enzyme activity, the above experiments were repeated in the presence of the free trypanopain-Tb peptide. Free peptide should compete with the enzyme for binding to the antibody and so diminish the effect of the antibodies on trypanopain-Tb activity. Peptide was dissolved in 0.1% (m/v) Brij 35 to give 10,000:1 to 1:1 molar ratios of peptide:enzyme. The peptide solution (100 μl) was incubated with antibody (500 μl, final concentration 500 μg/ml, 15 min at 37°C). Trypanopain-Tb was added (15 min, 37°C) and activity against Z-Phe-Arg-AMC determined as before. A non-related peptide (poly-L-glutamic acid) was added in parallel experiments to further confirm the specificity of the interaction.

3. Results

3.1. Determination of anti-peptide antibody titre in rabbits and chickens

Both the free and the conjugated trypanopain-Tb peptide were immunogenic in rabbits and chickens. High titre anti-peptide antibodies were produced against the conjugated peptide within 3 weeks of the initial immunisation (Fig. 1), while similarly high titre antibodies were only produced against the free peptide 8 weeks after immunisation (Fig. 1). By 8 weeks post-immunisation, the titre of antibodies against the two forms of immunogen was comparable. Anti-peptide antibody titres in rabbits and chickens against corresponding immunogens were similar, as were titres of individual animals and species.

3.2. Recognition of whole trypanopain-Tb and cathepsin L by anti-peptide antibodies

In addition to recognising the free trypanopain-Tb peptide, the anti-peptide antibodies recognised whole

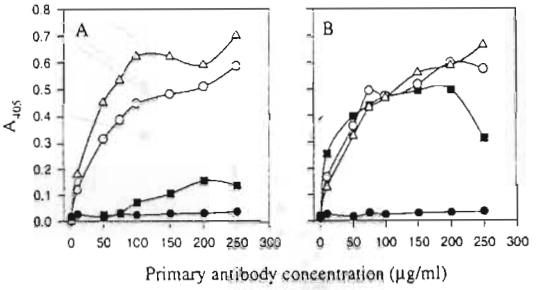

Fig. 1. Titre of rabbit antibodies produced against the free (A) and conjugated (B) trypanopain-Tb peptide at various weeks post-immunisation. Free peptide (5 μg/ml) was coated for 16 h at 4°C in PBS. Non-immune antibodies (●) and antibodies from week 3 (■), week 8 (○) and week 12 (△) post-immunisation were titrated between 250 μg/ml and 0.1 μg antibody/ml. Sheep anti-rabbit-HRPO was used as secondary antibody with ABTS/H_2O_2 as substrate.

trypanopain-Tb in an ELISA (Fig. 2). Antibodies against both forms of the immunogen (free and conjugated peptide) and from both species (rabbit and chicken) recognised the whole enzyme to a comparable extent, with titres below 31 μg antibody/ml. Similarly, both chicken and rabbit anti-free peptide and anti-conjugate antibodies recognised

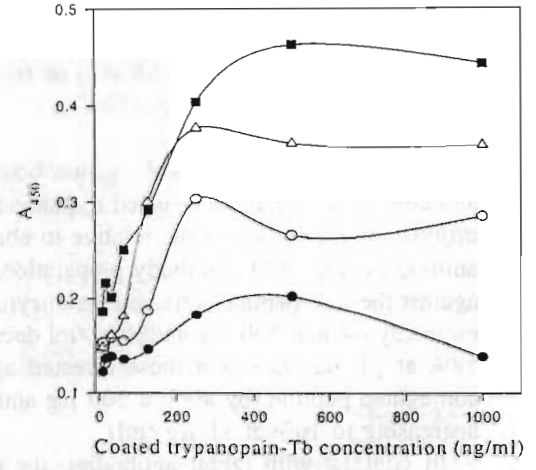

Fig. 2. ELISA showing recognition of whole trypanopain-Tb by chicken anti-free peptide and anti-conjugated peptide antibodies. Trypanopain-Tb (serial doubling dilutions from 1 μg/ml) was coated for 16 h at 4°C in 40 mM carbonate buffer, pH 6.0. A fixed concentration of test antibody (50 μg antibody/ml) was then applied. Symbols represent non-immune antibodies (●), anti-free peptide antibodies from chicken I (■) and II (○) and anti-conjugated peptide antibodies from chicken III (△). Rabbit anti-chicken-HRPO was used as secondary antibody with TMB/H_2O_2 as substrate.

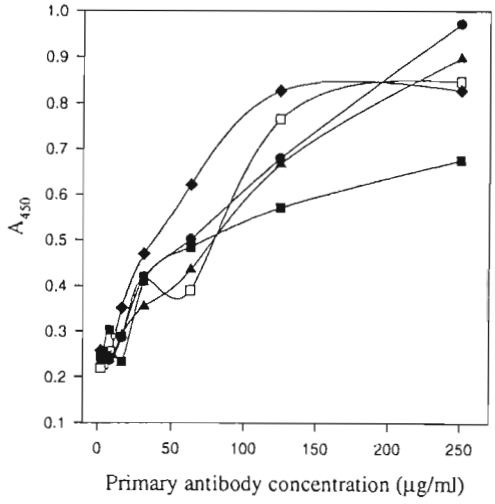

Fig. 3. Cross-reactivity between rabbit anti-trypanopain-Tb anti-peptide antibodies and sheep cathepsin L in an ELISA. Sheep cathepsin L (1 μg/ml) was coated for 16 h at 4°C in 40 mM carbonate buffer, pH 6.0. Rabbit non-immune antibodies (■), anti-free peptide antibodies from rabbits I (□) and II (●) and anti-conjugated peptide antibodies from rabbits III (▲) and IV (◆) were titrated from 250 μg/ml to 2 μg/ml. Sheep anti-rabbit-HRPO secondary antibodies and TMB/H_2O_2 were used for detection.

cathepsin L in an ELISA (Fig. 3), albeit weakly when compared to recognition by the non-immune antibody.

3.3. Effect of anti-peptide antibodies on trypanopain-Tb activity against Z-Phe-Arg-AMC

Rabbit antibodies directed against both the free and conjugated peptide enhanced trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC relative to non-immune antibodies (Fig. 4A). Antibody preparations directed against the free peptide activated the enzyme slightly more (by 64% at 500 μg antibody/ml decreasing to 19% at 31 μg/ml) than those directed against the conjugated peptide (by 49% at 500 μg antibody/ml decreasing to 16% at 31 μg/ml).

In contrast with rabbit antibodies, the effects of chicken antibodies on trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC were more complex. Individual chickens produced both activity-enhancing and activity-inhibiting antibodies at different stages during their immune response. This effect was observed both in chickens immunised with the free peptide and those immunised with the conjugated peptide. Up to 176% activation (by 500 μg/ml week 8 anti-free peptide antibodies) and 85% inhibition (by 250 μg/ml week 10 anti-conjugate antibodies) was observed (Fig. 5). Antibodies produced soon after immunisation/boosting appeared to enhance activity while those produced a few weeks later appeared to be inhibitory.

3.4. Incubation with free peptide reduced antibody effect on trypanopain-Tb activity

Pre-incubation of anti-peptide antibodies with the trypanopain-Tb peptide diminished the effect these antibodies subsequently had on trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC. For example, week 8 chicken anti-free peptide antibodies alone enhanced trypanopain-Tb activity by 276% at 500 mg/ml, but in the presence of a 10,000:1 molar ratio of peptide:enzyme, only 46% enhancement of trypanopain-Tb activity was observed, which represents an 83% reduction in the antibody effect (Table 1).

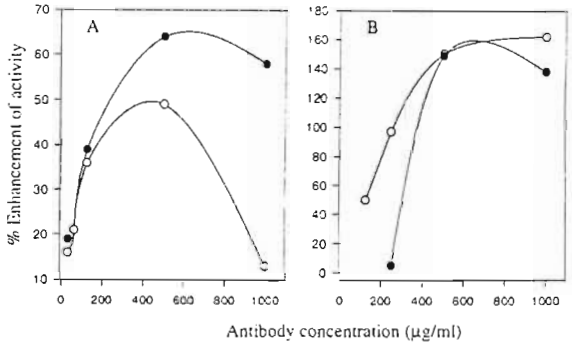

Fig. 4. Anti-peptide antibody-mediated enhancement of trypanopain-Tb activity. A: Effect of conjugation on anti-peptide antibody enhancement of activity: Trypanopain-Tb (1.5 ng) was incubated with rabbit anti-peptide antibodies (31.25 to 1000 μg antibody/ml) at pH 8.0 (15 min. 37°C) and trypanopain-Tb activity against Z-Phe-Arg-AMC then determined. Enhancement mediated by antibodies raised against the free peptide (●) and the conjugated peptide (○) are expressed relative to enzyme activity in the presence of the same concentration of non-immune antibodies. B: Antibody-mediated enhancement of activity of purified trypanopain-Tb and *T.b. bruce* lysates. Z-Phe-Arg-AMC-hydrolysing activity in lysates of *T.b. brucei* (●) and by purified trypanopain-Tb (○) was enhanced by the addition of chicken anti-free peptide antibodies (week 8, 1000 to 31.25 μg antibody/ml).

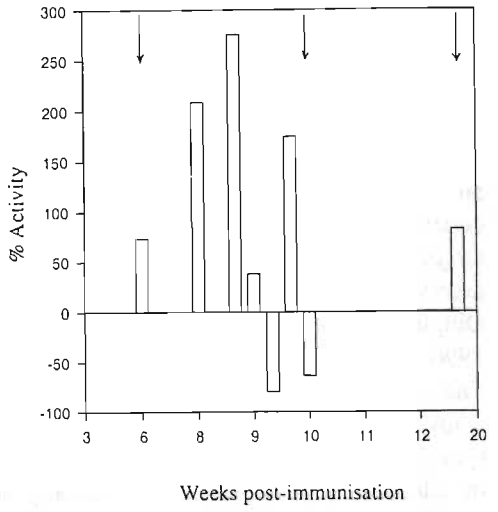

Fig. 5. Modulation of trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC by chicken anti-free peptide antibodies. Trypanopain-Tb (1.5 ng) was incubated with chicken anti-free peptide antibodies (500 μg/ml) at pH 8.0 (15 min, 37°C). Enzyme activity against Z-Phe-Arg-AMC was then determined. Activity is expressed relative to that observed after incubation with non-immune antibodies (500 μg/ml) with % enhancement of activity expressed as positive values and % inhibition of activity expressed as negative values. Chickens were immunised at weeks 0, 1, 2, 6, 10 and 20 (indicated by arrows).

In contrast with chicken anti-free peptide antibodies produced in week 8, antibodies produced during week 10 by the same chicken inhibited trypanopain-Tb activity by 80%. Pre-incubation of these antibodies with the free trypanopain-Tb peptide reduced the inhibition observed to the extent that at 10,000:1 molar ratio of peptide:enzyme, no inhibition was seen (Table 1). This represents a 100% decrease in the antibody effect by addition of free peptide.

Addition of the free peptide to rabbit activity-enhancing anti-peptide antibodies produced a similar reduction in the antibody effect (Table 1). Addition of a non-related peptide (poly-L-glutamic acid) did not alter antibody effects on enzyme activity (results not shown).

3.5. Effect of anti-peptide antibodies on trypanopain-Tb activity in T.b. brucei lysates

Chicken anti-peptide antibodies modulated trypanopain-Tb activity in lysates at levels comparable to those seen with the purified enzyme (Fig. 4B). For example, chicken antibodies that enhanced activity of the purified enzyme (anti-free peptide week 8) also enhanced Z-Phe-Arg-AMC hydrolysis in crude lysates of *T.b. brucei*. Comparable enhancement of the purified enzyme and of enzyme activity in lysates was observed at 1 and 0.5 mg antibody/ml. Below this concentration, lower enhancement was seen in lysates than with the purified enzyme at equal antibody concentrations.

Chicken anti-conjugated peptide antibodies (week 10) that inhibited pure trypanopain-Tb also inhibited Z-Phe-Arg-AMC hydrolysis in *T.b. brucei* lysates. For example, up to 82% inhibition was observed with the purified enzyme at 500 μg antibody/ml, while up to 70% inhibition was seen in lysates at the

Table 1
Effects of pre-incubation of rabbit (IgG) and chicken (IgY) antibodies with the free trypanopain-Tb peptide on activity against Z-Phe-Arg-AMC

	Peptide:enzyme ratio				
	No peptide	1:1	100:1	1000:1	10 000:1
Chicken activity-enhancing antibodies					
% Enhancement of activity	276	131	99	73	46
% Reduction of enhancement by peptide	0	59	64	74	83
Chicken activity-inhibiting antibodies					
% Inhibition of activity	80	59	54	56	0
% Reduction of inhibition by peptide	0	26	33	31	100
Rabbit activity-enhancing antibodies					
% Enhancement of activity	64	57	48	28	15
% Reduction of enhancement by peptide	0	11	25	56	77

same antibody concentration. However, rabbit anti-free and anti-conjugated peptide antibodies that enhanced the activity of pure trypanopain-Tb, mediated no such enhancement in lysates. Even at high concentrations of antibody (1 mg/ml) no enhancement of activity was detectable.

4. Discussion

4.1. Immunogenicity of peptide and antibody production

The trypanopain-Tb peptide was found to be equally immunogenic in rabbits and chickens. By 8 weeks post-immunisation comparably high titre antibodies were produced against the conjugated and free peptide. The free peptide was thus clearly immunogenic in its own right, in agreement with previous reports regarding similar peptides by Coetzer (1993).

In addition to recognising the free peptide, all the anti-peptide antibodies recognised whole trypanopain-Tb in an ELISA. This shows that at least a proportion of the conformations adopted by the highly mobile peptide in solution mimic those that occur in the corresponding constrained region of the parent proteir. Anti-conjugate and anti-free peptide antibodies cross-reacted equally well with trypanopain-Tb, showing that conjugation of the peptide to a carrier protein had no discernible effect on the degree of cross-reactivity with the parent molecule. Such cross-reactivity between anti-peptide antibodies and their corresponding protein has been previously reported, for example, cathepsin L and D (Coetzer et al., 1991) and cytochromeP-450IA2 (Edwards et al., 1990), but absence of cross-reactivity has also been found e.g. for cathepsin B (Coetzer et al., 1991).

Anti-trypanopain-Tb peptide antibodies not only recognised whole trypanopain-Tb in an ELISA, but also cross-reacted weakly with whole cathepsin L in an ELISA. This is thought to be a reflection of the homology between the peptide from the parasite proteinase and the corresponding region of the active site of the mammalian host proteinase, cathepsin L (Ritonja et al., 1996). The effect of anti-trypanopain-Tb antibodies on cathepsin L activity against protein substrates is being investigated.

4.2. Effects of anti-peptide antibodies on trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC

Previous studies have shown that anti-enzyme antibodies can enhance or inhibit enzyme activity, depending on the enzyme under study, the region targeted by the antibody and the substrate used to assess the antibody effects (Arnon and Shapira, 1967; Dingle et al., 1971; Richmond, 1977; Edwards et al., 1990; Dennison and Pike, 1991; Coetzer et al., 1992). The anti-peptide antibodies produced in the present study affected trypanopain-Tb activity, showing that they do recognise and bind to native trypanopain-Tb in addition to recognising the partially denatured form present on ELISA plates. However, the effects on trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC were varied.

All of the rabbit anti-free and anti-conjugated peptide antibodies produced enhanced trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC. Such immunoenhancement of enzyme activity is thought to occur by antibody induction of a more active enzyme conformation (Richmond, 1977). Rabbit antibodies directed against the free peptide enhanced enzyme activity slightly more than those directed against the conjugated peptide, suggesting that the titre of antibodies recognising the native form of trypanopain-Tb is slightly higher in anti-free peptide antibody preparations than in those directed against the conjugated peptide. A similar observation was made for anti-cathepsin H peptide antibodies where anti-free peptide antibodies decreased enzyme activity to a greater extent than their conjugated counterparts (Coetzer, 1993).

The effects of chicken antibodies on trypanopain-Tb were more varied, with individual chickens producing activity-enhancing and activity-inhibiting antibodies at different stages in their immune response. Antibodies produced shortly after each immunisation appear to enhance trypanopain-Tb activity, while those produced a few weeks later appear inhibitory. Since Z-Phe-Arg-AMC is a small substrate, it is considered likely that antibody binding induces a less active enzyme conformation, rather than sterically hindering substrate binding.

The production of both activity-enhancing and -inhibiting antibodies during the immunisation of a single test animal is thought to be a function of the

polyclonal nature of the evoked antibody response. Since a peptide can adopt numerous conformations in solution, some may elicit activity-enhancing antibodies and some activity-inhibiting antibodies. The varied effects of antibodies observed in this study could thus reflect different B-cell clones being dominant at different stages in the immune response.

The effects of these antibodies on trypanopain-Tb activity were shown to be specific by the fact that pre-incubation of the antibodies with their target peptide reduced antibody effects on trypanopain-Tb, while pre-incubation with an unrelated peptide (poly-L-glutamic acid) had no such effect. Complete abolition of the antibody effects was not seen in these assays, possibly as a consequence of the high dilution of antibody and enzyme required by this sensitive fluorometric assay.

4.3. Effects in lysates

Anti-peptide antibodies were added to lysates of *T.b. brucei* to assess the potential effects of other trypanosomal factors on interactions of these antibodies with trypanopain-Tb. Chicken anti-peptide antibodies had the same effect in lysates as with the purified enzyme i.e. some antibodies mediated inhibition and others enhancement of trypanopain-Tb hydrolysis of Z-Phe-Arg-AMC. The degree of enhancement/inhibition observed with the purified enzyme and enzyme activity in lysates was comparable, suggesting that these antibodies have the potential to modulate trypanopain-Tb activity in vivo. In contrast, rabbit antibodies which substantially activated the purified enzyme, had no effect in lysates even at 1 mg antibody/ml. This suggests that the interaction between this antibody and trypanopain-Tb is reduced by some factor in trypanosome lysates which does not affect interactions with chicken antibodies.

Depending on the epitope against which antibodies are raised, the host species and the substrate used to assess the effects of the antibodies on enzyme activity, some anti-trypanopain antibodies are thus able to modulate trypanopain activity in vitro. The physiological importance of such modulation is unclear, because it is highly unlikely that trypanopain-Tb remains active in the mammalian bloodstream since it is effectively inhibited by cystatins (Troeberg et al., 1996). Additionally, no trypanopain-Tb-like activity is detectable in the bloodstream of infected rats (Troeberg et al., 1996). While Lonsdale-Eccles et al. (1995) reported that a kininogen-like moiety increases trypanopain-Tb activity in gelatin SDS-PAGE analyses, the in vivo occurrence of such interactions is unclear. Assuming that trypanopain-Tb is effectively controlled in the host bloodstream, the correlation between production of anti-trypanopain antibodies and tolerance to trypanosomiasis in certain cattle breeds (Authiè et al., 1993) is puzzling. While this correlation may just be a function of a more surveillant immune system in tolerant breeds, it is tempting to speculate that the anti-trypanopain antibodies are more directly protective. Experiments are currently under way to assess the in vivo effects of anti-trypanopain peptide antibodies more directly, by determining their effects on trypanopain-Tb hydrolysis of proteins and their effects on trypanosomes in cell culture.

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References

Arnon, R., Shapira, E., 1967. Antibodies to papain. A selective fractionation according to inhibitory capacity. Biochemistry 6, 3942–3950.

Authiè, E., Duvallet, G., Robertson, C., Williams, D., 1993. Antibody response to a 33 kDa cysteine protease of *Trypanosoma congolense*: Relationship to trypanotolerance in cattle. Parasite Immunol. 15, 465–474.

Cármenes, R.S., Freije, J.P., Moline, M.M., Martin, J.M., 1989. Predict7, a program for protein structure prediction. Biochem. Biophys. Res. Commun. 159, 687–693.

Coetzer, T.H.T., 1993. Type IV collagenase and cathepsins L and H: Proteinases involved in tumour invasion. Ph.D. thesis, University of Natal, Pietermaritzburg.

Coetzer, T.H.T., Elliott, E., Fortgens, P.H., Pike, R.N., Dennison, C., 1991. Anti-peptide antibodies to cathepsins B, L and D and type IV collegenase. J. Immunol. Methods 136, 199–210.

Coetzer, T.H.T., Pike, R.N., Dennison, C., 1992. Localisation of an immunoinhibitory epitope of the cysteine proteinase, cathepsin L. Immunol. Invest. 21, 495–506.

Dennison, C., Pike, R.N., 1991. A peptide antibody that specifically inhibits cathepsin L. In: Atassi, M.Z. (Ed.), Immunobiol-

ogy of Proteins and Peptides VI. Plenum Press, New York, pp. 285–288.

de Souza, A.C.M., Persechini, P.M., de Souto Padron, T., de Souza, W., Guimarães, J.A., Scharfstein, J., 1990. Structural and functional identification of GP 57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase. Mol. Biochem. Parasitol. 43, 27–38.

Dingle, J.T., Barrett, A.J., Weston, P.D., 1971. Cathepsin D. Characteristics of immunoinhibition and the confirmation of a role in cartilage breakdown. Biochem. J. 123, 1–13.

Drenth, J., Janonius, J.N., Koekoek, R., Wolthers, B.G., 1971. The structure of papain. Adv. Protein Chem. 25, 79.

Edwards, R.J., Singleton, A.M., Murray, B.P., Sesardic, D., Rich, K.J., Davies, D.S., Boobis, A.R., 1990. An anti-peptide antibody targeted to a specific region of rat cytochrome P-450IA2 inhibits enzyme activity. Biochem. J. 266, 497–504.

Hopp, T.P., Woods, K.R., 1981. Prediction of protein antigenic determinants from amino acid sequences. Proc. Natl. Acad. Sci. USA 78, 3824–3828.

Kitagawa, T., Aikawa, T., 1976. Enzyme coupled immunoassay of insulin using a novel coupling reagent. J. Biochem. 79, 233–236.

Kyte, J., Doolittle, R.F., 1982. A simple method for displaying the hydropathic character of a protein. J. Mol. Biol. 157, 105–132.

Lerner, R.A., 1984. Antibodies of predetermined specificity in biology and medicine. Adv. Immunol. 36, 1–44.

Liu, F.-T., Zinnecker, M., Hamaoka, T., Katz, D.H., 1979. New procedure for preparation and isolation of conjugates of proteins and a synthetic copolymer of D-amino acids and immunochemical characterisation of such conjugates. Biochemistry 18, 690–697.

Lonsdale-Eccles, J.D., Mpimbaza, G.W.N., Nkhungulu, Z.R.M., Olobo, J., Smith, L., Tosomba, O.M., Grab, D.J., 1995. Trypanosomatid cysteine protease activity may be enhanced by a kininogen-like moiety from host serum. Biochem. J. 305, 549–556.

Mottram, J.C., North, M.J., Barry, M.J., Coombs, G.H., 1989. A cysteine protease cDNA from *Trypanosoma brucei* predicts an enzyme with an unusual C-terminal extension. FEBS Lett. 258, 211–215.

Muller, S., 1988. Peptide-carrier conjugation. In: Burdon, R.H., Van Knippenberg, P.H. (Eds.) Laboratory Techniques in Biochemistry and Molecular Biology, vol. 19, Synthetic peptides as antigens. Elsevier, Amsterdam, pp. 131–141.

Polson, A., Coetzer, T.H.T., Kruger, J.S., Von Maltzahn, E., Van der Merwe, K.J., 1985. Improvements in the isolation of IgY from the yolks of eggs laid by immunised hens. Immunol. Invest. 14, 323–327.

Richmond, M.H., 1977. b-Lactamase/anti-b-lactamase interactions. In: Salton, M.R.J. (Ed.), Immunochemistry of Enzymes and their Antibodies. Wiley, New York, pp. 39–55.

Ritonja, A., Coetzer, T.H.T., Pike, R.N., Dennison, C., 1996. The amino acid sequences, structure comparisons and inhibition kinetics of sheep cathepsin L and stefin B. Comp. Biochem. Physiol. 114B, 193–198.

Troeberg, L., Pike, R.N., Morty, R.E., Berry, R.K., Coetzer, T.H.T., Lonsdale-Eccles, J.D., 1996. Proteases from *Trypanosoma brucei brucei*: Purification, characterisation and interactions with host regulatory molecules. Eur. J. Biochem. 238, 728–736.

Van Regenmortel, M.V.H., 1986. Which structural features determine protein antigenicity?. TIBS 11, 36–39.

Eur. J. Biochem. *238*, 728–736 (1996)

Proteases from *Trypanosoma brucei brucei*
Purification, characterisation and interactions with host regulatory molecules

Linda TROEBERG, Robert N. PIKE, Rory E. MORTY, Ronald K. BERRY, Theresa H. T. COETZER and John D. LONSDALE-ECCLES

Department of Biochemistry, University of Natal, Scottsville, South Africa

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African trypanosomes contain proteases that may be released into the bloodstream of their infected hosts. This paper describes a novel, combined isolation of a cysteine proteinase (called trypanopain-Tb) and a serine oligopeptidase (which we call oligopeptidase-Tb) from *Trypanosoma brucei brucei*, as well as a comparison of the activities of these two enzymes against several host regulatory molecules.

The enzymes differed in various respects. Firstly, purified trypanopain-Tb was shown to readily cleave proteins such as gelatin maximally at acidic pH. In contrast, oligopeptidase-Tb, which is optimally active at alkaline pH, did not hydrolyse proteins larger than 4 kDa. However, it readily hydrolysed various polypeptides, including neurotensin and atrial natriuretic factor.

The interaction of the two enzymes with mammalian protease inhibitors also differed. Cystatins and α_2-macroglobulin effectively inhibited trypanopain-Tb, with the K_i values for cystatin C and low-molecular-mass kininogen ($\approx 10^{-11}$ M) predicting that trypanopain-Tb is likely to be effectively controlled by these inhibitors if released into the host bloodstream. In contrast, oligopeptidase-Tb was not inhibited by serpins or α_2-macroglobulin, suggesting that it may remain active if released into the host bloodstream. In support of these *in vitro* results, the blood of trypanosome-infected rats displayed no trypanopain-Tb-like activity, but exhibited high oligopeptidase-Tb-like activity. Thus, while trypanopain-Tb seems likely to be confined to an intracellular role within the parasite, oligopeptidase-Tb has the potential to remain active in the host bloodstream and so contribute directly to pathogenesis.

Keywords: *Trypanosoma brucei*; cysteine proteinase; oligopeptidase; cystatin.

African trypanosomes are protozoan parasites which cause the diseases nagana and sleeping sickness in cattle and humans, respectively. These diseases are characterised by intermittent fever, progressive anaemia, general loss of condition and, ultimately, the death of the infected host. Nagana is still of great economic importance in Africa, where it prevents livestock farming in many areas, and certain parts of Africa are experiencing a large resurgence in human trypanosomiasis (Walgate, 1994). While the parasite's major lysosomal cysteine proteinase (trypanopain) is considered a potentially important factor in the development of the disease (Authié et al., 1993; Russo et al., 1994), the role of a cytoplasmic serine oligopeptidase (which we call oligopeptidase-Tb, OP-Tb) has not yet been explored.

Trypanopain is lysosomally located (Mbawa et al., 1991) and, as such, is likely to be centrally involved in intracellular digestive and catabolic proteolysis. Additionally, the enzyme is proposed to help the parasite escape opsonisation by degrading internalised antibody-variant surface glycoprotein complexes (Russo et al., 1994). Enzyme released into the host bloodstream has also been proposed to contribute to pathogenesis more directly by degrading various host proteins. Also, since trypanotolerant cattle infected with *Trypanosoma congolense* produce antibodies to trypanopain-Tc, while susceptible cattle do not, immune targeting of the enzyme may help protect infected hosts (Authié et al., 1993). While the cDNA of trypanopain-Tb from *Trypanosoma brucei brucei* has been sequenced (Mottram et al., 1989), no purification of the enzyme to electrophoretic homogeneity, or characterisation of its basic enzymatic properties, has been reported. The interactions of trypanopain-Tb with mammalian cysteine proteinase inhibitors and other host regulatory molecules also have not been reported before.

While OP-Tb has previously been partially purified from *T. b. brucei* (Huet et al., 1992; Kornblatt et al., 1992) and *Trypanosoma cruzi* (Ashall, 1990), the role of this enzyme in pathogenesis has not been investigated. Previous data indicates that the enzyme is a serine protease, but its sensitivity to reagents which modify cysteine residues suggests that reduction of a cysteine residue is required for full enzyme activity (Kornblatt et al., 1992). The enzyme also appears to be restricted in its action, cleaving only small peptide substrates. Both these facets of the enzyme's activity require further investigation.

Here we report the joint purification of trypanopain-Tb and OP-Tb from *T. b. brucei*, the effects of various potential *in vivo* inhibitors on the enzymes and the action of the enzymes on potentially relevant host peptide and protein substrates.

Correspondence to T. H. T. Coetzer, Department of Biochemistry, University of Natal, Private Bag X01, Scottsville, South Africa 3209

Fax: +27 331 260 5462.

Phone: +27 331 260 5467.

Abbreviations. α_2-M, α_2-macroglobulin; Boc, butoxycarbonyl; E-64, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane; $H_2NEtPhSO_2F$, 4-(2-aminoethyl)-benzenesulfonyl fluoride; iPr_2P-F, di-isopropyl fluorophosphate; L-kininogen, low-molecular-mass kininogen; MeO-Suc, methoxysuccinyl; NHMec, aminomethyl coumarin; OP-Tb, trypanosomal oligopeptidase; $PhMeSO_2F$, phenylmethylsulfonyl fluoride; rTM, rat trypanopain modulator; Suc, succinyl; SBTI, soybean trypsin inhibitor; $TosLysCH_2Cl$, tosyllysylchloromethane; $TosPheCH_2Cl$, tosylphenylalanylchloromethane; Z, benzyloxycarbonyl.

MATERIALS AND METHODS

Materials. HiLoad™ Q-Sepharose and HiTrap™ Blue Sepharose were from Pharmacia. DEAE-cellulose was from What-

man. 4-(2-Aminoethyl)-benzenesulfonyl fluoride ($H_2NEtPhSO_2F$), BSA and α_2-macroglobulin (α_2-M) were from Boehringer Mannheim. Chymostatin, leupeptin and antipain were from Cambridge Research Chemicals (UK) and di-isopropyl fluorophosphate (iPr_2P-F) was from Fluka. [1-^{14}C]Acetic anhydride was from Amersham. All other inhibitors, benzyloxycarbonyl phenylanlanyl-arginyl-aminomethyl coumarin (Z-Phe-Arg-NHMec), Z-Arg-Arg-NHMec, pepstatin-A−Sepharose, poly-(L-lysine)-Sepharose, fibrinogen, rabbit IgG, Percoll™ and Sephacryl S-100 HR were from Sigma. The C18 HPLC column was from Vydac. Sheep stefin B was purified as described by Pike et al. (1992). Human recombinant stefin A was from Cal-Biochem and human low-molecular-mass kininogen (L-kininogen) was purified from human plasma as described by Gounaris et al. (1984). Recombinant human L-kininogen domain 3 was a gift from Dr Ennes Auerswald (Ludwig-Maximilians-Universität München) human cystatin C was a gift from Dr Magnus Abrahamson (University of Lund, Malmö General Hospital) and serpins were a gift from Dr Jan Potempa (Jagiellonian University, Cracow).

Growth and purification of trypanosomes. *T. b. brucei* (clone ILTat 1.1) was grown in rats and purified from the infected blood by a combination of Percoll™ gradient centrifugation (Grab and Bwayo, 1982) and anion-exchange chromatography on DEAE-cellulose (Lanham and Godfrey, 1970). Purified trypanosomes were stored at −70°C until used.

Enzyme and protein assays. Trypanopain-Tb activity against 5 μM Z-Phe-Arg-NHMec at 37°C was initially measured as described by Barrett and Kirschke (1981) using an assay buffer of 170 mM sodium acetate, 30 mM acetic acid, 2 mM Na_2EDTA, 3 mM dithiothreitol, pH 5.5. Following enzyme characterisation, the assay buffer was amended to 200 mM sodium phosphate pH 7.0 containing 30 mM cysteine and 2 mM Na_2EDTA. OP-Tb was similarly assayed against 5 μM Z-Arg-Arg-NHMec in 100 mM Tris/HCl, 10 mM dithiothreitol, pH 8.0 (Kornblatt et al., 1992).

Protein assays were conducted according to the methods of Bradford as modified by Read and Northcote (1981) and Smith et al. (1985).

Isolation of trypanopain-Tb and OP-Tb. Trypanosomes (≈5.5×10^9 parasites) were thawed and lysed by addition of 0.1% (by vol.) Triton X-100 (final concentration). Three-phase partitioning (Pike and Dennison, 1989) was performed on the lysate, with all enzyme activity precipitating between 10−25% (mass/vol. of the total volume) ammonium sulfate (results not shown). The pellet was redissolved in buffer A (20 mM sodium acetate, 1 mM Na_2EDTA, pH 5.5) and loaded onto HiLoad™ Q-Sepharose (26×110 mm, flow rate 1 ml · min^{-1}) equilibrated in buffer A with 100 mM NaCl. After elution of the unbound material (which contained OP-Tb and was retained for OP-Tb purification), a linear gradient of 0.1−1 M NaCl in buffer A was applied over five column volumes. Fractions active against Z-Phe-Arg-NHMec, but inactive against Z-Arg-Arg-NHMec and therefore identified as containing trypanopain-Tb, were pooled and dialysed against buffer A. Since preliminary studies had identified pepstatin A as a weak inhibitor of trypanopain-Tb, this inhibitor was considered to be a potential affinity chromatography ligand. Active samples were therefore loaded onto pepstatin-A−Sepharose (13×10 mm, flow rate 0.26 ml · min^{-1}) equilibrated in buffer A containing 500 mM NaCl. Following elution of the unbound material, trypanopain-Tb was eluted with 5% (mass/vol.) Brij-35 in buffer A with 500 mM NaCl. Fractions active against Z-Phe-Arg-NHMec were applied to HiTrap™ Blue Sepharose (9×23 mm, flow rate 1 ml · min^{-1}) equilibrated in 50 mM Tris/HCl pH 8.0 to remove suspected albumin contamination. Active fractions were concentrated and stored at −20°C mixed with glycerol (1:1). The purity of the enzyme was analysed by tricine/SDS/PAGE (Shägger and von Jagow, 1987).

The unbound material from HiLoad™ Q-Sepharose was active against Z-Arg-Arg-NHMec and Z-Phe-Arg-NHMec, but insensitive to L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64), indicating that it contained OP-Tb (Kornblatt et al., 1992). This fraction was dialysed against buffer B (50 mM Tris/HCl, 1 mM dithiothreitol, pH 8.0) and applied to *p*-aminobenzamidine−Sepharose (15×50 mm, flow rate 0.3 ml · min^{-1}). After the elution of unbound material, bound OP-Tb was eluted with a step gradient of 250 mM NaCl in buffer B. Following dialysis against buffer B, the sample was applied to HiLoad™ Q-Sepharose equilibrated in buffer B containing 100 mM NaCl, and a five-column-volume gradient of 0.1−1 M NaCl applied. The active fractions were diluted four-fold in buffer B and applied to poly-(L-lysine)−Sepharose (55×15 mm, flow rate 1 ml · min^{-1}) equilibrated in buffer B containing 100 mM NaCl. A 10-column-volume gradient of 0.1−1.5 M NaCl in buffer B was applied to elute the bound material. Active fractions were concentrated and applied in buffer B to a Sephacryl S-100 HR gel filtration column (15×850 mm, flow rate 10 ml · h^{-1}). Following analysis of enzyme purity by tricine/SDS/PAGE, the purified enzyme was concentrated and stored at −20°C mixed with glycerol (1:1).

Characterisation of enzymes. The activity of trypanopain-Tb and OP-Tb in the presence of various inhibitors was investigated by incubating the enzymes with each inhibitor for 15 min at 37°C before assaying residual activity against Z-Phe-Arg-NHMec or Z-Arg-Arg-NHMec, respectively, as described above. OP-Tb experiments were conducted in the presence of either 10 mM dithiothreitol (indicated as + dithiothreitol in Table 2) or in residual dithiothreitol remaining from purification (≈40 μM dithiothreitol, indicated as − dithiothreitol in Table 2). Since chloromethanes and alkylating agents are inactivated by reducing agents, care was taken to minimise the relative amount of reducing agents used with these inhibitors. Concentrated enzymes were thus activated (10 min at 37°C in 30 mM cysteine for trypanopain-Tb; 10 mM dithiothreitol for OP-Tb), before being diluted to the required working concentration in assay buffer containing inhibitor (final reducing agent concentrations of 3 mM cysteine for trypanopain-Tb, 1 mM dithiothreitol for OP-Tb).

The effects of various reducing agents on trypanopain-Tb and OP-Tb activity were investigated by adding dithiothreitol, cysteine · HCl, reduced glutathione or 2-mercaptoethanol (1−100 mM for trypanopain-Tb and 1−25 mM for OP-Tb) to the relevant assay buffer. The pH profile of each enzyme against synthetic substrates was investigated by substituting assay buffer with constant-ionic-strength acetate/Mes/Tris buffers (100 mM acetate, 200 mM Tris, 100 mM Mes, 4 mM Na_2EDTA) of pH 4.0−9.0 (Ellis and Morrison, 1982). Trypanopain-Tb hydrolysis of [^{14}C]gelatin at various pH values was assessed using acetate/Mes/Tris buffers as described above, with the [^{14}C]gelatin prepared using [1-^{14}C]acetic anhydride as described by Cawston and Barrett (1979). The pH stability of the enzymes was determined by incubating the enzymes in acetate/Mes/Tris buffers (25 mM acetate, 50 mM Tris, 25 mM Mes, 1 mM Na_2EDTA) for 1 h at 37°C before addition of a pH-7.0 assay buffer and Z-Phe-Arg-NHMec for trypanopain-Tb, or pH-8.0 assay buffer and Z-Arg-Arg-NHMec for OP-Tb. The initial incubation was done either in the presence or absence of reducing agents (30 mM cysteine for trypanopain-Tb and 10 mM dithiothreitol for OP-Tb).

Following determination of the concentration of active trypanopain-Tb by titration with E-64 (Barrett and Kirschke, 1981),

trypanopain-Tb (0.05 pmol) was incubated with readily hydrolysed synthetic substrates (25−100 μM) for 10 min at 37°C in continuous assays, or with poorly hydrolysed synthetic substrates for 4 h at 37°C in stopped time assays. K_m, V_{max} and k_{cat} were determined from the direct linear plot (Eisenthal and Cornish-Bowden, 1974) using the software package Hyper 1.01 (© 1992-1993, J. S. Easterby, UK).

Digestion of protein and peptide substrates by trypanopain-Tb and OP-Tb. Fibrinogen, BSA and rabbit IgG were digested with trypanopain-Tb at 37°C over 1 h at various molar ratios of enzyme/substrate in 100 mM Tris/HCl, 30 mM cysteine, pH 7.4. After the reactions were stopped by the addition of 1 mM E-64, samples were boiled in reducing treatment buffer and analysed by electrophoresis on 10% tricine gels (Shägger and von Jagow, 1987). Trypanopain-Tb digestion of serpins was analysed at 37°C at 1:100 and 1:10 molar ratios of enzyme/inhibitor in 25 mM Tris, 10 mM cysteine, pH 7.4. Reactions were stopped by the addition of 20 μM E-64. To determine the extent of serpin degradation, remaining inhibition of trypsin or chymotrypsin was determined (Potempa et al., 1986) and the degradation products analysed by tricine/SDS/PAGE.

OP-Tb was incubated with oligopeptide substrates at various molar ratios of enzyme/substrate in 0.1 M Tris/HCl, 10 mM dithiothreitol, pH 8.0 for 1−16 h at 37°C, after which the reaction was stopped by acidification with an equal volume of 5% (by vol.) trifluoroacetic acid. The samples were placed on ice until analysed by HPLC on a C18 column (flow rate 1 ml · min^{-1}) using a gradient of 10−90% (by vol.) acetonitrile in water containing 0.1% (by vol.) trifluoroacetic acid.

Association with cystatins and α_2*-M.* The kinetics of trypanopain-Tb inhibition by various cystatins were determined as described by Salvesen and Nagase (1992) with data treatment as described by Henderson (1972). Trypanopain-Tb and OP-Tb association with α_2-M was assessed by comparing the elution volumes from Sephacryl S-100 (for trypanopain-Tb) and Sephacryl S-200 (for OP-Tb) (both 15×300 mm, flow rate 10 cm · h^{-1}, equilibrated with 50 mM Tris/HCl pH 8.0 containing 1 mM dithiothreitol for OP-Tb only) of each enzyme alone with that for each enzyme previously incubated with bovine α_2-M (≈ 1:1 molar ratio, Mason, 1989).

Measurement of enzyme activity in the blood of infected rats. The blood of trypanosome-infected rats was harvested by cardiac puncture at peak parasitaemia, and centrifuged (3000×*g*, 25 min, 4°C). The supernatant was microscopically confirmed to be free of both parasites and blood cells, and then assayed against Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec in the presence of various inhibitors. Since OP-Tb, rat plasma kallikrein and trypanopain-Tb all hydrolyse Z-Phe-Arg-NHMec, soybean trypsin inhibitor (SBTI, 20 μM), $H_2NEtPhSO_2F$ (1.85 mM) and E-64 (1.7 mM) were used to discriminate between the activities of the three enzymes. SBTI inhibits blood plasma kallikrein (Coleman and Bagdasarian, 1976) but not OP-Tb, while $H_2NEtPhSO_2F$ inhibits both kallikrein and OP-Tb, so these inhibitors were used to discriminate between the two activities. Hydrolysis of Z-Arg-Arg-NHMec in the presence of these inhibitors was additionally investigated to confirm the presence of OP-Tb.

RESULTS

Proteinase isolation. As far as we are aware, this is the first time trypanopain-Tb and OP-Tb have been completely and demonstrably purified from *T. b. brucei* (Fig. 1). Three-phase partitioning proved to be a very efficient crude purification method, removing large quantities of extraneous trypanosomal proteins

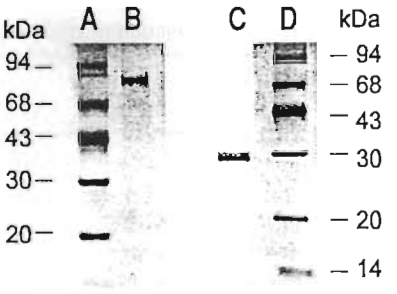

Fig. 1. Silver-stained tricine/SDS/PAGE of trypanopain-Tb and OP-Tb. OP-Tb (lane B, 250 ng) and trypanopain-Tb (lane C, 250 ng) were treated with non-reducing buffer and electrophoresed on a 10% tricine/SDS/PAGE gel prior to silver staining (Blum et al., 1987). Molecular mass markers, shown in lanes A and D, are phosphorylase *b* (94 kDa), BSA (68 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), SBTI (20 kDa) and α-lactalbumin (14.4 kDa).

(Table 1). Additionally, the salt remaining from this procedure reduced the amount of protein binding to HiLoad™ Q-Sepharose at pH 5.5, and thus increased the efficiency of this step for trypanopain-Tb purification. This initial chromatography step on HiLoad™ Q-Sepharose also separated OP-Tb from trypanopain-Tb (Fig. 2), taking advantage of the unusual affinity of trypanopain-Tb for this anion exchanger at pH 5.5 and making it possible for the two enzymes to be purified from the same trypanosome preparation. The current method is thus more economical and convenient than previously reported approaches.

Various affinity chromatography resins were tested for the purification of trypanopain-Tb. The high affinity with which inhibitors, such as stefin B and chicken egg white cystatin, bound trypanopain-Tb rendered these ligands unsuitable for affinity chromatography purposes. While pepstatin A typically inhibits aspartic proteinases, it surprisingly also inhibits trypanopain-Tb weakly (Pamer et al., 1989) and was thus considered a potentially suitable affinity chromatography ligand. Pepstatin-A−Sepharose chromatography proved to be a very successful purification step, with electrophoresis of the column eluate showing that trypanopain-Tb was contaminated only by a 68-kDa protein, suspected to be rat serum albumin. This contaminant was successfully removed by HiTrap™ Blue Sepharose. The speed of the trypanopain-Tb purification procedure was found to be critical for maintenance of enzyme activity, with activity irreversibly lost if more than one day was taken for purification.

Two affinity chromatography steps were used in the purification of OP-Tb, namely *p*-aminobenzamidine−Sepharose and poly-(L-lysine)−Sepharose chromatography. Since benzamidine is a fairly weak inhibitor of OP-Tb (62% inhibition, Table 2), OP-Tb could be eluted from this column using fairly low salt concentrations (250 mM NaCl). Poly-(L-lysine)-Sepharose, on the other hand, bound OP-Tb more strongly, with approximately 450 mM NaCl required to elute the enzyme. While *p*-aminobenzamidine−Sepharose was particularly effective in removing large quantities of contaminating protein (increasing the specific activity 30-fold, Table 1), poly-(L-lysine)−Sepharose was essential for removing persistent contamination by a 68-kDa protein, shown by amino acid analysis to be rat albumin, as well as other minor contaminants.

Trypanopain-Tb characterisation. Trypanopain-Tb activity against Z-Phe-Arg-NHMec was optimal across a plateau between pH 5.5 and 8.0 (Fig. 3). The enzyme was optimally stable between pH 4.0 and 7.0 in the presence of cysteine, and between pH 4.0 and 8.0 in the absence of cysteine. At all pH values tested, more trypanopain-Tb activity was maintained after a 1-h

Table 1. Purification table for the isolation of trypanopain-Tb and OP-Tb from *T. b. brucei*

Enzyme	Fraction	Total protein	Total activity	Specific activity	Purification	Yield
		mg	pmols · s^{-1}	pmol · s^{-1} · mg^{-1}	-fold	%
Trypanopain-Tb	Lysate	213	4562	22	1	100
	Three-phase partitioning	9	1144	127	6	25
	HiLoad™ Q-Sepharose	0.2	397	1 985	90	9
	Pepstatin-A−Sepharose	0.04	252	6 300	286	5.5
	HiTrap™ Blue Sepharose	0.01	245	24 500	1114	5.3
OP-Tb	Lysate	213	3245	15	1	100
	Three-phase partitioning	9	2044	227	15	63
	HiLoad™ Q-Sepharose (pH 5.5)	8.6	1947	226	15	60
	p-Aminobenzamidine−Sepharose	0.211	1429	6 773	452	44
	HiLoad™ Q-Sepharose (pH 8)	0.134	1428	10 657	710	44
	Poly(L-lysine)−Sepharose	0.104	941	9 048	603	29
	Sephacryl S-100	0.068	909	13 368	891	28

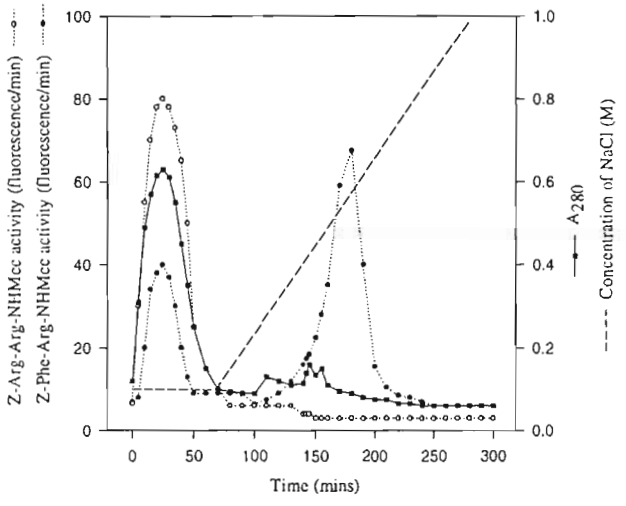

Fig. 2. Elution profile of HiLoad™ Q-Sepharose showing separation of trypanopain-Tb and OP-Tb. HiLoad™ Q-Sepharose (26×110 mm, flow rate 1 ml · min^{-1}) was equilibrated in loading buffer (100 mM NaCl in 20 mM sodium acetate, 1 mM Na_2EDTA, 100 mM NaCl, 0.02% (mass/vol.) NaN_3, pH 5.5). The 10–25% $(NH_4)_2SO_4$ fraction of the three-phase partitioning was loaded onto the column and the bound material eluted with a five-column-volume gradient of 0.1–1 M NaCl. While OP-Tb fractions (unbound) were active against both Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec, trypanopain-Tb-containing fractions (bound) were active against only Z-Phe-Arg-NHMec.

Table 2. Effects of various inhibitors on trypanopain-Tb activity against Z-Phe-Arg-NHMec and OP-Tb activity against Z-Arg-Arg-NHMec at 37°C. Enzymes were incubated with each inhibitor for 15 min at 37°C and then assayed as usual. OP-Tb experiments were conducted in the presence of either 10 mM dithiothreitol (indicated as + dithiothreitol) or in residual dithiothreitol remaining from purification (≈ 40 µM dithiothreitol, indicated as − dithiothreitol). Where different concentrations of inhibitor were used for OP-Tb and trypanopain-Tb, the concentrations for trypanopain-Tb are given first. IAA, iodoacetate; IAN, iodoacetamide; NEM, *N*-ethylmaleimide; nd, not determined.

Inhibitor	Concentration	Activity of		
		Trypanopain-Tb	OP-Tb activity	
			− dithiothreitol	+ dithiothreitol
	mM	% of control		
E-64	0.001/0.2	0	70	85
IAA	0.1/10	0	8	59
NEM	10	0	21	16
IAN	10	0	90	67
Oxidised glutathione	10	100	182	100
$H_2NEtPhSO_2F$	0.5	100	0	3
iPr_2P-F	10	100	14	9
$PhMeSO_2F$	10	100	100	95
$TosLysCH_2Cl$	1	0	5	5
$TosPheCH_2Cl$	1	0	78	89
Leupeptin	0.1	0	3	2
Antipain	0.1	0	8	8
Chymostatin	0.1	1	35	37
Benzamidine	10	nd	33	38
Aprotinin	0.03	nd	56	36
SBTI	0.01	100	100	100
EDTA	10	100	154	103
Pepstatin A	0.1	78	100	100

incubation in the absence of cysteine than in its presence, indicating that the active enzyme undergoes some autocatalysis. Trypanopain-Tb was active against [^{14}C]gelatin over a far narrower and more acidic pH range than against Z-Phe-Arg-NHMec, with hydrolysis being optimal at pH 5.7 (Fig. 3). While activity decreased sharply on either side of this pH, trypanopain-Tb was still 44% active at pH 7.4, suggesting that it would be active under physiological pH conditions.

Trypanopain-Tb was only active in the presence of reducing agents, thus displaying typical cysteine proteinase latency. Cysteine · HCl was the most effective of the reducing agents tested, with an optimal final concentration of 30 mM. Dithiothreitol was maximally effective at 20 mM, with 2-mercaptoethanol reaching optimum efficiency at much higher concentrations (250 mM). Reduced glutathione was a comparatively poor activator. All subsequent trypanopain-Tb assays against Z-Phe-Arg-NHMec were thus performed at pH 7.0 (in the middle of the optimal pH range) using 200 mM sodium phosphate containing 30 mM cysteine and 2 mM Na_2EDTA (final concentrations).

Typical cysteine proteinase inhibitors (including E-64 and iodoacetate) inhibited trypanopain-Tb completely, while typical serine and metalloproteinases inhibitors such as iPr_2P-F and EDTA, respectively, had no effect on enzyme activity (Table 2). Leupeptin, antipain and chymostatin were all effective inhibitors of the enzyme (99–100% inhibition). Interestingly, pepstatin A (22% inhibition) also inhibited trypanopain-Tb.

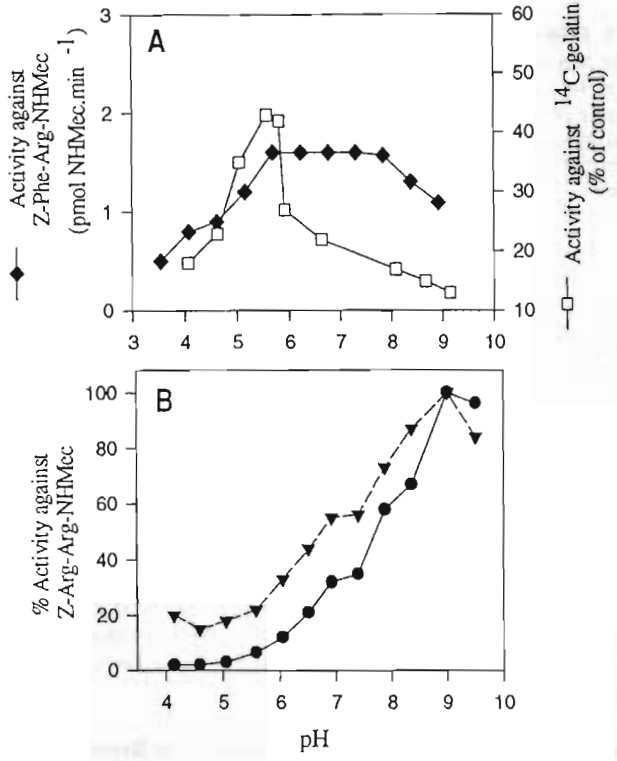

Fig. 3. pH profile of trypanopain-Tb and OP-Tb activity at 37°C. Enzyme activity at various pH values was assessed using constant-ionic-strength acetate/Mes/Tris buffers (Ellis and Morrison, 1982) of pH 4.0–9.0. (A) Trypanopain-Tb (1.5 ng) activity against 5 μM Z-Phe-Arg-NHMec (–◆–) and [^{14}C]-gelatin (–□–); (B) OP-Tb (2 ng) activity against 5 μM Z-Arg-Arg-NHMec in the presence (●) or absence (▼) of 10 mM dithiothreitol.

Table 3. Kinetic constants for trypanopain-Tb hydrolysis of various NHMec substrates. Values are calculated from 10-min or 4-h stopped-time assays. Constants were calculated at 37°C using the direct linear plot (Eisenthal and Cornish-Bowden, 1974). No hydrolysis occurred in 4 h with Suc-Ala-Ala-Phe-NHMec, Glu-Gly-Gly-Phe-NHMec, Z-Gly-Pro-NHMec, Gly-NHMec, Leu-NHMec, Ac-Ala-Ala-Tyr-NHMec, Ac-Ala-Ala-Pro-Ala-NHMec, Z-Pro-Arg-NHMec, Glu-Gly-Arg-NHMec, Z-Gly-Gly-Arg-NHMec, Boc-Val-Gly-Arg-NHMec, Boc-Leu-Gly-Arg-NHMec, Boc-Ile-Glu-Gly-Arg-NHMec, Z-Arg-NHMec, Z-Ala-Arg-Arg-NHMec or Arg-NHMec.

Substrate	Time	K_m	k_{cat}	k_{cat}/K_m
		μM	s^{-1}	s^{-1} μM^{-1}
Z-Arg-Arg-NHMec	4 h	12	0.126	0.01
Z-Phe-Arg-NHMec	10 min	1.2	13.4	11.3
Pro-Phe-Arg-NHMec	10 min	4.2	4.2	1
Val-Leu-Lys-NHMec	10 min	25	8.2	0.3
Ala-Leu-Lys-NHMec	10 min	27	6.5	0.24
Suc-Ala-Phe-Lys-NHMec	10 min	42	0.006	0.002
Ala-Phe-Lys-NHMec	10 min	28	5.2	0.19
Suc-Leu-Tyr-NHMec	4 h	54	0.05	0.001
MeOSuc-Asp-Tyr-Met-NHMec	4 h	68	0.09	0.001
MeOSuc-Gly-Trp-Met-NHMec	4 h	4	0.04	0.011

Trypanopain-Tb degradation of synthetic substrates and proteins. Of the synthetic substrates tested, Z-Phe-Arg-NHMec was most readily cleaved by trypanopain-Tb, with a K_m of 1.2 μM and k_{cat} of 13.4 s^{-1} (Table 3). Pro-Phe-Arg-NHMec was also readily hydrolysed. All other substrates tested, including Z-Arg-Arg-NHMec used to monitor OP-Tb isolation, were comparatively poorly hydrolysed.

Trypanopain-Tb hydrolysed fibrinogen, BSA and rabbit IgG at physiological pH. At a 1:100 molar ratio of enzyme/substrate, both the α and β chains of fibrinogen were substantially degraded within 15 min at pH 7.4, while the γ chain remained resistant over the test period (1 h). However, at a 1:1000 molar ratio of enzyme/substrate, no degradation occurred. Trypanopain-Tb also readily degraded BSA (1:500 and 1:1000 molar ratio) and IgG (1:100 and 1:1 000 molar ratio) within 15 min, hydrolysing the latter predominantly in the heavy-chain region.

Characterisation of OP-Tb. OP-Tb activity was substantially increased by the presence of reducing agents; dithiothreitol was the most effective, increasing activity 10-fold at 25 mM and 6-fold at 1 mM. Cysteine was almost as effective as dithiothreitol at lower concentrations, but activity was lowered at concentrations above 5 mM. Glutathione was about half as effective as dithiothreitol.

Irrespective of the presence or absence of reducing agents, OP-Tb was found to be most active between pH 8 and 9.5 at 25°C (Fig. 3). In contrast, the pH stability of the purified enzyme was slightly affected by the presence of a reducing agent, with the enzyme being most stable in the absence of dithiothreitol in the pH range 6–7.5 and in the presence of dithiothreitol at pH 8–9. These differences, however, were all ranged between 80–100% activity and it was only below pH 6 and above pH 9 that stability decreased significantly.

The serine proteinase inhibitors iPr_2P-F and $H_2NEtPhSO_2F$ completely inhibited OP-Tb, supporting the hypothesis that OP-Tb is a serine protease (Table 2). Leupeptin and antipain were very effective inhibitors of OP-Tb, as was tosyllysylchloromethane (TosLysCH_2Cl). However, a basic residue was required for the chloromethane to be active, as shown by the lack of inhibition tosylphenylalanylchloromethane (TosPheCH_2Cl). The enzyme was not inhibited by phenylmethylsulfonyl fluoride ($PhMeSO_2F$) or Z-Phe-Phe-CHN_2. Benzamidine was found to be a weak inhibitor of the enzyme, possibly explaining the success of *p*-aminobenzamidine–Sepharose affinity chromatography as a step in the isolation of OP-Tb. EDTA increased OP-Tb activity, indicating that the enzyme is not stabilised by divalent cations. While E-64 had no effect on activity, iodoacetate, iodoacetamide and *N*-ethylmaleimide, which modify cysteine residues, all reduced OP-Tb activity to various degrees (Table 2).

Cleavage of protein and peptide substrates by OP-Tb. OP-Tb did not hydrolyse fibrinogen, BSA, ovalbumin, carbonic anhydrase, SBTI, lysozyme, cystatin C or cytochrome *c* at pH 8.0. Therefore, as speculated by Kornblatt et al. (1992), the enzyme appears to be an oligopeptidase rather than a proteinase. We thus examined a range of substrates of increasing molecular mass in order to characterise the minimum size of substrates excluded from the active site of the enzyme. Since OP-Tb cleaves most efficiently after arginine residues (Kornblatt et al., 1992), only peptides containing such residues were tested. Where possible, peptides containing two consecutive basic residues were chosen to increase the likelihood of hydrolysis. Additionally, peptides with regulatory roles in the host system were chosen in order to shed light on the potential physiological relevance of OP-Tb.

OP-Tb was found to completely cleave reduced [Arg^8]vasopressin (1084.2 Da, CYFQNCPRG) and [Lys^8]vasopressin (1056.2 Da; CYFQNCPKG; 1:100 molar ratios) in 4 h, while the oxidised forms of these peptides remained uncleaved after 16 h. Neurotensin (1672.9 Da, ELYENKPRRPYIL) was also completely hydrolysed in 4 h at a 1:500 molar ratio of enzyme/

Table 4. Equilibrium constant for inhibition, association and dissociation rate constants for cystatin inhibition of trypanopain-Tb (0.05 nM) at 30C. Note that the associations of cystatin C and L-kininogen domain 3 with trypanopain-Tb was too rapid for accurate measurement and the value given is an estimate.

Inhibitor	K_i	k_{on}	Predicted k_{off}
	nM	$M^{-1} \cdot s^{-1}$	s^{-1}
Human stefin A	0.045	2.13×10^7	9.6×10^{-4}
Sheep stefin B	0.004	6.9×10^7	2.8×10^{-4}
Human cystatin C	0.001	$\approx 1.7 \times 10^8$	1.7×10^{-4}
Human L-kininogen	0.0035	2.5×10^7	8.8×10^{-5}
Human L-kininogen domain 3	0.0044	$\approx 1.1 \times 10^8$	4.8×10^{-4}

Table 5. Effect of inhibitors on Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec activity in the blood of rats infected with *T. b. brucei*. The blood of infected rats was assayed against synthetic substrates in the absence or presence of various inhibitors for 30 min at 37°C. Values are in arbitrary units.

Substrate	Rats	Total activity with			
		no inhibitors	E-64 (1.67 mM)	SBTI (20 μM)	$H_2NEtPhSO_2F$ (1.85 mM)
Z-Phe-Arg-NHMec	infected	1521	1585	1573	297
	uninfected	234	177	193	193
Z-Arg-Arg-NHMec	infected	2244	2372	2284	97
	uninfected	104	97	74	55

peptide. The point of cleavage in this instance was determined to be after the second arginine residue in the sequence, reinforcing the specificity of OP-Tb for two consecutive basic residues in a given sequence. Atrial natriuretic factor (3080.5 Da, SLRRSSCFGGRMDRIGAQSGLGCNSFRY) was effectively cleaved at a 1:100 molar ratio, with the reduced form of the peptide undergoing more cleavage than the oxidised form. The importance of the state of substrate reduction suggests that substrate conformation is critical for OP-Tb activity. Peptides such as angiotensin I (1296.5 Da, DRVYIHPFHL), substance P (1347.6 Da, RPKPQQFFGLM) and oxidised insulin B chain (3495.9 Da, FVNQHLCGSHLVEALYLVCGERGFFYTPKA; 1:100 molar ratios) were not cleaved after 16 h, possibly due to the particular amino acid residues surrounding the basic residues present in these peptides (Kornblatt et al., 1992), although it is possible that a size restriction was the reason for non-cleavage of the insulin B chain. Pancreatic polypeptide (4181.7 Da) and growth-hormone-releasing factor (5039.7 Da) were also not cleaved, possibly due to the sizes of these peptides. OP-Tb thus does not appear to cleave peptides larger than 4 kDa. Further investigation of peptides between 3–4 kDa which contain suitable consecutive basic residues is needed to clarify the exclusion limit more finely.

Interaction of trypanopain-Tb and OP-Tb with host proteinase inhibitors. k_{on} and predicted k_{off} values for trypanopain-Tb association with various cystatins were in the $10^7-10^8\ M^{-1} \cdot s^{-1}$ and $10^{-4}\ s^{-1}$ range, respectively, while K_i values were in the 10^{-11} M range (Table 4). Cystatin C and L-kininogen were the most effective inhibitors. Interestingly, very little difference was observed between the inhibition by whole L-kininogen and by domain 3 of the molecule, suggesting that this domain is likely to be predominantly responsible for the inhibition observed with the whole molecule. Assuming a plasma concentration of 7.5 μM for L-kininogen and 80 nM for cystatin C (Abrahamson, 1993), trypanopain-Tb would be expected to associate very rapidly with these inhibitors: the half-life (Bieth, 1980) of the enzyme in the presence of L-kininogen can be calculated as 0.006 s and with cystatin C, 0.08 s. The inhibitor-enzyme complexes can also be predicted to dissociate slowly, with a half-life of 131 min for L-kininogen and 67 min for cystatin C. Trypanopain-Tb is thus likely to be inhibited effectively by cystatin C and (especially) L-kininogen *in vivo*. Additionally, trypanopain-Tb bound to α_2-M ($\approx$ 1:1 molar ratio) and is thus also likely to be effectively controlled by this inhibitor *in vivo*.

Trypanopain-Tb was found to cleave the serpin inhibitor antithrombin III, but only at enzyme/inhibitor ratios of 1:10, so it is unlikely that such hydrolysis would occur *in vivo*. Trypanopain-Tb did not cleave other serpins, such as α_1-proteinase inhibitor, α_1-antichymotrypsin and α_2-antiplasmin.

OP-Tb did not interact with any of the tested host system inhibitors, including serpins, cystatins and α_2-M, presumably due to the limited interaction of the active site with higher-molecular-mass proteins in general.

Measurement of enzyme activity in infected rat blood. High levels of activity against both Z-Phe-Arg-NHMec and Z-Arg-Arg-NHMec were detected in the blood of infected rats, while little activity was detectable in the blood of control, uninfected animals (Table 5). This activity was insensitive to E-64 and SBTI, but entirely eliminated by $H_2NEtPhSO_2F$. OP-Tb is thus likely to be predominantly responsible for the hydrolysis observed, suggesting that OP-Tb may be released into the bloodstream of infected rats. Secondly, this study supports the conclusion arrived at from *in vitro* studies with the purified enzymes, namely that while trypanopain-Tb is likely to be effectively controlled in the bloodstream, OP-Tb seems to remain active.

DISCUSSION

Isolation of trypanopain-Tb and OP-Tb. A cysteine proteinase has previously been isolated from *T. congolense* (Mbawa et al., 1992) but we have now purified one from *T. b. brucei*. OP-Tb from *T. b. brucei* has been partially purified and characterised (Kornblatt et al., 1992), but here we report the purification of the enzyme to homogeneity as well as a more complete characterisation of certain aspects of its activity. The linked isolation of these proteinases as reported here has the added advantage of maximising the yield of both enzymes from a single parasite preparation.

Characterisation of trypanopain-Tb. Trypanopain-Tb was shown to be a fairly typical cysteine proteinase, similar to trypanopain-Tc from *T. congolense* (Mbawa et al., 1992) and cruzipain from *T. cruzi* (Cazzulo et al., 1989). Interestingly, the aspartic proteinase inhibitor pepstatin A inhibits trypanopain-Tb slightly (Table 2; Pamer et al., 1989). In contrast, pepstatin A inhibits trypanopain-Tc by only 9.7% (Mbawa et al., 1992) and does not affect cruzipain (Murta et al., 1990; Cazzulo et al., 1989). Pepstatin A appears to be a competitive inhibitor of trypanopain-Tb (data not shown), and may act as a substrate analogue. This unusual inhibition was successfully exploited in the purification of trypanopain-Tb, using pepstatin-A–Sepharose.

Trypanopain-Tb was only active in the presence of reducing agents, and appeared to be quite similar to cathepsin L (Dennison et al., 1992) in its requirement of fairly high concentra-

tions of reducing agent for optimal activity. It was shown to have cathepsin-L-like specificity for synthetic substrates, hydrolysing Z-Phe-Arg-NHMec and Pro-Phe-Arg-NHMec most rapidly of the substrates tested. Generally, only substrates with basic residues such as Arg or Lys in P_1 and hydrophobic residues such as Phe or Leu in P_2 were hydrolysed (Table 3). The presence of an acidic residue in P_2 appeared to be unacceptable. Similar specificity has been reported for trypanopain-Tc (Mbawa et al., 1992), cruzipain (Cazzulo et al., 1990) and mammalian cathepsin L (Barrett and Kirschke, 1981; Dufour and Ribadeau-Dumas, 1988). Minimal hydrolysis of Z-Arg-Arg-NHMec was observed in the current study (4-h incubation required to demonstrate hydrolysis), allowing this substrate to be used for monitoring OP-Tb isolation. This contradicts a previous report by Pamer et al. (1991) that trypanopain-Tb cleaves Z-Arg-Arg-NHMec readily, with a K_m of 3.2 μM, although it must be noted that only partially purified trypanopain-Tb was used in their study. Trypanopain-Tc does not hydrolyse Z-Arg-Arg-NHMec (Mbawa et al., 1992), while cruzipain cleaves it poorly (K_m 41 μM; Lima et al., 1992).

Of the serpins tested, only antithrombin III was cleaved by trypanopain-Tb. Interestingly, cathepsin L, which has been found to be one of the closest mammalian analogues to trypanopain-Tb (Mottram et al., 1989), cleaves α_1-proteinase inhibitor very effectively (Johnson et al., 1986). Cathepsin L also cleaved antithrombin III and α_2-antiplasmin very effectively at much lower enzyme/substrate ratios than did trypanopain-Tb (data not shown). This indicates that these two enzymes, while very similar in substrate specificity, are not always comparable.

Effect of host proteinase inhibitors on trypanopain-Tb. It has been postulated that if released into the host bloodstream, trypanopain-Tb could contribute to pathogenesis by degrading various host proteins. For this to occur, the enzyme would have to remain active in the presence of host proteinase inhibitors such as cystatins, kininogens and α_2-M. Trypanopain-Tb is, however, effectively inhibited by kininogens, various cystatins and α_2-M *in vitro* (Table 4). The physiological concentrations of cystatin C and L-kininogen are more than 10 times K_i, so these inhibitors are likely to inhibit any trypanopain-Tb secreted or released by trypanosomes *in vivo* (Bieth, 1980). Interestingly, the most abundant cysteine proteinase inhibitors in blood are also the most effective *in vitro* inhibitors of the purified enzyme. Since cystatins are relatively conserved between species (Barrett et al., 1986) and the trypanosomal cysteine proteinases sequenced to date are highly similar to each other (Mottram et al., 1989; Pamer et al., 1990; Eakin et al., 1992; Fish et al., 1995), this *in vitro* approach implies that trypanopains from various trypanosome species are likely to be effectively inhibited by cystatins in the bloodstream of a variety of mammalian hosts. This conclusion is supported by Nwagwu et al. (1988), who showed that addition of rat serum to isolated parasites inhibited a trypanopain-Tb-like activity. Additionally, the Z-Phe-Arg-NHMec activity detected in the blood of infected rats in the present study was not E-64-inhibitable (Table 5), suggesting that there are not significant concentrations of active trypanopain-Tb in the blood of infected animals. Thus while trypanopain-Tb readily degrades IgG and BSA *in vitro*, it appears that this is unlikely to occur in the host bloodstream.

In apparent conflict with this conclusion, Lonsdale-Eccles et al. (1995) reported that a kininogen-like molecule from rat serum enhances trypanopain-Tb activity in fibrinogen zymograms of *T. b. brucei* lysates. This molecule (called rat trypanopain modulator, rTM) cross-reacts to some extent with anti-(human kininogen) antibodies and has many other features in common with kininogens, such as its size (68 kDa), its acid and heat stability, and the presence of 15-kDa proteinase-sensitive domains. Additionally, L-kininogen was shown to enhance trypanopain activity on fibrinogen zymograms in a similar manner to rTM. The current study, however, clearly indicates that purified human L-kininogen is an effective inhibitor of purified trypanopain-Tb with a K_i of 0.0035 nM. Pike et al. (1992) reported a similar apparent contradiction in the interaction between a cysteine proteinase and its putative inhibitor. While purified stefin B inhibited purified cathepsin L as expected, active covalent complexes of the enzyme and inhibitor were shown to form under certain circumstances. An unidentified factor, which may be absent *in vitro* studies using purified components, may contribute to the formation of these active complexes *in vivo*. It is possible that this putative factor modifies the interaction between trypanopain-Tb and L-kininogen *in vivo*, resulting in the formation of active complexes as reported by Lonsdale-Eccles et al. (1995). Such an interaction may therefore interfere with effective control of trypanopain in the host bloodstream.

Effect of reducing agents and cysteine proteinase inhibitors on OP-Tb. Because OP-Tb is inhibited by iPr_2P-F and $H_2NEtPhSO_2F$, it is likely to be a serine protease. However, the enzyme is not inhibited by some other serine protease inhibitors, namely $PhMeSO_2F$ and SBTI. The lack of inhibition by $PhMeSO_2F$ is not entirely unexpected in view of the subsite specificity of the enzyme as discussed by Kornblatt et al. (1992). SBTI (20.1 kDa), on the other hand, is probably ineffective because it is too large to gain complete access to the active site of the enzyme.

Additionally, previous work on this enzyme (Kornblatt et al., 1992) and a similar enzyme from *T. cruzi* (Ashall, 1990; Ashall et al., 1990) indicated that some compounds which are generally considered to be cysteine protease inhibitors reduce OP-Tb activity. Here we have been able to delineate more clearly the effect of various cysteine protease inhibitors on this enzyme. While OP-Tb is unaffected by the common cysteine protease inhibitor E-64, the enzyme is inactivated by compounds such as iodoacetate, iodoacetamide and *N*-ethylmaleimide, that covalently modify cysteine residues. It is interesting to note that these three compounds act in rather contrasting manners, depending on the presence or absence of dithiothreitol, possibly reflecting the particular micro-environment of the cysteine residue involved (Table 2). This data, together with the fact that OP-Tb is activated by reducing agents, suggests that the enzyme contains a cysteine residue which must be reduced and available for maximum activity and thus involved in the control of OP-Tb activity. This may provide an alternative means of controlling this enzyme *in vivo*, which would be of particular importance in light of the insensitivity of OP-Tb to more typical means of control by host inhibitors, including serpins and α_2-M.

Hydrolysis of peptides by OP-Tb. OP-Tb successfully cleaved reduced [Arg^8]vasopressin, [Lys^8]vasopressin, neurotensin and atrial natriuretic factor, but was found to be inactive against substrates of more than 4 kDa. The enzyme is likely to cleave only small peptides *in vivo*. The fact that atrial natriuretic factor, [Arg^8]vasopressin and [Lys^8]vasopressin were more readily cleaved when reduced suggests that access to the OP-Tb active site is probably dependent on conformation as well as size. A similar enzyme from *T. cruzi* was found to be vital for the infectivity of this organism, since it cleaves and activates a cytoplasmic factor involved in a calcium signalling mechanism which mediates the entry of trypanosomes into cells (Burleigh and Andrews, 1995). While a similar function for OP-Tb is unlikely since African trypanosomes are not intracellular parasites.

OP-Tb may play a role in the activation of other hormone-like peptides in *T. b. brucei*.

OP-Tb was not inhibited by any mammalian protein proteinase inhibitor used in this study. In view of the restricted access to the active site of the proteinase, this is not entirely unexpected. Upon release of this enzyme from the parasite, as we have shown is likely to occur during infections, the enzyme will thus be free to act upon small regulatory molecules in its immediate environment. The demonstration here that the enzyme is able to cleave native neurotensin and atrial natriuretic factor, which have diverse roles in the complex mechanisms of control over bodily functions, means that this enzyme has the potential to seriously disrupt control mechanisms of the host system. Symptoms of sleeping sickness are difficult to define in general terms, but altered blood volume, and eventual disruption of neurological systems, is not inconsistent with the general symptoms found (Tizard et al., 1978); thus inappropriate cleavage of regulatory molecules by OP-Tb may have profound implications for the host. It will be of interest to determine the levels of various hormones in hosts infected by *T. b. brucei* in order to investigate this possibility more directly.

In conclusion, this study suggests that OP-Tb is more likely than trypanopain-Tb to have an extracellular, pathological role in African trypanosomiasis, making OP-Tb an attractive target for future studies in drug design.

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REFERENCES

Abrahamson, M. (1993) Cystatins-protein inhibitors of papain-like cysteine proteinases, *Cienc. Cult. Soc. Bras. Progr. Cienc. 445*, 299–304.

Ashall, F. (1990) Characterisation of an alkaline peptidase of *Trypanosoma cruzi* and other trypanosomatids, *Mol. Biochem. Parasitol. 38*, 77–88.

Ashall, F., Harris, D., Roberts, H., Healy, N. & Shaw, E. (1990) Substrate specificity and inhibitor sensitivity of a trypanosomatid alkaline peptidase, *Biochim. Biophys. Acta 1035*, 293–299.

Authié, E., Duvallet, G., Robertson, C. & Williams, D. J. L. (1993) Antibody response to a 33 kDa cysteine protease of *Trypanosoma congolense*: relationship to 'trypanotolerance' in cattle, *Parasite Immunol. 15*, 465-474.

Barrett, A. J. & Kirschke, H. (1981) Cathepsin B, cathepsin H and cathepsin L, *Methods Enzymol. 80C*, 535–561.

Barrett, A. J., Rawlings, N. D., Davies, M. E., Machleidt, W., Salvesen, G. & Turk, V. (1986) Cysteine proteinase inhibitors of the cystatin superfamily, in *Proteinase inhibitors* (Barrett, A. J. & Salvesen, G., eds) pp. 515–569, Elsevier, Amsterdam.

Bieth, J. G. (1980) Pathophysiological interpretation of kinetic constants of protease inhibitors, *Bull. Eur. Physiopathol. Respir. 16*, 183–195.

Blum, H., Beier, H. & Gross, H. J. (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels, *Electrophoresis 8*, 93–99.

Burleigh, B. A. & Andrews, N. W. (1995) A 120-kDa alkaline peptidase from *Trypanosoma cruzi* is involved in the generation of a novel Ca^{2+}-signalling factor for mammalian cells, *J. Biol. Chem. 270*, 5172–5180.

Cawston, T. E. & Barrett, A. J. (1979) A rapid and reproducible assay for collagenase using [1-^{14}C]acetylated collagen, *Anal. Biochem. 99*, 340–345.

Cazzulo, J. J., Couso, R., Raimondi, A., Wernstedt, C. & Hellman, U. (1989) Further characterization and partial amino acid sequence of a cysteine proteinase from *Trypanosoma cruzi*, *Mol. Biochem. Parasitol. 33*, 33–42.

Cazzulo, J. J., Cazzulo Franke, M. C., Martínez, J. & Franke de Cazzulo, B. M. (1990) Some kinetic properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*, *Biochim. Biophys. Acta 1037*, 186–191.

Coleman, R. W. & Bagdasarian, A. (1976) Human kallikrein and prekallikrein, in *Methods Enzymol. 45B*, 303–322.

Dennison, C., Pike, R., Coetzer, T. & Kirk, K. (1992) Characterisation of the activity and stability of single-chain cathepsin L and of proteolytically active cathepsin L/cystatin complexes, *Biol. Chem. Hoppe-Seyler 373*, 419–425.

Dufour, E. & Ribadeau-Dumas, B. (1988) Proteolytic specificity of chicken cathepsin L on bovine β-casein, *Biosci. Rep. 8*, 185–191.

Eakin, A. E., Mills, A. A., Harth, G., McKerrow, J. H. & Craik, C. S. (1992) The sequence, organisation and expression of the major cysteine protease (cruzipain) from *Trypanosoma cruzi*, *J. Biol. Chem. 267*, 7411–7420.

Eisenthal, R. & Cornish-Bowden, A. (1974) The direct linear plot. A new graphical procedure for estimating enzyme kinetic parameters, *Biochem. J. 139*, 715–720.

Ellis, K. J. & Morrison, J. F. (1982) Buffers of constant ionic strength for studying pH-dependent processes, *Methods Enzymol. 87C*, 405–426.

Fish, W. R., Nkhungulu, Z. M., Muriuki, C. W., Ndegwa, D. M., Lonsdale-Eccles, J. D. & Steyaert, J. (1995) The cDNA and deduced amino acid sequence of a cysteine protease from *Trypanosoma (Nannomonas) congolense* metacyclic forms, *Gene 161*, 125–128.

Gounaris, A. D., Brown, M. A. & Barrett, A. J. (1984) Human plasma α-cysteine proteinase inhibitor, *Biochem. J. 221*, 445–452.

Grab, D. J. & Bwayo, J. J. (1982) Isopycnic isolation of African trypanosomes on Percoll gradients formed *in situ*, *Acta Trop. 39*, 363–366.

Henderson, P. J. F. (1972) A linear equation that describes the steady-state kinetics of enzymes and subcellular particles interacting with tightly bound inhibitors, *Biochem. J. 102*, 193–202.

Huet, G., Richet, C., Demeyer, D., Bisiau, H., Soudan, B., Tetaert, D., Ki Han, K. & Degand, P. (1992) Characterisation of different proteolytic activities in *Trypanosoma brucei brucei*, *Biochim. Biophys. Acta 1138*, 213–221.

Johnson, D. A., Barrett, A. J. & Mason, R. W. (1986) Cathepsin L inactivates -proteinase inhibitor by cleavage in the active site region, *J. Biol. Chem. 261*, 14748–14751.

Kornblatt, M. J., Mpimbaza, G. W. N. & Lonsdale-Eccles, J. D. (1992) Characterisation of an endopeptidase of *Trypanosoma brucei brucei*, *Arch. Biochem. Biophys. 293*, 25–31.

Lanham, S. M. & Godfrey, D. G (1970) Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose, *Exp. Parasitol. 25*, 521–534.

Lima, A. P. C. A., Scharfstein, J., Storer, A. C. & Ménard, R. (1992) Temperature-dependent substrate inhibition of the cysteine proteinase (GP57/51) from *Trypanosoma cruzi*, *Mol. Biochem. Parasitol. 56*, 335–338.

Lonsdale-Eccles, J. D., Mpimbaza, G. W. N., Nkhungulu, Z. R. M., Olobo, J., Smith, L., Tosomba, O. M. & Grab, D. J. (1995) Trypanosomatid cysteine protease activity may be enhanced by a kininogen-like moeity from host serum, *Biochem. J. 305*, 549–556.

Mason, R. W. (1989) Interaction of lysosomal cysteine proteinases with α_2-macroglobulin: conclusive evidence for the endopeptidase activities of cathepsins B and H, *Arch. Biochem. Biophys. 273*, 367–374.

Mbawa, Z. R., Webster, P. & Lonsdale-Eccles, J. D. (1991) Immunolocalisation of a cysteine protease within the lysosomal system of *Trypanosoma congolense*, *Eur. J. Cell Biol. 56*, 243–250.

Mbawa, Z. R., Gumm, I. D., Shaw, E. & Lonsdale-Eccles, J. D. (1992) Characterisation of a cysteine protease from bloodstream forms of *Trypanosoma congolense*, *Eur. J. Biochem. 204*, 371–379.

Mottram, J. C., North, M. J., Barry, J. D. & Coombs, G. H. (1989) A cysteine protease cDNA from *Trypanosoma brucei* predicts an enzyme with an unusual C-terminal extension, *FEBS Lett. 258*, 211–215.

Murta, A. C. M., Persechini, P. M., de Souto Padron, T., de Souza, W., Guimarães, J. A. & Scharfstein, J. (1990) Structural and functional identification of GP57/51 antigen of *Trypanosoma cruzi* as a cysteine proteinase, *Mol. Biochem. Parasitol. 43*, 27–38.

Nwagwu, M., Okenu, D. M. N., Olusi, T. A. & Molokwu, R. I. (1988) *Trypanosoma brucei* releases proteases extracellularly, *Trans. R. Soc. Trop. Med. Hyg. 82*, 577.

Pamer, E. G., So, M. & Davis, C. E. (1989) Identification of a developmentally regulated cysteine protease of *Trypanosoma brucei*. *Mol. Biochem. Parasitol. 33*, 27–32.

Pamer, E. G., Davis, C. E., Eakin, A. & So, M. (1990) Cloning and sequencing of the cysteine protease cDNA from *Trypanosoma brucei rhodesiense*, *Nucleic Acids Res. 18*, 6164.

Pamer, E. G., Davis, C. E. & So, M. (1991) Expression and deletion analysis of the *Trypanosoma brucei rhodesiense* cysteine proteinase in *Escherichia coli*, *Infect. Immun. 59*, 1074–1078.

Pike, R. N. & Dennison, C. (1989) Protein fractionation by three-phase partitioning (TPP) in aqueous/*t*-butanol mixtures, *Biotech. Bioeng. 33*, 221–228.

Pike, R. N., Coetzer, T. H. T. & Dennison, C. (1992) Proteolytically active complexes of cathepsin L and a cysteine proteinase inhibitor: purification and demonstration of their formation *in vitro*, *Arch. Biochem. Biophys. 294*, 623–629.

Potempa, J., Watorek, W. & Travis, J. (1986) The inactivation of human plasma alpha-1-proteinase inhibitor by proteinases from *Staphylococcus aureus*, *J. Biol. Chem. 261*, 14330–14334.

Read, S. M. & Northcote, D. H. (1981) Minimization of variation in the response to different proteins of the Coomassie Blue dye-binding assay for protein, *Anal. Biochem. 116*, 53–64.

Russo, D. C. W., Williams, D. J. L. & Grab, D. J. (1994) Directional movement of variable surface glycoprotein-antibody complexes in *Trypanosoma brucei*, *Parasitol. Res. 80*, 487–492.

Salvesen, G. & Nagase, H. (1992) Inhibition of proteolytic enzymes. in *Proteolytic enzymes: a practical approach* (Benyon, R. J. & Bond, J. S., eds) pp. 83–104, IRL Press, Oxford.

Shägger, H. & von Jagow, G. (1987) Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa, *Anal. Biochem. 166*, 368–379.

Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J. & Klenk, D. C. (1985) Measurement of protein using bicinchoninic acid, *Anal. Biochem. 150*, 76–85.

Tizard, I., Nielsen, K. H., Seed, J. R. & Hall, J. E. (1978) Biologically active products from African trypanosomes. *Microbiol. Rev. 42*, 661–681.

Walgate, R. (1994) Sleeping sickness on the boil in Zaire, *TDR News 46*, 6.

Please note: I legally changed my surname from Smith to Troeberg in 1996.

Trypanosomatid cysteine protease activity may be enhanced by a kininogen-like moiety from host serum

John D. LONSDALE-ECCLES,*‡ George W. N. MPIMBAZA,*†§ Zeres R. M. NKHUNGULU,†‖ Joseph OLOBO,¶ Linda SMITH,* Omalokoho M. TOSOMBA*† and Dennis J. GRAB†**

*Department of Biochemistry, University of Natal, P.O. Box 375, Pietermaritzburg 3200, South Africa,
†International Laboratory for Research on Animal Diseases, P.O. Box 30709, Nairobi, Kenya, and ¶Institute of Primate Research, P.O. Box 24481, Karen, Kenya

African trypanosomes contain cysteine proteases (trypanopains) the activity of which can be measured by *in vitro* digestion of fibrinogen, after electrophoresis in fibrinogen-containing SDS/polyacrylamide gels. When assessed by this procedure, trypanopain from *Trypanosoma brucei* (trypanopain-Tb) is estimated to have a molecular mass of 28 kDa. However, two additional bands of trypanopain activity (87 kDa and 105 kDa) are observed if serum is added to the trypanopain before electrophoresis. Formation of the 87 and 105 kDa bands is frequently accompanied by a reduction in the intensity of the 28 kDa activity which suggests that the extra bands are complexes of the 28 kDa trypanopain-Tb and a molecule from rat serum called rat trypanopain moledulator (rTM). The rTM-induced activation of cysteine proteases is not restricted to *T. brucei* as it is also observed with proteases from other protozoan parasites such as bloodstream forms of *Trypanosoma congolense* and the mammalian-infective *in vitro*-derived promastigote forms of *Leishmania donovani* and *Leishmania major*. The physical properties of rTM resemble those of the kininogen family of cysteine protease inhibitors. rTM is an acidic (pI 4.7) heat-stable 68 kDa glycoprotein with 15 kDa protease-susceptible domains. This resemblance between rTM and kininogens was confirmed by the positive, albeit weak, immunoreactivity between anti-(human low-molecular-mass kininogen) antibody and rTM as well as anti-rTM antibody and human low-molecular-mass kininogen. Furthermore, commercial preparations of human-low-molecular-mass kininogen and chicken egg white cystatin mimicked rTM by forming extra bands of proteolytic activity in the presence of trypanopain-Tb. In some instances, low-molecular-mass kininogen was also observed to increase the rate of hydrolysis of 7-(benzyloxycarbonyl-phenylalanyl-arginyl-amido)-4-methylcoumarin by live *T. brucei*. Although this effect was rather erratic, in no instance was significant inhibition observed when this putative cysteine protease inhibitor was used under these conditions. The activation of parasite cysteine proteases by commonly accepted cysteine protease inhibitors is unexpected and may have important pathological repercussions.

INTRODUCTION

Bloodstream and metacyclic forms of African trypanosomes such as *Trypanosoma brucei* and *Trypanosoma congolense* endocytose exogenous proteins through their flagellar pockets. These molecules are then delivered, via a complex endosomal network, into the lysosomes of the parasites (Langreth and Balber, 1975; Webster and Grab, 1988; Webster, 1989) where they are assumed to be hydrolysed by proteases (Lonsdale-Eccles and Grab, 1987a; Mbawa et al., 1992). Although the function of the proteases has not been clearly defined, they may play a role in parasite evasion of the host immune system. For example, bloodstream forms of *T. brucei* that were incubated with antibodies specific for the variant surface glycoprotein were shown to endocytose and degrade the immunoglobulins (Lonsdale-Eccles et al., 1993; Russo et al., 1993). However, when the parasites were incubated in the presence of protease inhibitors, there was a massive accumulation of IgG within the endosomal/lysosomal system of the parasites. Procyclic forms of *T. brucei*, which do not possess a variant surface glycoprotein, have minimal or no lysosomal cysteine protease activity (Lonsdale-Eccles and Mpimbaza, 1986; Pamer et al., 1989; Mbawa et al., 1991b).

Lysosomal cysteine proteases have been found in a wide variety of protozoans (e.g. North et al., 1983; Bontempi et al., 1984, 1989; Grab et al., 1987; Lonsdale-Eccles and Grab, 1987a; Cazzulo et al., 1989; Mbawa et al., 1991a) and the importance of these proteases to protozoan viability has been established in a number of systems. For example, specific inhibitors of lysosomal cysteine proteases kill American and African trypanosomes (Ashall et al., 1990; Mbawa et al., 1992) and block the development of *Plasmodium falciparum* (Rosenthal et al., 1989; Rockett et al., 1990). The molecular-mass values of the cysteine protease from *T. brucei* (trypanopain-Tb) and *T. congolense* (trypanopain-Tc) are about 28 and 32 kDa respectively. However, the molecular-mass values of crude trypanopain preparations have proved to be variable when analysed by either contact print zymography (Rautenberg et al., 1982) or electrophoresis in substrate-containing (e.g. fibrinogen) SDS/polyacrylamide gels [Fbg/SDS/PAGE (Lonsdale-Eccles and Mpimbaza, 1986)]. Some of this variability may be caused by the presence of molecules derived from host plasma or serum in the parasite preparations. Indeed, the pattern obtained varies according to the animal species from which the serum is obtained (Lonsdale-Eccles and Grab, 1987a). As well as changing the zymographic

Abbreviations used: Trypanopain-Tb, lysosomal cysteine protease from *Trypanosoma brucei*; trypanopain-Tc, lysosomal cysteine protease from *Trypanosoma congolense*; Fbg/SDS/PAGE, SDS/PAGE on fibrinogen-containing gels; rTM, rat trypanopain modulator; Z-Phe-Arg-NH-Mec, 7-(benzyloxycarbonyl-phenylalanyl-arginyl-amido)-4-methylcoumarin; E64, *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane.

§ Present address: P.O. Box 5170, Kampala, Uganda.
‖ Present address: Veterinary Department, University of Zambia, P.O. Box 32379, Lusaka, Zambia.
** Present address: Tulane Regional Primate Center, Tulane University, 18703 Three Rivers Road, Covington, LA 70112, U.S.A.

molecular-mass patterns, the addition of serum to the trypanosome lysates causes an apparent increase in the total amount of proteolysis when the mixture is analysed by Fbg/SDS/PAGE. Here we describe the isolation and partial characterization of a serum-derived molecule that actuates this modulation of trypanopains.

MATERIALS AND METHODS

Materials

Dithiothreitol, fibrinogen, Mes, Hepes, BSA, cystatin, trypsin, and phospholipase C were obtained from Sigma Chemical Co. Glycosidases were from Seikagaku Fine Biochemicals and kininogens from Protogen AG. Methanol, acetic acid and glycine were purchased from BDH Chemical Co. Tris (hydroxymethyl)-aminomethane and Coomassie Brilliant Blue were from E. Merck. SDS and poly(ethylene glycol) were obtained from Serva. Ampholytes were from LKB-Produktor AB and Sephacryl S-200 and the molecular-mass markers (phosphorylase *b*, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soya-bean trypsin inhibitor, 21.1 kDa; α-lactalbumin, 14.4 kDa) were from Pharmacia Fine Chemicals Co. Matrix Gel Blue A (lot JD-1462) was purchased from Amicon Corp. Linoleic acid and DE-53 (DEAE-cellulose) were obtained from Aldrich Chemical Co. and Whatman respectively. [7-^{14}C]Phenylglyoxal (855 MBq/mmol) was from Amersham. Human high- and low-molecular-mass kininogens and anti-(human low-molecular-mass kininogen) antibodies were purchased from Protogen AG or Calbiochem-Novabiochem AG. 7-(Benzyloxycarbonyl-phenylalanyl-arginyl-amido)-4-methylcoumarin (Z-Phe-Arg-NH-Mec), leupeptin and *trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E64) were from Cambridge Research Biochemicals.

Sample preparation and analysis

Bloodstream forms of the African trypanosomes *T. brucei*, MITat 1.2, and *T. congolense*, clone IL 3000 (Fish et al., 1989), were grown in lethally irradiated randomly bred Sprague–Dawley rats (600–900 rad). The parasites were isolated on the day 3 after infection and purified by centrifugation through Percoll (Grab and Bwayo, 1982) and by DEAE-cellulose chromatography in PBS/gluocose buffer, pH 8.0 [prepared as described by Lanham and Godfrey (1970)] containing 100 μM hypoxanthine (Lonsdale-Eccles and Grab, 1987b). After elution from the DEAE-cellulose, the parasites were washed three times with the hypoxanthine-containing PBS/glucose, pH 8.0, suspended in the same buffer and stored in 1 ml portions of 2×10^8 parasites/ml at −70 °C.

Leishmania donovani (NLB-065) and *Leishmania major* (NLB-144) promastigotes were grown by *in vitro* culture in Schneider's *Drosophila* medium (Gibco Laboratories) (Childs et al., 1978) and medium 199 (Flow Laboratories) (Hendricks et al., 1978) respectively. The parasites were harvested at the stationary phase of growth, washed three times in PBS/glucose and resuspended at a final concentration between 5×10^8 and 1×10^9 parasites/ml and either used directly or stored at −70 °C until required.

Proteolytic activity in the parasite lysates was detected by Fbg/SDS/PAGE (Lonsdale-Eccles and Mpimbaza, 1986). Analysis of rat trypanopain modulator (rTM) during its purification was performed by mixing the rTM-containing samples (20–100 μl) with a trypanosome lysate (4×10^6 trypanosomes in 20 μl) and subjecting the mixture to Fbg/SDS/PAGE. The molecular masses of the active bands were determined from a calibration curve of relative mobility against log (molecular mass) prepared using Bio-Rad molecular-mass markers as standards. To test serum albumin, kininogens and cystatin for their ability to modulate trypanopain activity, 20 μl containing 4×10^6 trypanosomes mixed with 20 μl of the protein (1.05 mg/ml) was analysed by Fbg/SDS/PAGE. In the case of the *Leishmania* samples, 20 μl of 5.2×10^6 promastigotes/ml was used. After electrophoresis (40 mA per gel at 12 °C) of the mixtures, the Fbg/SDS/polyacrylamide gels (1.5 × 135 mm × 145 mm) were rinsed in distilled water for 15 min, followed by overnight incubation in a shaking water bath (37 °C) in a buffer mixture containing 1 mM dithiothreitol, 50 mM each of Mes, Hepes and citric acid (pH 6.0). The gels were stained in 0.1% (w/v) Coomassie Brilliant Blue R250 in methanol/acetic acid/water (3:1:6, by vol.) and destained in the same solution without Coomassie Brilliant Blue. Quantitative analysis of the gels was performed using a Molecular Dynamics personal densitometer.

Purification of rTM

Rat serum (1 ml) was added to a 5 ml packed volume of Matrex Gel Blue A or Matrex Gel Green A that had been prewashed in 50 mM Tris/HCl, pH 8.0, and then resuspended in 5 ml of the same buffer. The serum and beads (total volume 11 ml) were mixed on a rotary mixer at 4 °C for 2 h to remove serum albumin. Samples were then centrifuged in a bench Minifuge set at 4 °C for 20 min at 3000 *g*. The supernatant was used immediately or stored at −20 °C until required.

A total of 15 ml of Matrex Gel Blue A-treated serum supernatant was applied to a column of DE-53 DEAE-cellulose (65 ml) equilibrated in 50 mM Tris/HCl buffer, pH 8.0. The column was washed with 250 ml of the same buffer. A 200 ml linear gradient (0–500 mM NaCl in 50 mM Tris/HCl, pH 8.0) was then applied to the column to fractionate proteins bound to the column. The chromatographic fractions were analysed for protein by SDS/PAGE (Laemmli, 1970), and rTM was analysed by Fbg/SDS/PAGE (Lonsdale-Eccles and Mpimbaza, 1986). Fractions containing rTM activity were pooled and dialysed against water (2 × 2 litres for 4 h each).

The pooled and dialysed fractions from the DE-53 columns were subjected to isoelectric focusing in 1% (v/v) ampholytes (pH range 3.5–10) in a 5–50% (w/v) sucrose gradient for 18 h at 4 °C and 20 W constant power (final voltage 2000 V). The fractions containing rTM were pooled, dialysed against 4 × 2 litres of 10 mM Tris/HCl buffer, pH 8.0, over 8 h, lyophilized and reconstituted in double-distilled deionized water (to give a final concentration of Tris/HCl of 50 mM). The pooled material was loaded on to a Sephacryl S-200 gel-filtration column (100 cm × 1.6 cm, equilibrated in 100 mM Tris/HCl buffer, pH 8.0) and the sample was eluted with the same buffer. Fractions containing rTM were concentrated by lyophilization, reconstituted in 10 ml of water, dialysed against water (4 litres for 4 h) and stored at −70 °C. The purified rTM was used to prepare anti-rTM in rabbits (Harlow and Lane, 1988).

Characterization of rTM

Reduced and unreduced samples of rTM were analysed by SDS/PAGE using the discontinuous buffer system described by Laemmli (1970). The gels were stained with 0.1% Coomassie Brilliant Blue R250 in methanol/acetic acid/water (3:1:6, by vol.), and then destained in the same solution without Coomassie Brilliant Blue.

Enzymic digestion of rTM was performed by incubating 7.5 μg of rTM with trypsin, glycosidase or phospholipase C (0.1 μg each) for 1 h at 37 °C in a total volume of 10 μl of 25 mM Tris/HCl, pH 8.0, before boiling for 5 min to inactivate the

enzymes. The samples were cooled to room temperature, added to a lysate of 4×10^6 trypanosomes/ml and subjected to Fbg/SDS/PAGE. Samples of purified rTM (7.5 μg in 5 μl) were also added to various concentrations of linoleic acid (0–750 μM). The mixtures were incubated for approx. 1 min and then lysates of trypanosomes, in sample buffer, were added before the samples were analysed by Fbg/SDS/PAGE. The areas corresponding to the high-molecular-mass bands were scanned using the LKB laser densitometer. For Western-blot analysis, samples of purified rTM, human low molecular-mass kininogen and human high-molecular-mass kinogen were subjected to SDS/PAGE before immunoblotting (Harlow and Lane, 1988).

Arginine residues on the purified rTM were radiolabelled by treatment with [7-^{14}C]phenylglyoxal by the procedure of Takahashi (1968). To 100 μg of rTM in 200 μl of 50 mM Tris/HCl, pH 8.0, was added 2.16 μmol (50 μCi) of [7-^{14}C]phenylglyoxal for 90 min at room temperature. The reaction by-products from the above were separated from radiolabelled rTM by chromatography through Sephadex G-25 (medium grade) equilibrated in PBS. The radiolabelled rTM was stored at −70 °C until required.

Z-Phe-Arg-NH-Mec hydrolysis by live *T. brucei*

T. brucei (1.8×10^6 parasites/ml; stored on ice until required) were incubated at 25 °C and 37 °C with the fluorogenic trypanopain substrate Z-Phe-Arg-NH-Mec (5 μM) in PBS/glucose, pH 7.4 (Lanham and Godfrey, 1970) containing 100 μM hypoxanthine. Z-Phe-Arg-NH-Mec, which is an excellent substrate for trypanopains (Mbawa et al., 1991b, 1992), permits the *in situ* assessment of the effect of various inhibitors on trypanosomal proteases without the need to purify reaction products from the parasites. Hydrolysis of Z-Phe-Arg-NH-Mec was monitored continuously for up to 5 min on an Hitachi F-200 spectrofluorimeter (excitation wavelength 370 nm; emission wavelength 460 nm) in the presence and absence of E64 (0.1 mg/ml), leupeptin (0.1 mg/ml) or low-molecular-mass kininogen (0.1 mg/ml).

RESULTS

Lysates of *T. brucei* contain lysosomal cysteine protease activity, called trypanopain-Tb, which has a molecular mass of 28 kDa when analysed by Fbg/SDS/PAGE. The addition of a trace (0.01 %) of heated (boiled for 5 min at 93 °C; altitude 1800 m) or unheated rat plasma or serum to such a lysate of *T. brucei* results in the generation of two (87 kDa and 105 kDa) or more extra bands of proteolytic activity when analysed by Fbg/SDS/PAGE (not shown). Similar results are obtained with serum from different animal species, although the apparent molecular masses of the additional band(s) of activity vary according to the particular animal species studied (Lonsdale-Eccles and Grab, 1987a). When the trypanosome lysates are boiled, no activity is observed in either the presence or absence of added serum. Thus rat serum contains a heat-stable molecule (rTM) that seems to alter the apparent molecular mass and activity of *T. brucei* proteolytic activity.

The initial steps of the purification of rTM involved the removal of serum albumin by chromatography on Amicon Matrex Gel Blue A; rTM does not bind to Amicon Matrex Gel Blue A whereas serum albumin does (Fulton, 1980). Further purification of rTM was then accomplished by a combination of gel-filtration and ion-exchange column chromatography and isoelectric focusing.

Fgb/SDS/PAGE of the fractions obtained from Sephacryl S-200 chromatography of rTM showed that rTM was eluted slightly before a 68 kDa molecule that might be serum albumin

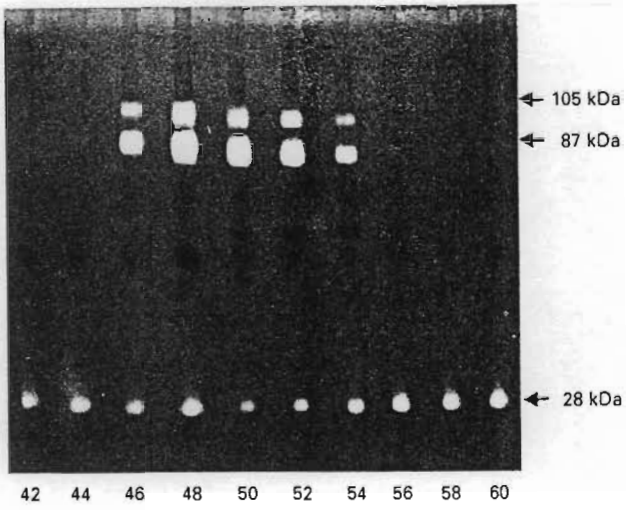

Figure 1 Chromatography of rTM on Sephacryl S-200

A column of Sephacryl S-200 was equilibrated and run in 100 mM Tris/HCl (pH 8.0; 4 °C). Fractions of volume 5 ml were collected and 50 μl samples assayed with 20 μl of lysates of 2×10^8 trypanosomes/ml by Fbg/SDS/PAGE. The fraction number is indicated at the bottom of the Figure. The peak of rTM activity, fraction 48, corresponded to a molecular mass of 120 kDa.

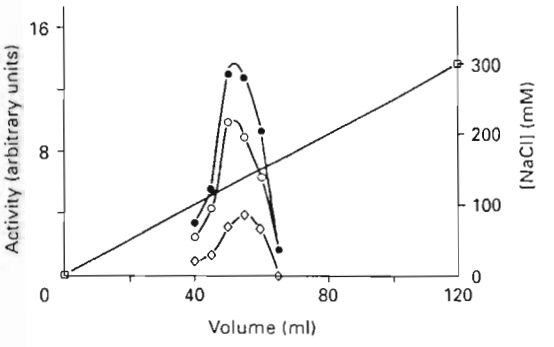

Figure 2 Chromatography of rTM on DE-53 cellulose

After equilibration in 50 mM Tris/HCl (pH 8.0), a 16 cm × 2.5 cm column of DE-53 cellulose was loaded with rTM. The column was washed with 4 bed vol. of 50 mM Tris/HCl, and then a 200 ml gradient of 0–500 mM NaCl in 50 mM Tris/HCl was applied. Fractions (2.5 ml) were collected and assayed by Fbg/SDS/PAGE as described in the Materials and methods section. The destained areas of the gel, corresponding to the peaks of proteolytic activity, were measured by densitometric scanning. Areas lacking rTM activity were not scanned. ○, 87 kDa activity band; ◇, 105 kDa activity band; ●, 87 kDa + 105 kDa activity. rTM was eluted between 110 and 160 mM NaCl.

(Figure 1). The peak of rTM activity from the Sephacryl column corresponded to a molecular mass of 120 kDa and may be a dimer of an approx. 70 kDa molecule. Figure 1 also illustrates the dramatic enhancement of proteolytic activity that can occur in the presence of rTM.

A fairly broad peak of rTM was eluted from DE-53 cellulose at approx. 140 mM NaCl (Figure 2). A trace amount of an approx. 70 kDa protein, which may be serum albumin, was also eluted from the same column slightly after the peak of rTM activity at about 150 mM NaCl (not shown). Fbg/SDS/PAGE of rTM that had been purified by isoelectric focusing indicated that it has a pI of approx. 4.7 (not shown). SDS/PAGE shows

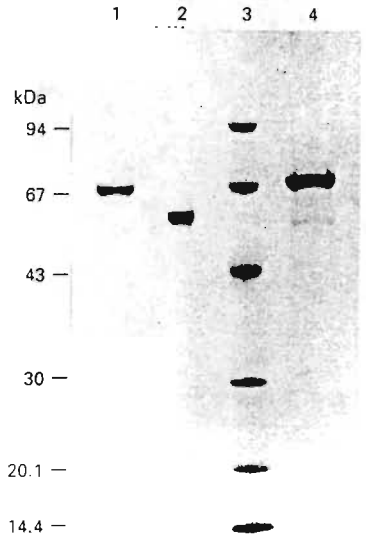

Figure 3 Coomassie-stained SDS/polyacrylamide gel of purified rTM

Lane 1, reduced rTM (7.5 μg of rTM/lane; the sample was boiled in sample buffer for approx 3 min); lane 2, non-reduced unboiled rTM; lane 3, molecular-mass standards; lane 4, purified rat serum albumin.

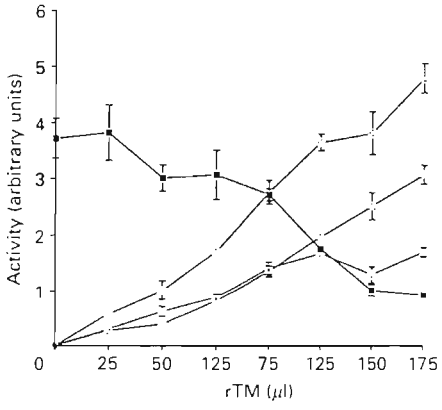

Figure 4 Dose–response of rTM with trypanosome lysate

Increasing volumes of rTM (2.6 μg/ml) were added to 20 μl containing 4×10^6 trypanosomes. The mixtures were then subjected to Fbg/SDS/PAGE. The gel was stained and destained with Coomassie Brilliant Blue and photographed to obtain a negative image. The negative was then scanned and the relative amounts of enzyme activity in each band determined. △, 87 kDa band of activity; ▽, 105 kDa band of activity; ◇, 37 + 105 kDa band of activity; ■, 28 kDa band of activity.

that the molecular mass of rTM is very similar to that of bovine and rat serum albumin (Figure 3). Reduction and boiling increases the apparent molecular mass of purified rTM when analysed by SDS/PAGE but provides no evidence for the presence of different subunits that may be held together by disulphide bridges.

The addition of increasing amounts (volumes) of rTM to trypanopain-Tb results in saturable increases in higher-molecular-mass proteolytic activity accompanied by a decrease in the 28 kDa band. In the dose–response curve (Figure 4), the 87 kDa band is more prominent than the 105 kDa band at high doses of

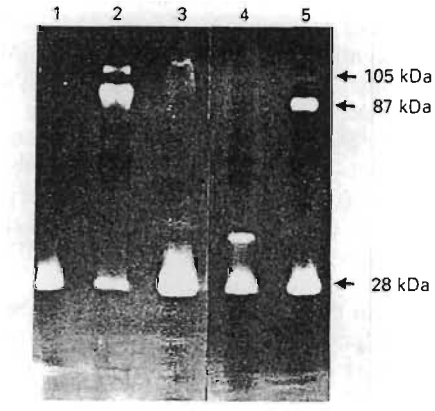

Figure 5 Effect of enzymes on rTM

Purified rTM (5 μl; 7.5 μg) was incubated for 1 h at 37 °C before Fbg/SDS/PAGE with the enzymes indicated. Lane 1, trypanosome lysate alone; lane 2, trypanosome lysate + purified rTM; lane 3, trypanosome lysate + rTM treated with trypsin (5 μl of 0.01%, w/v); lane 4, trypanosome lysate + rTM treated with glycosidase-S (5 μl of 0.01%, w/v; 1 h; 37 °C); lane 5, trypanosome lysate + rTM treated with phospholipase C (5 μl of 0.01%, w/v).

rTM, whereas at low doses the reverse applies. Although the observation of activation is reproducible, precise quantification of the enhancement of the trypanopain proteolytic activity in the presence of rTM has not yet been achieved despite the knowledge that the densitometric readings are proportional to the amount of activity applied to the gel (Lonsdale-Eccles and Mpimbaza, 1986). This is because of the variation that occurs between different preparations of rTM and parasite extracts. Nevertheless, extrapolation of an inverse plot of a similar experiment, in which less than 10% diminution of the 28 kDa band was observed, suggested that the activation may approach 20-fold. This is five-fold greater than that reported for the cystatin activation of cathepsin L (Dennison et al., 1992) but is similar to the 24-fold activation of papain by a protein in blood (Senthilmohan and Topping, 1994) and the 28-fold increase in proteolytic activity of an *Entamoeba histolytica* cysteine protease when released into serum-containing medium (Avila and Calderon, 1993).

The ability of trypsin or a mixture of glycosidases to cause partial breakdown of the active protein with the high molecular mass (Figure 5) suggests that the activator is a glycoprotein. When the high-molecular-mass bands were excised and subjected again to Fbg/SDS/PAGE, several additional discrete bands of activity were found between about 105 kDa and 30 kDa (Table 1). Thus the rTM–enzyme complex appears to undergo self-catalysed limited proteolysis. If, as suggested above, the high-molecular-mass bands of activity are composed of a complex of the trypanopain and rTM, then the apparent molecular-mass values of the fragmentation products appear to correspond to cleavages at about 14–15 kDa intervals in the complex.

In order to assess the nature of the rTM–trypanopain complex, rTM was treated with radiolabelled phenylglycoxal which modifies arginine residues. The specific radioactivity of the [7-^{14}C]phenylglyoxal-labelled protein was greater than 7400 d.p.m./μg of protein. However, the treatment of rTM with this reagent had no obvious inhibitory or enhancing effect on the interaction of rTM with trypanopain-Tb (not shown). In contrast, hydrophobic interactions would appear to be an important component of the complex-formation. Linoleic acid, when added to a final concentration of 500 μM, completely prevented the formation of the high-molecular-mass bands of activity. A 50%

Table 1 Molecular masses of trypanopain–rTM complexes and auto-digestion products

The value α is obtained by subtracting the molecular mass of the native cysteine protease (28 kDa for trypanopain-Tb and 32 kDa for trypanopain-Tc) from the observed molecular mass of the proteolytically active fragments. The integer (β) corresponds to a hypothetical repetitive domain structure that could accommodate the observed molecular mass. Numbers in parentheses refer to bands of weak proteolytic activity.

	Molecular mass (kDa)	α	β	α/β
T. congolense	106	74	5	14.8
	88	56	4	14.0
	(74)	42	3	14.0
	(61)	29	2	14.5
	46	14	1	14.0
	32	0	0	–
			$\bar{x}$ =	14.3
T. brucei	105	77	5	15.4
	87	59	4	14.8
	60	32	2	16.0
	43	15	1	15.0
	28	0	0	–
			$\bar{x}$ =	15.3

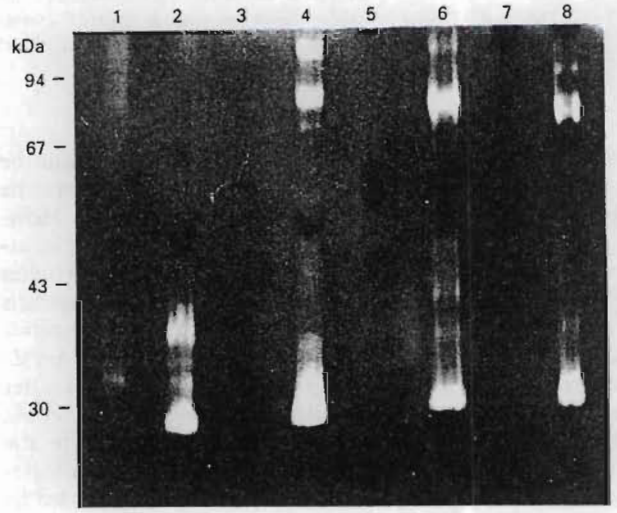

Figure 6 Cystatin and kininogen interaction with trypanopain-Tb

Chicken cystatin and human kininogen were subjected to Fbg/SDS/PAGE in the presence and absence of trypanopain-Tb. Lane 1, cystatin alone; lane 2, cystatin + trypanopain-Tb; lane 3, low-molecular-mass kininogen alone; lane 4, low-molecular-mass kininogen + trypanopain-Tb; lane 5, rat serum alone; lane 6, rat serum + trypanopain-Tb; lane 7, human serum alone; lane 8, human serum + trypanopain-Tb.

inhibition of the production of the high-molecular-mass bands, as assessed by a 50% reduction in the areas of the scanned bands, occurred between 100 and 250 μM linoleic acid. SDS also completely inhibited the formation of the 87 and 105 kDa bands of activity when used at 0.25% (8.7 mM). This is just above its critical micelle concentration of 8.2 mM (Helenius and Simons, 1975). However, when used at the concentrations employed in SDS/PAGE (350 μM), SDS does not prevent the interaction.

The ability of rTM to interact with cysteine proteases, coupled with its acidic isoelectric point and approx. 70 kDa molecular mass, suggested to us that it might be a kininogen-like molecule.

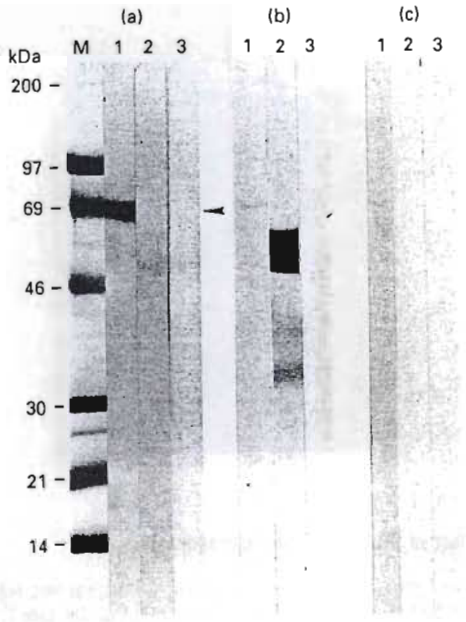

Figure 7 Western-blot analysis of rTM and kininogens

Purified rTM (lane 1), human-low-molecular-mass kininogen (lane 2) and human high-molecular-mass kininogen (lane 3) were subjected to SDS/PAGE and Western blotting with anti-rTM (**a**) and anti-(human low-molecular-mass kininogen) antibodies (**b**). (**c**) Negative control serum blot. Lane M, molecular-mass markers. The arrowhead indicates the position of rTM. Strong reactivity is observed in this position using anti-rTM and a weak but positive reaction with anti-(human low-molecular-mass kininogen).

We therefore investigated whether kininogen and/or cystatin cause similar activation. Both did so (Figure 6). The molecular mass of the additional bands from the kininogen were in the same range as that of rTM whereas that of cystatin, as expected, was considerably smaller. Serum albumins also have similar molecular masses (Figure 3) and isoelectric points to low-molecular-mass kininogens, but neither rat serum albumin (see Figure 8) nor BSA (not shown) caused formation of the additional bands of Fbg/SDS/PAGE-detectable activity. [Interestingly, a commercial preparation of high-molecular-mass kininogen was observed to contain endogenous leupeptin-inhibitable Fbg/SDS/PAGE-detectable activity (results not shown) which precluded its use for the analysis of rTM-like activity.] Further indication that rTM may be a kininogen-like molecule was provided by Western-blot analysis using anti-(human low-molecular-mass) and anti-rTM antibodies (Figure 7). As might be expected, the cross-reactivity between the different species was rather weak. Nevertheless, anti-rTM antibody reacted with a commercial human kininogen preparation to give two weak bands, and anti-(human low-molecular-mass kininogen) antibody produced a single 68 kDa cross-reactive band against the purified rTM.

Very variable results were obtained with live parasites. Suspensions of live *T. brucei* hydrolyse the fluorogenic peptide Z-Phe Arg-NH-Mec (see also Lonsdale-Eccles et al., 1989). In the presence of leupeptin the hydrolysis of Z-Phe Arg-NH-Mec by live *T. brucei* was inhibited by almost 100%. In contrast, in the presence of E64 only about 50% of the activity was inhibited. This difference probably reflects the presence of two predominant but distinct typanosomal enzymes that hydrolyse Z-Phe-Arg-NH-Mec; leupeptin inhibits both enzymes but E64 only the cysteine protease (Lonsdale-Eccles, 1991). In our preliminary

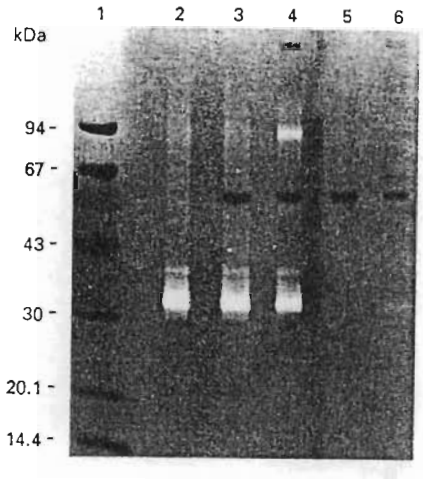

Figure 8 Effect of rTM on purified trypanopain-Tc

Trypanopain-Tc was purified rapidly on a column of cystatin–Sepharose as described by Mbawa et al. (1991a), and subjected to Fbg/SDS/PAGE in the presence of rTM. Lane 1, molecular-mass markers; lane 2, trypanopain-Tc alone; lane 3, trypanopain-Tc + commercial rat serum albumin; lane 4, trypanopain-Tc + rTM; lane 5, commercial rat serum albumin; lane 6, rTM alone.

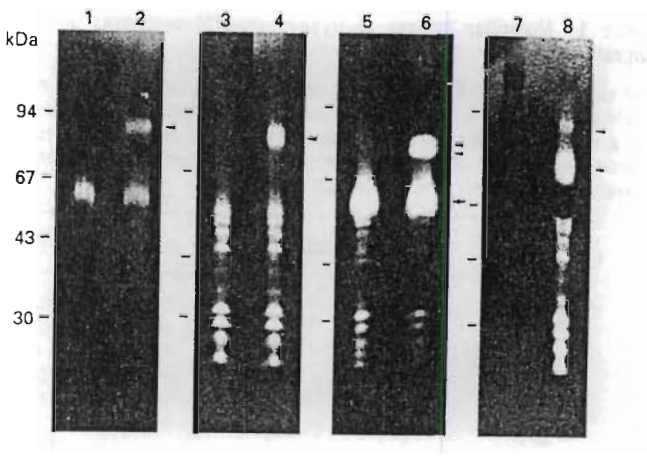

Figure 9 Effect of rTM and human serum on *Leishmania* sp

Lysates of *L. donovani* and *L. major* were subjected to Fbg/SDS/PAGE in the absence of rTM (lanes 1, 3 and 5) and in its presence (lanes 2, 4 and 6). Lane 7 shows human serum alone and lane 8 human serum in the presence of *L. major*. The *Leishmania* samples in lanes 1–4 and 8 were freshly prepared whereas those in lanes 5 and 6 were stored frozen at −70 °C for several days. Lanes 1 and 2, *L. donovani* (7.6×10^6 parasites/ml); lane 3 and 4, *L. major* (1.7×10^6 parasites/ml); lanes 5, 6 and 8, *L. major* (10.3×10^6 parasites/ml). The arrowheads indicate the positions of the additional bands of activity in the presence of rTM or human serum and the arrow indicates the induction of an approx. 60 kDa band of activity observed in *L. major* after storage. The dashes on the left of each panel indicate the positions of the 94, 67, 43 and 30 kDa molecular-mass markers from top to bottom respectively.

experiments we observed an approx. 200 % increase in the rate of hydrolysis of Z-Phe-Arg-NH-Mec by the parasites in the presence of low-molecular-mass kininogen. Unfortunately, subsequent attempts to reproduce this effect showed minimal activation (approx. 10–20 %) or no significant activation. It should be noted, however, that we saw no significant inhibition of the hydrolysis of Z-Phe-Arg-NH-Mec by live parasites at 37 °C when using this putative cysteine protease inhibitor. The variability in activation is similar to the variability observed using the Fbg/SDS/PAGE procedure with differing purified rTM and/or trypanopain sources.

In order to determine whether the protease-activating effect of rTM is restricted to bloodstream forms of *T. brucei*, bloodstream forms of *T. congolense* were also assayed for interaction with rTM (Figure 8). A trypanopain-Tc preparation was prepared from *T. congolense* by rapid affinity chromatography on cystatin–Sepharose (Mbawa et al., 1991a). This result shows that activation can occur with purified as well as crude preparations of trypanosomal enzymes. The result also shows that rTM can interact with a cysteine protease of a different species of African trypanosome.

The formation of similar additional bands of high-molecular-mass activity was observed when rTM was added to lysates of stationary-phase (metacyclic) promastigote forms of either *L. donovani* or *L. major* (Figure 9). Because *Leishmania* are human infective parasites, we investigated whether human serum could mimic rTM by generating additional bands of proteolytic activity. Human serum alone exhibited no proteolytic activity (Figure 9, lane 7), but formed two extra bands of higher-molecular-mass proteolytic activity when added to *Leishmania* extracts (Figure 9, lane 8). Thus human serum must contain a similar molecule to rTM that can modulate the proteolytic activity of the *L. major* parasites.

The protease profiles of *L. major* promastigotes, like those of *Leishmania mexicana* (Robertson and Coombs, 1990; Combs et al., 1991), were considerably more complex than those of *L. donovani* or the African trypanosomes. We have not specifically characterized each of the bands of proteolytic activity from the *Leishmania* and so one or more of these bands of activity may be related to the surface metalloprotease of these parasites. However, over 90 % of the protease activity in *Leishmania* is attributable to cysteine proteases (Coombs et al., 1991) and studies on the metalloproteases from *L. major*, *Leishmania amazonensis* and *L. mexicana* show that these proteases are optimally active at neutral to basic pH and that the metalloprotease from *L. amazonensis* is not able to digest fibrinogen at acid pH after SDS/PAGE (Etges et al., 1986; Chaudhuri et al., 1989; Ip et al., 1990). Thus, although we cannot unequivally eliminate the possibility that some of the bands of activity may be metalloprotease, this seems unlikely, particularly in view of the acidic pH in which our assays are performed.

After storage of *L. major* at −70 °C for 24 h or more, enhanced proteolytic activity was observed at an approx. molecular mass of 60 kDa that was only moderately apparent in the freshly prepared sample (compare lanes 3 and 4 with lanes 5 and 6 respectively of Figure 9). Indeed, the stored sample had to be diluted about 6-fold in order to obtain similar levels of proteolytic activity to those observed before storage. The extra activity may result from activation of the zymogen-like precursors, predicted from cDNA sequence analysis of the structure of several protozoan cysteine proteases [reviewed in Coombs et al. (1991)], but this remains to be shown.

DISCUSSION

African trypanosomal cysteine proteases have molecular-mass values of approx. 30 kDa when assayed by Fbg/SDS/PAGE in the absence of serum. However, if a trace amount of serum is added to the trypanosome lysates, there is an increase in total proteolytic activity that can be correlated with the formation of additional high-molecular-mass bands of activity (Lonsdale-Eccles and Grab, 1987a). The appearance of the additional high-

molecular-mass bands of activity (87 and 105 kDa) frequently occurs with a concomitant decrease in the amount of the 30 kDa band which suggests that the additional enzyme activity is formed by the binding of the rTM to the 30 kDa trypanopain, presumably by causing the trypanopain to change its conformation into a more active and/or stable form. The mechanism by which this proteolytic activity is increased is not clear as it may be an allosteric process that resembles the activation of intracellular proteases by ATP, fatty acid or a Ca^{2+}-dependent protease regulator (De Martino and Goldberg, 1979; De Martino and Blumenthal, 1982; Dahlmann et al. 1985). Nor have we eliminated the possibility that rTM may enhance the minimal endogenous activity found in some zymogens (Lonsdale-Eccles et al., 1978) but, in such a case, the rTM preparation must also cause the inhibition of the 30 kDa enzyme. This seems unlikely, particularly in view of the recent report of the activation of papain by a molecule in blood (Senthilmohan and Topping, 1994).

Interaction between rTM and trypanopains appears to reduce the pH optimum of the enzyme activity from about pH 5.3 to about pH 4.8 (Lonsdale-Eccles and Mpimbaza, 1986). This lower value is close to the intralysosomal pH (4.2) reported for an African trypanosome (Mbawa et al., 1992) and may result in enhanced lysosomal proteolytic activity *in vivo*.

The ability of rTM to form an rTM–trypanopain complex is dependent on the handling of the various preparations. High-molecular-mass bands of activity were not seen with highly purified enzyme that had been isolated from lysates of *T. brucei* by slow conventional chromatography on DE-52 cellulose and Sephacryl S-200. Similarly, repeated freezing and thawing of the trypanosome lysates also results in a diminished capacity to generate the high-molecular-mass bands of activity. However, when the cysteine protease from *T. congolense* was purified rapidly by affinity chromatography, complex-formation was observed with the pure enzyme, although the activities of the high-molecular-mass bands were weak. Similar erratic activation was observed with live parasites. The reason for such variability, which has made it difficult to quantify the extent of activation, is not known and will be the subject of future study. Possibly the domain to which the rTM binds is very susceptible to (self) proteolysis. Limited proteolysis may also explain why the 105 kDa band is frequently less intense than the 87 kDa band.

Early work on the cysteine proteases of African trypanosomes led to the suggestion that trypanopains may be composed of several 14–15 kDa structural domains (Lonsdale-Eccles, 1985) but subsequent sequence studies provided no support for such a repetitive structure within the proteases themselves (Mottram et al., 1989; Pamer et al., 1990). It now seems likely that the early studies were performed on enzyme–rTM complexes and so the repetitive structural motifs may be present in the rTM itself rather than in the enzyme. These domains could be analogous to the similar-sized cystatin-like domains of kininogens (Ohkubo et al., 1984; Müller-Esterl et al., 1985) with the cleavages occurring in the 'hyperfrangible regions I and II' (Vogel et al., 1988) between domains 1 and 2 and domains 2 and 3 (Salvesen et al., 1986). The association of the enzyme with fragments obtained by cleavage at each of these sites would be sufficient to explain the autodigestion products seen with rTM and trypanopain-Tb (Table 1).

Although the idea that a cysteine protease inhibitor may interact with its target enzyme to form an active complex seems counterintuitive, it should be born in mind that not all cysteine proteases (e.g. bromelain) are inhibited by kininogens (Barrett et al., 1986). Furthermore, purified chicken cystatin and human kininogen react in a similar manner with trypanopain to form active complexes like those seen with trypanopain and rTM. The cross-reactivity between anti-(human kininogen) and rTM, and between anti-rTM and human kininogen, lends credence to the idea that rTM contains amino acid sequence domains that are common to those in human kininogen. Also the active inhibitor–protease complexes are similar to those observed with cystatin (or stefin B) and cathepsin L (Pike et al., 1992; Dennison et al., 1992). The similarity between rTM and kininogens is also supported by the observations that α_1-cysteine protease inhibitor, T-kininogen and thiostatin (Esnard and Gauthier, 1983; Enjyoji et al., 1988; Rusiniak et al., 1991) are single-chain glycoproteins with similar molecular masses (68 kDa) and similar pIs (pI 4.75) to rTM, and the carbohydrate moiety is as important for the interaction between thiostatin and papain as it is for the interaction between trypanopain-Tb and rTM.

The results in this study are based primarily on SDS/PAGE analyses and so the phenomena may be artifacts, a consequence of the proteins interacting under partially denaturing conditions. Nevertheless, we have seen that, in the presence of live *T. brucei*, hydrolysis of the small substrate Z-Phe-Arg-NH-Mec may also be enhanced by kininogen, albeit erratically. Furthermore, recent studies with cystatin or kininogen showed that proteolytically active complex-formation is possible between those inhibitors and cathepsin L in the absence of SDS (Dennison et al., 1992; Pike et al., 1992; Dennehy et al., 1994).

An activator of trypanopains has been observed in the serum of several mammalian species (Lonsdale-Eccles and Grab, 1987a), and rat TM enhances the *in vitro* activity of cysteine proteases in *T. brucei*, *T. congolense*, *L. major* and *L. donovani*. However, we have been unable to demonstrate any reactivity between rTM and the cysteine protease activities in rat skin or liver, the proteolytic activity of which is 100–200-fold lower than that present in the trypansomes (J. D. Lonsdale-Eccles and G. W. N. Mpimbaza, unpublished work). The activation or stabilization of the trypanosomatid cysteine proteases by mammalian kininogen-like cysteine protease inhibitors may have profound implications with respect to disease pathology as a consequence of the inappropriate control of these proteases when they are released into the hot bloodstream and tissues (Nwagwu et al., 1988; Authié et al., 1992). It has been proposed that these cysteine protease inhibitors contribute to the body's defence against foreign organisms that invade by using cysteine proteases (Green et al., 1984), and yet our observations suggest that this may not always be the case. Recently, papain was reported to be activated 24-fold by a molecule in blood (Senthilmohan and Topping, 1994), and Avila and Calderon (1993) have reported that the proteolytic activity of a cysteine protease of *E. histolytica* was increased 28-fold when released into medium containing 15% bovine serum. Despite the strong inhibition of some cysteine proteases by the cystatin/kininogen family of proteins [e.g. the inhibition of papain by the acute-phase reactant α_1-cysteine proteinase inhibitor; $K_i = 5 \times 10^{-11}$ M (Esnard and Gauthier, 1993)], these reports and our observations strengthen the argument that a reassessment of the regulation of cysteine protease activity in blood is probably in order.

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REFERENCES

Ashall, F., Angliker, H. and Shaw, E. (1990). Biochem. Biophys. Res. Commun. **170**, 923–929

Authié, E., Muteti, D. K., Mbawa, Z. R., Lonsdale-Eccles, J. D., Webster, P. and Wells, C. (1992) Mol. Biochem. Parasitol. **56**, 103–116
Avila, E. E. and Calderon, J. (1993) Exp. Parasitol. **76**, 232–241
Barrett, A. J., Rawlings, N. D., Davis, M. E., Machleidt, W., Salvesen, G. and Turk, V. (1986) in Proteinase Inhibitors (Barrett, A. J. and Salvesen, G., eds.), pp. 515–569, Elsevier Science Publishers, Amsterdam
Bontempi, E., Franke de Cazzulo, B. M., Ruiz, A. M. and Cazzulo, J. J. (1984) Comp. Biochem. Physiol. **77B**, 599–604
Bontempi, E., Martinez, J. and Cazzulo, J. J. (1989) Mol. Biochem. Parasitol. **33**, 43–48
Cazzulo, J. J., Couso, R., Raimondi, A., Wernstedt, C. and Hellman, U. (1989) Mol. Biochem. Parasitol. **33**, 33–42
Childs, G. E., Foster, K. A. and McRoberts, M. J. (1978) Int. J. Parasitol. **8**, 255–258
Chaudhuri, G., Chaudhuri, M., Pan A. and Chang, K.-P. (1989) J. Biol. Chem. **264**, 7483–7489
Coombs, G. H., Robertson, C. D. and Mottram, J. C. (1991) in Biochemical Protozoology (Coombs, G. N. and North, M. J., eds.), pp. 208–220, Taylor and Francis, London
Dahlmann, B., Rutschmann, M., Kuehn, L. and Reinauer, H. (1985) Biochem. J. **228**, 171–177
De Martino, G. N. and Blumenthal, D. K. (1982) Biochemistry **21**, 4297–4303
De Martino, G. N. and Goldberg, A. L. (1979) J. Biol. Chem. **254**, 3712–3715
Dennehy, K. M., Dennison, C. and Coetzer, T. H. T. (1994) Proceedings of the 12th Congress of the South African Biochemical Society, p. 176
Dennison, C. D., Pike, R., Coetzer, T. and Kirk, K. (1992) Biol. Chem. Hoppe-Seyler **373**, 419–425
Enjyoji, K., Kato, H., Hayashi, I., Oh-ishi, S. and Iwanaga, S. (1988) J. Biol. Chem. **263**, 973–979
Esnard, F. and Gauthier, F. (1983) J. Biol. Chem. **258**, 12443–12447
Etges, R., Bovier, J. and Bordier, C. (1986) J. Biol. Chem. **261**, 9098–9101
Fish, W. R., Muriuki, C., Muthiani, A., Grab, D. J. and Lonsdale-Eccles, J. D. (1989) Biochemistry **28**, 5415–5421
Fulton, S. (1980) Amicon Corp. Publication 512A, Dye-Ligand Chromatography, C36–C38
Grab, D. J. and Bwayo, J. J. (1982) Acta Trop. **39**, 363–366
Grab, D. J., Webster, P., Ito, S., Fish, W. R., Verjee, Y. and Lonsdale-Eccles, J. D. (1987) J. Cell Biol. **105**, 737–746
Green, G. D. J., Kembhavi, A. A., Davies, M. E. and Barrett, A. J. (1984) Biochem. J. **218**, 939–946
Harlow, E. and Lane, D. (1988) in Antibodies: A Laboratory Manual, pp. 435–450, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
Helenius, A. and Simons, K. (1975) Biochim. Biophys. Acta **415**, 29–79
Hendricks, L. D., Wood, D. E. and Hajduk, M. E. (1978) Parasitology **76**, 309–316
Ip, H. S., Orn, A., Russell, D. G. and Cross, G. A. M. (1990) Mol. Biochem. Parasitol. **40**, 163–172
Laemmli, U. K. (1970) Nature (London) **227**, 680–685
Langreth, S. G. and Balber, A. E. (1975) J. Protozool. **22**, 40–53
Lanham, S. M. and Godfrey, D. G. (1970) Exp. Parasitol. **28**, 521–534
Lonsdale-Eccles, J. D. (1985) Prog. Clin. Biol. Res. **180**, 229–231
Lonsdale-Eccles, J. D. (1991) in Biochemical Protozoology (Coombs, G. and North M., eds.), pp. 200–207, Taylor and Francis, London
Lonsdale-Eccles, J. D. and Grab, D. J. (1987a) Eur. J. Biochem. **169**, 467–475
Lonsdale-Eccles, J. D. and Grab, D. J. (1987b) J. Protozool. **34**, 405–408
Lonsdale-Eccles, J. D. and Mpimbaza, G. W. N. (1986) Eur. J. Biochem. **155**, 469–473
Lonsdale-Eccles, J. D., Neurath, H. and Walsh, K. A. (1978) Biochemistry **17**, 2805–2809
Lonsdale-Eccles, J. D., Mpimbaza, G. W. N., Verjee, Y. and Webster, P. (1989) in International Laboratory for Research on Animal Diseases, 1989. Protein Traffic in Parasites and Mammalian Cells: Proceedings of a Workshop Held at the International Laboratory for Research on Animal Diseases, Nairobi, Kenya, 29 August to 1 September 1988, pp. 94–99, ICRAD, Nairobi
Lonsdale-Eccles, J. D., Grab, D. J., Russo, D. C. W. and Webster, P. (1993) in Molecular mechanisms of membrane traffic (NATO ASI Ser **74**) (Morre D. J., Howell, K. E. and Bergeron, J. J. M., eds.), pp. 315–316, Springer-Verlag, Berlin and Heidelberg
Mbawa, Z. R., Webster, P. and Lonsdale-Eccles, J. D. (1991a) Eur. J. Cell Biol. **56**, 243–250
Mbawa, Z. R., Gumm, I. D., Fish, W. R. and Lonsdale-Eccles, J. D. (1991b) Eur. J. Biochem. **195**, 183–190
Mbawa, Z. R., Gumm, I. D., Shaw, E. and Lonsdale-Eccles, J. D. (1992) Eur. J. Biochem. **204**, 371–379
Mottram, J. C., North, M. J., Barry, J. D. and Coombs, G. H. (1989) FEBS Lett. **258**, 211–215
Müller-Esterl, W., Fritz H., Machleidt, W., Ritonja, A., Brzin, J., Kotnik, M., Turk, V., Kellermann, J. and Lottspeich, F. (1985) FEBS Lett. **182**, 310–314
North, M. J., Coombs, G. H. and Barry, J. D. (1983) Mol. Biochem. Parasitol. **9**, 161–180
Nwagwu, M., Okenu, D. M. N., Otusi, T. A. and Molokwu, R. I. (1988) Trans. R. Soc. Trop. Med. Hyg. **82**, 577
Ohkubo, I., Kurachi, K., Takasawa, T., Shiokawa, H. and Sasaki, M. (1984) Biochemistry **23**, 5691–5697
Pamer, E. G., So, M. and Davis, C. E. (1989) Mol. Biochem. Parasitol. **33**, 27–32
Pamer, E. G., Davis, C. E., Eakin, A. E. and So, M. (1990) Nucleic Acid Res. **18**, 6141
Pike, R. N., Coetzer, T. H. T. and Dennison, C. (1992) Arch. Biochem. Biophys. **294**, 623–629
Rautenberg, P., Schadler, R., Reinwald, E. and Risse, J. J. (1982) Mol. Cell. Biochem. **47**, 151–159
Robertson, C. D. and Coombs, G. H. (1990) Mol. Biochem. Parasitol. **42**, 269–276
Rockett, K. A., Playfair, J. H. L., Ashall, F., Targett, G. A. T., Angliker, H. and Shaw, E. (1990) FEBS Lett. **259**, 257–259
Rosenthal, P. J., McKerrow, J. H., Rasnick, D. and Leech, J. H. (1989) Mol. Biochem. Parasitol. **35**, 177–184
Rusiniak, M. E., Bedi, G. S. and Back, N. (1991) Biochem. Biophys. Res. Commun. **179**, 927–932
Russo, D. C. W., Grab, D. J., Lonsdale-Eccles, J. D., Shaw, M. K. and Williams, D. J. L. (1993) Eur. J. Cell Biol. **62**, 432–441
Salvesen, G., Parkes, C., Abrahamson, M., Grubb, A. and Barrett, A. J. (1986) Biochem. J. **234**, 429–434
Senthilmohan, T. and Topping, M. (1994) Chem. Br. **30**, 350
Takahashi, K. (1968) J. Biol. Chem. **243**, 6171–6179
Vogel, R., Assfalg-Machleidt, I., Esterl, A., Machleidt, W. and Müller-Esterl, W. (1988) J. Biol. Chem. **263**, 12661–12668
Webster, P. (1989) Eur. J. Cell Biol. **49**, 295–302
Webster, P. and Grab, D. J. (1988) J. Cell Biol. **106**, 279–288

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