Epitope Mapping of a Trypanosomal Cysteine Proteinase

by

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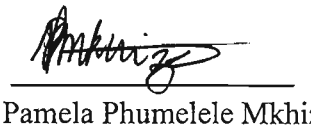
This dissertation is dedicated to my parents
Allison and Thobile Mkhize

Preface

The experimental work described in this dissertation was carried out in Biochemistry, School of Molecular and Cellular Biosciences, University of Natal, Pietermaritzburg from March 2001 to October 2003 under the supervision of Professor Theresa Coetzer. These studies represent original work by the author and have not been submitted in any other form to another university. Where use has been made of the work of others, it has been duly acknowledged in the text.



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Abstract

Trypanosomosis is a parasitic disease in man, domestic and wild animals and is of major economic importance in many parts of the world, particularly in Sub-Saharan Africa. *Trypanosoma congolense*, *T. vivax* and *T. brucei brucei* are the major pathogenic trypanosomes infecting cattle in sub-Saharan Africa. The parasite itself is not directly responsible for the disease, but rather causes illness through the release of pathogenic factors. One of the major pathogenic factors released by trypanosomes is proteinases.

Trypanotolerant cattle produce antibodies against a trypanosomal proteinase, congopain, that inhibit congopain activity. Congopain thus has vaccine potential. This study describes the mapping of immunogenic epitopes of congopain to identify peptide regions of the protein that induce enzyme inhibitory antibodies for inclusion in a trypanosome vaccine. This vaccine approach targets the disease, rather than the parasite by focusing on a pathogenic factor. These peptides also have potential for use in diagnostic assays. Peptides from the catalytic domain of a trypanosomal cysteine proteinase, congopain, were selected using an epitope prediction program. Peptides selected were from the two forms of congopain called CP1 and CP2. Antibodies against peptide-carrier conjugates were produced in chickens. The antibodies recognised native congopain, recombinant CP2 and the recombinant catalytic domain (C2). This suggests that the peptides selected have promise for use in vaccines.

The peptides were also used to determine whether they are natural immunogenic epitopes of CP2 and thus have potential for use in diagnostic assays. Antibodies in the sera from *T. congolense* infected cattle recognised all the peptides in an ELISA. Antibodies in the sera from C2-immunised, non-infected cattle recognised most of the peptides in an ELISA. In order to distinguish between *T. congolense* and *T. vivax* infection, two different peptides from the C-terminal extensions of CP2 and vivapain were used in ELISA tests with sera from infected cattle. Although anti-peptide antibodies produced against the two C-terminal extension peptides were specific for their respective peptides, thereby indicating the discriminatory power of the peptides selected, there was cross-reactivity by the sera from *T. congolense* and *T. vivax* infected cattle. Optimal antibody binding peptide sequences of these two peptides need to be identified by testing modified sequences of these two peptides to

improve the sensitivity of this assay.

In addition to attempting to define the epitopes of congopain, preliminary studies to increase the immunogenicity of congopain were also undertaken. Alpha 2-macroglobulin is a natural host inhibitor of proteinases. Inhibition occurs by entrapment of an active proteinase within the alpha 2-macroglobulin cage. In addition, it has been demonstrated that antigen complexed with alpha 2-macroglobulin becomes more immunogenic, resulting in enhanced antigenic presentation of an entrapped antigen. This study reports the interaction between congopain and alpha 2-macroglobulin. The preliminary results of this study showing congopain-alpha 2-macroglobulin interaction could be used to explore the possibility of increasing the immunogenicity of congopain and congopain epitopes by complexing these to alpha 2-macroglobulin. Congopain epitopes complexed with alpha 2-macroglobulin could be used to form a peptide-based vaccine.

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To my God; I can do all things through Christ who strengthens me.

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Abbreviations

A_{280}	Absorbance at 280 nm
ABTS	2,2′-azinobis [3-ethyl-2,3-dihydrobenzthiazole-6-sulfonate]
BCIP	5-bromo-4-chloro-3-indolyl phosphate
Bis	N,N′-methylenebisacrylamide
BSA	bovine serum albumin
BSA-TBS	bovine serum albumin in Tris-buffered saline
Bz	benzoyl
c	concentration
C2	truncated form of congopain 2 without carboxy terminal extension
CAMOR	carrier-modified residues
CP1	congopain type 1
CP2	congopain type 2
C-terminal	carboxy terminal
DDT	dichlorodiphenyltrichloroethane
DMF	dimethylformamide
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
ε	extinction coefficient
E-64	L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane
EDTA	disodium ethylene diaminetetraacetate dihydrate
ELISA	enzyme-linked immunosorbent assay
FCA	Freund's complete adjuvant
FIA	Freund's incomplete adjuvant
HRPO	horse-radish peroxidase
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
kDa	kilo-Dalton
kDNA	kinetoplast DNA
K_i	inhibition constant

K_m	Michaelis-Menten constant
L-kininogen	low molecular weight kininogen
α_2M	alpha-2-macroglobulin
MBS	*m*-maleimidobenzoyl-N-hydroxysuccinimide ester
MEC	molecular exclusion chromatography
M_r	molecular mass
N-terminus	amino terminus
NBT	nitroblue tetrazolium
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pI	isoelectric point
RT	room temperature
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide-gel electrophoresis
TBS	Tris-buffered saline
TEMED	N,N,N′,N′-tetramethylethylenediamine
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
VSG	variant surface glycoprotein
Z	carbobenzoxy

Chapter 1
Literature review

1.1 Introduction

Trypanosomosis[a] is a disease in man, domestic and wild animals that has a negative impact on food production and economic growth in many parts of the world, particularly in sub-Saharan Africa (Taylor, 1998). This disease is caused by trypanosomes, which are parasitic protozoa that live and multiply in the blood and other body fluids of their hosts. The disease is transmitted to man and animals by the bite of a tsetse fly (*Glossina palpalis*) infected with trypanosomes (Grant, 2001). The hosts for trypanosomes are not only mammals (man, cattle, horses, sheep, camels) but also fish, amphibians, reptiles and birds (Hall, 1977). Trypanosomes cause the disease nagana and sleeping sickness in cattle and humans, respectively.

None of the methods currently available for trypanosomosis control are completely successful. Furthermore, there have been many difficulties and challenges in the development of a vaccine against the trypanosome parasite, for example, one difficulty is the ability of the parasite to cyclically change its surface coat and thus evade the host immune response (Turner, 1997). However, it has been suggested for trypanosomosis, as well as other parasitic infections that the parasite itself is not directly responsible for the disease, but rather causes illness through the release of pathogenic factors which are toxic to the host (de Souza et al., 1990; Mbawa et al., 1991a; b; Authié et al., 1993a; b; Troeberg et al., 1999). If the activity of these parasitic components could be disturbed, the host's condition might improve. Elimination of the parasite would therefore not be an absolute necessity. One way of inhibiting such pathological factors could be through immunisation with parasite proteinases (Authié et al., 1993a; 1993b; Troeberg et al., 1997). This study attempts to use this approach to tackle trypanosomosis, the ultimate aim being to develop an anti-disease vaccine, as opposed to an anti-parasite vaccine.

1.2 Trypanosomosis

1.2.1 Cattle trypanosomosis

Trypanosoma congolense, T. vivax and *T. brucei brucei* are the tsetse transmitted trypanosome

[a]Standardised nomenclature of animal parasitic diseases (Kassai et al., 1988)

species of primary importance in causing trypanosomosis, also known as nagana in domestic livestock (Mattioli et al., 1999). *T. congolense* is the dominant species in East Africa, while *T. vivax* is dominant in West Africa, although both species occur all over Africa and cause infection and a milder form of the disease where they are not dominant (Ukoli, 1984). *T. congolense* has not been found in cerebro-spinal fluid but it has been localized in the capillaries of the brain. Parasitaemia is usually low in the blood and may be missed if only blood films are examined. The disease is characterised by chronic anaemia, weight loss, infertility and increased susceptibility to other pathogens due to poor immune responses (Taylor, 1998). Mixed infection of cattle with *T. congolense*, *T. vivax* and *T. b. brucei* is very common and makes it very difficult to design diagnostic and therapeutic procedures (Mattioli et al., 1999). Because of the immunosuppression in infected cattle, secondary bacterial infections may occur and this complicates the disease, leading to a higher death rate.

Nagana is a major obstacle to increased livestock production, and to agricultural development in most countries of sub-Saharan Africa. The presence of tsetse in tropical Africa has a large impact on cattle breeding and there is a big difference in nutritional income between Africa and the European Union due to nagana (Tables 1 and 2, Opperdoes, 1997). The estimated direct losses in cattle alone are between US $6 000 million and US $12 000 million per year (Taylor, 1998). In Africa alone, the total cost of the disease is estimated to be US $1.3 billion per year (Lalmanach et al., 2002). The disease can result in the death of about 3 million cattle per year (Hursey and Slingenbergh, 1995).

Different breeds of cattle have varying susceptibilities to trypanosome infection. For example, Boran cattle are prone to trypanosomosis, while N'Dama cattle are known to be 'trypanotolerant' (Authié et al., 1993a). Trypanotolerance is defined as the genetically determined ability to limit multiplication of the parasite and to resist the pathogenic effects of infection (Murray and Dexter, 1988; Trail et al., 1989). The existence of an association between immune recognition of a trypanosome antigen and resistance to trypanosomosis might lead to new methods of immuno-prophylaxis in susceptible cattle. In addition, by providing possible methods for selection of resistant cattle, it could contribute to genetic improvement of trypanotolerance in cattle (Authié et al., 1993a).

Table 1. Differences in nutritional income between Africa and the European Union due to nagana (Opperdoes, 1997).

	European Union	Tropical Africa
Nutritional income	>3.400 cal / day	<2000 cal / day
Proteins from cattle meat	66%	20%
Calories from cattle meat	33%	6%

Table 2. Influence of tsetse on cattle breeding in Africa (Opperdoes, 1997).

African region	Biomass (mass of cattle / inhabitant)
Outside tsetse belt	136 kg
Inside tsetse belt	26 kg

1.2.2 Trypanosomosis in other livestock

Trypanosomosis in other livestock caused by *T. evansi* and *T. equinum* is called surra. Surra is acutely fatal to horses and chronically fatal to camels (González, 2001). *T. evansi* can also affect cattle, pigs and a variety of other domesticated and wild animals (Cazzulo, 1997). *T. evansi* and *T. equinum* are transmitted by biting flies such as *Tabanus*, *Stomoxys* and *Lyperosia* and they are therefore far more widespread than trypanosomes which rely on tsetse transmission. Their distribution range extends throughout North Africa, the Middle East, the Indian sub-continent and also Central and South America (Purnell, 1985). *T. equiperdum* affects horses causing a disease called dourine. The parasite is transmitted during coitus and no insect vector is involved (González, 2001). Other trypanosomes such as *T. vivax*, *T. congolense*, *T. b. brucei* and *T. simiae* have also been reported to be pathogenic for sheep, goats, dogs, pigs, antelope, camels and horses (Purnell, 1985; González, 2001).

1.2.3 Human trypanosomosis

African human trypanosomosis, also called sleeping sickness, is caused by two sub-species of *T. brucei,* viz. *T. b. gambiense* and *T. b. rhodesiense*, which are transmitted by the bite of two species of tsetse fly, viz. *Glossina palpalis* (*T. b. gambiense*) and *G. morsitans* (*T. b. rhodesiense*) (Welburn et al., 2001). Clinical signs and symptoms of sleeping sickness may be suggestive but not conclusive (Kuzoe, 1993). Therefore, a demonstration of trypanosomes in

the blood, lymph or cerebrospinal fluid is necessary to confirm diagnosis. The early stage of the disease is characterised by fever, headache and joint pains followed in later stages by neurological symptoms and endocrinal disorders (Welburn et al., 2001). *T. b. gambiense* and *T. b. rhodesiense* are morphologically indistinguishable but the *gambiense* form is chronic and slow in onset and its course may last from a few months to several years, while the *rhodesiense* form is acute and rapid in onset and may last for only a few weeks or months (Welburn et al., 2001).

South American trypanosomosis, also called Chagas' disease, is caused by *T. cruzi* which is transmitted to man, wild and certain domestic animals by the bite of *Rhodnius prolixus* and *Triatoma infestans* (Purnell, 1985). Transmission is effected by contamination of mucous membranes or the skin with infected excreta. During the blood meal, or soon after, defaecation occurs and metacyclic, infective trypomastigotes present in the hindgut and rectum of the vector may be deposited onto the skin with the faeces and can penetrate the skin barrier through the site of the bite, or the mucosae of the eye or lips or through other local abrasions (Van Meirvènne and Le Ray, 1985). The disease can also be acquired with mother's milk and by blood transfusion. Animals are infected by eating the vectors or licking their bites (Purnell, 1985). At the site of trypanosome penetration into a new host, the trypanosome invades surrounding cells, especially macrophages, and multiplies locally. Spreading of infection is ensured by migrating parasitised macrophages and by free trypomastigotes. The latter invade blood and body fluids and penetrate muscle and reticulo-endothelial cells before further multiplication (Van Meirvenne and Le Ray, 1985). The disease is characterised by fever, enlarged glands, anaemia and disturbances of the nervous system (Purnell, 1985).

1.3 Classification and life-cycle of trypanosomes

Trypanosomatids are allocated to a number of genera based on morphological features and host range (Momen, 2001; Figure 1). The major pathogenic species in the genus Trypanosoma are *T. congolense*, *T. vivax* and *T. b. brucei*, that belong to the section salivaria, and *T. cruzi* that belongs to the section stercoria (Mattioli et al., 1999). Salivarian trypanosomes, like most parasites, may appear in different forms at different stages of their complex life cycles.

Completion of the life cycle depends upon the transmission between the host and the tsetse fly

vector (Figure 2). The parasite undergoes two major transformations during its journey through the two hosts, resulting in two different life cycle forms. Trypanosomes ingested by the tsetse fly undergo a series of biochemical and structural changes in the fly's midgut. The parasite residing in the salivary glands of the fly is in the procyclic form, due to its procyclin (a glycosylated protein) coat. Here, it differentiates into the metatrypanosome form, the form that can infect the host. When the fly bites a mammal, metacyclic trypanosomes are introduced into the host's bloodstream where they rapidly differentiate into a form that can proliferate (Turner, 1997). These rapidly proliferating forms divide by binary fission in the bloodstream, lymphatic and cerebrospinal fluid (Markell et al., 1992). These rapidly dividing trypomastigote forms are long and slender but they become short and stumpy in the later stages after infection (Markell et al., 1992).

Trypanosomes can invade many sites in the host. One of these sites is the brain, where they can cause tissue damage that leads to lethargy, confusion and, ultimately, death (Turner, 1997). Trypanosomes can be ingested once again by a tsetse fly when it bites the host, where it will be taken into the gut of the fly along with the blood. Here it will transform back into the procyclic form, undergo sexual reproduction and move into the salivary glands for the cycle to be repeated.

1.4 Prevention and treatment methods for trypanosomosis

1.4.1 Prevention methods

Current methods of trypanosomosis control in Africa are based on chemoprophylaxis, chemotherapy and the elimination of vectors using insecticides (Grant, 2001). In Africa there were widespread attempts at tsetse control by clearing the vegetation where the flies rested, and shooting the wild animals on which the flies fed. However, such ecologically unsound methods are no longer accepted (Schofield and Maudlin, 2001). Large-scale use of insecticides has been criticised for its effects on non-target organisms (Aksoy, 2001). One way of solving this problem was the development of odour-baited traps and targets impregnated with insecticides, whereby flies could be killed without severe threat of environmental pollution (Schofield and Maudlin, 2001). However, absolute control cannot be achieved with these technologies and they require frequent maintenance. Clearly, new approaches that are

environmentally acceptable, efficacious and affordable are needed for the control of tsetse vector populations.

Aksoy et al. (2001) have introduced new prospects for the control of trypanosomosis. Their approach is to focus on molecular genetic approaches to modulate tsetse vector competence. Their technology aims to eliminate the ability of insects to transmit pathogens by introducing and expressing foreign genes with anti-pathogenic properties that interfere with pathogen viability, development or transmission. These genetically engineered insects can then be driven into natural populations to replace their susceptible counterparts (Aksoy et al., 2001).

1.4.2 Treatment methods

There are currently only three trypanocides available for controlling tsetse-transmitted trypanosomosis in domestic ruminants. These are isometamidium and homidium, which have both prophylactic and therapeutic effects, and diminazene, which has only therapeutic properties (Geerts et al., 2001). Present chemotherapies are inadequate, toxic or both. The trypanocides used to control tsetse transmitted trypanosomosis in domestic animals in Africa have been in use for over 40 years and consequently resistance of trypanosomes to these drugs has emerged (Geerts et al., 2001). Development of new drugs is not a priority for international pharmaceutical companies because there is a limited market in Africa and the cost of developing and licensing new drugs is high (Geerts et al., 2001). It is therefore unlikely that new chemotherapeutic agents will be available anytime soon.

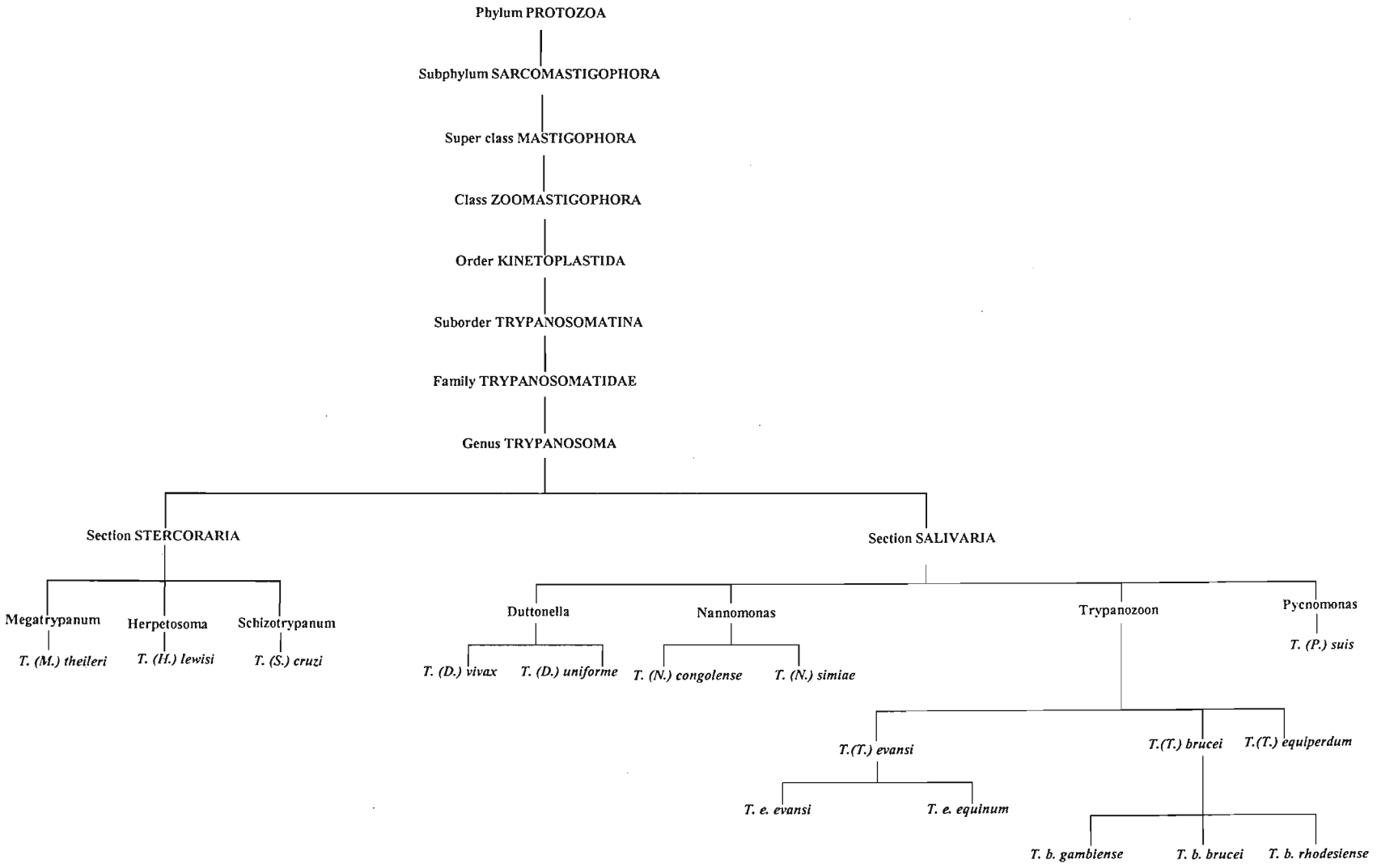

Figure 1. Schematic representation of the *T. b. brucei* phylogenetic tree (Troeberg, 1997).

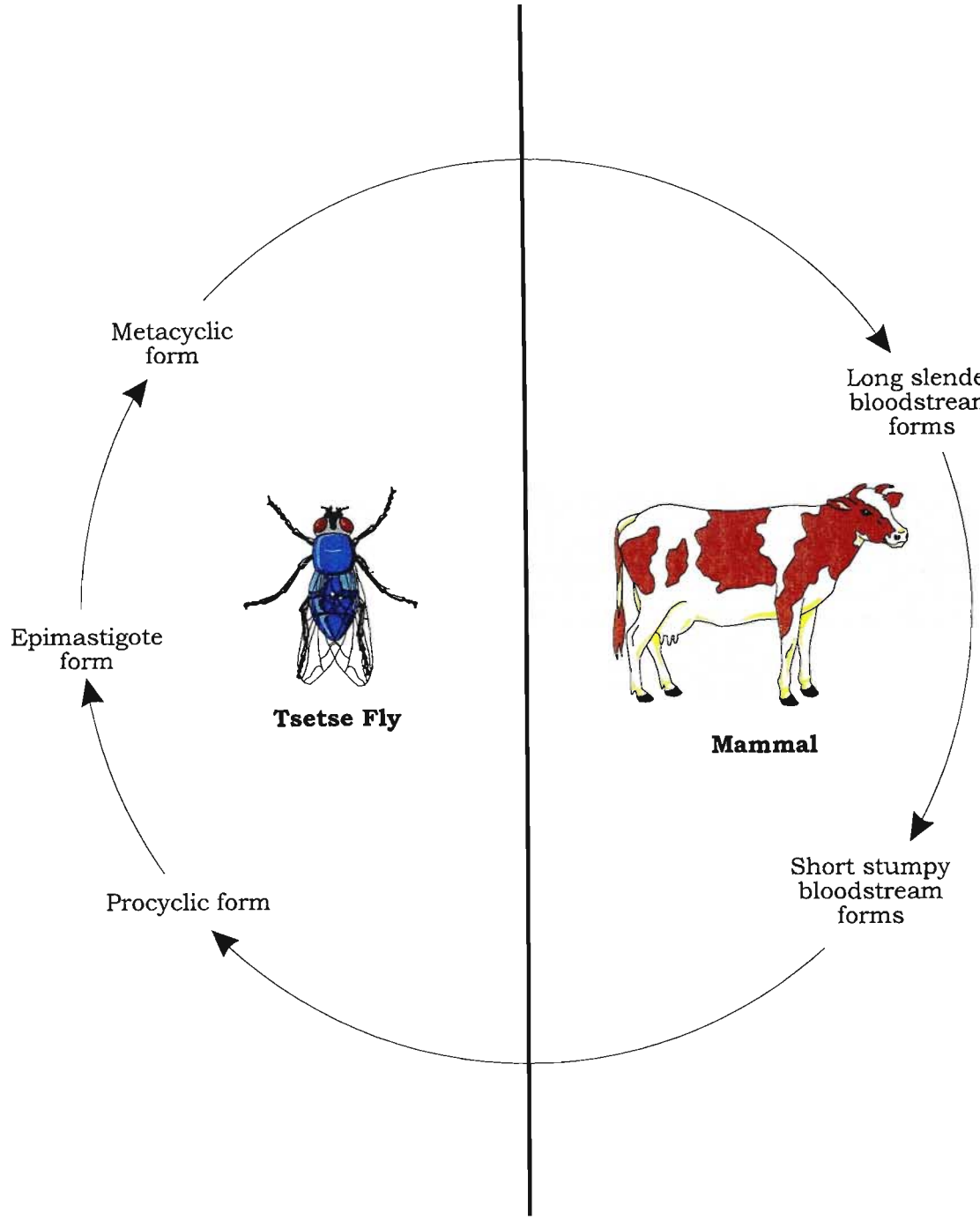

Figure 2. Life cycle of *T. b. brucei* showing morphological stages present in mammalian and tsetse fly hosts (Troeberg, 1997).

1.5 Vaccine strategies

Vaccination is done to stimulate the host's normal immune response so that it can provide an efficient immunity against an invading pathogen (Roitt, 1997). There are a number of crucial

conditions, which must be satisfied for a vaccine to work. The antigens chosen as part of the vaccine must be able to produce a protective response that can be maintained over a long period of time without the requirement of boosters, readily available, stable and cheap (Roitt, 1997). Furthermore, antigens should be safe and be able to produce a good, protective response and not other non-protective mechanisms which might mask the response. The host should be immunocompetent.

There are many challenges which are faced in developing successful parasite vaccines. Unlike bacteria and viruses, parasitic organisms are very complex, having complex life cycles and the effect of parasites on the host's immune system is not fully understood. It is essential to understand the protective mechanisms of parasites because it is necessary to know what immune response is required of a vaccine. Furthermore, the development of new vaccines may require high cost technology (Van Regenmortel, 1990c).

Vaccination against trypanosomosis has long been thought to be the most desirable control method (Taylor, 1998). Experimental vaccines consisting of attenuated forms of the parasite or parasite lysates or partially purified variable surface glycoprotein (Duxbury et al., 1972; Wellde et al., 1975; Morrison et al., 1982; Wells et al., 1982; Bosombrio, 1990; Bosombrio et al., 1993; Onah and Wakelin, 1999; Paiva et al., 1999), flagellar pocket antigen (Mkunza et al., 1995; Wrightsman, 1995; Miller et al., 1996; Wrightsman and Manning, 2000) and naked DNA (Pereira-Chioccola et al., 1999) have been tested in laboratory animals with varying degrees of success. Several of these studies showed that susceptible hosts may be partially protected against death by vaccination before challenge but individual antigens capable of ensuring survival against lethal challenge have not been identified (Wrightsman, 1995). The development of a conventional vaccine capable of preventing trypanosome infection in immunised animals seems to be very difficult (Lalmanach et al., 2002). None of the vaccines tested against trypanosomosis have yet met all the requirements for a successful vaccine. The success of experimental vaccination becomes highly questionable when protection is monitored with highly sensitive methods such as complement mediated lysis (CML) which detects antibodies that lyse bloodstream trypomastigotes in the presence of complement (Kierszenbaum, 1989). Lytic antibodies associated with active infection have been detected with the CML method in spite of vaccination (Kierszenbaum, 1989).

1.5.1 Vaccines based on variable surface glycoprotein (VSG)

Variable surface glycoprotein (VSG) is the most abundant, exposed and immunologically dominant antigen of trypanosomes in the mammalian host (Donelson, 1996). Within the mammalian host, a VSG coat covers the cell membrane of the bloodstream form trypanosome. One approach used to protect against trypanosomosis was the immunisation of cattle with purified VSG (Duxbury et al., 1972; Wellde et al., 1975; Morrison et al., 1982; Wells et al., 1982, Lutje et al., 1995). Trypanosome-infected trypanotolerant Cape buffalo developed antibodies that are specific for the VSG of the infecting trypanosomes and can lyse parasites, indicating their capacity to fix complement (Black et al., 2001). Antibody responses to surface exposed epitopes of the VSG in *T. congolense*-infected N'Dama (trypanotolerant) and Boran (susceptible) cattle were investigated (Williams et al., 1996). It was found that trypanosusceptible Boran cattle had low IgG1 responses to cryptic VSG epitopes but high IgM responses to non-trypanosome antigens. In contrast, the N'Dama cattle had significantly higher IgG1 responses to cryptic VSG epitopes and negligible responses to non-trypanosome antigens (Williams et al., 1996).

These results seemed to show that anti-VSG antibody responses play a role in trypanotolerance. However, VSG proteins cannot be used as vaccine targets due to their high variability. The number of different kinds of VSGs displayed by trypanosomes is very great and has been estimated to be up to 10^7 (Cross, 1990). When an animal is invaded by a pathogen, the immune system of the host animal recognises some part of the pathogen as foreign and makes antibodies to attack this foreign part (antigen). The antibodies bind to the antigen and either neutralise the effects of disease-producing microorganisms or help to destroy them. Trypanosomes, however, avoid total destruction by the host's immune response by repeatedly changing VSGs displayed on their surface coat membrane (Donelson, 1996). Antigenic variation is the primary mechanism that prevents most domestic animals from developing effective immunity against trypanosomosis. The control and function of the bloodstream VSG expression sites in *T. b. brucei* is under investigation in the hope of identifying factors which play a role in their control (Borst and Ulbert, 2001; Vanhamme et al., 2001). Cattle immunised with purified VSG are protected against challenge with homologous but not heterologous trypanosomes. Thus, the potential of trypanosomes to express different VSG genes reduces the likelihood of an effective VSG based vaccine (Taylor, 1998).

1.5.2 Killed or live, attenuated trypanosomes as vaccines

Killed or live, attenuated trypanosome parasites have been used as vaccines in many studies (Basombrio, 1990; Basombrio et al., 1993; Gomez et al., 1996). In many instances the immunity given by killed vaccines, even when given with adjuvant, is often inferior to that resulting from infection with live organisms (Roitt, 1997). Attenuation is done to damage the parasite in some way so that they may not develop fully, resulting in a live parasite with a short life span but which survives long enough to stimulate the host's immune system (Roitt, 1997). The preventive effect of a vaccine consisting of cultured *T. cruzi* killed by freezing and thawing in the presence of saponin was tested in mice and guinea pigs (Bosombrio, 1990). It was found that after an exposure time of about four months, natural *T. cruzi* infection occurred in 55% of the controls and 33% of the vaccinated group (Bosombrio, 1990). The results showed that immunisation was only partially protective in animals. In another study, live attenuated *T. cruzi* were used for vaccination (Bosombrio et al., 1993). The results showed that vaccination failed to completely prevent natural infection (Bosombrio et al., 1993). These two studies showed that for trypanosomes, the use of attenuated trypanosomes, compared to the use of killed trypanosomes is not significantly better since in both studies only partial protection was achieved. It has been proven that vaccination with live trypanosomes does not cause any T-cell abnormalities (Paiva et al., 1999). However, this method is not sufficient because it does not give complete immunity. Furthermore, trypanosomes appear in different forms at their different life-cycle stages and it may not be possible to get enough infective stages to make this type of vaccine effective.

1.5.3 Flagellar pocket antigens

The flagellar pocket of the trypanosome is an area where receptors are used for specific host macromolecule uptake and this area is therefore thought to be invariant. For this reason, a vaccine against flagellar pocket proteins may provide immunity. Experimental vaccines consisting of flagellar pocket proteins have been tested and seem to show promise. Cattle were vaccinated in Kenya with flagellar pocket antigens derived from *T. b. rhodesiense* and then infected with *T. congolense* and *T. vivax*. It was found that the infection was reduced from 13% to 0.9% (Mkunza et al., 1995). Unlike most other studies, this experiment was carried out in an environment of natural exposure, with a naturally infectious trypanosome species. Most other studies have used a murine model, which is subject to disease based immunosupression

(Mkunza et al., 1995). Pure paraflagellar rod proteins have been used alone, or in combination with adjuvant, in many studies and most results seemed to show protection in immunised animals (Wrightsman, 1995; Miller, 1996; Wrightsman and Manning, 2000).

1.5.4 Immunisation with naked DNA

Immunisation with naked DNA is an alternative strategy for the development of vaccines against different infections, including those by protozoan parasites (Pereira-Chioccola, 1999). Immunisation with a plasmid containing the trans-sialidase gene, a parasite antigen, resulted in protective immunity against *T. cruzi* infection in BALB/c mice. Immunisation using either plasmid in a mouse strain, highly susceptible to infection, generated high levels of antibody and decreased the mortality caused by acute infection (Pereira-Chioccola et al., 1999).

1.5.5 Congopain: anti-disease approach to immunological control of livestock trypanosomosis

Most of the vaccine strategies mentioned above are aimed at developing vaccines against trypanosomes. However, the ability of trypanosomes to escape host immune mechanisms through antigenic variation makes it difficult to obtain a vaccine that would prevent trypanosome infection. An alternative approach of vaccine research is to eliminate the pathogenicity of the parasite, not the infection itself. Eradication of the infectious agent is not always the most practical goal. For example, in malaria, the blood-borne form releases molecules which stimulate production of tumour necrosis factor (TNF) and other cytokines from monocytes and the secretion of these mediators is responsible for the disease symptoms. Therefore, an antibody response targeted to the released antigens, with structurally conserved epitopes, may be more effective than targeting the parasites with variable surface antigens (Roitt, 1997). This has led to the development of vaccines which are composed of synthetic, recombinant, or highly purified antigens which target the disease rather than targeting the whole parasite. This form of vaccine is called a subunit vaccine.

Congopain is a major proteolytic enzyme from *T. congolense*, which displays pathogenic effects *in vivo* and is released into the bloodstream of infected cattle (Authié et al., 1992). Trypanotolerant cattle, originating from West Africa, have a genetically determined ability to limit multiplication of the parasite and to resist the pathogenic effects of infection (Roelants,

1986; Murray and Dexter, 1988; Trail et al., 1989). Studies have shown that congopain may play a role in the different levels of tolerance (Authié et al., 1993a, 1993b, Authié et al., 2001, Lalmanach et al., 2002). The important role played by congopain in pathogenicity was evident in a study where recombinant congopain was used to immunise susceptible cattle (Authié et al., 2001). Congopain-immunised cattle, together with a control group of non-immunised cattle, were infected via tsetse fly bites with *T. congolense*. During the acute phase of the disease, no major difference was observed in the state of health of the animals in the congopain-immunised group compared to the control group. At the later stage of the disease, however, immunised cattle maintained or gained weight, exhibited less severe anaemia, developed prominent humoral immune responses to trypanosome antigens and their haematocrit and leukocyte counts showed a tendency to recovery after two to three months of infection (Authié et al., 2001). A more efficient immune response to congopain may thus ameliorate the disease in trypanotolerant cattle (Authié, 1994).

1.6 Adjuvants and α_2-macroglobulin

New vaccines which require less immunogen, and which can be produced more economically, are under development (Cianciolo et al., 2002). When a whole parasite or virus is used in a vaccine formulation it will contain, in addition to the immunogens or epitopes that induce a protective response, numerous additional epitopes that may have a suppressive effect on the immune response. A more effective response may be induced by using the isolated protective immunogen to produce what is known as a subunit vaccine. Congopain and its epitopes may be used in the subunit vaccine concept. Subunit vaccines are usually of high purity and may thus lack self-adjuvanting components which are found in attenuated or killed vaccines. Consequently, subunit vaccines may have weaker immunogenicity (Vogel, 2003). Adjuvants are used to ensure high quality and quantity, memory-enhanced antibody responses. Formulation of vaccines with potent adjuvants can improve the immunogenicity of subunit vaccines.

A number of adjuvants have become available for use in experimental animals. However, many are not suitable because of their high level of toxicity (Cianciolo et al., 2002). The benefits of incorporating adjuvants into vaccine formulations must be weighed against the risk of these antigens causing undesirable reactions (Vogel, 2003). The most commonly used

adjuvant is Freund's complete adjuvant. This consists of a non-metabolisable mineral oil, a surfacant (Arlacel A) and *Mycobacterium tuberculosis* or *M. butyricum* organisms or components of the organism (Penney et al., 1998). It is used as a water-in-oil emulsion with water-soluble immunogen. The antigen is released from the emulsion slowly over a period of time, therefore effectively increasing the chances for interaction with macrophages. Freund's complete adjuvant is effective but there are many disadvantages associated with its use. Responses to it are antigen dependent and so it is not always the best choice. It should be used when only small amounts of antigen are available and only for initial immunisations, because chronic inflammatory responses may occur upon second exposure of the experimental animal to *Mycobacteria* (Penney et al., 1998). Furthermore, this adjuvant is not safe for laboratory personnel because of the presence of harmful mycobacteria (*M. tuberculosis* or *M. butyricum*) which respond poorly to antibiotic treatment (Hanly, 1995).

Freund's incomplete adjuvant is composed of the same oil/surfactant mixture as Freund's complete adjuvant but does not contain harmful mycobacteria. However, this adjuvant favours humoral immunity without cell-mediated immunity and is therefore usually used in booster injections (Hanly, 1995). It can also cause inflammatory responses but to a lesser extent than Freund's complete adjuvant.

Other, effective adjuvants also have their limitations. Alternative adjuvants with their shortcomings are summarised in Table 3. Reactions to adjuvants in experimental animals include malaise, fever, adjuvant arthritis and anterior ureitis (Allison and Byars, 1991). A safe, effective, more potent adjuvant than those currently available would help in the development of new vaccines.

Alpha-2-macroglobulin (α_2M) is a natural protein inhibitor of proteinases and has been suggested to have another important functional role, i.e. of effectively enhancing delivery of antigens to macrophages. The excellent adjuvanticity of α_2M has been proven in a small number of studies. Evidence for dramatically increased immunogenicity of hen egg lysozyme (Chu and Pizzo, 1993; Chu et al., 1994), prostate-specific antigen (Otto et al., 1998), α_2M-bound peptides (Binder et al., 2001) and hepatitis B virus surface antigen (Cianciolo et al., 2002), incorporated into α_2M, have been reported.

Table 3. Commonly used alternative adjuvants with their shortcomings (adapted from Hanly, 1995 and Penney et al., 1998).

Adjuvant	Composition	Disadvantage
Montanide incomplete Seppic (Seppic, Paris, France)	Different surfactants combined with either non-metabolisable, metabolisable, mineral oil or mixtures	Chronic inflammatory response may occur
Ribi's (Ribi ImmunoChem Research, Inc., Hamilton, MT, USA)	Oil, detoxified endotoxin and mycobacterial cell wall components in 2% squalene	Limited to certain antigens and species Bias antibody response to epitopes of native protein rather than to epitopes of denatured protein
Hunter's TiterMax (CytRx Corp., Norcross, GA)	Oil/surfactant-based	Inflammatory reaction may occur
Aluminum salt (Accurate Chemical and Scientific Co., Westbury, NY)	$Al(OH)_3$	Less effective than emulsion adjuvants Frequent boosting required
Nitrocellulose-adsorbed protein	Nitrocellulose paper	Poor immune response Frequent boosting required Antibodies raised may not react well with native protein
Encapsulated antigens	Antigen entrapped in liposomes, ethylene-vinyl acetate copolymer Poly (DL-lactide-co-glycolide)	Preparation is complex
Gerbu (Gerbu Biotechnik GMbH, Gaiberg, Germany)	Immunostimulators and zinc proline	Frequent boosting required
Syntex (Syntex, Palo Alto, CA)	Oil, Tween 80 and pluronic polyoxyethlene	Activates complement by the alternate pathway and is said to bias the humoral immune response to IgG2a in the mouse

A method which allows incorporation of a variety of subunit vaccine candidates into α_2M was reported by Cianciolo et al. (2002). Complexes of antigens and α_2M could be used as effective vaccines since antigens complexed with α_2M showed enhanced immunogenicity compared to antigens delivered with other adjuvant systems (Liao et al., 2002). As an adjuvant α_2M is entirely natural and there have been no risks reported with its use. Antigens incorporated into α_2M are sequestered and therefore protected from degradation until delivered to macrophages (Liao et al., 2002).

α_2M inhibits proteinases by a 'trapping' mechanism (Barrett et al., 1979). Initially, α_2M cleaves proteinases and the cleavage triggers a conformational change in α_2M that traps the proteinase. Conformational changes of α_2M-protein complexes expose receptor recognition sites on α_2M. This allows rapid delivery of α_2M-protein complexes to cells which have α_2M receptors, such as macrophages (Chu and Pizzo, 1994). Non-proteolytic immunogens can also be incorporated into α_2M by the method introduced by Cianciolo et al. (2002). In this method, α_2M is incubated with ammonia and this leads to the cleavage of the bait region of α_2M without any proteolysis step. Consequently, α_2M is converted to receptor-recognised α_2M. The non-proteolytic antigen is heated together with α_2M-NH_3 to 50°C and upon cooling to RT the non-proteolytic immunogen is incorporated into α_2M (Cianciolo et al., 2002).

It has been demonstrated that α_2M interacts with the trypanosomal cysteine proteinase, cruzipain (Ramos et al., 1997; 2002). It is possible that it also interacts with other trypanosomal proteases, such as congopain. It was thus necessary to determine whether α_2M interacts with congopain before the possibility of enhancing the immunogenicity of congopain and its epitopes by complexing with α_2M could be explored. This result could then be compared with those obtained with other adjuvants such as RWL (proprietary adjuvant from Smith Kline Beecham), which has been used successfully in the immunisation of cattle with congopain (Authié et al., 2001). The structure of α_2M and its mode of interaction with congopain and other proteases will be detailed in Chapter 5.

1.7 Proteases in parasites

The five major classes of proteases (aspartic, serine, cysteine, metallo and threonine) catalyse the hydrolysis of polypeptide bonds. The protease enzymes control protein synthesis, turnover and function and consequently regulate physiological processes such as digestion, fertilisation, growth, differentiation, cell signalling, immunological defence, wound healing and apoptosis (Leung et al., 2000). These functions extend from the cellular level to the organ and organism level, to produce a number of systems like haemostasis and inflammation, and complex processes at all levels of physiology and pathophysiology.

Proteases of parasitic protozoa are also involved in different aspects of host-parasite interactions. They facilitate the invasion of host tissues and allow nutrition as well as the

survival of the parasite in its host. They also participate in the parasite's evasion of the host's immune response (Trap and Boireau, 2000; Paugam et al., 2003).

The function of proteinases in parasitic protozoa is generally not well known. Members of all five major classes of proteases have been identified in parasitic protozoa. For example, *T. cruzi*, contains cysteine, serine, threonine, and metallo proteinases (Cazzulo, 2002). Several proteinases of 25-55 kDa were identified from *in vitro* cultures of muscle larvae of *Trichinella spiralis*; these were serine, cysteine and metalloproteinases active at pH 5-7 (Todorova, 2000). Serine proteinases are present in African trypanosomes (Kornblatt et al., 1992; Troeberg et al., 1996; Morty et al., 1999). A serine oligopeptidase has been purified from *T. congolense* (Morty et al., 1999), *T. b. brucei* (Troeberg et al., 1996) and *T. cruzi* (Ashall, 1990).

The class of trypanosomal protease most widely studied is the cysteine proteinases. Homologous enzymes of trypanopain-Tc or congopain from *T. b. brucei* and *T. cruzi* are called trypanopain-Tb and cruzipain respectively. These seem to be important proteinases in pathogenesis because they readily digest host proteins (Coetzer, 1998).

Proteinases of many parasites are under investigation as potential contributors to pathogenesis and as possible targets for novel anti-parasite drugs, with special attention focused on the cysteine proteinases. Parasite proteinases studied in this context include those from protozoa; *Trypanosoma* (Lonsdale-Eccles and Grab, 2002); *Leishmania* (Rafati et al., 2001; 2002); *Plasmodium* (Raphael et al., 2000; Rosenthal et al., 2002); *Trichinella spiralis* (Todorova 2000); four helminths species; *Schistosoma*, *Fasciola*, *Taenia* and *Heamanchus* (Trap and Boireau, 2000); *Entamoeba histolytica* (Que and Reed, 2000) and the nematodes (Zang and Maizels, 2001). In all these organisms, cysteine proteinases have been found to be the major proteinases and located in the lysosomes. Cysteine proteinases of protozoan parasites are considered to have a high potential as targets for novel anti-parasite agents (Rafati et al., 2002).

1.7.1 Cysteine proteinases

The cysteine proteinases of various pathogens have received attention as potential targets for chemotherapeutic intervention. Cysteine proteinases of malaria parasites offer potential new chemotherapeutic targets. Cysteine protease inhibitors block parasite haemoglobin hydrolysis indicating that cysteine proteinases play a major role in haemoglobin degradation, a necessary function for development of erythrocyte trophozoites (Rosenthal et al., 2002). Cysteine proteinases are a key virulence factor of *E. histolytica* and play a role in intestinal invasion by degrading the extracellular matrix and circumventing the host's immune response (Que and Reed, 2000).

For trypanosomes, cysteine proteinase inhibitors have been shown to kill *T. cruzi* (Cazzulo et al., 2001), *T. congolense* (Mbawa et al., 1992; McKerrow et al., 1999) and *T.b. brucei* (Troeberg et al., 1999). Current knowledge of the parasite enzyme has arisen largely from analysis of enzyme activities, many of which have been found to show variation between life-cycle stages, or to occur in unusual locations such as the blood brain barrier. Parasites contain many cysteine proteinases. Although they perform many different roles, there may be some overlap between the actions of the individual enzymes (Robertson et al., 1996).

The essential amino acid residues in the active site of cysteine proteinases are cysteine, histidine and asparagine in positions 25 and 159 and 175 respectively, based on the numbering of papain. These three amino acid residues form a catalytic triad involved in the catalytic function of the enzyme (Turk et al., 1998). Cysteine proteinases are most reactive at slightly acidic pH values but some are also active at basic pH values (Dehrmann et al., 1995). The active site cysteine residue acts as a nucleophile that attacks the carbonyl carbon of the susceptible peptide bond of the substrate. The active site histidine residue forms a thiolate imidazolium ion pair with active site cysteine residue. During hydrolysis a covalent acyl-enzyme intermediate is formed (Storer and Menard, 1996).

Congopain, trypanopain-Tb and cruzipain are thought to have active sites similar to that of papain. These proteinases belong to the papain superfamily. Papain has a large active site which extends over about 25Å and can be divided into seven "subsites" each accommodating one amino acid residue of the peptide substrate. The substrates are lined up in the active site in such a way that the scissile bond always occupies the same place (close to the catalytic site).

The amino acid residues that occupy adjacent subsites are those towards the NH_2–end, (called $P_1$to P_4), occupying subsites S_1 to S_4, and those towards the COOH-end (called P_1' to P_3') occupying subsites S_1' to S_3' (Figure 3) (Schechter and Berger, 1967). The substrate is visualised as fitting into the pocket, binding to several subsites of specific geometry (Figure 3).

The protease activity in *T. congolense*, *T.b. brucei* and *T. vivax* changes from one life cycle stage to another (Mbawa et al., 1991a). The highest level of protease activity is in the bloodstream forms of the parasite and, therefore, proteases are thought to play an important role in pathogenesis (Mbawa et al., 1991a).

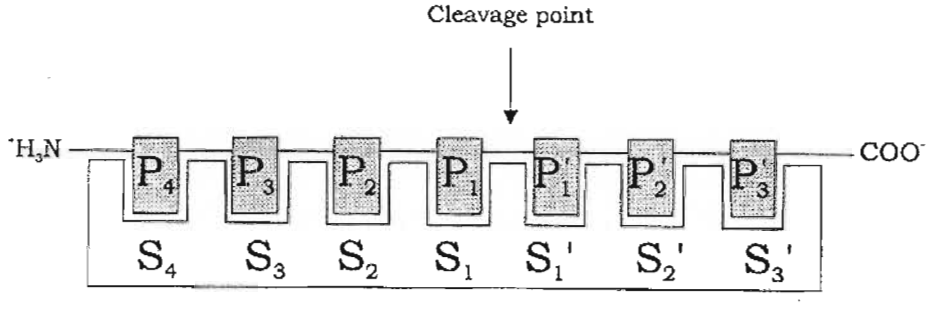

Figure 3. Schematic representation of the enzyme-substrate complex for cysteine proteinases (Schechter and Berger, 1967). (S) represents the subsite of the cysteine proteinases active site and (P) represents the corresponding substrate amino acid residue. The scissile bond is indicated by an arrow.

1.7.2 Congopain

T. congolense has at least two families of closely related cysteine proteinases, named CP1 (Fish et al., 1995, EMBL accession number Z25813) and CP2 (Jaye et al., EMBL accession number L25130). The deduced amino acid sequences of the two cysteine proteinases are 90% identical (Figure 4) but they differ in their N-terminal sequences and in their catalytic domains (Boulangé et al., 2001). Congopain is a 33 kDa cysteine proteinase purified from *T. congolense* bloodstream form lysates (Authié et al., 1992) and has been shown by N-terminal sequencing to belong to the CP2 type of enzymes (Lalmanach et al., 2002). This enzyme is equivalent to the trypanopain-Tc, identified by Lonsdale-Eccles and Grab (1987) and Mbawa et al. (1991a), which has an apparent molecular weight of 32 kDa when analysed by SDS/PAGE and 66 kDa when analysed by molecular exclusion chromatography (MEC). No CP1-type of enzyme has yet been isolated from *T. congolense*. Among *T. congolense* cysteine

proteinases, only congopain (CP2) has been studied in detail.

Congopain shares a high sequence identity with the cathepsin L-like enzyme from *T. cruzi*, cruzipain (McKerrow et al., 1995). The alignment of trypanosome cysteine proteases from *T. congolense*, *T. vivax*, *T. b. brucei* and *T. cruzi* with mouse cathepsin L and plant papain is shown in Figure 4. Like mammalian cysteine proteinases, congopain is synthesised as a zymogen that is converted to the mature form by proteolytic release of the pro-region. This maturation can also be effected by autolysis *in vitro* under acidic conditions (Boulange et al., 2001). The transcript of congopain can be divided into four parts, viz. a pre-region, a pro-region, a catalytic domain and a 130 amino acid residue C-terminal extension that is joined to the catalytic domain by a proline-rich hinge region (Lalmanach et al., 2002). The hinge region forms a flexible region readily accessible to proteolytic cleavage.

The C-terminal extension is a feature that distinguishes cysteine proteases of trypanosomatids from other cysteine proteases of the papain superfamily. The function of the C-terminal extension is as yet unknown (Stoka et al., 2000). It has been suggested that it may have a role in immune evasion, as the extension is highly immunogenic and could potentially divert the host's immune response (Lalmanach et al., 2002).

Previous studies showed that the C-terminal extension is not required for protein folding, enzyme trafficking or catalysis (Stoka et al., 2000). Unlike many mammalian lysosomal cysteine proteases, congopain and cruzipain are stable at both acidic and alkaline pH (Lalmanach et al., 2002). Initially, it was proposed that the C-terminal extension may play a role in the stability of trypanosomal cysteine proteinases. However, Stoka et al. (2000) showed that truncated cruzipain, devoid of the C-terminal domain, has the same stability as the full length enzyme. This proved that the different stabilities of congopain and cruzipain, compared to related mammalian proteases, may be due to differences in the number, nature and distribution of charged residues within the catalytic domain and not due to the presence of the C-terminal domain. These authors reached this conclusion from studies on cruzipain stability using different, same-molarity buffer systems with appropriate pKa values over the pH range of 2.25 to 12, without taking account of the change in ionic strength. For a constant molarity phosphate buffer, ionic strength increases sigmoidally between pH 5.5 to 8.5, with the most dramatic change at the pKa (pH 7.21). Stoka et al. (2000) used phosphate over the range of 5.7

to 7.5. It was shown by Dehrmann et al. (1995) that failing to take account of the effect of ionic strength, overstates the effect of increasing pH on the activity and stability of e.g. cysteine proteinases. Hence it is erroneously generally accepted that mammalian cysteine proteinases are not significantly active and stable at neutral pH. It would be interesting to repeat the experiments of Stoka et al. (2000) using acetate-MES-Tris buffers of constant ionic strength (Ellis and Morrison, 1982) over the pH range tested.

A 3D model of congopain has been proposed, based on the sequence homology (68% identical) between cruzipain and CP2 (Lecaille et al., 2001). The model has a structure similar to that of most papain-like cysteine proteinases (Figure 5). It folds into two domains the L-domain and the R-domain. The catalytic triad (Cys 25, His 159 and Asn 175; papain numbering) are located in the cleft between the R-domain and the L-domain (Lecaille et al., 2001).

Dithiothreitol and 2-mercaptoethanol are the most effective agents for the activation of isolated congopain, while glutathione and cysteine are only moderately effective (Mbawa et al., 1992). This means that the activity of isolated congopain is enhanced by the presence of reducing agents. Congopain has an optimal activity at pH 6.0 with a broad peak of activity extending well into the alkaline range (pH from 4.0 to 8.5) (Boulangé et al., 2001). This is similar to the characteristics of cysteine proteases of protozoan parasites in general (North et al., 1990b).

Congopain is a Z-Phe-Arg-NHMec-hydrolysing enzyme that has a noticeable preference for aromatic residues at P2. Lalmanach et al. (1993) observed that the hinge region between the catalytic domain and the C-terminal extension has cystatin-like motifs. Furthermore, cystatin-derived peptides have been identified as congopain (and other papain-like proteases) sensitive. Based on these findings, the amino acid sequence LVG, which constitutes the N-terminal portion of the inhibitory site of cystatin C, and QVG, which constitutes the central portion of cystatin C, have been used to form internally quenched fluorogenic substrates of the series Abz-peptide-EDDnp (Abz, O-amino-benzoyl; EDDnp, ethylenediamine 2-4-dinitrophenoyl). Abz-His-Pro-Gly-Pro-Gln-EDDnp is hydrolysed by trypanosomal cysteine proteinases below pH 6.8. Furthermore, it competitively inhibits cruzipain and congopain above pH 6.8 and mammalian cathepsins B and L at all pH values (Serveau et al., 1999).

It is more likely that CP2, rather than CP1, plays pathogenic roles in the host. The catalytic domains of CP1 (referred to as C1) and of CP2 (referred to as C2) were expressed and characterised (Boulangé et al., 2001). C2 appears to be stable over a wide pH range, while C1 is rapidly inactivated at neutral and alkaline pH and strictly requires acidic pH for its activity. Therefore, CP1 is unlikely to be active at physiological pH to interact with host proteins. Kinetic studies confirmed the relatedness between C2 and native congopain isolated from *T. congolense* bloodstream forms, but did not relate C1 to any of the described trypanosomal cysteine proteinases (Boulangé et al., 2001). The results obtained by Authié et al. (2001) indicated that both CP1 and CP2 may contribute to the mechanism of anaemia, while mainly CP2 appears to be involved in trypanosome induced immunosuppression. A predominant role of CP2 over CP1 in pathology would be consistent with the capacity of CP2, but not of CP1, to degrade protein substrates at physiological pH. It has been hypothesised, therefore, that these closely related but distinct enzymes perform different roles *in vivo* in the parasite and in the host-parasite relationships (Boulangé et al., 2001).

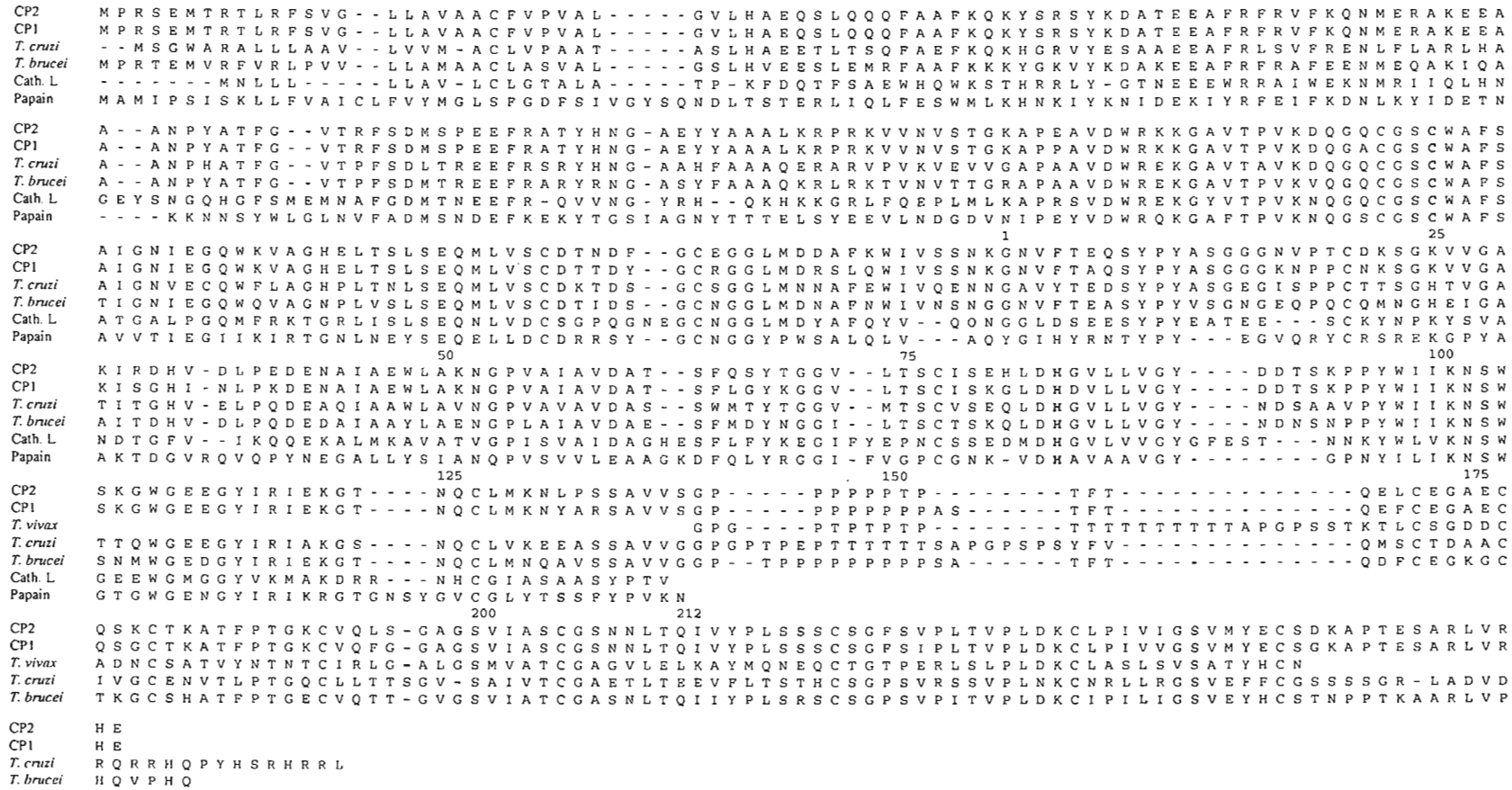

Figure 4. Alignment of the *T. congolense* cysteine proteinases, CP2 and CP1, with related cysteine proteinases. The deduced amino acid sequences of CP2 (Jaye et al., EMBL accession number L25130) and CP1 (Fish et al., 1995) are aligned with the deduced amino acid sequences of the cysteine proteinases from *T. cruzi* (Eakin et al., 1992) and *T. brucei* (Mottram et al., 1989), human cathepsin L (Ritonja et al., 1988), papain (Cohen et al., 1986) and the C-terminal extension of a cysteine proteinase from *T. vivax* (Prof. Theo Baltz, University of Bordeaux, France, personal communication). The numbering system for mature papain (Drenth et al., 1971) is shown.

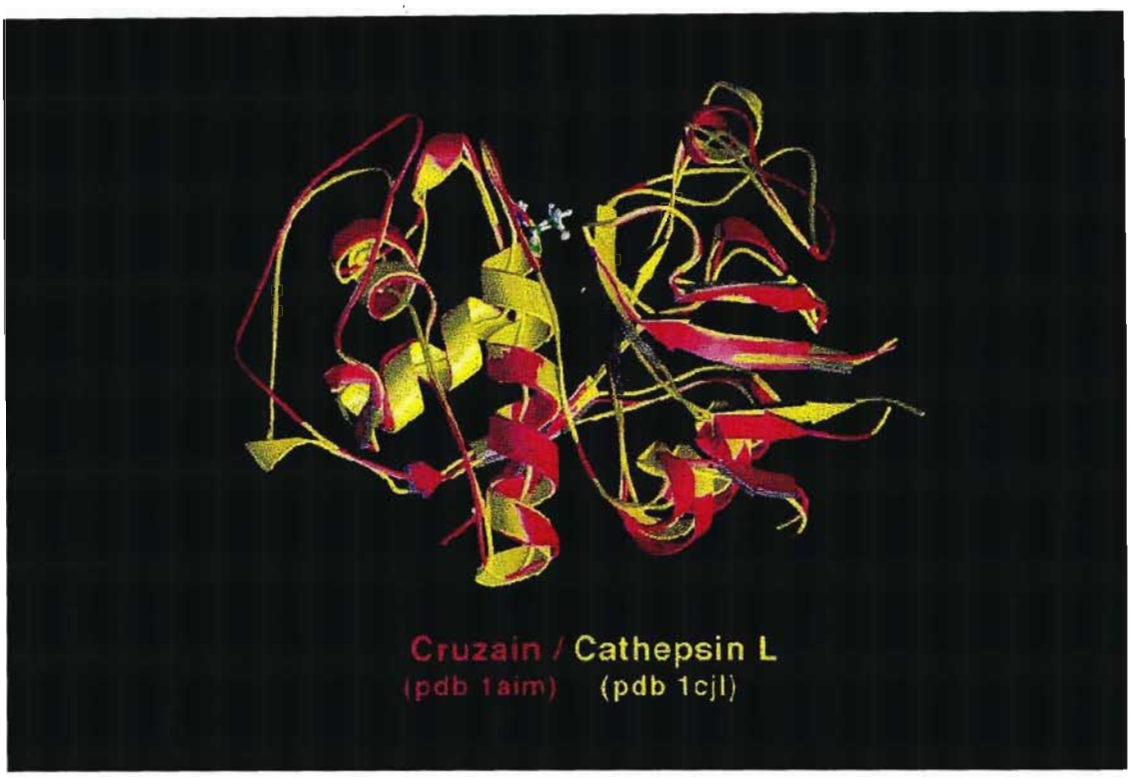

Figure 5. The homology-based model of the catalytic domain of congopain (Lecaille et al., 2001). The X-ray crystallographic structure of cruzain is represented in pink and that of cathepsin L in yellow. The catalytic triad (Cys 25, His 159 and Asn 175; papain numbering) is located between the L-domain (helical) and the R-domain (anti-parallel sheets).

Several important roles are played by congopain (CP2) in the survival of the parasites, as evidenced by the finding that potent congopain inhibitors, Z-Leu-Leu-Met-CHN_2, Z-Leu-Met-CHN_2 and Z-Leu-Lys-CHN_2 were trypanocidal (Mbawa et al., 1992). Furthermore, trypanotolerant cattle have anti-congopain antibodies, whereas susceptible cattle do not (Authié et al., 1993b). The lysosomal location of congopain makes it likely that the enzyme is involved in digestion of exogenous protein taken up into the cell by pinocytosis as a source of nutrition for the parasite (Mbawa et al., 1991b). Congopain may also protect the parasite against attack by the host immune system because it is involved in the intralysosomal processing or turnover of the VSG proteins (Mbawa et al., 1991a).

Congopain (CP2) catalyses the limited proteolysis of a variety of protein substrates such as fibrinogen and serum albumin *in vitro* and this could present a mechanism by which the enzyme contributes to disease pathology in the host bloodstream (Mbawa et al., 1992). Thus inhibition of CP2 by specific chemical inhibitors or specific antibody produced by

trypanotolerant cattle during infection, or artificially elicited by immunisation, may result in higher resistance to trypanosomosis.

1.8 Anti-trypanosomal therapy based on congopain

1.8.1 Chemotherapy

Many parasites are sensitive to cysteine proteinase inhibitors (Ashall et al., 1990; Wasilewski et al., 1996; Irvine et al., 1997). Peptidyl chloromethyl ketones, dimethyl formamides and diazomethyl ketones are effective irreversible inhibitors of congopain (Mbawa et al., 1992). Congopain has a preference for an aromatic residue such as Phe at P_2 and for Pro at P_2' (Lalmanach et al., 2002). Peptidyl diazomethanes which have non-polar residues in P_2 and P_3 were found to be potent inhibitors for the enzyme. They were about 100-fold more effective inhibitors than the classical cysteine protease inhibitor, E-64 (Mbawa et al., 1992a). However, reversible inhibitors are preferred for use *in vivo* because an irreversible inhibitor may eventually inhibit proteinases it reacts with only slowly, whereas a reversible inhibitor will not interfere with the activity of an untargeted proteinase when that inhibitor is at a concentration above the K_i for the reaction (Salvesen and Nagase, 1989).

Congopain is inhibited by cystatins and kininogens with K_i values in the pM range, similar to the inhibition constants obtained for native cruzipain (Lalmanach et al., 2002). However, complexes between congopain and their natural inhibitors have been shown to have enzymatic activity (Lalmanach et al., 2002). This implies that even in the presence of natural inhibitors, congopain may retain its activity, and this may explain its role in pathogenicity.

Isolated propeptides may still bind and inhibit their mature enzyme even when released from the proenzyme (Fox et al., 1992; Mach et al., 1994). The ability of prodomains of trypanosomal cysteine proteinases to inhibit their active form was studied using a set of 23 overlapping 15-mer peptides covering the whole pro-sequence of congopain (Lalmanach et al., 1998). Three consecutive peptides with a common 5-mer sequence YHNGA were found to be competitive inhibitors of congopain (Lalmanach et al., 1998). These results agreed with the findings of Tao et al. (1994). Pro-peptides of mammalian and plant cysteine proteinases were found to take part in the proper folding, intracellular trafficking or secretion of the mature

proteinases, and in the control of proteolytic activity by blocking the active site (Tao et al., 1994). The YHNGA-containing peptides specifically inhibited cysteine proteinases from other trypanosomal species but not mammalian lysosomal cathepsins. The K_i values of these pro-region derived peptides are too high to make these peptides efficient inhibitors. Nevertheless, these YHNGA containing peptides represent a promising new feature that may contribute to the design of proteinase-directed anti-parasitic drugs of therapeutic interest (Lalmanach et al., 1998).

1.8.2 Antibody therapy

Another approach for interfering with the activity of proteases is the use, in passive immunity, of anti-catalytic antibodies produced in experimental animals. Congopain was identified as a major antigen recognised in cattle and human infections (Authié et al., 1993a). Immune recognition of parasite factors involved in pathogenesis may be one of the processes by which trypanotolerant animals control the disease. Antibodies against congopain are not likely to be internalised by trypanosomes, therefore they are not likely to affect the survival of the parasite itself. Anti-congopain antibodies may, however, be useful in ameliorating the disease since congopain is likely to be the main pathogenic factor of *T. congolense* (Lalmanach et al., 2001). An IgG-antibody response may be important either in inhibiting some of the enzyme activity, or in removal of circulating proteases via immune complexes. Thus, animals which are not able to induce an antibody response may be exposed to more of the potential harmful effects of the protease (Authié et al., 1993b). For this reason, congopain was used for immunisation in cattle, and it was found that congopain is antigenic in both trypanotolerant and susceptible cattle. Antibody response to congopain appeared to be associated with higher resistance to disease (Authié et al., 1992, Authié et al., 1993a). However, the switch from IgM to IgG was delayed in susceptible cattle during infection (Authié et al., 1993a).

An immunisation trial was conducted where trypanosusceptible cattle were immunised with a recombinant catalytic domain of CP2, called C2 (Authié et al., 2001). Cattle immunised with C2 developed features typical of trypanotolerant cattle, such as development of antibodies against other trypanosome antigens such as VSG. Antibodies against C2 had an inhibitory effect on congopain, although the inhibition was not as effective as that of antibodies from *T. congolense* infected N'Dama (trypanotolerant) cattle (Authié et al., 2001). These results show

that it may be possible to increase resistance to trypanosomosis in susceptible cattle through immunisation against pathogenic factors of the parasite.

Both anti-whole congopain antibodies (Authié et al., 1993b) and anti-C2 antibodies (Authié et al., 2001) had an inhibitory effect on congopain activity. This suggests that the essential epitopes for inducing inhibitory antibodies are likely to be located in the catalytic domain of the enzyme. It may thus be possible to produce antibodies against a particular peptide selected from the catalytic domain that will inhibit congopain activity. The production of anti-peptide antibodies is preferred because anti-peptide antibodies have specificity more similar to that of monoclonal antibodies than to anti-protein polyclonal antibodies (Lerner, 1984). Furthermore, anti-peptide antibodies may recognise the target sequence in many different conformations and all have different binding sites because they bind overlapping regions of the peptide, thus allowing the enzyme to be recognised in both its native and denatured forms (Muller, 1990b). A peptide consisting of a sequence of 10 or more amino acids is likely to produce specific antibodies because there is a good chance that that peptide would be unique to that protein (Briand et al., 1985).

The peptides critical for the induction of effective immunity against congopain (congopain epitopes) have not been identified. To raise effective anti-congopain peptide antibodies, immunogenic epitopes in congopain need to be identified. Such epitopes can not only be used in raising anti-peptide antibodies that could be used for passive immunotherapy, but they could also be used in the development of a synthetic peptide-based vaccine (subunit vaccine) composed of purified components and devoid of substances that could carry the risk of the disease. These peptides could possibly also be used for diagnostic assays.

1.9 Objectives of the study.

Previous studies showed that the production of anti-congopain antibodies have some correlation with trypanotolerance (Authié et al., 1993a,b; Authié 1994; Authié et al., 2001). This implies that there are epitopes in congopain responsible for inducing antibodies that inhibit enzymatic activity. This type of epitope was previously identified in our laboratory for trypanopain-Tb and the resulting anti-peptide antibodies were shown to bind to the whole parent enzyme and to inhibit enzyme activity (Troeberg at al., 1997). Such epitopes could

replace whole recombinant congopain, currently under evaluation by collaborators of this laboratory, as a subunit vaccine. The major objective of this study was thus to map epitopes of congopain that could form the basis of a subunit vaccine. To evaluate the immunogenicity of the peptides selected from congopain, anti-peptide antibodies were raised against the peptides conjugated to a carrier protein. The resulting anti-peptide antibodies were examined for their ability to recognise whole congopain in ELISA and western blot assays and to inhibit enzyme activity (Chapter 3).

Epitopes of major pathogenic factors, such as those of congopain, may also have a use in diagnostics. As techniques commonly applied in the detection of trypanosome infections have poor sensitivity, they often fail to detect a high proportion of chronic and often aparasitemic infections (Masake et al., 2002). A diagnostic method for trypanosomosis which is sensitive, specific, simple and inexpensive is thus required. The peptides identified in Chapter 3 were tested for their ability to be recognised by sera from *T. congolense* infected cattle, C2 immunised, uninfected cattle and rabbits immunised with native congopain or with C2. Attempts were also made to identify peptides that would discriminate between *T. congolense* and *T. vivax* infections. These studies are described in Chapter 4.

One of the main determinants of protection elicited by a vaccine is the adjuvant vehicle (Cianciolo et al., 2002). The availability of a new, entirely natural adjuvant might assist in the efforts to exploit the use of a trypanosomal subunit vaccine. The general protease inhibitor, alpha-2-macroglobulin (α_2M) has potential as such an adjuvant. In this regard this study also aimed to first ascertain whether there is interaction between congopain and α_2M. α_2M was isolated from bovine plasma and the interaction between congopain and α_2M was demonstrated by inhibition assays, described in Chapter 5. The preliminary results of this study showing congopain-α_2M interaction paves a way for the possibility of using α_2M as an alternative natural adjuvant that could enhance the immunogenicity of congopain as well as congopain peptides. These peptides could be complexed with α_2M by the chemistry described by Cianciolo et al. (2002) which involves incubation of α_2M with ammonia, changing α_2M to a receptor recognised "fast" form in which the thiol ester of α_2M has been cleaved without any proteolysis step. This was, however, beyond the scope of this study.

Chapter 2
Materials and methods

2.1 Introduction

The various experimental methods described in this Chapter are the common biochemical techniques used throughout this study. These are mainly general protein methods, chromatographic techniques and immunochemical techniques. More specific experiments are described in their appropriate sections.

2.2 Materials

The following list includes specialised products that were used in this study. All general chemicals were of analytical reagent standard. Sephacryl S-300, imidodiacetic acid-epoxy-activated Sepharose 4B, Coomassie brilliant blue, sodium dodecyl sulfate (SDS), L-cysteine.HCl, ovalbumin, m-maleimidobenzoyl-N-hydroxysuccinimide ester (MBS), N-Benzoyl-Pro-Phe-Arg-p-nitroanilide hydrochloride (Bz-Pro-Phe-Arg-pNA), Carbobenzoxy-Phe-Arg-7-amino-4-methylcoumarin (Z-Phe-Arg-NHMec), dialysis tubing (12 kDa cut-off), rabbit albumin, azocasein and Freund's complete and incomplete adjuvants were from Sigma (St Louis, USA). 2,2′-Azinobis [3-ethyl-2, 3-dihydrobenzthiazole-6-sulphonate] (ABTS), bovine serum albumin (BSA), 5-bromo-4-chloro-3-indolylphosphate (BCIP), nitroblue tetrazolium (NBT), α_2-macroglobulin (α_2M) and dithiothreitol (DTT) were from Roche diagnostics (Mannheim, Germany). Protein M_r standards, Sephadex G-100 and Sephadex G-25 were from Pharmacia LKB Biotechnology (Lund, Sweden). Dry dimethyl formamide (DMF), dimethyl sulphoxide (DMSO) and glutaraldehyde solution were from Fluka (Buchs, Switzerland). Serva blue G-dye was from Serva (Heidelberg, West Germany). Gelatin was from Difco (Michigan, USA). Whatman No. 1 filter paper was from Whatman International Ltd (Maidstone, England). Tween 20 and Brij 35 were from Merck (Darmstadt, Germany). Nunc-Immuno Maxisorp F96 plates and FluorNunc® 96 well plates were from AEC Amersham (SA). Disposable polystyrene columns were from Biorad (Hercules, California). SulfoLink™ and AminoLink™ coupling gels were from Pierce (Rockford, USA). Distilled H_2O and deionised water was produced by Milli-Q Plus ultra pure water system (Millipore,

Marlboro, USA). Purified recombinant CP2 and C2 expressed in *Pichia pastoris* were provided by Edith Authié [International Livestock Research Institute (ILRI), Nairobi, Kenya].

2.3. Bradford dye binding assay

This is a rapid and sensitive method for the quantification of protein (Bradford, 1976). Coomassie brilliant blue G-250 exists in two different colour forms, red and blue. This dye binds to most basic amino acid side chains, primarily to arginine, and to a lesser extent to histidine and lysine and also to tyrosine, tryptophan and phenyalanine. Upon binding of the dye to protein, the red cationic form, which absorbs maximally at 470 nm, is converted to the blue anionic form, which absorbs maximally at 595 nm (Compton and Jones, 1985).

A micro assay, which quantifies small amounts of protein, described by Read and Northcote (1981) was used in this study. This is a modification of the method of Bradford (1976) and uses Serva Blue G dye to minimise variation in the response to different standard proteins. However, the sensitivity is decreased (Read and Northcote, 1981).

2.3.1 Reagents

Dye reagent. Serva blue G dye (50 mg) was dissolved in 88% phosphoric acid (50 ml) and 99.5% ethanol (23.5 ml). The solution was made up to 500 ml with distilled H_2O, stirred for 30 min on a magnetic stirrer, filtered through Whatman No. 1 filter paper and stored in an amber coloured bottle at room temperature. It is stable for several weeks but during this time the dye may precipitate and so the stored reagent was inspected for precipitate, filtered if necessary and recalibrated before use.

Standard protein solution (1 mg/ml ovalbumin). Ovalbumin (10 mg) was dissolved in distilled H_2O (10 ml). This stock solution was diluted to 100 µg/ml before use for the micro-assay.

2.3.2 Procedure

Protein standard (0-50 µl of the 100 µg/ml solution, i.e. 1-5 µg) or sample was diluted to 50 µl with distilled H_2O in 1.5 ml polyethylene microfuge tubes. Dye reagent (950 µl) was added, and mixed in by inversion. The absorbance at 595 nm was determined in 1 ml plastic micro-

cuvettes, against reagent blank, between 2 min and 1 h after mixing. Mean absorbance values of four replicates were used to construct a standard curve. The concentration of the sample of unknown concentration was calculated from an equation generated by linear regression of the standard curve values.

2.4 Concentration of protein samples

2.4.1. Dialysis against PEG

Dilute samples, usually obtained after protein purification steps, were concentrated before molecular exclusion chromatography using dialysis against polyethylene glycol (PEG) M_r 20 000. The dialysis tubing pore size prevents the protein from escaping (Bollag et al., 1996). Water from the dilute sample moves along a concentration gradient out of the dialysis bag to dissolve dry polymer, thereby concentrating the sample without affecting its activity.

2.4.1.1 Procedure

The dialysis bag was soaked in distilled H_2O for 10 min before use. The dilute protein sample was placed in the dialysis bag, which was surrounded by solid PEG 20 000. The dialysis bag was covered with solid PEG and stored at 4°C. The bag was rinsed with distilled H_2O after concentration, and the sample was squeezed out.

2.4.2 SDS/KCl precipitation

Dilute samples were sometimes concentrated, before reducing SDS-PAGE, by precipitation with sodium dodecylsulfate (SDS) and KCl. A negatively charged complex of protein and anionic detergent, SDS, is formed. This complex is precipitated out of water solution by reacting with potassium which precipitates KDS (Hames, 1981). The concentrated protein sample has a high salt concentration and this method is therefore only suitable for reducing SDS-PAGE.

2.4.2.1 Reagents

5% (w/v) SDS. SDS (0.5 g) was dissolved in 10 ml of distilled H_2O.

3 M KCl. KCl (2.24 g) was dissolved in 10 ml of distilled H_2O.

2.4.2.2 Procedure

5% SDS (10 µl) was added to the sample (100 µl) in a polyethylene microfuge tube. The solution was mixed by inverting the tube. 3 M KCl (10 µl) was added. The mixture was again inverted and centrifuged (12 000 x g, 2 min, RT), the supernatant discarded and the precipitate dissolved in stacking gel buffer (10 µl) and reducing treatment buffer (10 µl) (Solution G, Section 2.5.1) added.

2.5 SDS-PAGE

The disc electrophoresis method for analysing proteins consists of two gels, a small pore running gel (with higher pH) overlain by a large-pore stacking gel (with lower pH). The protein sample is layered on top of the stacking gel. The gels contain Tris buffer with a counter ion of high mobility (e. g. Cl^-), called the leading ion (Ornstein, 1964). The electrode compartments on the other hand contain Tris buffer with a counter ion of relatively low, but pH-dependent mobility (e. g. glycinate$^+$), called the trailing ion. Upon application of the electrical potential, a sharp interface is formed between the leading and the trailing ion as they migrate downwards, and the proteins of intermediate mobility are swept up and concentrated into a stack of thin bands between the leading and trailing ions. When the stack of proteins reach the running gel, a change in pH causes an increase in the mobility of the trailing ion and this passes the protein bands. Proteins are left behind to separate in a uniform voltage gradient (Ornstein, 1964).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) is a low cost, reproducible, and rapid method for comparing and characterising proteins. In this technique proteins are reacted with the anionic detergent, sodium dodecyl sulfate (SDS), to form negatively charged complexes (Makowski and Ramsby, 1997). SDS dissociates non-covalently bound polypeptide chains and unfolds these proteins to form rod-like complexes. SDS binds to most proteins at a constant ratio of 1.4 gram SDS per gram of protein (Reynolds and Tanford, 1970). The negative charge of SDS is sufficient to mask the charge on the protein. As a result, all proteins acquire the same charge/mass ratio and an anodic migration

(Makowski and Ramsby, 1997). The separation of proteins is thus only due to differences in size, controlled by the 'sieving effect' of the gel. The molecular weight of a protein is estimated by comparing the relationship between size and mobility of a protein band with that of standard proteins of known size (Makowski and Ramsby, 1997).

SDS-PAGE can be conducted under non-reducing or reducing conditions. In reducing SDS-PAGE, protein samples are combined with a treatment buffer which contains 2-mercaptoethanol. Mercaptoethanol reduces disulfide bonds. In the case of proteins in which disulfide bonds remain intact, the amount of SDS bound per unit mass of protein is less than normal because the protein is constrained and cannot unfold sufficiently to form the characteristic rod-like structure (Makowski and Ramsby, 1997). Glycerol increases the density of the sample and therefore aids in loading of the sample. A drop of saturated bromophenol blue, which migrates with the buffer front, is added to each sample before loading to allow monitoring of electrophoresis progress. The SDS-PAGE method described by Laemmli (1970) was employed in this study.

2.5.1 Reagents

Solution A: Monomer solution [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]. Acrylamide (73 g) and bis-acrylamide (2 g) were dissolved together and made up to 250 ml with distilled H_2O. The solution was filtered through Whatman No. 1 filter paper and stored in an amber coloured bottle at 4°C.

Solution B: Separating gel buffer (1.5 M Tris-HCl buffer, pH 8.8). Tris (45.37 g) was dissolved in approximately 200 ml of distilled H_2O, titrated to pH 8.8 using HCl and made up to 250 ml. The solution was filtered through Whatman No. 1 filter paper and stored at 4°C.

Solution C: Stacking gel buffer (0.5 M Tris-HCl buffer, pH 6.8). Tris (3 g) was dissolved in approximately 40 ml of distilled H_2O, titrated to pH 6.8 using HCl and made up to 50 ml. The solution was filtered through Whatman No. 1 filter paper and stored at 4°C. The pH of this solution was checked before use, because of its poor buffering capacity at 2.1 pH units below its pKa at 4°C (Pharmacia products catalogue).

Solution D: 10% (w/v) Sodium dodecyl sulfate (SDS). SDS (10 g) was dissolved in 100 ml of H_2O with gentle heating if necessary.

Solution E: Initiator [10% (w/v) ammonium persulfate]. Ammonium persulfate (0.2 g) was made up to 2 ml. This solution is stable for a week at 4°C.

Solution F: Tank buffer [0.25 M Tris, 0.192 M glycine, 0.1% (w/v) SDS, pH 8.3]. Tris (15 g) and glycine (72 g) were dissolved and made up to 5 l with distilled H_2O. Prior to use, 2.5 ml of SDS stock (solution E) was added to 250 ml for use in the Bio-Rad Mini-Protean® II appartus.

Solution G: Reducing treatment buffer [0.125 M Tris-HCl buffer, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with distilled H_2O and stored in aliquots at –20 °C.

Solution H: Non-reducing treatment buffer [0.125 M Tris-HCl buffer, 4% (w/v) SDS, 20% (v/v) glycerol, pH 6.8]. Buffer C (2.5 ml), 10% SDS (4 ml) (solution D) and glycerol (2 ml) were made up to 10 ml with distilled H_2O and stored at 4°C.

Table 4. Preparation of running and stacking gels of different acrylamide concentrations.

Reagent	Volume (ml)			
	Running gel (%)			Stacking gel (%)
	15.0	12.5	5.0	4.0
A	7.5	6.25	2.5	0.94
B	3.75	3.75	3.75	0
C	0	0	0	1.75
D	0.15	0.15	0.15	0.07
E	0.75	0.75	0.75	0.35
Distilled H_2O	3.5	4.75	8.5	4.3
TEMED	0.075	0.075	0.075	0.015

2.5.2 Procedure

The gel sandwich was assembled according to the manufacturer's instructions. The gel plates and spacers were cleaned with 70% ethanol. The gel plates and spacers were carefully placed against a flat surface before tightening the clamp to avoid misalignment which might result in a leak. The running gel solution was introduced into the gel sandwich, using a pipette, to a depth of 3 cm from the top of the front plate. The running gel solution was over-layered with distilled H_2O to allow for even polymerisation. When the gel polymerised (about 45-60 min.), a distinct interface appeared between the running gel and the water layer. The water was poured off. The stacking gel solution was poured into the gel sandwich on top of the separating gel until the solution reached the top of the front plate. A comb was inserted to form sample application wells. Once the gel had set (about 30 min.) the comb was removed and the wells were rinsed with distilled H_2O to remove unpolymerised acrylamide.

The gels were attached to the electrode assembly before inserting into the electrophoresis tank. The electrophoresis buffer (solution F) was added to the inner and outer reservoirs, ensuring that both top and bottom of the gels are immersed in buffer. For reducing SDS-PAGE, samples were combined with an equal volume of reducing treatment buffer (solution G) and incubated in a boiling waterbath for 2 min. The samples were cooled on ice before loading. For non-reducing SDS-PAGE, samples were combined with half their volume of non-reducing treatment buffer (solution H) before loading. Gels were run at 18 mA per gel until the bromophenol blue tracker was 0.5 cm from the bottom of the running gel. The gels were

removed from the glass plate sandwich and stained (Section 2.6) or subjected to electro blotting (Section 2.7).

2.6 Protein staining procedures

2.6.1 Coomassie blue staining

This method of staining is simple and rapid. It can detect up to 0.1 μg of protein in a single band. This method is based on the principle that dyes tend to be attracted to positively charged groups (Lys, Arg) in proteins. Consequently, basic proteins tend to stain more strongly than acidic proteins (Scopes, 1982). This method can, however, be used when high sensitivity is not required because of its simplicity.

2.6.1.1 Reagents

Stain stock solution [1% (w/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in distilled H_2O (100 ml) by magnetic stirring for 1 h. The solution was filtered through Whatman No. 1 filter paper. The solution was stored at RT.

Staining solution [0.125% (w/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 distilled H_2O. The solution was stored at RT.

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 distilled H_2O. The solution was stored at RT.

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 distilled H_2O. The solution was stored at RT.

2.6.1.2 Procedure

Gels were removed from the electrophoresis unit using gloves and transferred to a small container containing staining solution (50 ml, 4 h). The staining solution was poured out. Following rinsing with a few changes of distilled water, gels were placed into destain I (100 ml, overnight). Gels were soaked in destain II until the background had cleared fully and

stored hydrated in polythene zip-seal bags until photographed.

2.6.2 Silver staining

The method of silver staining was first introduced by Switzer et al. (1979). It provides detection sensitivity between 20-200 times higher than methods using Coomassie brilliant blue R-250, being able to detect about 0.1 ng protein/band (Switzer et al., 1979). In silver staining, ionic silver is reduced to its metallic form by formaldehyde made alkaline with either sodium carbonate or NaOH. Thiosulfate chemically dissolves salts by complexation. The improved method of silver staining of proteins introduced by Blum et al. (1987) utilizes thiosulfate and reduces non-specific background staining without loss of sensitivity and contrast.

2.6.2.1 Reagents

All reagents were stored at RT.

Fixative [50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) formaldehyde]. Methanol (100 ml), acetic acid (24 ml) and 37% formaldehyde (0.1 ml) were mixed and made up to 200 ml with deionised H_2O.

Wash solution [50% (v/v) ethanol]. Ethanol (100 ml) was made up to 200 ml with deionised H_2O.

Pre-treatment solution [0.02% (w/v) $Na_2S_2O_3$]. $Na_2S_2O_3.5H_2O$ (40 mg) was dissolved in 200 ml of deionised H_2O.

Impregnation solution [0.2% (w/v) $AgNO_3$, 0.075% (v/v) formaldehyde]. $AgNO_3$ (400 mg) and 37% formaldehyde (0.15 ml) were dissolved in 200 ml of deionised H_2O.

Developer [6% (w/v) Na_2CO_3, 0.05% (v/v) formaldehyde, 0.0004% (w/v) $Na_2S_2O_3.5H_2O$]. Na_2CO_3 (12 g), 37% formaldehyde (0.1 ml) and pre-treatment solution (4 ml) were combined and made up to 200 ml with deionised H_2O.

Stop solution [50% (w/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (12 ml) were diluted to 100 ml with deionised H_2O.

2.6.2.2 Procedure

The developing procedure was carried out on a Belly Dancer® agitator (Stovall Life Sciences, Greensboro, USA) and in scrupulously clean glass containers to minimize background staining. Gels were soaked in fixing solution (100 ml, 1 h or overnight) and washed in washing solution (3 × 20 min) to remove acetic acid. Gels were soaked in pre-treatment solution (1 min). Gels were washed in deionised H_2O (3 × 20 s) followed by soaking in impregnation solution (20 min). After washing in deionised H_2O (3 × 20 s), the developer was added. When the bands became visible, the developing solution was immediately replaced with deionised H_2O and the gels were washed until the bands were fully developed. Development was stopped by soaking the gels in stopping solution (10 min). Gels were stored hydrated in polythene zip-seal bags, until photographed.

2.7 Western and dot blotting

Western blotting is an electrophoretic technique that allows testing of recognition of individual protein bands separated on a SDS-PAGE gel with an antibody raised against a specific antigen (Towbin et al., 1979). This method is usually used to verify the identity of a protein band. In western blots, proteins are transferred electrophoretically from the SDS-PAGE gel to a sheet of nitrocellulose, which binds proteins well. The unoccupied sites on the nitrocellulose sheet are blocked with a non-antigenic protein, for example, non-fat milk to prevent non-specific immunoglobulin binding to the nitrocellulose. The nitrocellulose is treated with the primary antibody raised against the target protein and next with an enzyme linked secondary antibody that binds to the primary antibody. The marker enzymes most commonly used for detection of the antigen-antibody interaction are alkaline phosphatase and horseradish peroxidase (Copeland, 1994). Enzyme activity is visualized by incubating the nitrocellulose with an appropriate chromogenic substrate that is converted to a coloured, insoluble product (Kemeny and Chantler, 1988).

A dot blot assay is very similar to western blotting. The only difference is that, in dot blots, antigen is applied directly to the nitrocellulose in the form of a small dot without being transferred electrophoretically. This method was particularly useful when determining the

optimum secondary antibody dilution to use in a western blot or an ELISA by observing the relative intensity of the dots that resulted from label development.

2.7.1 Reagents

Blotting buffer [0.05 M Tris, 0.19 M glycine, 5% (v/v) methanol, 10% (w/v) SDS]. Tris (27.23 g) and glycine (64.8 g) were dissolved in approximately 3.5 litre of distilled H_2O, and methanol (900 ml) was added. The volume was made up to 4.5 litres and stored at 4°C. Just before use, 10% (w/v) SDS (4.5 ml solution D, Section 2.5.1) was added to 450 ml of blotting buffer.

0.1% (w/v) Ponceau S. Ponceau S (0.1 g) was dissolved in 1% (v/v) glacial acetic acid. The solution was stored at RT.

Tris buffered saline (TBS) (0.02 M Tris-HCl buffer, 0.2 M NaCl, pH 7.4). Tris (2.42 g) and NaCl (11.69 g) were dissolved in approximately 950 ml of distilled H_2O, titrated to pH 7.4 with HCl, and made up to 1 litre. This buffer was stored at 4°C.

5% Low fat milk (w/v) in TBS. Low fat milk powder (5 g) was dissolved in 100 ml of TBS.

0.5% (w/v) BSA-TBS. BSA (0.5 g) was dissolved in TBS (100 ml).

1 M $MgCl_2$. $MgCl_2$ (9.5 g) was dissolved in 100 ml of distilled H_2O.

0.05 M Tris-HCl buffer, pH 9.5. Tris (3.0 g) was dissolved in approximately 450 ml of distilled H_2O, titrated to pH 9.5 with HCl, made up to 500 ml and stored at 4°C.

5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NTB) alkaline phosphatase substrate. NTB (30 mg) was dissolved in 70% (v/v) dry dimethyl formamide (DMF) (1 ml) and BCIP (15 mg) was dissolved in 70% (v/v) DMF (1 ml). The two solutions were combined and 1 M $MgCl_2$ (500 µl) was added. The mixture was made up to 100 ml with 0.05 M Tris-HCl buffer, pH 9.5.

4-chloro-1-naphthol substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2]. 4-chloro-1-naphthol (0.03 g) was dissolved in methanol (10 ml). Two ml of this solution was mixed with 30% H_2O_2 (4 µl) and diluted to 10 ml with TBS.

2.7.2 Procedure

Western blotting After separation of proteins by SDS-PAGE (Section 2.5), usually on duplicate gels, one gel was stained directly and the other one used for immunoblotting. The Bio-Rad Mini Trans-Blot transfer cell was used. Gloves were worn when handling nitrocellulose. Nitrocellulose was cut to a suitable size and floated on top of the blotting buffer before immersion to prevent entrapment of air-bubbles. Three pieces of wetted blotting paper (NW 218 B, Macherey-Nagel, Germany) were placed on a well-soaked fibre pad on the black panel of the transfer cassette which in turn was submerged in blotting buffer. Blotting buffer was used for soaking. The gel was carefully placed on the wet filter paper. The immersed nitrocellulose was placed on top of the gel, and bubbles removed by rolling a small test tube over the nitrocellulose. Three more pieces of wetted blotting paper were placed over the nitrocellulose. The sandwich was covered with the second well-soaked fibre pad and the transfer cassette was closed.

Blotting buffer was added to a buffer chamber until half full and the stirrer bar was inserted. The cooling unit was inserted. The transfer cassette was slid into the electrode insert in the buffer tank, keeping the black panel of the cassette on the same side as the black panel of the electrode assembly to ensure that the nitrocellulose is connected to the anode. The buffer tank was filled with blotting buffer. The electrodes were attached and the entire Trans-Blot apparatus was placed on a magnetic stirrer and stirred throughout the process to allow even distribution of cooling. Electrotransfer of the proteins from the gel to the nitrocellulose was done at 30 V for 16 h with unlimiting current.

Following blotting, molecular weight markers were visualized by transient staining of the nitrocellulose sheet with 0.1% (w/v) Ponceau S, and with background stain removed with distilled H_2O. The positions of molecular weight markers were marked on the nitrocellulose in pencil and the blot was completely destained by the addition of a few drops of 0.5 M NaOH to the distilled H_2O.

Dot blotting. The protein samples were applied to a nitrocellulose sheet in the form of small dots. The visualisation steps for both western blotting and blot dotting are the same.

Development of blots. The nitrocellulose was air dried for about 1.5 h. Unoccupied nitrocellulose binding sites were blocked with 5% (w/v) low fat milk powder in TBS for 1 h. The nitrocellulose was washed in TBS (3 × 5 min) and incubated for 2 h with primary antibody in 0.5% BSA-TBS. Following washing in TBS (3 × 5 min), the nitrocellulose was incubated in rabbit anti-chicken IgG-HRPO linked secondary antibody (Jackson Immunoresearch laboratories inc, Pennsylvania, USA), rabbit anti-chicken IgG-alkaline phosphatase conjugate (Sigma, St Louis, USA) or goat anti-rabbit IgG-alkaline phosphatase conjugate (Sigma, St Louis, USA) in 0.5% BSA-TBS for 1 h, and again washed in TBS (3 × 5 min). When HRPO-linked secondary antibody was used, the nitrocellulose sheet was immersed in 4-chloro-1-naphthol substrate solution and when alkaline phosphatase linked secondary antibody was used, the nitrocellulose sheet was immersed in BCIP/NBT alkaline phosphatase substrate and reacted in the dark until bands (for western blotting) or dots (for dot blotting) were clearly evident. The reaction was stopped by washing the blots in distilled H_2O. The blots were kept between filter paper discs to preserve the bands for photography.

2.8 Immunisation of chickens

Immunisation of experimental animals for antibody production is done by injecting the immunogen mixed with an adjuvant. A good immune response depends on three variables: antigen, adjuvant and the immunised animal (Schwarzkopf et al., 2001). Antibody production is promoted by adjuvants by increasing both humoral and cellular immune responses and by inducing immunological memory (Schwarzkopf et al., 2001). Adjuvants stimulate an immune response by forming an immunogen depot at the immunisation site, from where the immunogen is released slowly over a long period of time. Adjuvants activate macrophages by inducing the release of co-stimulators in macrophages (Sharon, 1998). Adjuvant stimulation is independent of the kind of antigen used and is therefore non-specific.

The choice of adjuvant depends on the type of immune response desired, the animal species to be immunised, the route of administration of the immunogen and the tolerance of an animal

for any side effects caused by an adjuvant (Allison and Byars, 1994). Classes of adjuvant include aluminium salts, bacterial derivatives [*Mycoplasma tuberculosis* in Freund's complete adjuvant (FCA)], surface-active agent (saponins), vehicles (syntax adjuvant formulation), slow release agents [Freund's incomplete adjuvant (FIA)] and liposomes (Allison and Byars, 1994). Undesirable side effects of adjuvants often limit their use. FCA can induce injection site granulomas and results in the animal becoming tuberculin sensitive. This is avoided by injecting animals once only with FCA, followed by FIA in subsequent injections. Alpha-2-macroglobulin (α_2M) is a promising new adjuvant. It is safe, effective and more potent than any other currently available adjuvants (Cianciolo et al., 2002). Incorporation of antigens into α_2M can be used to convert a poorly immunogenic immunogen into one that is strongly immunogenic (Cianciolo et al., 2002). The mode of operation of this adjuvant will be explained further in Chapter 5.

The choice of animal for immunisation is important and can determine the success of the immunisation. It would have been impractical in the present study to use cattle to test the immunogenicity of the congopain peptides. Usually, rabbits are preferred because they are relatively cheap, easy to care for, able to withstand intensive immunisation and they are fairly easy to bleed. Chickens are easier to handle than rabbits and have some additional benefits. Chickens do not require bleeding. The hen transfers her serum IgG to the egg yolk and gives immunity to her offspring and, because of this, chicken antibodies can be readily isolated from the egg yolks (Polson et al., 1980). The use of eggs instead of bleeding animals, represents a more humane, non-invasive treatment of experimental animals. The antibodies are called IgY, because Y represents their location in the egg yolk. There is a high amount of IgY in egg yolk compared to IgG obtained from rabbit serum (Erhard and Schade, 2001). One egg yolk yields between 60 and 100 mg of IgY whereas the same amount of IgG is obtained from 5-8 ml rabbit serum (10-16 ml blood). While chickens lay between 5-7 eggs per week, only 50 ml blood can safely be taken from a rabbit per month. A further benefit in using chickens is found in the evolutionary distance between the avian immune system and the mammalian or parasite source of immunogens under study leading to very good immune responses against these proteins.

2.8.1 Procedure

The methods used for the preparation of immunogens (conjugation of peptides to a carrier protein) will be given in Chapter 3, because the background information on the selection of peptides and details of the peptides selected are given in Chapter 3.

Peptide-carrier conjugates were mixed with an equal volume of adjuvant. A stable water-in-oil emulsion was prepared by trituration using a syringe. The peptide-carrier conjugates contained 200 μg of peptide per inoculation and were mixed with FCA for the first immunisation and with FIA at weeks 2, 4 and 6. Chickens were injected on either side of the chest into the breast muscle. Eggs were collected before the first immunisation (non-immune control IgY) and daily thereafter until 16 weeks post immunisation.

2.9 Isolation of antibodies from chicken egg yolks

The yolk in a hen's eggs consists of dry matter and water in nearly equal proportions. The dry matter is made up of proteins, fats, carbohydrates and inorganic matter. The protein in the yolk can be divided into four fractions: lipovitellin, vitellin, phosvitin and livetin. The livetin fraction consists of alpha-livetin (the equivalent of plasma albumins), beta-livetin (the equivalent of α_2-glycoprotein) and gamma-livetin (the equivalent of serum gamma-globulins together with transferrin). IgY constitutes the gamma-livetin fraction (Staak et al., 2001).

A number of methods have been described for the extraction of IgY (Jensenius et al., 1981). Various procedures for isolating IgY from the yolk of immunised eggs may be used, based on the following criteria: yield and purity of IgY isolated and antibody activity. The use of polyethylene glycol (PEG, M_r 6000) for IgY isolation was introduced by Polson et al. (1980). This method can be regarded as a standard technique because it is the most widely accepted method (Staak et al., 2001). PEG is a hydrophilic polymer and it is thought to precipitate proteins by excluding the protein from the solvent (Dennison, 1999). Protein is brought to its solubility limit with an increase in protein concentration. The PEG method is suitable for the isolation of large proteins since they can easily be brought to their solubility limit. The precipitation method used in this study was a modification of the method by Polson et al. (1985).

2.9.1 Reagents

0.1 M Na-phosphate buffer, 0.02% (w/v) NaN_3, pH 7.6. $NaH_2PO_4.2H_2O$ (15.7 g) and NaN_3 (0.2 g) were dissolved in 950 ml of distilled H_2O, titrated to pH 7.6 using NaOH, and made up to 1l.

2.9.2 Procedure

IgY was isolated from eggs from weeks 0-16 post immunisation. Two chickens were used for antibody production against each peptide. Eggs from chickens immunised with the same peptide were kept separate to compare the responses of the two chickens. Egg yolks were separated from egg white and washed under running water. The yolk sac was punctured and the yolk volume determined in a measuring cylinder. The yolk was mixed with 2 volumes of 0.1 M phosphate buffer, pH 7.6. PEG (3.5%) was added and dissolved by stirring. The mixture was centrifuged (4 420 x g, 30 min, RT), forming two phases. The watery phase containing the IgY and other chicken serum proteins were filtered through absorbent cotton wool. The solid phase containing lipoproteins and fatty substances was discarded. The chicken serum proteins that remained were separated from IgY by increasing the PEG concentration to 12% (w/v) [i.e. 8.5% (w/v) was added to the supernatant], the solution was mixed until the PEG was dissolved and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the pellet of IgY was dissolved 0.1 M phosphate buffer pH 7.6, in a volume equal to the volume of the original egg yolk. IgY was purified further by precipitation once more with 12% (w/v) PEG. The solution was stirred thoroughly and centrifuged (12 000 x g, 10 min, RT). The supernatant was discarded and the final IgY pellet was dissolved in a volume of 0.1 M phosphate buffer, pH 7.6 equivalent to 1/6 of the original yolk from which IgY was derived. The antibodies were stored at 4°C.

2.9.3 Determination of IgY concentration

Antibody solution (20 µl) was added to phosphate buffer (980 µl) making a 1 in 50 antibody dilution. The absorbance at 280 nm was determined and the concentration of IgY was calculated using the equation: $A = \varepsilon \times l \times c$

where A = absorbance

ε = extinction coefficient

l = the length of the light path (cm)

c = concentration of the absorbing solution.

The dilution factor was taken into account for the calculation of the concentration of the undiluted sample. The extinction coefficient of IgY, $\varepsilon_{280\,nm}^{1\,mg/ml}$ =1.25 (Coetzer, 1985).

2.10 Enzyme-linked immunosorbent assay (ELISA)

The immunoreactivity (Section 3.4.3) and the specificity (Section 3.4.5) of the isolated antibodies were tested using ELISA. The assay allows detection of a partially denatured antigen with great accuracy and sensitivity (Kerr and Thorpe, 1994). Antigens are adsorbed directly onto the wells of microtitre plates. The unoccupied sites are blocked with a non-cross reacting protein to prevent non-specific binding of reagents used in subsequent steps. The antibodies to be quantitated are added. These are detected by the addition of an anti-species enzyme linked detection ("secondary") antibody which, upon addition of a substrate, produces a coloured product which can be quantified photometrically (Figure 6).

When antibodies are raised against peptide-carrier conjugates, antibodies are produced against the peptide, the carrier protein and the carrier residues modified by the conjugation reaction (Briand et al., 1985). In order to detect the presence of antibodies produced specifically against the peptide, an unconjugated peptide should therefore be used to coat the wells of the microtitre plates. The subpopulation of anti-peptide antibodies that is able to cross-react with the whole CP2 enzyme was measured by coating the microtitre plates with either the whole CP2 enzyme or the catalytic domain C2 (Section 3.4.5).

2.10.1 Reagents

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 1 l of distilled H_20. The pH of this buffer does not require adjustment to pH 7.2.

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

0.5% (w/v) Bovine serum albumin (BSA) – PBS. BSA (0.5 g) was dissolved in 100 ml PBS.

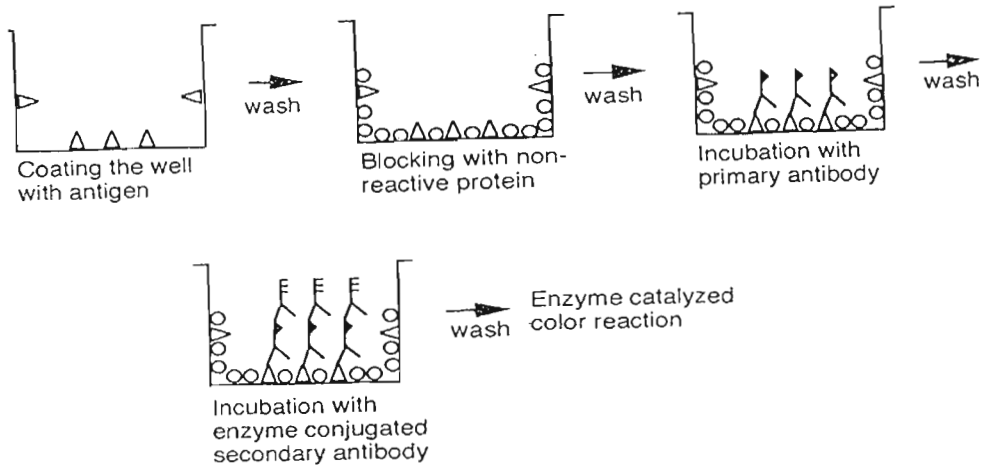

Figure 6. An illustration of a non-competitive indirect ELISA (Lauritzen et al., 1994).

0.1% (v/v) PBS-Tween. Tween 20 (1 ml) was made up to 1 l in PBS.

0.15 M Citrate phosphate buffer, pH 5.0. A 0.15 M solution of citric acid. H_2O (21.0 g/l) was titrated with a 0.15 M solution of $Na_2HPO_4.2H_2O$ (35.6 g/l) to pH 5.0.

Substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in citrate phosphate buffer]. ABTS (7.5 mg) and H_2O_2 (7.5 µl) were dissolved in citrate phosphate buffer, pH 5.0 (15 ml).

Stopping buffer [citrate-phosphate buffer; 0.1% (w/v) NaN_3]. NaN_3 (0.1 g) was dissolved in citrate phosphate buffer, pH 5.0 (100 ml).

2.10.2 Procedure

The peptides (5 µg/ml in PBS) or, CP2 or C2 (1 µg/ml, in 50 mM carbonate buffer, pH 6.0) were coated (150 µl, 16 h at 4°C or 3 h at 37°C). Non-specific binding of antibody was prevented by blocking the wells with 0.5% BSA-PBS (200 µl, 1 h at 37°C) and the plates washed three times with 0.1% (v/v) PBS-Tween, a non-ionic detergent which blocks hydrophobic interactions. Appropriate dilutions of IgY were prepared in duplicate in 0.5% BSA-PBS and incubated in the coated wells of the plate (100 µl, 2 h at 37°C). The plates were washed three times with 0.1% (v/v) PBS-Tween. The rabbit anti-chicken IgG-HRPO-linked

secondary antibody (Jackson ImmunoResearch laboratories inc, Pennsylvania, USA) at a dilution of 1/30 000 in 0.5% BSA-PBS, was added to each well and incubated (120 µl, 1 h at 37°C) and the plates washed three times with 0.1% (v/v) PBS-Tween. Substrate solution (150 µl) was added and the colour was allowed to develop in the dark against the background of the negative controls to check non-specific binding (usually 10-20 min). The enzyme reaction was stopped by the addition of 0.1% (w/v) NaN_3 in citrate-phosphate buffer (50 µl per well) and the absorbance determined at 405 nm in a Molecular Devices Versamax microplate reader.

2.11 Immuno-affinity purification of anti-peptide antibodies

Affinity chromatography is a method of purification based on biological recognition between ligand and receptor molecules and is mainly used for the purification of biologically active molecules (Kruger and Hammond, 1988). Affinity chromatography takes advantage of the reversible binding interactions that occur on a protein's surface. The specific interaction between antibodies and their antigens may be exploited in the immuno-affinity purification of antibodies specific for a peptide from other contaminating antibodies such as those against a carrier protein.

Specific antigens or antibodies are covalently coupled to an insoluble matrix such as cellulose, agarose beads or glass beads (Tharakan, 1994). In this study, peptides were used as affinity ligands and were covalently coupled to agarose beads. The careful selection of the matrix is crucial to the successful operation of any affinity process (Tharakan, 1994). The physical and chemical stability of bonds linking the support should be considered to ensure minimal leakage of an immobilised protein or peptide. In this study, the peptides had to have the same orientation on the affinity matrix as they had on the carrier protein during immunisation. Peptides with N- or C-terminal cysteine residues were therefore coupled to Pierce's SulfoLink™ through their free cysteine residues. The peptide which did not have an internal lysine group was coupled to Pierce's AminoLink™ through its free amino group.

2.11.1 Preparation of peptide-Sulfolink™ affinity matrices

To couple a peptide to a SulfoLink™ gel, the peptide with a cysteine residue at the N- or C-

terminal end is first reduced to make sulfhydryls, which react efficiently with an alkylating agent, available. Peptides with sulfhydryls bind specifically to a SulfoLink^{TM} gel because the gel has an immobilised iodoacetyl which reacts with the sulfhydryls (Pierce SulfoLink^{TM} data sheet).

2.11.1.1 Reagents

0.1 M Na-phosphate buffer, 0.005 M EDTA, pH 6.0. $NaH_2PO_4.2H_2O$ (1.56 g) and EDTA (0.186 g) were dissolved in 90 ml of distilled H_2O, titrated to pH 6.0 with NaOH and made up to 100 ml.

0.05 M Tris-HCl buffer, 0.005 M EDTA, pH 8.5. Tris (3.03 g) and EDTA (0.939 g) were dissolved in 450 ml of distilled H_2O, titrated to pH 8.5 with HCl and made up to 500 ml.

0.01 M Dithiothreitol (DTT). DTT (7.71 mg) was dissolved in 5 ml of Tris-HCl buffer, pH 8.5.

0.05 M Na-phosphate buffer, pH 7.0. $NaH_2PO_4.2H_2O$ (3.9 g) was dissolved in 450 ml of distilled H_2O, titrated to pH 7.0 with NaOH and made up to 50 ml.

0.01 M Ellman's reagent. 5,5 –dithiobis-2-nitrobenzoic acid (DTNB) (0.004 g) was dissolved in 10 µl of methanol and diluted to 1 ml with 0.05 M phosphate buffer, pH 7.0.

0.05 M cysteine. Cysteine (0.0176 g) was dissolved in 2 ml of Tris-HCl buffer, pH 8.5.

1 M NaCl, 0.05% NaN_3. NaCl (58.44 g) and NaN_3 (0.5 g) were dissolved in 1 litre of distilled H_2O.

0.1 M Na-phosphate buffer, 0.02% NaN_3, pH 6.5. $NaH_2PO_4.2H_2O$ (7.8 g) and NaN_3 (0.1 g) were dissolved in 450 ml of distilled H_2O, titrated to pH 6.5 with NaOH and made up to 500 ml.

2.11.1.2 Procedure

CP1 (10 mg) and CP2 (7.5 mg) peptides were individually dissolved in the minimum amount of DMSO (approximately 100 µl) and made up to 1 ml with Tris-HCl buffer, pH 8.5. The peptides were reduced by adding 10 mM DTT (1 ml) slowly to the dissolved peptide with stirring. The mixture was incubated for 1.5 h at 37°C. The reduced peptide was separated from excess DTT on a Sephadex G-10 column (130 mm × 10 mm, flow rate 0.13 ml/min), pre-equilibrated with 50 mM Tris-HCl buffer, pH 8.5. Fractions (500 µl) were collected and the elution profile was determined by the addition of 0.01 M Ellman's reagent (10 µl) to an equal sample volume from each fraction. A yellow colour was indicative of the elution of the reduced peptide peak, which was separated from the unreacted DTT peak, which gave an intensely yellow colour.

The SulfoLink™ gel was supplied as a 50% slurry, 4 ml of which was packed into a Biorad disposable polystyrene column. The liquid was drained from the gel until the wet cake remained. The gel was washed with 6 column volumes (12 ml) of Tris-HCl buffer, pH 8.5. The reduced peptide was added to the gel. The gel was mixed at RT by gentle end-over-end rotation for 15 min and incubated at RT for 30 min without mixing. The buffer was drained. The column was washed with 3 column volumes (6 ml) of Tris-HCl buffer, pH 8.5. The non-specific binding sites were blocked by applying 2 ml of cysteine to the column. The gel was mixed at RT by gentle end-over-end rotation for 15 min and incubated at RT for 30 min without mixing. The column was washed with 16 column volumes (32 ml) of NaCl, 2 column volumes (6 ml) of Na-phosphate buffer, pH 6.5, and stored at 4°C until use.

2.11.2 Preparation of a peptide-AminoLink™ affinity matrix

The AminoLink™ coupling gel has many advantages. It uses a coupling chemistry that is active over a wide pH range. More than 85% of coupling with AminoLink™ support is obtained throughout the pH range of 4-10. It can couple proteins of different molecular masses and isoelectric points with high efficiency. The AminoLink™ gel has no additional charge on the matrix, thus reducing non-specific binding of proteins and allowing greater purification of proteins (PIERCE, Immuno Pure instruction manual). The AminoLink™ coupling gel consists of a 4% cross-linked beaded agarose matrix, activated to form aldehyde functional groups,

suitable for coupling peptides and proteins using reductive amination. The aldehyde groups on the matrix react with the primary amine group of the peptide to form a labile Schiff base. Reduction of the Schiff base intermediate with sodium cyanoborohydride ($NaCNBH_4$) results in the formation of a stable secondary amine bond between the matrix and the peptide or protein. $NaCNBH_4$ is the preferred reducing agent because of its specificity toward the Schiff base structure and because it is more gentle, especially when antibodies are immobilised (Hermanson, 1996). The unreacted aldehyde groups on the coupling gel are quenched with Tris-HCl. The Schiff base intermediate formed between the Tris primary amine and coupling gel is reduced with a further addition of $NaCNBH_4$ to form a stable Schiff base. The mechanism for the coupling of a peptide to an AminoLink™ matrix is shown in Figure 7.

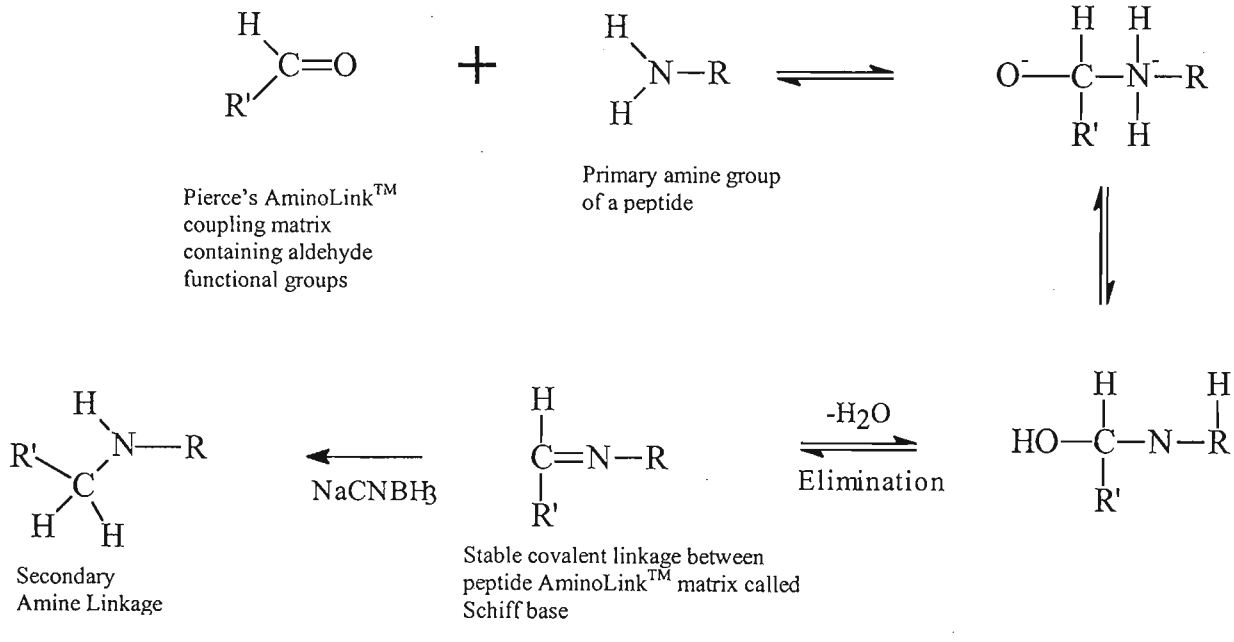

Figure 7. Reductive amination mechanism for coupling of a peptide to an AminoLink™ matrix. The mechanism was adapted from Ginsburg (1967) and Hermanson (1996). Pierce's AminoLink™ agarose containing aldehyde functional groups react with primary amine to form labile Schiff bases which are reduced to form amine linkages.

2.11.2.1 Reagents

0.1 M Na-phosphate buffer, 0.05% NaN_3, pH 7.0. $NaH_2PO_4.2H_2O$ (7.8 g) was dissolved in 450 ml of distilled H_2O, titrated to pH 7.0 with NaOH, NaN_3 (0.25 g) added and made up to 500 ml.

1 M Tris–HCl buffer pH 7.4. Tris (60.75 g) was dissolved in 450 ml of distilled H_2O, titrated to pH 7.4 with HCl and made up to 500 ml.

1 M Na-cyanoborohydride. $NaCNBH_3$ (0.0623 g) was dissolved in 1 ml of distilled H_2O. This solution was prepared 1 h before use.

1 M NaCl. NaCl (29.22 g) was dissolved in 500 ml of distilled H_2O.

0.05% NaN_3. NaN_3 (0.25 g) was dissolved in 500 ml of distilled H_2O.

2.11.2.2 Procedure

Peptide #3 [Section 3.4.1 (10 mg)] was coupled to the AminoLink™ coupling gel (2 ml) according to the method in the PIERCE, Immuno Pure instruction manual. To prepare a peptide solution, peptide (10 mg) was dissolved in Na-phosphate buffer, pH 7.0 (2 ml). AminoLink™ gel (4 ml), supplied as a 50% slurry, was packed into a Biorad disposable polystyrene column. The liquid was drained from the gel until only the wet gel cake remained. The gel was washed with Na-phosphate buffer, pH 7.0 (2 ml). The peptide solution was added to the column, followed by addition of $NaCNBH_3$ solution (200 µl) in a fume hood. The column was mixed by gentle end-over-end rotation (2 h, RT). The reduction amination reaction proceeded at RT for an additional 4 h. The gel was washed with Tris-HCl buffer, pH 7.4 (2 ml). $NaCNBH_3$ solution (200 µl) was added to the column in a fume hood. The column was mixed by gentle end-over-end rotation (30 min, RT). The gel was drained and washed with 10 column volumes (20 ml) of NaCl solution followed by washing with 10 column volumes (20 ml) of NaN_3 solution. NaN_3 solution (2 ml) was added to the column which was stored at 4°C until use.

2.11.3 Affinity purification of anti-peptide antibodies on SulfoLink-^{TM} or AminoLink-^{TM} peptide matrices

2.11.3.1 Reagents

0.1 M Na-phosphate buffer, 0.02% NaN_3, pH 6.5. As per Section 2.11.1.1

0.1 M Na-phosphate buffer, 0.05% NaN_3, pH 7.0. As per Section 2.11.2.1

0.1 M Glycine-HCl, pH 2.8. Glycine (3.7535 g) was dissolved in 450 ml of distilled H_2O, titrated to pH 2.8 with HCl and made up to 500 ml.

1 M Na-phosphate buffer, pH 8.5. $NaH_2PO_4.2H_2O$ (78.005 g) was dissolved in 450 ml of distilled H_2O, titrated to pH 8.5 with NaOH and made up to 500 ml.

2.11.3.2 Procedure

For the affinity purification of antibodies, the appropriate column was first brought to RT. The column was equilibrated with 10 column volumes (20 ml) of 0.1 M Na-phosphate buffer, pH 6.5 (SulfoLink^{TM} matrix) or pH 7.0 (AminoLink^{TM} matrix) at a flow rate of 0.13 ml min^{-1}. Chicken antibodies (IgY) (maximum volume of 60 ml) were circulated through the column overnight. The column was washed with 10 column volumes (20 ml) of 0.1 M Na-phosphate buffer, pH 6.5 (SulfoLink^{TM} matrix) or pH 7.0 (AminoLink^{TM} matrix). Anti-peptide antibodies (900 µl fractions) were eluted using glycine-HCl buffer, pH 2.8. Samples were collected into tubes containing 100 µl of 1 M Na-phosphate buffer, pH 8.5, to increase the pH of eluted samples to pH 7.2. The elution of anti-peptide antibodies was monitored by reading absorbance values at 280 nm. The fractions containing anti-peptide antibodies were pooled. The column was regenerated with 0.1 M Na-phosphate buffer, pH 6.5 (SulfoLink^{TM} matrix) or pH 7.0 (AminoLink^{TM} matrix).

2.12 Inhibition of catalytic activity of CP2 using antibodies

Fluorogenic substrates contain fluorescent leaving groups conjugated to a short peptide which has selectivity and specificity for the protease under study (Knight, 1995). In this study, Z-

phenylalanyl-arginyl-aminomethyl coumarin (Z-Phe-Arg-AMC) was used. Z-Phe-Arg-AMC seemed appropriate to use because congopain is similar to cathepsin L (Mbawa et al., 1992) and it was used successfully in the CP2 inhibition assay (Authie et al., 2001). Furthermore, 7-amino-4-methyl coumarin is a sensitive, safe and convenient leaving group to use in enzyme assays (Barrett and Kirschke, 1981). Both the substrate and the product are intensely fluorescent, but with different excitation and emission wavelengths (Knight, 1995). 7-amino-4 methylcoumarin has a λ_{ex} of 345 nm and λ_{em} of 445 nm. The release of 7-amino-4-methylcoumarin can be measured against low background fluorescence of unhydrolysed substrate at a λ_{ex} of 370 nm and λ_{em} of 460 nm.

2.12.1 Reagents

Assay buffer [400 mM Na-phosphate buffer, 4 mM Na_2EDTA, 2 mM DTT, pH 7.0]. $NaH_2PO_4.2H_2O$ (3.12 g) and Na_2EDTA (0.075 g) were dissolved in 40 ml of distilled H_2O and titrated to pH 7.0 with NaOH, NaN_3 (0.01 g) added and made up to 50 ml. DTT (3 mg) was added to 10 ml of buffer just before use.

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 distilled H_2O. This was also kept at 4°C.

0.1% (w/v) Brij 35 diluent. Brij 35 (0.1 g) was dissolved in 100 ml of distilled H_2O.

Stopping reagent [100 mM monochloroacetate, 30 mM sodium acetate, 70 mM acetic acid, pH 4.3]. Monochloroacetate (4.275 g), sodium acetate (2.04 g) and glacial acetic acid (2 ml) were dissolved in 475 ml of distilled H_2O, titrated to pH 4.3 with NaOH and made up to 500 ml with distilled H_2O.

Antibody diluting buffer [100 mM Na-acetate, 1 mM Na_2EDTA, 0.02% (w/v) NaN_3, 0.1% (v/v) Tween-20, pH 8.0. Glacial acetic acid (2.85 ml) and $Na_2EDTA.2H_2O$ (0.185 g) were dissolved in 450 ml of distilled H_2O and Tween-20 (500 µl) was added. The solution was

titrated to pH 8.0 with NaOH, NaN_3 (0.1 g) added and made up to 500 ml with distilled H_2O.

2.12.2 Procedure

Serial two fold dilutions of anti-peptide antibodies (from 500 µg/ml, in antibody diluting buffer, 25 µl per well) were mixed with CP2 or C2 [20 ng in 0.1% (w/v) Brij 35, 15 min. 37°C]. The assay buffer was added (25 µl, 1 min, 37°C) followed by substrate (25 µl, 10 min, 37°C). The reaction was terminated by the addition of stop reagent (100 µl). Fluorescent product was determined (excitation 370 nm and emission 460 nm) in a Cambridge Technology 7620 Microplate Fluorometer. The inhibition or activation of an enzyme by antibody was expressed as a percentage of the activity detected after incubation with the same concentration of non-immune antibody.

2.13 Azocasein assay for determination of CP2 proteolytic activity

Azocasein is formed by coupling of diazotised sulfanilic acid or sulphanilamide to tyrosyl and histidyl side chains with in alkali (Barrett and Kirschke, 1981). Azocasein is a suitable substrate for determining proteolytic activity in crude samples (Barrett and Kirschke, 1981). When azocasein is proteolytically degraded, peptides which are soluble in dilute trichloroacetic acid are released. The soluble peptides are intensely yellow as a result of the presence of the azo-groups. The yellow colour can be quantified at 366 nm. Azocasein is mixed with urea because this is known to selectively depress the activity of other proteases and enhances that of cathepsin L (Barrett and Kirschke, 1981). CP2 is a cathepsin L-like enzyme (Lalmanach et al., 2002) and azocasein/urea is therefore a suitable substrate. In this study, the azocasein assay was used to determine the proteolytic activity of free CP2 in comparison with the proteolytic activity of CP2-α_2M complexes (Section 5.3.1.2). The assay was based on the method of Ramos et al. (2002).

2.13.1 Reagents

Assay buffer [0.1 M Na-acetate, 0.001 M Na_2EDTA, 0.02% (w/v) NaN_3, 1 µg/ml pepstatin, 0.04 M cysteine, pH 6.0]. Glacial acetic acid (2.86 ml) and $Na_2EDTA.2H_2O$ (0.185 g) were dissolved in 450 ml of distilled H_2O, titrated to pH 6.0 with NaOH, NaN_3 (0.1 g) and pepstatin (500 µg) were added and the solution made up to 500 ml. Just before the assay, cysteine.HCl

(0.04 g) was added to 5 ml of the buffer.

6% (w/v) Azocasein. Azocasein (3 g) was dissolved in 50 ml of distilled H_2O with gentle magnetic stirring at RT for approximately 1 h.

Azocasein/3 M urea solution. Urea (54 g) was dissolved in 6% azocasein solution (50 ml) by stirring on a magnetic heater stirrer at less than 30°C. The volume was made up to 150 ml with the assay buffer without cysteine.

5% (w/v) TCA. TCA (25 g) was dissolved in 500 ml of distilled H_2O.

2.13.2 Procedure

Enzyme (100 µl) was mixed with assay buffer (100 µl). The mixture was activated for 5 min at 37°C. Azocasien/urea solution (200 µl) was added. A sample (200 µl) of the mixture was immediately withdrawn and mixed with 5% TCA (1 ml) in a polyethylene microfuge tube. This served as the blank for the reaction. The remainder of the mixture was incubated for 2 h at 37°C. The samples (200 µl) were mixed with 5% TCA (1 ml). All samples were centrifuged (12 000 × g, 5 min, RT). Absorbance values were read at 366 nm using glass micro cuvettes with blacked-out sides. Proteolytic activity was determined by measuring the hydrolysis of substrate at 366 nm compared to the blank.

Chapter 3

Production and characterisation of anti-CP1 and CP2 peptide antibodies.

3.1 Introduction

Anti-peptide antibodies may be considered to be site directed probes for proteins (Hancock et al., 1998). They may recognise the corresponding peptide in the whole protein molecule, provided that some conformations, observed in the peptide, are similar to those that occur in the corresponding constrained regions of the parent protein (Troeberg et al., 1997). Anti-peptide antibodies in the antiserum that cross-react with the complete protein are regarded as antibodies of predefined specificity, since they react with a single antigenic region of the protein (Muller, 1990b). Unlike conventional anti-protein polyclonal antibodies, anti-peptide polyclonal antibodies share with monoclonal antibodies the property of binding to a discrete region of the protein antigen (Muller, 1990b). Monoclonal antibodies are commonly used because of their high specificity, but they have a disadvantage of being very expensive and their production is time consuming compared to anti-peptide antibodies. An immune response is usually generated against the peptide but the main problem with anti-peptide antibodies is that they may not recognise the whole protein molecule. It is therefore very important to carefully select a peptide in order to increase the chances of success and to minimize the cost. In this study, anti-peptide antibodies were used to study their effect on CP2 activity.

Antibodies against both CP1 and CP2 have previously been raised in cattle (Authié et al., 2001). However, it may be more advantageous to have antibodies against peptides corresponding to specific regions of these proteases. Such antibodies would potentially possess the desired specificities since they are "site directed" (Bulinski and Gundersen, 1986). Antibodies against a sequence of 10-20 residues are more likely to be specific for the protein of interest because it is very probable that a sequence of this length will be unique to a particular protein (Coetzer et al., 1991).

Peptides from both CP1 and CP2 were selected for immunisation (Sections 3.2 and 3.4.1). The CP1 and CP2 peptides selected were conjugated to rabbit albumin via M-maleidobenzoyl-N-

hydroxysuccinimide ester (MBS) or glutaraldehyde (Section 3.3.2 or 3.3.3) prior to inoculation into chickens. Antibody production against the respective peptides was monitored by ELISA (Section 3.4.3) prior to affinity purification (Section 3.4.4). Recognition of whole CP2 by the anti-peptide antibodies was determined by ELISA and western blotting (Sections 3.4.5 and 3.4.6). Finally, the effect of anti-peptide antibodies on CP2 activity was investigated (Section 3.4.7).

3.2 Prediction of immunogenic peptides in proteins

The position of an epitope in an intact protein may be detected by limited enzymatic digestion of the antigen, followed by immunoblotting of the protein fragments (Westwood and Hay, 2001). Once the fragment is identified, it is sequenced and the epitope is characterised. This method requires high purity of the protein and the data is not always precise. Alternatively, peptides of different lengths are produced for sequencing using cyanogen bromide that cleaves peptides at the carbonyl group of methionine residues, hydroxy-amine that cleaves asparagine-glycine bonds, or 2-nitro-5-thiocyanobenzoate that cleaves on the amine side of cysteine residues. The advantage of using chemicals over enzymes for protein cleavage is that chemicals are not prone to steric hindrance and can easily reach the target sites deep within the tertiary structure (Westwood and Hay, 2001).

There are several methods used in identifying immunogenic peptides, including predictive algorithms that look for certain features in the primary structure of a protein. These features are mainly hydrophilicity, segmental mobility and surface probability. The predicted peptides are then synthesised and screened. The amino acid sequence of the protein of interest should be known when using this method. The major disadvantage of this method is that the predictions are not always accurate (Sumar, 2001). Predict 7, a program for protein structure prediction, uses seven different algorithms simultaneously. The algorithms used include: secondary structure analysis, hydrophilicity, side chain flexibility, surface probability, antigenicity and the location of putative N-glycosylation sites (Cármenes et al., 1989).

Another epitope mapping method is called multiple pin peptide scanning ('Pepscan') technology. In this method, solid phase synthesis of peptides on polystyrene pins, in conjunction with ELISA, assist in the identification of epitopes of an antigen. One of the main

differences between this method and the above method is that peptides are synthesised and do not result from cleaving of a protein sequence. Linear peptides (for example, 12-mers with an overlap of four residues) spanning the whole protein sequence are synthesised on pins which are attached to a plastic support. The polystyrene pins are arranged in blocks of 8 x 12 (96 pins), which are immersed into the wells of a microtitre plate. The pins are then incubated with sera or antibodies of interest and with species-specific secondary antibody conjugated with an enzyme. The substrate is added and the colour is developed and measured in an ELISA reader (Sumar, 2001). The ELISA results show whether serum antibodies bind to the peptide or not and therefore help in the identification of peptides which are epitopes.

The three dimensional structure of the protein can also be inspected to map surface regions which are easily accessible to antibodies and are therefore more likely to be epitopes. In this study, a very quick and yet fairly accurate method was required. In view of the high cost of the other methods, the Predict 7 program remained attractive to use.

3.2.1 Structural parameters analysed by the Predict 7 program

Predict 7, is a program which analyses protein sequences (Cármenes et al., 1989). The main features analysed by this program are: hydrophilicity, segmental mobility and surface probability.

3.2.1.1 Hydrophilicity

Hydrophobic amino acids tend to be buried within the native structure of globular proteins, while hydrophilic side-chains are on the exterior where they can interact with water. Most hydrophilic segments of a protein tend to correspond to continuous epitopes (Hopp and Woods, 1981). Local maxima in hydrophilicity plots were found to correspond to segments that were exposed at the surface of the molecule and at the same time were part of epitopes (Van Regenmortel, 1990a). However, for predicting the positions of continuous epitopes, it is preferable to consider only the highest peaks appearing in hydrophilicity plots (Van Regenmortel, 1990a). Some hydrophilic amino acid sequences may be buried in water pockets forming inter or intra molecular bonds. Hydrophilicity is required, but not sufficient for selecting peptides. There is a correlation between secondary structure of a protein and hydrophilicity. Methods that predict the position of loops or turns, at the same time predict

regions of highest hydrophilicity

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3.2.1.2 Segmental mobility

X-ray and NMR studies indicated that amino acid residues at the surface of a protein molecule have higher mobility (Westwood and Hay, 2001). The more flexible a stretch of amino acids, the more likely it is to be immunogenic (Gullick, 1994). Regions of highest mobility correspond with highly accessible segments at the surface of the molecule. In several proteins, a correlation between the peaks in the mobility plot and the position of epitopes was found (Gullick et al., 1986). The induced fit model proposes that antigen and antibody interaction largely depends on side-chain movements of the antigen and some movements of the backbone conformation. This model highlights the importance of the observed link between antigenicity and local mobility of the peptide main chain (Van Regenmortel, 1989).

3.2.1.3 Surface probability

Antibodies bind to the surfaces of proteins, and therefore it is expected that epitopes will usually consist of residues exposed at the surface of a protein (Van Regenmortel, 1990a). Regions of proteins exposed on the surface, were found to correlate with the position of continuous epitopes and correlated well with side-chain flexibility, but there was less correlation with hydrophilicity (Thornton et al., 1986). Any accessible part of a molecule may be considered to be potentially antigenic but some accessible parts are naturally more immunogenic than others (Westwood and Hay, 2001).

3.2.2 Selection of peptides using the 3-dimensional structure of a protein

When this study was started, the three dimensional structure of CP2 was not known. It was published later after the selection of peptides (Lecaille et al., 2001). CP2 shares a high sequence homology with papain, whose three dimensional structure is known (Drenth et al., 1971). The three-dimensional structure of papain was therefore used to ascertain the position of the peptides selected using Predict 7 parameters. The papain structure was an appropriate structure to use, because, as expected, the subsequently published three dimensional model of CP2 exhibits a framework common to most papain-like cysteine proteinases (Lecaille et al., 2001).

The peptides were selected based on the results obtained from the epitope prediction plots and also on their location in the three dimensional structure of papain. In addition to this, they were selected for the following reasons: firstly, it is likely that antibodies against the active site would inhibit enzyme activity compared to antibodies against other regions of the enzyme. Anti-peptide antibodies which targeted the active site of trypanopain-Tb (Troeberg et al., 1997) and the active site of cathepsin L (Coetzer et al., 1991) inhibited enzyme activity. For this reason, peptides covering the active site of CP1 and CP2 were selected. Secondly, the peptides in the N-and C-termini of the proteins are usually good antibody targets, because they may more closely resemble the peptide immunogen than internal, more conformationally constrained sequences (Hancock et al., 1998). Thirdly, peptides in the C-terminal extensions of CP2 and vivapain are likely to be good immunogens because the C-terminal extension is thought to form an important part of antigenicity in the trypanosomal cysteine proteinases (Lalmanach et al., 2002). This was shown by high reactivity of infected sera with the recombinant C-terminal domain of CP2 (Lalmanach et al., 2002).

3.3 Considerations for coupling of peptides to carrier proteins

Short peptides can be poor immunogens, therefore it is essential to covalently conjugate them to carrier proteins to enhance the production of anti-peptide antibodies. Coupling of the peptide to a carrier protein can increase its immunogenicity by 100 fold (Mariani et al., 1987). The carrier-peptide complex has both T-cell epitopes on the carrier protein and B-cell epitopes on the peptide for interaction between T- and B- lymphocytes, an interaction which is necessary for antibody production (Roitt, 1997). The orientation of the peptide after conjugation to a carrier protein (i.e. whether the peptide-carrier link is at the peptide's N- or C-terminus) should be such that the most immunogenic residues are exposed to the experimental animal's immune system.

Choosing the appropriate conjugation procedure is important since the antigenic activity of a peptide may be affected by different coupling methods (Briand et al., 1985). Most coupling methods are based on the use of symmetrical or asymmetrical bifunctional reagents which become incorporated into the final conjugate. Alternatively, they activate certain reactive sites of the carrier protein molecule for subsequent linkage with the peptide (Muller, 1990a).

There is a wide range of coupling reagents available. Glutaraldehyde and MBS were used in this study. The coupling reaction should preferably not affect the configuration of amino acids which form the epitope (Muller, 1990a). Glutaraldehyde reacts mainly with N-terminal α-amine and lysine ε-amine groups. Glutaraldehyde is appropriate to use for conjugation of peptides which are more immunogenic towards their C-termini and which do not have an internal lysine residue. However, the majority of the peptides selected in this study had internal lysine residues whose ε-amines would cross-react with glutaraldehyde. This necessitated conjugation using MBS. For peptides which did not have a terminal cysteine, an additional cysteine was added at either the N- or C- terminus of the sequence, as appropriate, during peptide synthesis.

Rabbit albumin is a readily available protein with a molecular weight of 68 kDa which makes it big enough to be used as a carrier protein. Chickens were the animals of choice for immunisation in this study and their immune system is likely to recognise rabbit albumin as foreign. Rabbit albumin was therefore chosen as a carrier protein to be conjugated to peptides. Apart from peptide # 3 (Section 3.5.1), which was conjugated via glutaraldehyde, all peptides were conjugated via MBS.

3.3.1 Synthesis of peptides

Some modifications to the original sequences were made for the synthesis of peptides. For some of the peptides (peptides A, B, C, D and E, Section 3.4.1, Table 6) an additional Cys-residue was added either at the C-terminus or the N-terminus of the peptide for MBS conjugation. Since one of the peptides contained an internal Cys-residue, this residue was synthesised as 2-aminobutyric acid that is similar to the conformation of an internal Cys-residue in a disulfide bond without presenting an additional Cys-residue for unwanted conjugation (Muller, 1990a). All the peptides were synthesised as C-terminal amides so that they could mimic the peptide conformation in the native protein, since amidation renders the C-terminal residue uncharged as it would be in a peptide bond in a native protein (Multiple peptide systems technical bulletin).

3.3.2 Conjugation of peptides to rabbit albumin using MBS

MBS reacts in two steps, firstly by acylation of the amino groups of a carrier via the active N-

hydroxy succinimide ester and, secondly, by formation of a thioether bond through addition of a thiol group (from cysteine in the peptide) to the double bond of the maleimide (Figure 8), (Kitawa and Aikawa, 1976).

This coupling procedure is divided into three steps. Firstly, the peptide, which may be dimerised because of the cysteine residue at the N- or C-terminus, is reduced by incubation with the reducing agent dithiothreitol (DTT) and reduced peptide is separated from unreacted DTT using Sephadex G10 molecular exclusion chromatography (MEC). In the second step, the carrier protein is activated by MBS and activated carrier is separated from unreacted MBS using Sephadex G25 MEC. In the final step the reduced peptide and activated carrier protein are incubated together. Peptides A, B, C, D, E, F (Table 6) and 1, 2, 4, 5 (Table 7) were conjugated to the carrier via MBS. For the sake of brevity, the procedure followed has been described for only one peptide (peptide 1) as an example, and summarised for the remainder of the peptides in Table 5. A final molar ratio of carrier protein to MBS of 1:40 was used, and a molar ratio of peptide to carrier protein of 40:1 was used.

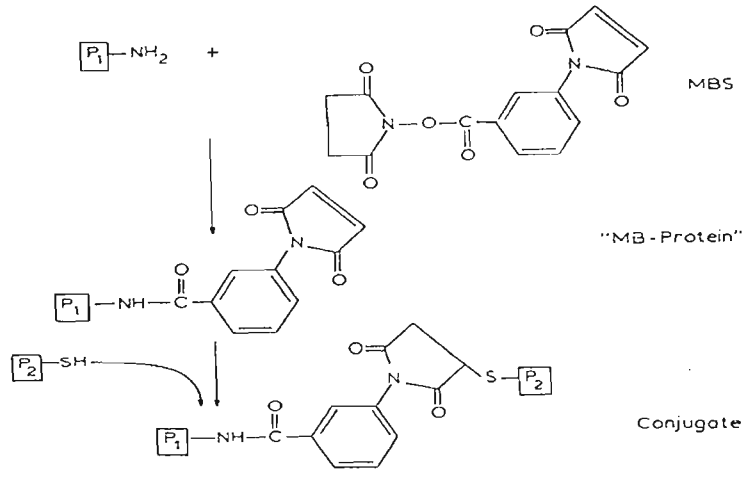

Figure 8. The mechanism for coupling of peptides to carrier proteins using the MBS conjugation method (Muller, 1990a). P_1 represents the carrier protein and P_2 represents the peptide.

Table 5. Conjugation of CP1 and CP2 peptides to rabbit albumin.

		Peptide			Carrier	
Peptide #	M_r	Purity[a] (%)	mg	µmoles	mg	µmoles
A	1578.73	91	4	2.53	4.32	0.06
B	1307.48	75	4	3.06	5.22	0.08
C	1282.63	73	4	3.12	5.32	0.08
D	1229.48	88	4	3.25	5.55	0.08
E	1124.29	90	4	3.56	6.07	0.09
F	2177.48	73	4	1.84	3.14	0.05
1	2382.70	87	4	1.68	2.86	0.04
2	2409.70	95	4	1.66	2.83	0.04
3	1969.00	>77	4	2.03	3.46	0.05
4	1798.00	>80	4	2.22	3.79	0.06
5	1621.00	>90	4	2.47	4.21	0.06

[a]Determined by RP-HPLC (see text)

3.3.2.1 Reagents

Peptides. Peptides A, B, C, D, E and F (CP1) were custom synthesised by Sigma Genosys, Cambridge, UK. Peptides 1 and 2 (CP2) were custom synthesised by Mimotopes, Clayton Victoria, Australia. Peptides 3, 4 (CP2) and 5 (vivapain) were custom synthesised by Auspep, Parkville, Australia. The purity of all peptides were 70% or greater as determined by reverse phase-high performance liquid chromatography (RP-HPLC) (Table 5). The molecular weight of each peptide was confirmed by mass spectral analysis by the suppliers. The selected peptides were modified, during synthesis, as described in Section 3.3.1.

0.01 M Ellman's reagent. As per Section 2.11.1.1

Reducing buffer [0.1 M Tris-HCl buffer, 0.001 M Na_2EDTA, 0.02% (w/v) NaN_3, pH 8.0]. Tris (1.21 g), Na_2EDTA (0.037 g) and NaN_3 (0.02 g) were dissolved in 90 ml of distilled H_20, titrated to pH 8.0 with HCl and made up to 100 ml with distilled H_2O.

0.01 M DTT in reducing buffer. DTT (1.5 mg) was dissolved in 1 ml of reducing buffer just before use.

Phosphate buffered saline (PBS) pH 7.2. As per Section 2.10.1

MEC buffer [0.1 M Na-phosphate buffer, 0.02% (w/v) NaN_3, pH 7.0]. NaH_2PO_4. H_2O (27.598 g) and NaN_3 (0.4 g) were dissolved in 950 ml of distilled H_2O, titrated to pH 7.0 with NaOH and made up to 2 l with distilled H_2O.

0.016 M MBS. MBS (5 mg) was dissolved in DMF (1 ml)

3.3.2.2 Procedure

Rabbit albumin (2.86 mg, 0.0419 μmoles, M_r 68 200) was dissolved in PBS (1.79 ml). MBS (105.6 μl 1.68 μmoles M_r 2382.7, giving a 1:40 molar ratio of carrier: MBS) was slowly stirred into the carrier solution. Carrier protein was acylated at RT for 30 min. Unreacted MBS was removed by MEC on a Sephadex G25 column (12 mm × 170 mm, flow rate 0.19 ml/min). Fractions (1 ml) were collected. The elution profile was monitored by reading absorbance values at 280 nm. Fractions constituting the first peak, which contained activated carrier, were pooled. The second peak, which contained unreacted MBS, eluted soon after the first peak.

Peptide #1 (4 mg, 1.68 μmoles, M_r 2382.7) was dissolved in DMSO (300 μl) and PBS (700 μl). 10 mM DTT in reducing buffer (1 ml) was added to the dissolved peptide with stirring and reduction was effected by incubation at 37 °C for 1.5 h. Reduced peptide was separated from excess DTT by MEC on a Sephadex G10 column (130 mm × 10 mm, flow rate 0.13 ml/min) pre-equilibrated with MEC buffer. Fractions (500 μl) were collected and the elution profile was determined by taking 10 μl of each fraction and mixing it with an equal volume of the Ellman's reagent. A yellow colour was an indication of the presence of the eluted peptide. Thereafter, fractions with excess DTT, indicated by an intensely yellow colour, were eluted. The reduced peptide was mixed with the activated carrier, giving a 40:1 molar ratio of peptide to activated carrier, and incubated for 3 h at RT.

To confirm the formation of a peptide-carrier conjugate, conjugates were analysed by non-

reducing SDS–PAGE gel (Section 2.5).

3.3.3 Conjugation of a peptide to rabbit albumin using glutaraldehyde

Peptide #3 (Section 3.4.1, Table 7) was conjugated via its free N-terminal α-amino group to rabbit albumin according to Briand et al. (1985).

3.3.3.1 Reagents

PBS, pH 7.2. As per Section 2.10.1

3.3.3.2 Procedure

Peptide (4 mg, 2.03 μmoles, M_r 1969) was added to a 1 mg/ml solution of rabbit albumin protein (3.464 mg, 0.051 μmoles, M_r 68 200, to give a molar ratio of peptide to carrier of 40:1) in PBS (3.46 ml). Glutaraldehyde (25% (v/v); 138.4 μl) was added slowly with stirring to the protein mixture, to give a final concentration of 1% (v/v) glutaraldehyde. The reaction was allowed to proceed for 1 h. The reaction was stopped by incubation for 1 h at 4°C after the addition of $NaBH_4$ (10 mg/ml). The mixture was dialysed against PBS (16 h, 3 changes) and stored at 4 °C.

3.4 Results

3.4.1 Selection of peptides

The epitope prediction plots for the complete CP1 and CP2 sequences and that for the C-terminal extension of vivapain were plotted (see Appendix). These plots were used for peptide selection. The prediction plots for peptides selected show local peaks of hydrophilicity, flexibility, surface probability and antigenicity. The prediction plots for peptides selected from the pro-region of congopain are shown in Figure 9, the CP1 catalytic domain in Figure 10 and the CP2 catalytic domain in Figure 11. The epitope prediction plots for the peptides in the C-terminal extensions of CP2 and vivapain are shown in Figure 12.

The CP1 peptides selected are shown in Table 6. The first three peptides (peptides A, B and C)

are from the pro-region of CP1. Antibodies against the peptides in the pro-region of CP cannot recognise the active, mature enzyme because the pro-region is cleaved upon maturation of the enzyme (Boulangé et al., 2001). Pro-peptides and antibodies against them can, however, be used to study inhibitory properties of the pro-region of trypanosomal cysteine proteinases and also to study the processing of the protease. For the purposes of this study, antibodies against peptides in the pro-region of congopain were raised to test the immunogenicity of the peptides chosen but these antibodies were not characterised further. The epitope prediction plots (Figure 9) show that peptides A and C are more immunogenic towards their N-termini and peptide B towards its C-terminus. Peptides A and C were therefore conjugated to the carrier protein via their C-termini and peptide B via its N-terminus.

An N-terminal peptide (D), a catalytic domain peptide (E) and an active site His-containing peptide (F) were selected from the catalytic domain of CP1 (Table 6). The epitope prediction plots (Figure 10) show that peptides D, E and F are more immunogenic towards their C-termini and were therefore conjugated to the carrier protein via their N-termini. An N-terminal peptide (#1), a catalytic domain peptide (#2) and an active site His-containing peptide (#3) selected from the catalytic domain of CP2 and peptides in the C-terminal extensions of CP2 (peptide 4) and vivapain (peptide 5) are shown in Table 7. The epitope prediction plots (Figure 11 and 12) show that peptides #1 and #5 are more immunogenic towards their N-termini and peptides #3 and #4 towards their C-termini. Peptides #1 and # 5 were therefore conjugated to the carrier protein via their C-termini and peptides #3 and #4 via their N-termini. The epitope prediction plot of peptide #2 (Figure 11) shows that this peptide is immunogenic at either end but this peptide was conjugated to the carrier protein via its N-terminus.

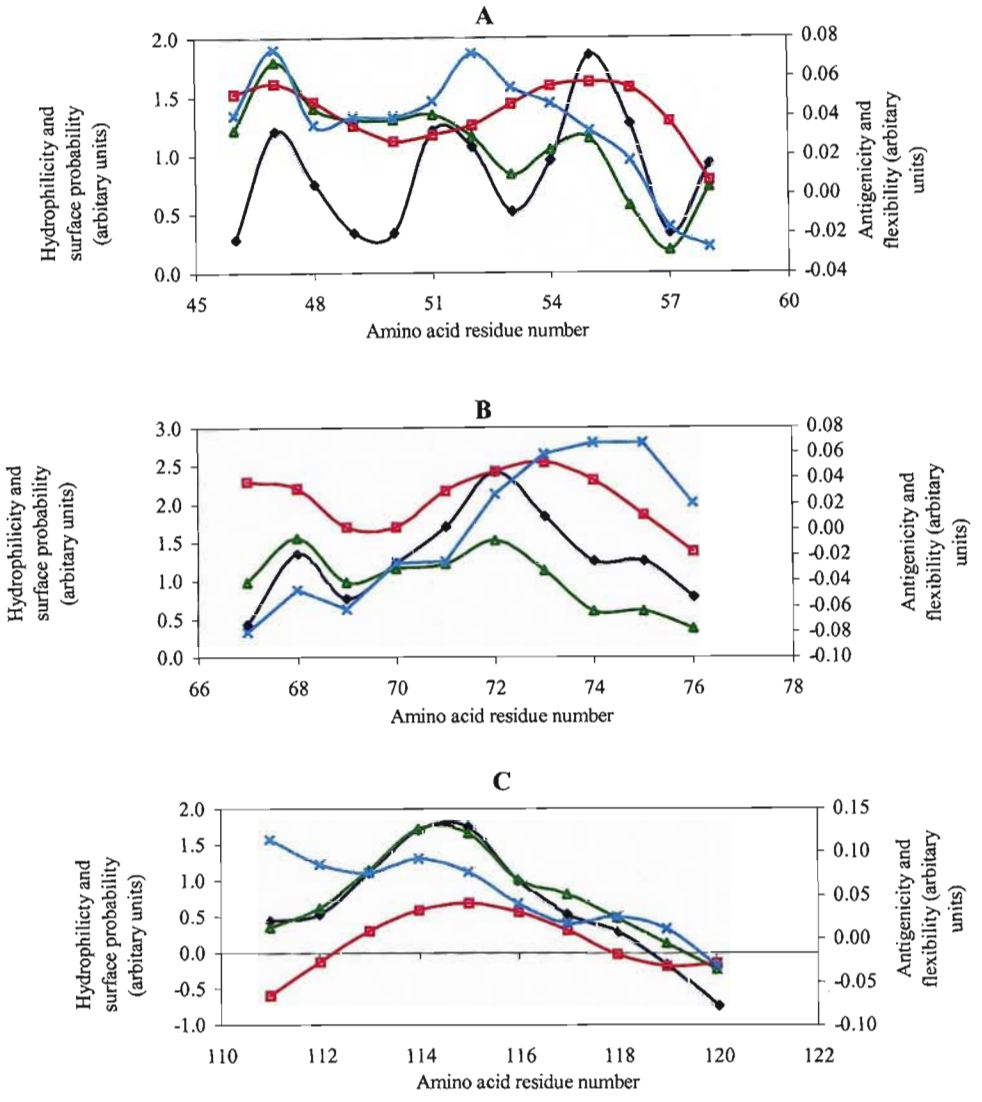

Figure 9. Epitope prediction plots for peptides selected from the pro-region of CP1. The CP1 amino acid sequence was deduced by Fish et al., 1995. Prediction plots of peptide A (panel A), residues 46-58; peptide B (panel B), residues 67-76 and peptide C (panel C), residues 111-120. The parameters plotted are: hydrophilicity (◇), surface probability (△), flexibility (□) and antigenicity (×). The computer program, Predict 7 (Cármens et al., 1989), was used to generate the data.

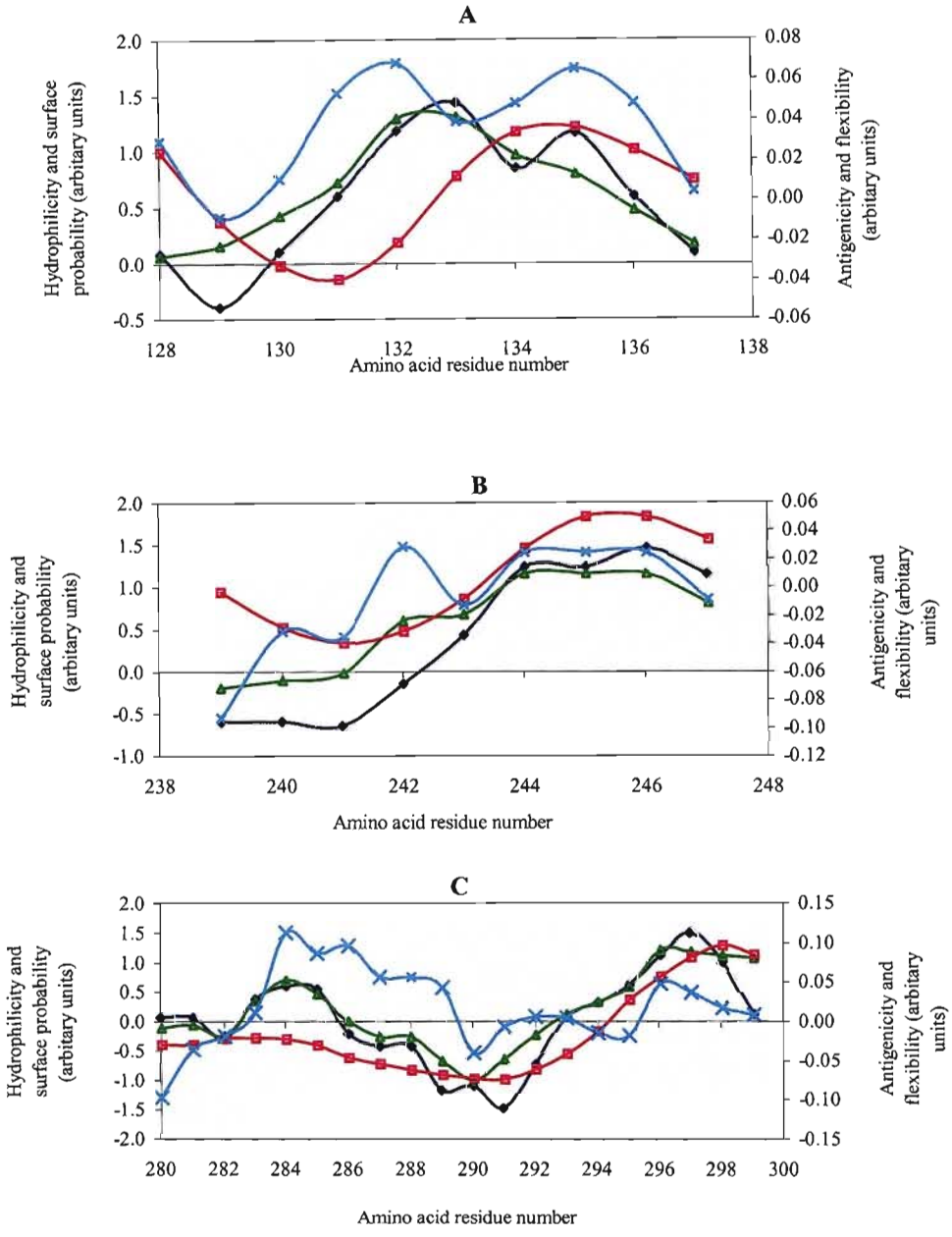

Figure 10. Epitope prediction plots for peptides selected from the catalytic domain of CP1. The CP1 amino acid sequence was deduced by Fish et al., 1995. Prediction plots of peptide D (panel A), residues 128-137; peptide E (panel B), residues 239-247 and peptide F (panel C), residues 279-299. The parameters plotted are: hydrophilicity (◇), surface probability (△), flexibility (□) and antigenicity (×). The computer program, Predict 7 (Cármens et al., 1989), was used to generate the data.

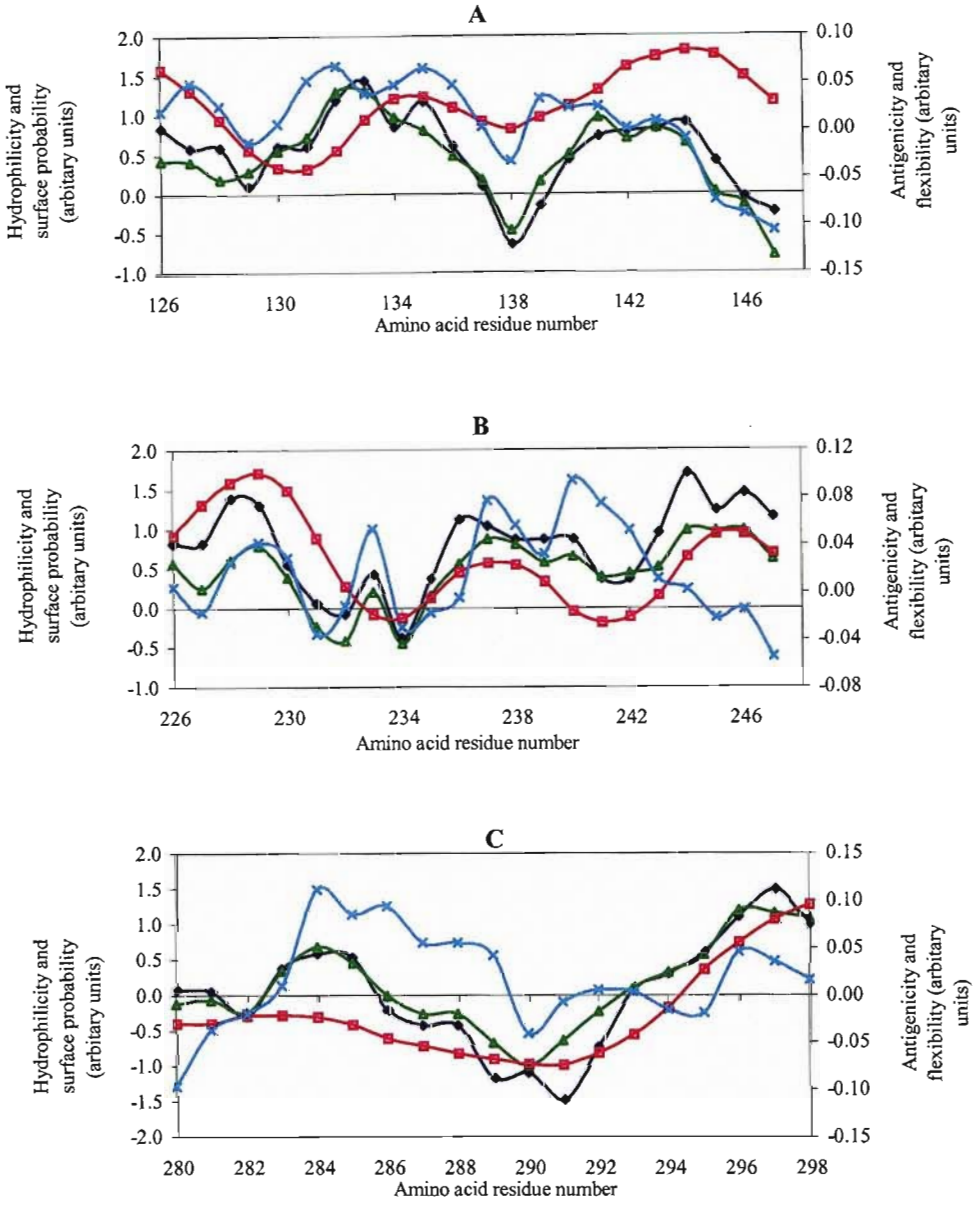

Figure 11. Epitope prediction plots for peptides selected from the catalytic domain of CP2. The CP2 amino acid sequence was deduced by Jaye et al., EMBL accession number L25130. Prediction plots of peptide #1 (panel A), residues 126-147; peptide #2 (panel B), residues 226-247 and peptide #3 (panel C), residues 281-298. The parameters plotted are: hydrophilicity (◇), surface probability (△), flexibility (□) and antigenicity (×). The computer program, Predict 7 (Cármens et al., 1989), was used to generate the data.

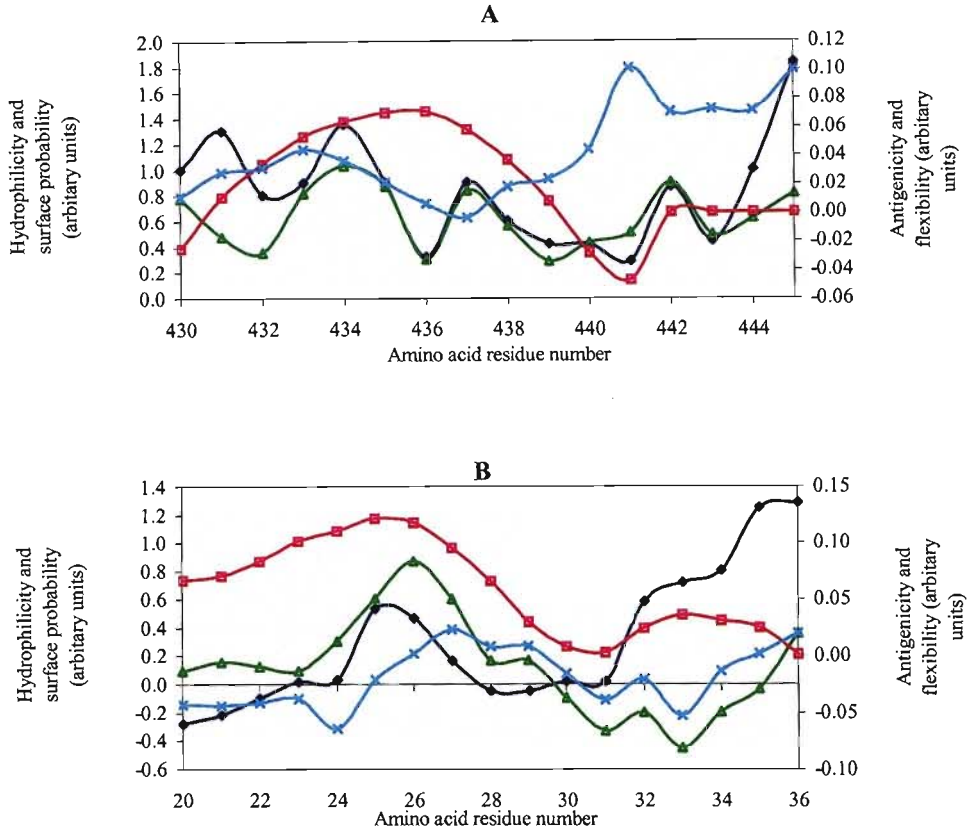

Figure 12. Epitope prediction plots for peptides selected from the C-terminal extensions of CP2 and vivapain. The CP2 amino acid sequence was deduced by Jaye et al., EMBL accession number L25130 and that of vivapain by Prof. Theo Baltz, University of Bordeaux, France, personal communication. Prediction plots of CP2, residues 430-445 (peptide #4, panel A) and residues 20-36 (peptide #5, panel B). The parameters plotted are: hydrophilicity (◇), surface probability (△), flexibility (□) and antigenicity (×). The computer program, Predict 7 (Cármens et al., 1989), was used to generate the data.

The catalytic domains of CP1 and CP2 have significant sequence homology (Figure 4). Consequently the amino acid sequence of peptide D is similar to its CP2 counterpart peptide #1. The same applies to peptide E and peptide #2 as well as peptide F and peptide #3. The alignment of the sequences of these peptides is shown in Figure 13. Because of the homology of CP1 peptides to CP2 peptides, antibodies against CP1 peptides are likely to cross-react with CP2 peptides and with native CP2.

Table 6. CP1 peptides chosen for anti-peptide antibody production

Residue #[a]	Peptide sequence	Region in CP1	Peptide #
46-58 (14)	Q-K-Y-S-R-S-Y-K-D-A-T-E-E-**C**[b]	Pro-region	A
67-76 (11)	**C**-Q-N-M-E-R-A-K-E-E-A	Pro-region	B
111-120 (11)	A-L-K-R-P-R-K-V-V-N-**C**	Pro-region	C
128-137 (11)	**C**-P-A-V-D-W-R-K-K-G-A	catalytic domain	D
239-247 (10)	**C**-G-H-I-N-L-P-K-D-E	catalytic domain	E
280-299 (20)	C-I-S-K-G-L-D-H-D-V-L-L-V-G-Y-D-D-T-S-K	catalytic domain	F

[a]Number of amino acid residues in peptide, including additional Cys residue is given in brackets. [b]Cys-residue shown in bold represents additional Cys-residues added during synthesis for MBS conjugation.

Table 7. CP2 peptides and the vivapain peptide chosen for anti-peptide antibody production

Residue #[a]	Peptide sequence	Region in CP2	Peptide #
126-147 (22)	A-P-E-A-V-D-W-R-K-K-G-A-V-T-P-V-K-D-Q-G-Q-C	catalytic domain	1
226-247 (22)	C-D-K-S-G-K-V-V-G-A-K-I-R-D-H-V-D-L-P-E-D-E	catalytic domain	2
279-298 (18)	I-S-E-H-L-D-H-G-V-L-L-V-G-Y-D-D-T-S	catalytic domain	3
430-445 (16)	C-S-D-K-A-P-T-E-S-A-R-L-V-R-H-E	C-terminal extension	4
20-36 (17)	T-A-P-G-P-S-S-T-K-T-L-C[b]-S-G-D-D-C	Vivapain C-terminal extension	5

[a]Number of amino acid residues in peptide given in brackets. [b]The internal Cys-residue was synthesised as a 2-aminobutyric acid that mimics the conformation of an internal disulfide bonded Cys residue.

The peptides selected appear to be on the surface of the 3-dimensional structure of papain, which has an analogous structure to that of congopain (Lecaille et al., 2001) (Figure 14 and Figure 15). The peptides selected seem accessible and could thus be good immunogens. Furthermore, peptide F and peptide 3 include the active site histidine residue, and antibodies against these peptides could be inhibitors of the enzyme. Analogous peptides that include the active site His-residue from cathepsin L (Coetzer et al., 1991) and trypanopain-Tb (Troeberg

et al., 1997) were previously selected to induce inhibitory antibodies.

Peptide D: *C-P-A-V-D-W-R-K-K-G-A

Peptide 1: A-P-E-A-V-D-W-R-K-K-G-A-V-T-P-V-K-D-Q-G-Q-C

Peptide E: C-G-H-I-N-L-P-K-D-E

Peptide 2: C-D-K-S-G-K-V-V-G-A-K-I-R-D-H-V-D-L-P-E-D-E

Peptide F: C-I-S-K-G-L-D-H-D-V-L-L-V-G-Y-D-D-T-S-K

Peptide 3: I-S-E-H-L-D-H-G-V-L-L-V-G-Y-D-D-T-S

Figure 13. Alignment of the peptide sequences selected from the catalytic domains of CP1 and CP2. Peptides D, E, and F are from the catalytic domain of CP1 and peptides #1, #2 and #3 are from the catalytic domain of CP2. Underlined residues denote differences between CP1 and CP2 peptide sequences. *Cys residues in bold were additional residues added at the N-terminus for conjugation using the MBS-method.

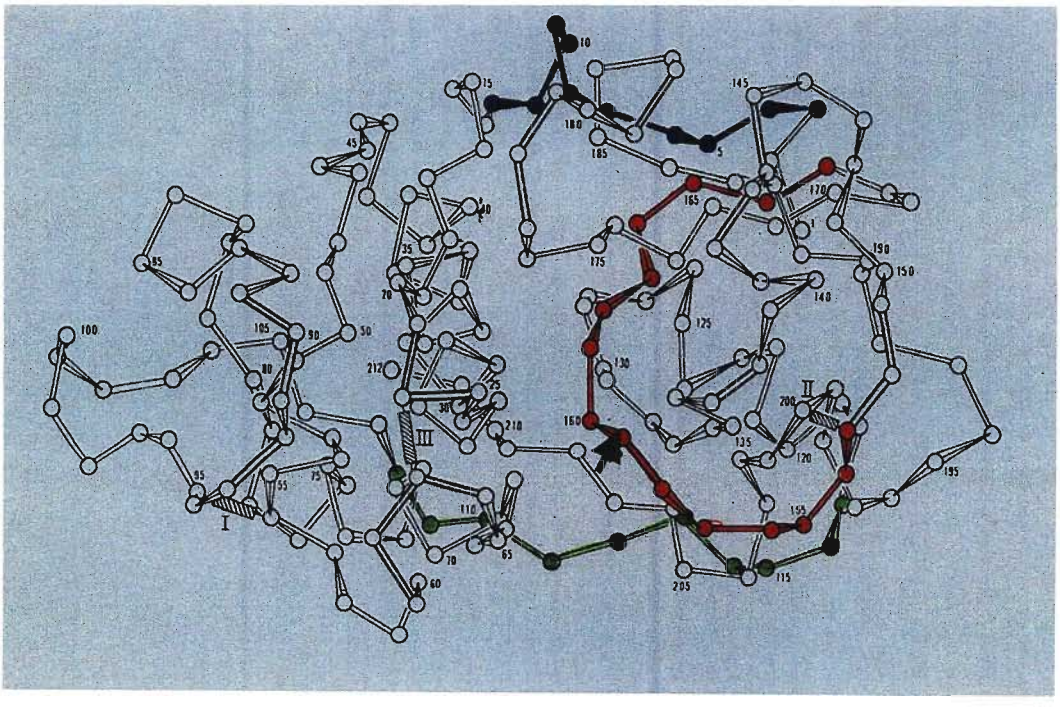

Figure 14. Papain structure (Drenth et al., 1971) showing the positions of peptides selected from the catalytic domain of CP1. Peptide D, CP1 residues 128-137 (•); peptide E, CP1 residues 239-247 (•) and peptide D, CP1 residues 280-299 (•). The arrow indicates the active site histidine residue.

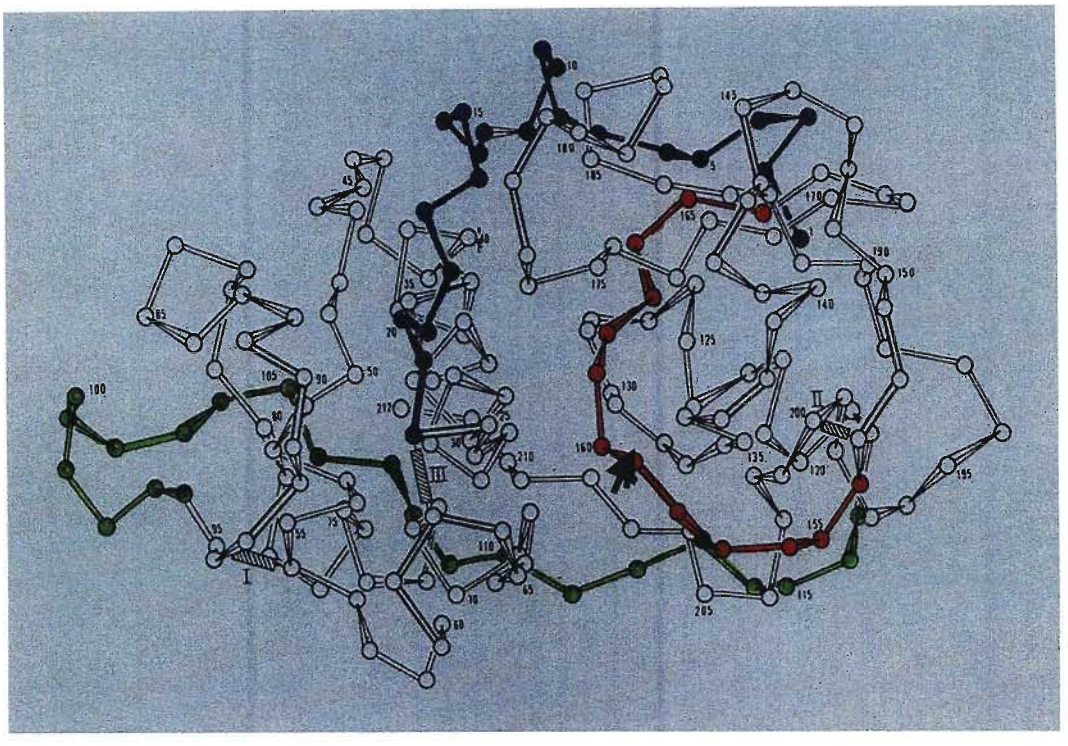

Figure 15. Papain structure (Drenth et al., 1971) showing the positions of peptides selected from the catalytic domain of CP2. Peptide #1, CP2 residues 126-147 (•); peptide #2, CP2 residues 226-247 (•) and peptide #3, CP2 residues 279-298 (•). The arrow indicates the active site histidine residue.

3.4.2 Evaluation of a conjugate by SDS-PAGE analysis

In order to confirm that peptide-carrier conjugates were formed, samples of carrier protein taken before and after conjugation were analysed on a non-reducing SDS-PAGE gel (Figure 16). Rabbit albumin alone gave a band at approximately 66 kDa (lane d) and MBS-activated rabbit albumin (lane c) gave a broad band of slightly greater and smaller size compared to lane d. The final conjugate (lane b) gave a band of slightly greater size compared to those of both lanes d and c, indicating successful conjugation.

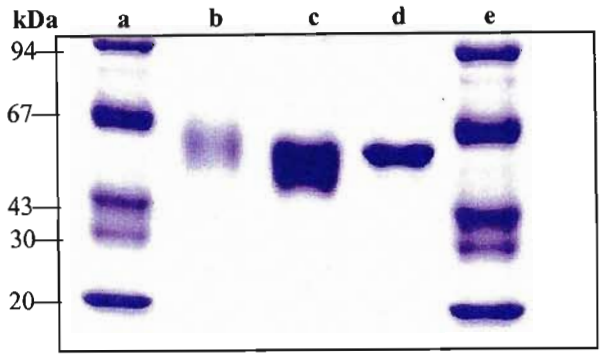

Figure 16. Non-reducing SDS-PAGE comparing protein sizes before and after peptide-carrier conjugation. Samples (10 µg) were electrophoresed on a 12.5% non-reducing SDS-PAGE gel (Section 2.5.2). Molecular mass markers (phosphorylase b, 94 kDa; BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa; soya-bean trypsin inhibitor, 20 kDa in lanes a and e); peptide-rabbit albumin conjugate (lane b); rabbit albumin-MBS (lane c) and rabbit albumin (lane d). The bands were visualised by Coomassie blue R-250 protein stain as described in Section 2.6.2.2.

3.4.3 Monitoring of antibody production by ELISA.

There was very little difference in the responses of the two chickens immunised with the same peptide, therefore results for only one chicken are shown. The ELISA results shown in Figures 17, 18 and 19 indicate that antibodies were raised in chickens against all the peptides when compared to non-immune antibody preparations. All the antibodies recognised the peptides very well except for peptide E (Figure 18 C) and peptide #3 (Figure 19 B) which gave low responses. Antibody production peaked between weeks 7 and 12 (Table 8). Most antibodies were produced between weeks 6 and 12. Although it may be speculated that the low titre of the anti-peptide E antibodies could be due to the small size (10 residues) of the peptide, peptide D (11 residues) produced antibodies of high titre. Their epitope prediction plots (panels A and B, Figure 10) did not suggest potential differences in immunogenicity. A portion of the immunogen was lost during preparation of the peptide #3 conjugate, so chickens received less immunogen. However, affinity purification of both anti-peptide E and anti-peptide #3 antibodies resulted in good targeting of the corresponding peptides in ELISA (Section 3.4.4).

Both peptides corresponding to sequences in the C-terminal extensions of congopain and vivapain gave fairly good immune responses (Figure 19 C and D). For the peptide in the C-terminal extension of CP2 antibody production was already high by week 6 post immunisation and for the peptide in the C-terminal extension of vivapain the highest antibody production

was at week 9 post immunisation. Table 8 gives a summary of the timing of peak antibody production against each peptide.

Table 8. Timing of peak of anti-CP peptide antibody and anti-vivapain C-terminal extension peptide antibody production.

Peptide #	Region in protease	Peptide length	Peak week
1	CP2 catalytic domain	22	12
2	CP2 catalytic domain	22	7
3	CP2 catalytic domain	18	7
4	CP2 C-terminal extension	16	7
5	Vivapain C-terminal extension	17	9
A	CP1 pro-region	14	7
B	CP1 pro-region	11	12
C	CP1 pro-region	11	9
D	CP1 catalytic domain	11	8
E	CP1 catalytic domain	10	10
F	CP1 catalytic domain	20	8

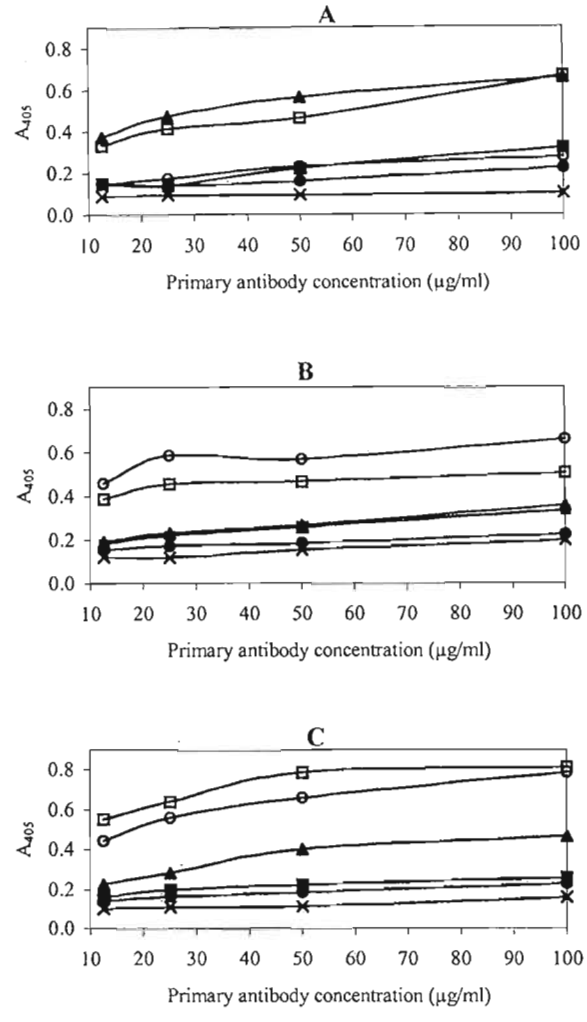

Figure 17. Recognition of peptides corresponding to sequences in the pro-region of CP1 in an ELISA by the anti-peptide antibodies raised in chickens at various weeks post immunisation. Plates were coated with peptides A (panel A), B (panel B) and C (panel C) (5 µg/ml in PBS, pH 7.2, 4 °C, 16 hours). See Table 6 for sequence details of peptides. Production of anti-peptide antibodies was determined at week zero (×), week 3 (■), week 6 (▲), week 9 (□), week 12 (○) and week 15 (●) post immunisation. All the anti-peptide antibodies were titrated between 100 µg/ml and 12.5 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

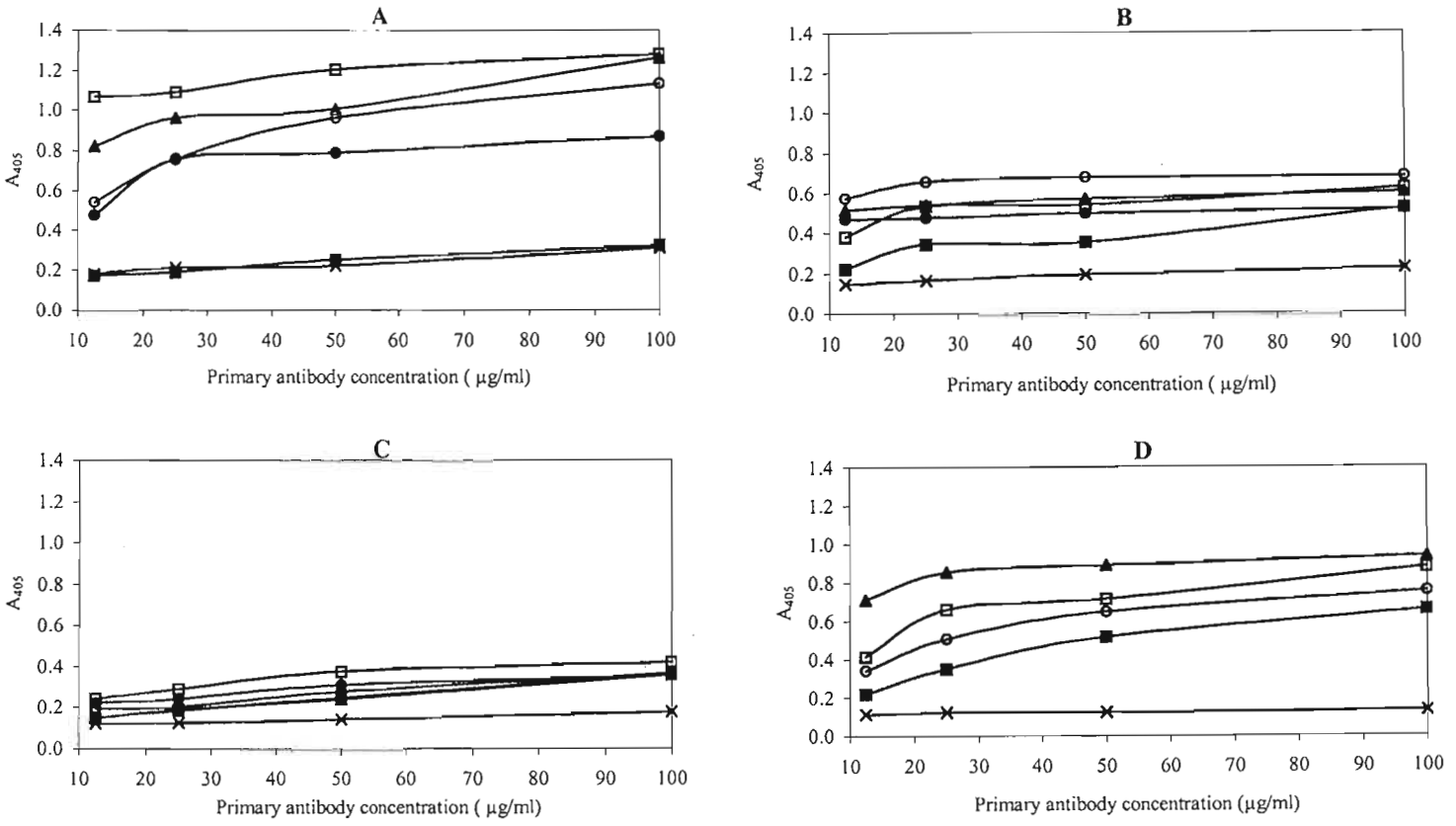

Figure 18. Recognition of N-terminal peptides and catalytic domain peptides selected from CP1 and CP2 in an ELISA by the anti-peptide antibodies raised in chickens at various weeks post immunisation. Plates were coated with peptides D (panel A), #1 (panel B), E (panel C) and #2 (panel D) (5 µg/ml in PBS, pH 7.2, 4 °C, 16 hours). See Tables 6 and 7 for sequence details of peptides. Production of anti-peptide antibodies was determined at week zero (×), week 3 (■), week 6 (▲), week 9 (□), week 12 (○) and week 15 (●) post immunisation. All the anti-peptide antibodies were titrated between 100 µg/ml and 12.5 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

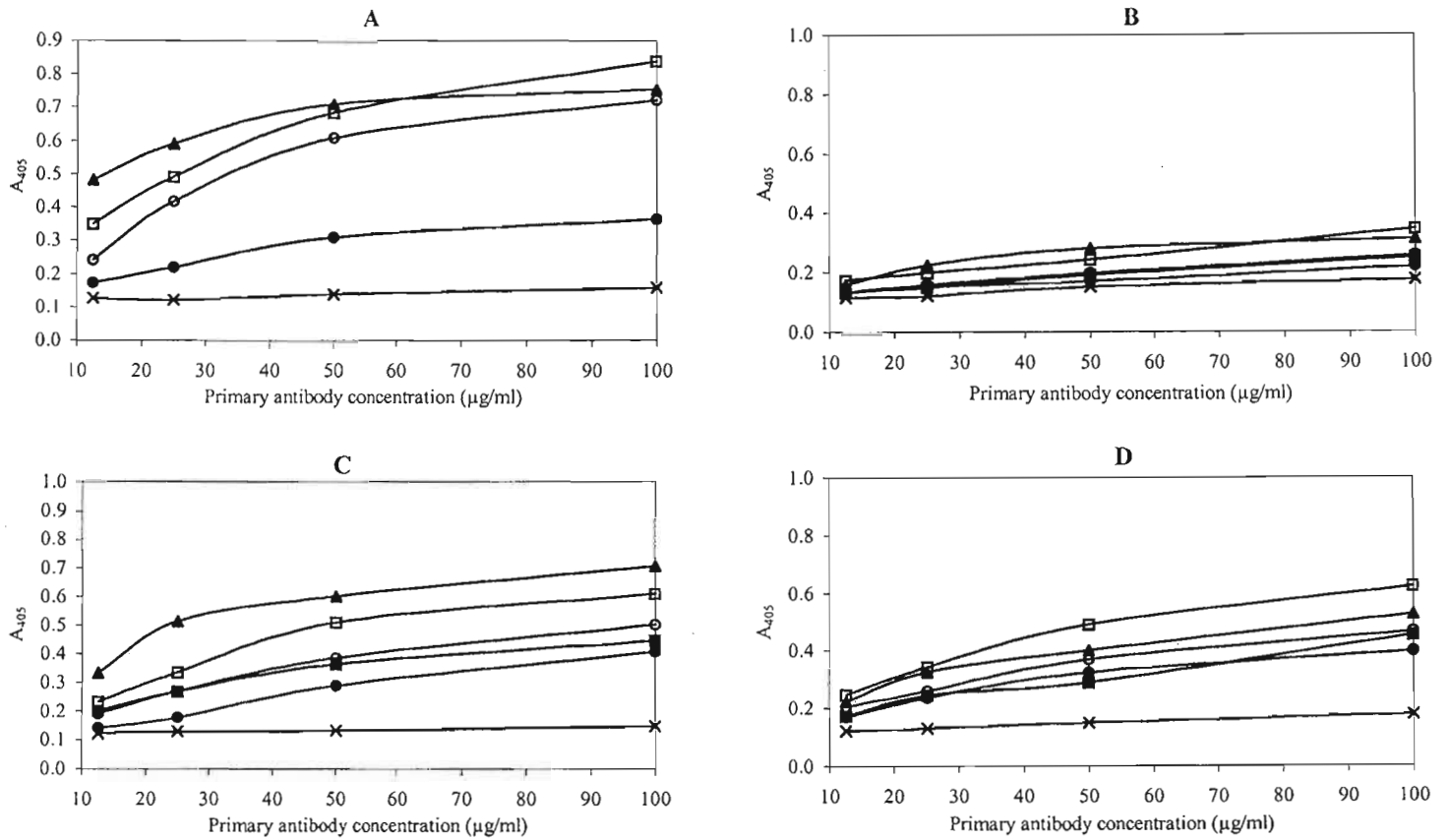

Figure 19. Recognition of active site-associated peptides from CP1 and CP2 and peptides from the C-terminal extension of CP2 and vivapain in an ELISA by the anti-peptide antibodies raised in chickens at various weeks post immunisation. Plates were coated with peptides F (panel A), #3 (panel B), #4 (panel C) and #5 (panel D) (5 µg/ml in PBS, pH 7.2, 4 °C, 16 hours). See Tables 6 and 7 for sequence details of peptides. Production of anti-peptide antibodies was determined at week zero (×), week 3 (■), week 6 (▲), week 9 (□), week 12 (○) and week 15 (●) post immunisation. All the anti-peptide antibodies were titrated between 100 µg/ml and 12.5 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

3.4.4 Affinity purification of anti-peptide antibodies

A representative elution profile for the affinity purification of anti-peptide antibodies is shown in Figure 20.

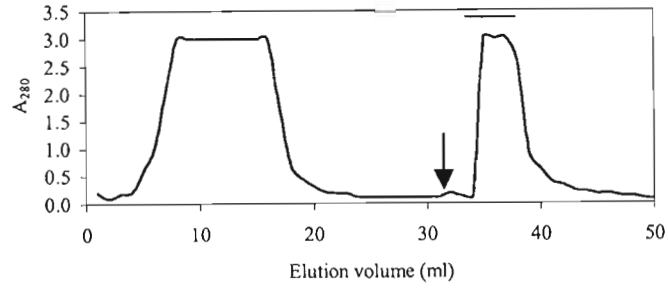

Figure 20. Elution profile for affinity purification of anti-peptide antibodies. The peptide affinity column was equilibrated in 0.1 M Na-phosphate buffer, 0.05% NaN_3, pH 7.0 (2 ml bed volume, 0.13 ml/min). After elution of unbound protein, bound protein was eluted by application of 0.1 M glycine-HCl, pH 2.8 (as described in Section 2.11.3.2), applied at the point indicated by the arrow (↓). Antibody fractions pooled (—).

To determine whether there is increased reactivity of the affinity purified antibodies with their respective peptides, ELISAs with immobilised peptide were done as described in Section 2.10.2. In the ELISAs the reactivity of the affinity purified antibodies, non-affinity purified antibodies, and the antibody fraction that did not bind to the peptide affinity column was compared. For each peptide, IgY samples were combined as follows: antibodies produced 2-6, 7-10, 11-12, 13-14 and 15-16 weeks after the first immunisation.

The affinity purified antibodies showed increased reactivity with the peptides compared to the non-affinity purified antibodies and the antibody fraction that did not bind to the peptide affinity column (Figures 21-23). Non-affinity purified antibodies recognised the peptides better than the antibody fraction that did not bind to the peptide affinity column. However, the reactivity of affinity purified antibodies targeting the N-terminal peptide (#1) and the catalytic domain peptide (#2) was only slightly higher than the reactivity of non-affinity purified antibodies and the antibody fractions that did not bind to the peptide affinity column (Figures 22A and 22B). This may be due to poor efficiency of the peptide affinity matrices where these two peptides were immobilised. On the other hand the reactivity of affinity purified antibodies targeting the active site-associated peptide (#3) and the vivapain C-terminal extension peptide (#5) were significantly higher than the non-affinity purified preparations (Figures 22C and

23B). In these cases the peptide matrices seemed to bind all the specific antibodies. In assays where these affinity purified antibodies were used, the development of the substrate colour was very quick and the whole reaction had to be stopped while the colour was still developing in wells containing non-affinity purified antibodies and the antibody fractions that did not bind to the peptide affinity column. This accounts for the low absorbance readings where non-affinity purified antibodies and the antibody fractions that did not bind to the peptide affinity column were used (Figure 22C and Figure 23B). The recognition of the active site His-containing peptide (#3) was significantly improved following affinity purification (Figure 22C).

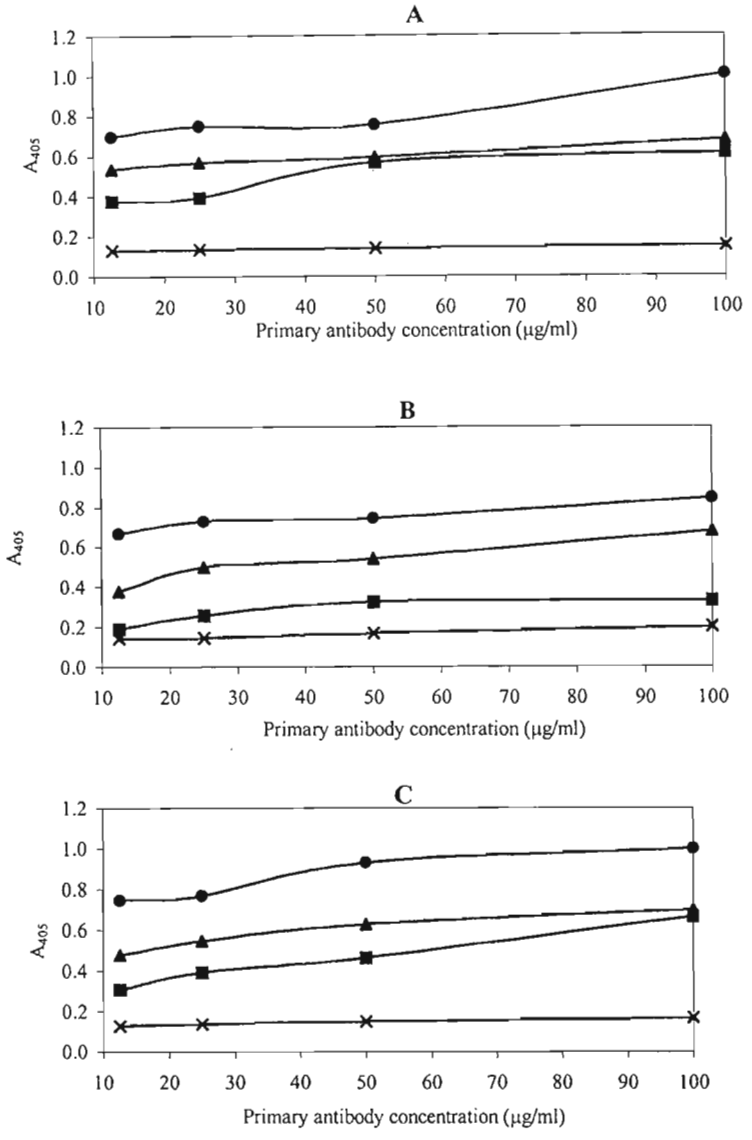

Figure 21. ELISA of affinity purified antibodies produced against peptides located in the catalytic domain of CP1. Plates were coated with N-terminal peptide D (panel A), catalytic domain peptide E (panel B) and active site-associated peptide F (panel C) (5 µg/ml in PBS, pH 7.2, 4 °C, 16 hours). The recognition of the peptides by non-immune IgY (×), antibody fraction that did not bind to the peptide affinity matrix (■), non-affinity purified antibodies (▲) and affinity purified antibodies (●) was determined. Antibodies were titrated between 100 µg/ml and 12.5 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

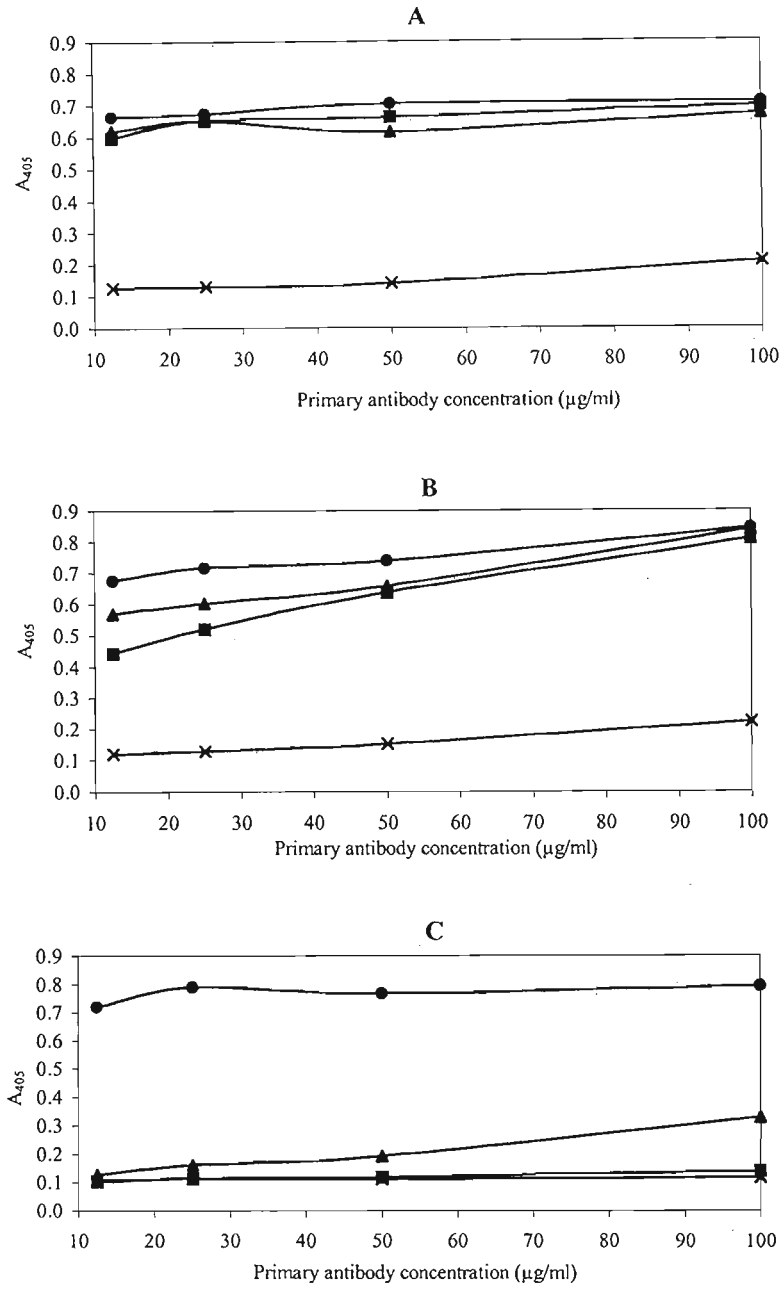

Figure 22. ELISA of affinity purified antibodies produced against peptides located in the catalytic domain of CP2. Plates were coated with N-terminal peptide #1 (panel A), catalytic domain peptide #2 (panel B) and active site-associated peptide #3 (panel C) (5 µg/ml in PBS, pH 7.2, 4 °C, 16 hours). The recognition of the peptides by non-immune IgY (×), antibody fraction that did not bind to the peptide affinity matrix (■), non-affinity purified antibodies (▲) and affinity purified antibodies (●) was determined. Antibodies were all titrated between 100 µg/ml and 12.5 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

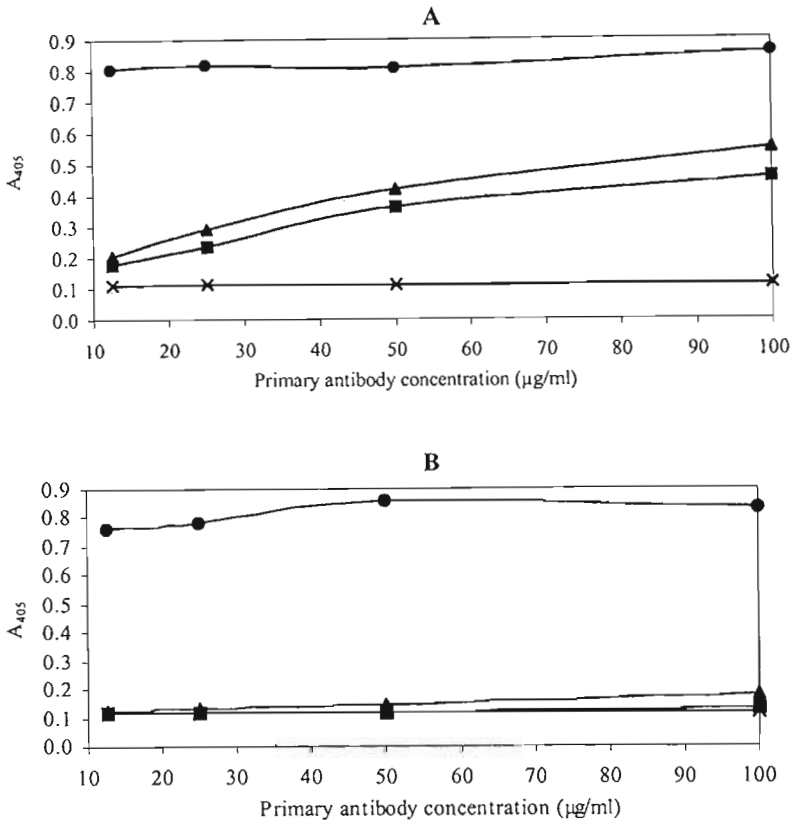

Figure 23. ELISA of affinity purified anti-C-terminal extension peptide antibodies. Plates were coated with congopain peptide #4 (panel A) and vivapain peptide #5 (panel B) (5 µg/ml in PBS, pH 7.2, 4 °C, 16 hours). The recognition of the peptides by non-immune IgY (×), antibody fraction that did not bind to the peptide affinity matrix (■), non-affinity purified antibodies (▲) and affinity purified antibodies (●) was determined. Antibodies were all titrated between 100 µg/ml and 12.5 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

3.4.5 Recognition of C2 and CP2 coated on ELISA plates by anti-peptide antibodies.

Anti-peptide antibodies were able to recognise CP2 in an ELISA. On comparing the recognition of CP2 by anti-CP2 peptide and anti-CP1 peptide antibodies, enhanced recognition was observed with the former. The reactivity of anti-CP2 peptide antibodies with CP2 in diminishing order of reactivity was: anti-active site peptide (#3) > anti-C-terminal extension peptide (#4) > anti-catalytic domain peptide (#2) > anti-N-terminal peptide (#1) (Figure 24A). The reactivity of anti-CP1 peptide antibodies with CP2 in diminishing order of reactivity was: anti-active site peptide (F) > anti-N-terminal peptide (D) > anti-catalytic domain peptide (E) (Figure 25A). Antibodies against both peptide #3 and peptide C recognised CP2 best and both peptides include the active site histidine residue.

Anti-peptide antibodies apparently reacted more strongly with C2 than CP2. The reactivity of anti-CP2 peptide antibodies with C2 in diminishing order of reactivity was: anti-N-terminal peptide (#1) > anti-catalytic domain peptide (#2) > anti-active site peptide (#3) >anti-C-terminal extension peptide (#4) (Figure 24B). The reactivity of anti-CP1 peptide antibodies with C2 in diminishing order of reactivity was: anti-active site peptide (F) > anti-N-terminal peptide (D) > anti-catalytic domain peptide (E) (Figure 25B).

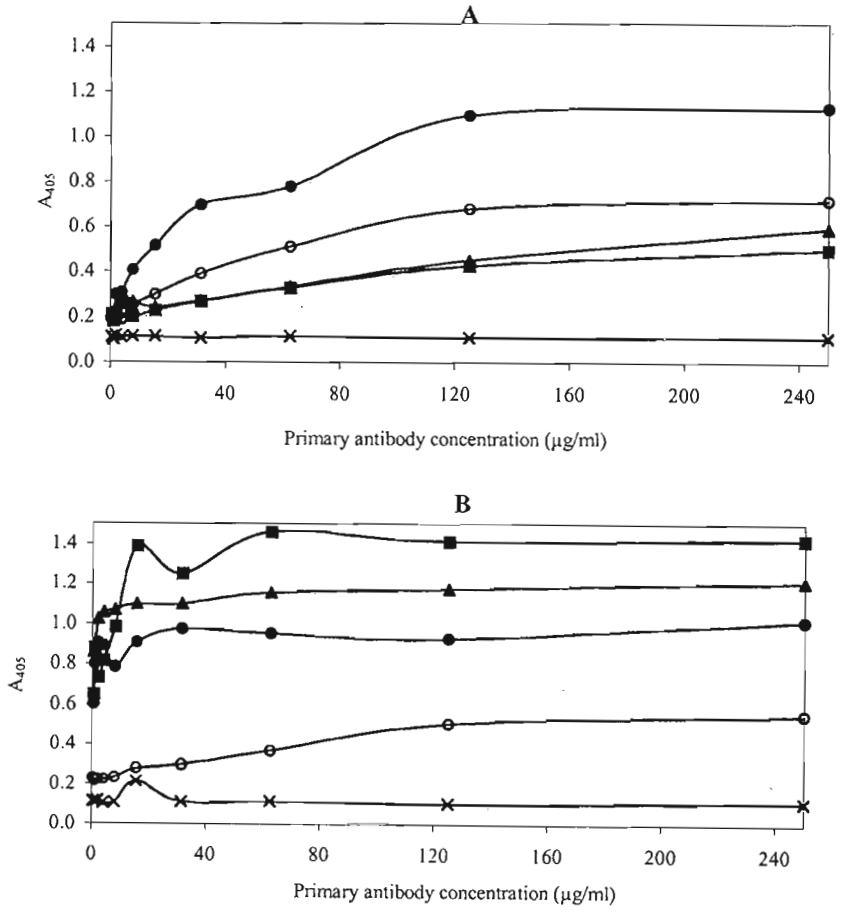

Figure 24. Recognition of CP2 and C2 by affinity purified anti-CP2 peptide antibodies in an ELISA. Plates were coated with CP2 (Panel A) or C2 (Panel B) (1 µg/ml for 16 hours at 4 ° C in 50 mM carbonate coating buffer, pH 6.0). Affinity purified antibodies against: N-terminal peptide #1(■), catalytic domain peptide #2 (▲), active site-associated peptide #3 (●), C-terminal extension #4 (○) and non-immune IgY (×) were titrated between 250 µg/ml and 0.49 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

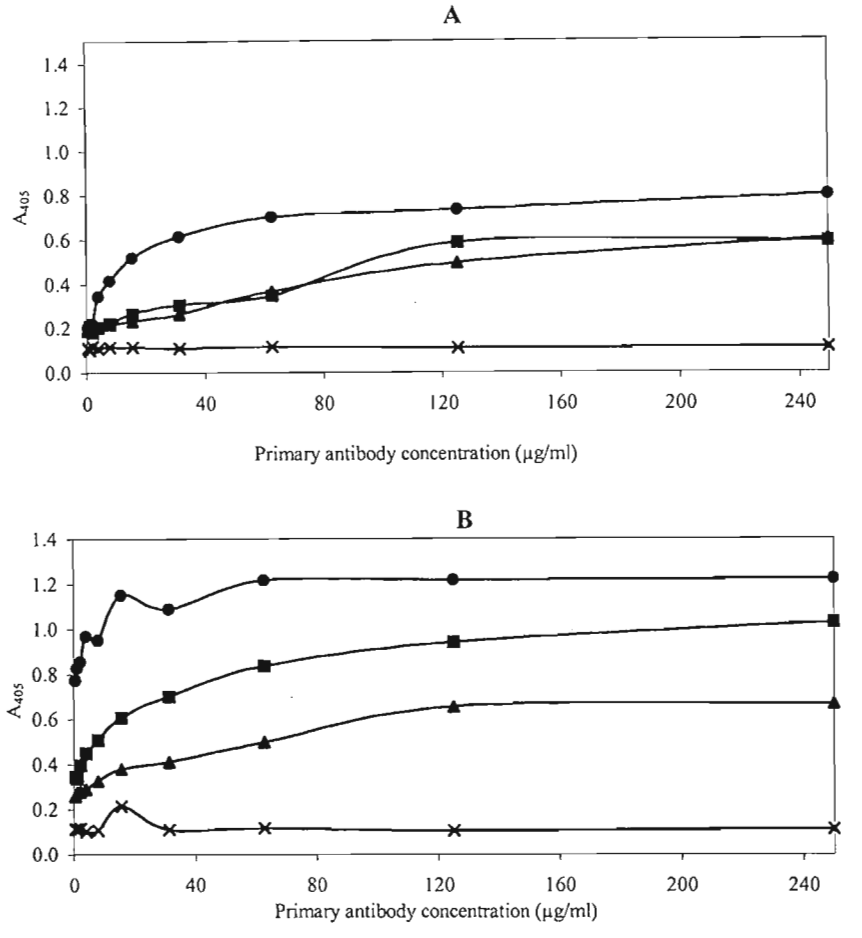

Figure 25. Recognition of CP2 and C2 by affinity purified anti-CP1 peptide antibodies in an ELISA. CP2 (Panel A) and C2 (Panel B) was coated at 1 µg/ml for 16 hours at 4 ° C in 50 mM carbonate coating buffer, pH 6.0. Affinity purified antibodies against N-terminal peptide D (■), catalytic domain peptide E (▲), active site-associated peptide F (●) and non-immune IgY (×) were titrated between 250 µg/ml and 0.49 µg/ml. Binding was visualised by incubation with rabbit anti-chicken HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate as described in Section 2.10.2. Each point is the mean absorbance at 405 nm of duplicate samples.

3.4.6 Recognition of C2 and CP2 by anti-peptide antibodies on western blots.

The homogeneity of the purified C2 and CP2 samples was determined in reducing Laemmli SDS-PAGE (Figure 26). Two bands were observed for CP2 on a silver stained gel, i.e. a very faint band at *ca.* 43 kDa and a prominent band at *ca.* 30 kDa (Figure 26). The ~ 43 kDa band is thought to be the dimeric form of congopain while the ~ 30 kDa band could be the monomeric form of congopain. The formation of monomeric and dimeric forms of congopain will be discussed in more detail in Section 3.5. For C2, only a single band was observed. The band had a molecular weight of approximately 30 kDa (Figure 26).

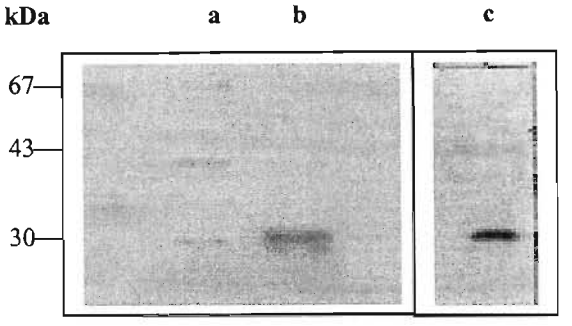

Figure 26. Reducing Laemmli SDS-PAGE evaluation of recombinant CP2 and C2. Molecular mass markers [BSA, 67 kDa; ovalbumin, 43 kDa; carbonic anhydrase, 30 kDa (2 µl, lane a)], CP2 (8 µg, lane b) and C2 (5 µg, lane c) were electrophoresed on a 12.5% reducing SDS-PAGE gel (Section 2.5.2). Protein bands were visualised by silver staining (Section 2.6.2.2).

To detect C2 in a western blot, anti-CP2 catalytic domain peptide antibodies were used. Since C2 was not recognised by antibodies against C-terminal extension peptide in ELISA (as expected), these antibodies were not used in western blots to conserve the small amount of C2 available. All the antibodies detected the M_r ~ 30 kDa band, except antibodies from the non-immunised chicken (Figure 27). In western blots of CP2 separated by reducing SDS-PAGE, both CP1 and CP2 anti-peptide antibodies detected both a 43 kDa band and a 30 kDa band (Figure 28). As can be seen in Figure 28, the ~ 43 kDa band (dimeric form) was recognised less well than the ~ 30 kDa band (monomeric form). Antibodies from the non-immunised chicken did not detect any of the bands, and neither did antibodies against the C-terminal extension peptide (#4) (Figure 28).

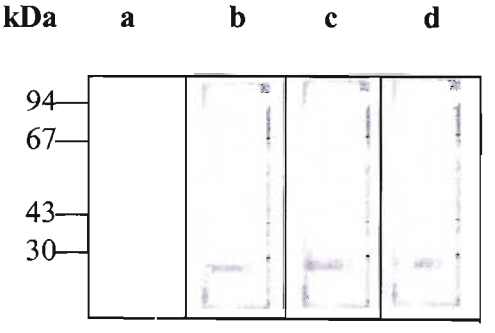

Figure 27. Recognition of C2 by the anti-CP2 peptide antibodies on western blots. C2 (5 µg) was electrophoresed on a 12.5% reducing SDS-PAGE gel (Section 2.5.2), electroblotted onto nitrocellulose and probed with non-immune IgY (lane a), anti-N-terminal peptide #1 (lane b), anti-catalytic domain peptide #2 (lane c) and anti-active site-associated peptide #3 (lane d). Immune reactions were detected using rabbit anti-chicken IgG-alkaline phosphatase conjugate (Sigma, St Louis, USA) and the substrate NBT/BCIP as described in Section 2.7.2.

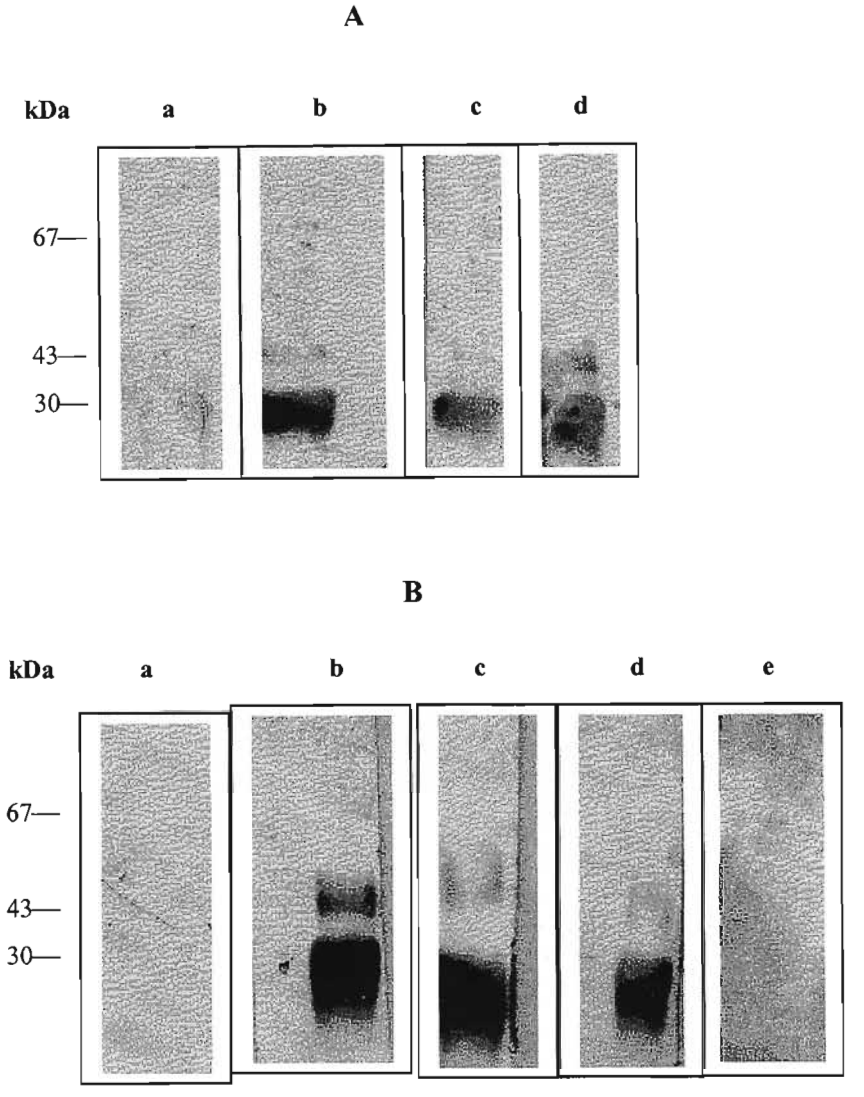

Figure 28. Recognition of CP2 by the anti-CP1 peptide and anti-CP2 peptide antibodies on western blots. CP2 (8 μg) was electrophoresed on a 12.5% reducing SDS-PAGE (Section 2.5.2), electroblotted onto nitrocellulose and probed with: anti-CP1 peptide antibodies (panel A): non-immune IgY (a), anti-N-terminal peptide D (b), anti-catalytic domain peptide E (c), anti-active site-associated peptide F (d) and anti-CP2 peptide antibodies (panel B): non-immune IgY (a), anti-N-terminal peptide #1 (b), anti-catalytic domain peptide #2 (c), anti-active site-associated peptide #3 (d) and anti-C-terminal extension peptide #4 (e). Immune reactions were detected using rabbit anti-chicken-HRPO conjugate (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and the substrate used was 4-chloro-1-naphthol/H_2O_2 as described in Section 2.7.2.

3.4.7 Inhibition of catalytic activity using antibodies.

Generally, anti-peptide antibodies had an activity enhancing effect compared to non-immune antibody (Figures 29 and 30). Antibodies produced in weeks 15 and 16 post immunisation usually had the highest activity enhancing effect.

Figure 29 shows the enhancement of CP2 activity by the anti-peptide antibodies. Low

concentrations (between 31.25 and 62.5 μg/ml) of anti-N-terminal peptide (#1) antibodies stimulated CP2 activity (up to 36.7%) but at higher concentrations, there was slight inhibition of CP2 activity (up to 1.3%). With an increase in antibody concentration their effect on enzyme activity diminished (Figure 29A). Lower concentrations of anti-catalytic domain peptide (#2) antibodies (between 31.25 and 250 μg/ml) enhanced CP2 activity (up to 12.7%) but at 500 μg/ml concentration there was inhibition of CP2 activity (up to 15.7%) (Figure 29B). Anti-active site peptide (#3) antibodies had a slight inhibiting effect (up to 2.6%) on CP2 activity at lower concentrations (between 31.25 and 62.5 μg/ml) but at higher concentrations they seemed to have no effect on CP2 activity, except for those produced in weeks 15 and 16 post immunisation. These antibodies had an activity enhancing effect (up to 23.9%) (Figure 29C). Anti-CP1 active site peptide (F) antibodies enhanced CP2 (up to 17.1%) and as the antibody concentration increased from 62.5 μg/ml to 259 μg/ml, activity was inhibited (up to 11.1%) (Figure 29D).

Most anti-peptide antibodies seemed to have a stimulating effect on C2 activity. The stimulation apparently increased with an increase in antibody concentration. Anti-N-terminal peptide (#1) antibodies stimulated C2 activity up to 27.6% (Figure 30A), anti-active site peptide (#3) antibodies up to 42.1% (Figure 30C) and anti-CP1 active site peptide (F) antibodies up to 29% (Figure 30D). Only anti-catalytic domain peptide (#2) antibodies seemed to give a biphasic response. For most antibodies, a biphasic response is obtained at different antibody concentrations whereby small amounts of sera stimulate activity, whereas higher antibody concentrations inhibit activity (Richmond, 1977). The enzyme activity was stimulated (up to 35.4%) at lower antibody concentrations (between 31.25 and 250 μg/ml), while activity was inhibited (up to 9.2%) at higher antibody concentrations (Figure 30B).

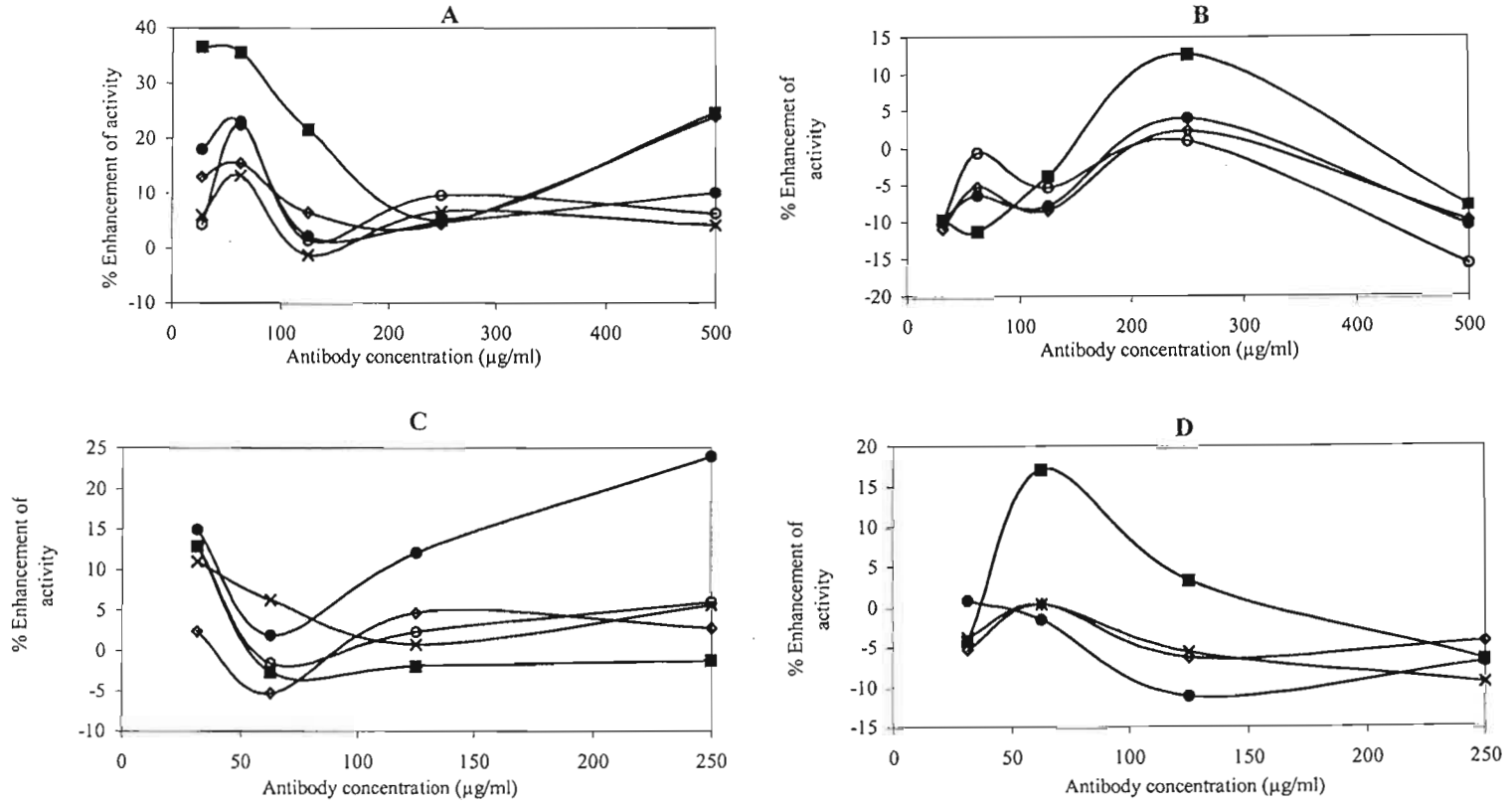

Figure 29. Effect of anti-peptide antibodies on Z-Phe-Arg-AMC hydrolysing activity of CP2. Serial two fold dilutions of anti-peptide antibodies raised against N-terminal peptide #1 (panel A), catalytic domain peptide #2 (panel B), active site-associated peptide #3 (panel C) and active site-associated peptide F (panel D) from weeks 2-6 (○), weeks 7-10 (■), weeks 11-12 (◇), weeks 13-14 (×) and weeks 15-16 (●) were pre-incubated (37 C, 15 min.) with CP2 (20 ng) before addition of the substrate (Section 2.12.2). Enhancement was expressed as a percentage of CP2 hydrolysis of Z-Phe-Arg-AMC relative to non-immune IgY.

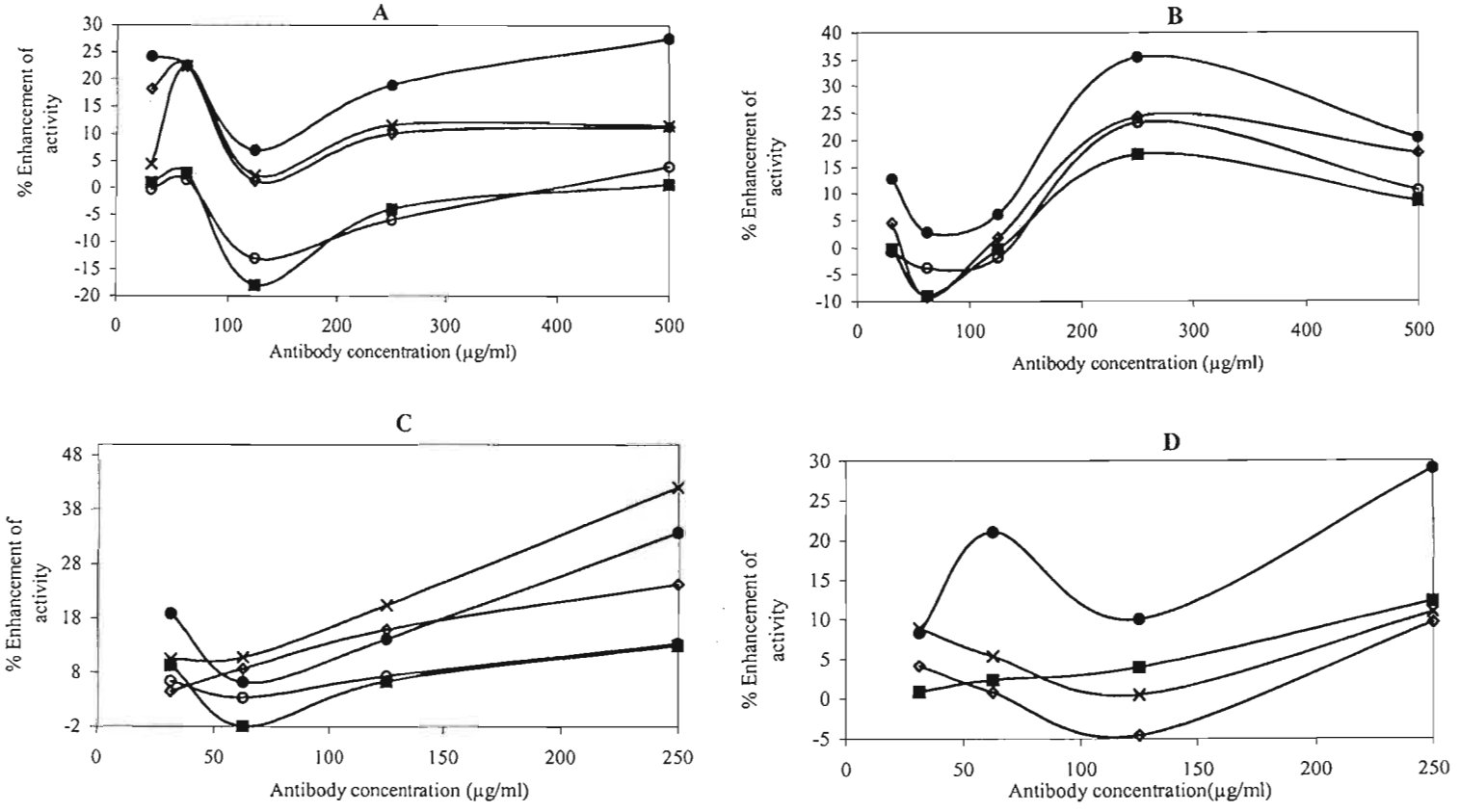

Figure 30. Effect of anti-peptide antibodies on Z-Phe-Arg-AMC hydrolysing activity of C2. Serial two fold dilutions of anti-peptide antibodies raised against N-terminal peptide #1 (panel A), catalytic domain peptide #2 (panel B), active site-associated peptide #3 (panel C) and active site-associated peptide F (panel D) from weeks 2-6 (O), weeks 7-10 (■), weeks 11-12 (◇), weeks 13-14 (×) and weeks 15-16 (●) were pre-incubated (37 °C, 15 min.) with C2 (20 ng) before addition of the substrate (Section 2.12.2). Enhancement was expressed as a percentage of CP2 hydrolysis of Z-Phe-Arg-AMC relative to non-immune IgY.

3.5 Discussion

In this study, antibodies against congopain peptides were raised, based on the hypothesis that congopain may contribute to pathogenesis and that an immune response to congopain may contribute to trypanotolerance (Authié et al., 2001). *T. congolense* possesses at least two families of closely related cysteine proteinases, named CP1 (Fish et al., 1995) and CP2 (Jaye et al., EMBL accession number L25130). The deduced amino acid sequences of the two cysteine proteinases are 90% identical but they differ in their N-terminal sequences (Boulangé et al., 2001). For this reason, peptides from both CP1 and CP2 were selected for the immunisation trials.

Peptides were chosen using a computer programme for epitope prediction, Predict 7 (Cármenes et al., 1989). The epitope prediction plots of the peptides selected showed overlapping peaks of hydrophilicity, flexibility, antigenicity and surface probability. The results also showed that the peptides selected should occur on the surface of the CP2 molecule. Sequences from the active site clefts of CP1 and CP2 were also selected, even though they did not appear to be on the surface of the enzyme molecule, because peptides from active site clefts are likely to elicit anti-catalytic antibodies (Troeberg et al., 1997). C-terminal extensions of trypanosomal cysteine proteinases are known to be antigenic (Lalmanach et al., 2002); for this reason peptides in the C-terminal extensions of congopain and vivapain were chosen. Also, when this study was undertaken only the sequence of the C-terminal extension of vivapain was known. Following the recent elucidation of the complete sequence of vivapain (Prof. Theo Baltz, University of Bordeaux, France, personal communication) a parallel study of the immunogenic epitopes in the catalytic domain of vivapain could be undertaken.

Mariani et al. (1987) showed that coupling of peptides to carrier proteins may result in increased immunogenicity of the peptide by up to 100 fold. Peptides were therefore conjugated to rabbit albumin via MBS or glutaraldehyde before immunisation into chickens, resulting in a molecular mass much higher than 5 kDa, which is regarded as the minimum molecular weight necessary to obtain an efficient immune response (Schwarzkopf et al., 2001). Additionally, adjuvants are known to enhance the immune response to antigen (Roitt, 1997), so Freund's adjuvant was used in this study. Chickens were ideal for immunisation because of their many advantages, mainly the high yield of antibodies obtained from egg yolk

(Schwarzkopf et al., 2001).

All the resulting anti-peptide antibodies were able to recognise their respective peptides in an ELISA compared to antibodies isolated from the eggs laid by non-immunised chickens. The antibody production peaked between weeks 6 and 12 post immunisation. Free peptides were used as antigens instead of conjugated peptides in ELISAs for monitoring antibody production. This is to ensure that only antibodies produced against peptides, and not those produced against the carrier protein, are detected in the ELISA. This obviates the need to prepare a second peptide-carrier conjugate with a different carrier protein for use as coating antigen.

It was essential to affinity purify the anti-peptide antibodies because non-affinity purified antibodies are only partially purified during PEG isolation. Unpurified preparations have antibodies against carrier protein, against epitopes created by coupling chemistry, termed carrier modified residues (CAMOR) regions (Briand et al., 1985), and against unrelated proteins. Therefore, not all the antibodies will react with the antigen of interest when using non-affinity purified antibodies. In the present study, most affinity purified anti-peptide antibodies showed an increased reactivity with the peptide compared to non-affinity purified antibodies. However, for some anti-peptide antibodies, the reactivity of the affinity purified antibodies and non-affinity purified anti-peptide antibodies was almost similar. This may be due to poor efficiency of the peptide affinity matrices, as a result of which not all the anti-peptide antibodies were bound to the column, even after repeating the purification procedure several times.

To measure the subpopulation of antibodies in each affinity purified anti-peptide antibody preparation able to cross-react with the whole enzyme, microtitre plates were coated with either the CP2 catalytic domain (C2) or the whole CP2 instead of peptide. This type of assay does not necessarily measure antibodies able to cross-react with native enzyme since proteins become partially denatured when they are adsorbed to a solid phase (Van Regenmortel, 1990b). A positive cross-reaction, however, is usually interpreted as an indication that the peptide has the potential of being an epitope of a protein. Both anti-CP1 and anti-CP2 peptide antibodies recognised the whole CP2 enzyme and C2 in ELISAs. This means that in solution peptides adopted a variety of interchangeable conformations, some of which mimicked those

found in the partially denatured protein adsorbed to the ELISA plate. The amino acid sequences of CP1 and CP2 are 90% identical (Boulangé et al., 2001). The cross-reactivity of anti-CP1 peptide antibodies with CP2 is not surprising but anti-CP1 peptide antibodies did not recognise CP2 as strongly as did the anti-CP2 peptide antibodies.

Anti-congopain peptide antibodies not only recognised whole CP2 and its catalytic domain, C2, in an ELISA, but were also shown to recognise their target sequences in fully denatured CP2 on a western blot. In CP2 blots two bands were observed, one slightly larger than 30 kDa and the other slightly smaller than 43 kDa. These are thought to be the monomeric and dimeric forms of congopain, respectively. These two forms of congopain occur as a result of a change of charge of side chains within the peptide (residues 38-45), resulting in either alpha helix (monomeric) or beta-turn (dimeric) forms (Dr Edith Authié, ILRI, Nairobi, Kenya, personal communication). The formation of such charges is pH dependent. Using short peptides it is possible to assess the susceptibility of the individual amino acid residues to form an alpha-helix or a beta-turn (Darby and Creighton, 1993). The group of amino acid residues which play a role in the formation of a dimeric form (beta turn) or a monomeric form (alpha-helix) is made up of the homologous pairs K-E in congopain, corresponding to R-D in cathepsin W (Figure 31). Depending on the relative charges of each amino acid within a pair, the sequence would either adopt an alpha-helix or beta-turn structure (Dr Johan Hoebeke, IBMC, Strasbourg, France, personal communication).

Congopain	(aa 38-45)	W **K** V A G **H** **E** L	dimer/monomer
Cruzipain	(aa 38-45)	W-F-L-A-G-H-P-L	monomer
Cathepsin W	(aa 38-45)	W-**R**-I-S-F-**W**-**D**-F	dimer

Figure 31. Peptide sequences of congopain, cruzipain and cathepsin W, used in determining their propensity to form monomers and dimers (Dr Johan Hoebeke, IBMC, Strasbourg, France, personal communication). The amino acid residues shown in bold are responsible for the formation of intramolecular bridges.

The alpha-helical structure is destabilised when oppositely charged residues are located at intervals of three to four residues, because this results in these residues occurring on the same face of the helix and thus forming salt bridges (Darby and Creighton, 1993). At pH 5.0, the

charges of the side chains of both lysine (K) and histidine (H) in congopain will be positive because the pH will be lower than their side chain pKa values (10.0 for K and 6.0 for H, Table 9) and the charge of the side chain of glutamic acid (E) will be negative because the pH will be higher than the side chain pKa value of E (pKa is 4.2, Table 9). It is therefore likely that at this pH, E^- will interact with the adjacent H^+ rather than with K^+. Since H and E are consecutive residues, there would be no salt bridge, and the alpha-helical structure (monomeric form) would remain stable. At pH 7.0, histidine will not be charged because the pH will be higher than the side chain pKa value, lysine will remain positive and glutamic acid will remain negative. K^+ is likely to interact with E^- at this pH, forming a salt bridge which destabilises alpha-helical structure, resulting in a beta turn (dimeric form). Cathepsin W remains in a dimeric form at both pH 5.0 and pH 7.0 because the charge of the side chain of arginine (R) remains positive at both pH 5.0 and pH 7.0 since the side chain pKa value of R is 12.5 (Table 9). Tryptophan (W) does not ionise and therefore remains neutral at any pH. The charge of the side chain of aspartic acid (D) remains negative at both pH 5.0 and pH 7.0 because the side chain pKa value of D is 3.9 (Table 9). The salt bridge between R^+ and D^- will be stable between pH 5.0 and pH 7.0, forming a beta turn (dimeric form). For cruzipain, both phenylalanine (F) and proline (P) do not ionise, therefore there will be no salt bridge and cruzipain will remain in a monomeric form.

Table 9. Side chain charges determining monomer or dimer formation in congopain, cruzipain and cathepsin W.

Protease	Residue	pKa (side chain)	Charge at pH 5	Monomer/ dimer	Charge at pH 7	Monomer/ dimer
Congopain	K	10.0	+		+	
	H	6.0	+		No charge	
	E	4.2	-	Monomer	-	Dimer
Cruzipain	F	-	No charge		No charge	
	P	-	No charge	Monomer	No charge	Monomer
Cathepsin W	R	12.5	+		+	
	W	-	No charge		No charge	
	D	3.9	-	Dimer	-	Dimer

In initial experiments anti-peptide antibodies recognised the monomer better than the dimer, consequently a relatively large amount of CP2 was loaded on the gel in order to increase the

visibility of the dimer band for scanning purposes. This resulted in broad monomer bands on western blots. Poor recognition of the dimer may be due to the fact that it has a compact structure and the epitopes recognised by the anti-peptide antibodies may have been less accessible or even 'hidden' in the dimer (Dr Edith Authié, ILRI, Nairobi, Kenya, personal communication). The dimeric form of C2 was also not recognised by anti-peptide antibodies. This may be due to the fact that the truncated dimer and the full length dimer do not share the same conformational characteristics. The C-terminal domain (present in CP2), may account for the different accessibilities of the anti-peptide antibodies (Dr Edith Authié, ILRI, Nairobi, Kenya, personal communication). This result still needs to be investigated further.

Various enzymes such as cathepsin L (Coetzer et al., 1991), trypanopain-Tb (Troeberg et al., 1997) and P450 enzyme (Schulz-Utermoehl et al., 2000) are inhibited by their anti-peptide antibodies. The use of anti-peptide antibodies that bind specifically to an enzyme and inhibit its activity is a useful approach in identifying regions that are involved in the catalytic function of the enzyme. IgG from *T. congolense* infected N'Dama cattle recognised the catalytic domain of CP2 and inhibited its Z-Phe-Arg-AMC hydrolysing activity (Authié et al., 2001). This suggests that critical CP2 epitopes are likely to be located in the catalytic cleft of CP2. In the present study antibodies against CP2 peptides were tested for their ability to inhibit the hydrolysis of Z-Phe-Arg-AMC by CP2 and C2. This investigation was not done for antibodies against CP1 peptides (except for antibodies against the peptide corresponding to a region of the CP1 active site) because of the limited amount of enzyme available. Furthermore, in a previous study, antibodies against the CP1 catalytic domain did not inhibit the activity of CP2 (Authié et al., 2001). Antibodies raised against the peptide in the C-terminal extension of CP2 were unlikely to have any effect on CP2 activity because the enzyme can remain active in the absence of the C-terminal extension. These antibodies were therefore not tested.

Previous studies have shown that antibodies may have complex effects on enzymes (Richmond, 1977; Dennison and Pike, 1991; Troeberg et al., 1997). Specific antibodies can inhibit, enhance or in certain cases, small amounts of antibodies stimulate enzyme activity, whereas higher antibody concentrations can neutralise enzyme activity (Pollock, 1964). In this study the effect of anti-peptide antibodies on C2/CP2 hydrolysis of Z-Phe-Arg-AMC was stimulatory at lower antibody concentrations and inhibitory at higher antibody concentrations but sometimes purely stimulatory. Stimulatory responses may be due to a conformational

change induced by antibodies. This may indicate that the conformation of an antigen is more important than its primary sequence in determining its reaction with an antiserum (Richmond, 1977). The effects of anti-peptide antibodies varied at different stages of the immune response. Generally, antibodies produced in weeks 7-10 and weeks 15 and 16 post immunisation enhanced enzyme activity more than the other antibodies. This shows that various batches of antibodies may vary in their properties, even when the same antigen preparation is used to raise antibodies and the same immunisation course is followed (Richmond, 1963).

Congopain was inhibited by IgG from cattle immunised with C2 (Authié et al., 2001). Congopain is active in a broad pH range, from pH 4.0 to 8.5, with an optimum at pH 6.0 (Boulangé et al., 2001). In the current study, the pH of the antibody diluting buffer was 8.0. This pH was also used by Troeberg et al. (1997) for trypanopain. Even though pH 8.0 falls within the active congopain pH range, it is realised that using the optimum pH of 6.0, or the physiologic pH might improve the results obtained in this study. Authié et al. (2001) used an antibody diluting buffer of pH 7.2 and the IgG used displayed an inhibitory effect over congopain. It is appreciated that the conditions used in this part of the experiment were not optimal for both the antibodies and congopain, but because of the time constraints and the limited amount of the CP2 and C2 enzyme available the experiment was not repeated. In future investigations, the effect of anti-peptide antibodies on congopain hydrolysis of Z-Phe-Arg-AMC will need to be investigated under the conditions used by Authié et al (2001).

The response of an enzyme to antibodies may vary depending on which substrate is present (Pollock, 1964). When Pollock (1964) tested the effects of the same sera on penicillinase activity using different substrates, they found that with some substrates antisera inhibited penicillinase, while with other substrates the response was biphasic and with a further group of substrates, antibodies enhanced enzyme activity. The results reported here only show the effect of anti-peptide antibodies when using the substrate, Z-Phe-Arg-AMC. One cannot assume that for every substrate, these anti-peptide antibodies would have an enzyme enhancing effect. Antibodies may have recognised their epitopes in congopain (in cases where the anti-peptide antibodies were not targeting the active site cleft) but since the Z-Phe-Arg-AMC substrate is small, it was easy for it to gain access to the active site where it was cleaved.

The effect of anti-trypanopain peptide antibodies on digestion of protein substrates by

trypanopain was investigated (Troeberg, 1997). It was expected that binding of anti-peptide antibodies to trypanopain would block the active site and thus inhibit the digestion of protein substrates. However, it was found that all anti-peptide antibodies tested enhanced the hydrolysis of ^{14}C-gelatin, while all antibodies inhibited hydrolysis of FITC-albumin. The possible explanation given for this result is that ^{14}C-gelatin and FITC-albumin might have different affinity constants for trypanopain. ^{14}C-gelatin might have a higher affinity for trypanopain than do the anti-peptide antibodies, and it is possible that the antibodies were displaced from the trypanopain active site upon addition of ^{14}C-gelatin, and the enhanced activity observed might be due to increased stabilising ability of specific antibodies compared to non-immune antibodies. On the other hand, FITC-albumin might have a higher affinity for trypanopain than do the anti-peptide antibodies, and as a result antibodies were not displaced from the trypanopain active site upon addition of FITC-albumin and trypanopain activity was therefore inhibited (Troeberg, 1997). This hypothesis was, however, not tested. It is apparent from these results that the substrate can largely determine the effects of anti-peptide antibodies on enzyme activity.

If the antibodies enhance enzyme activity, against protein substrates one would consider raising monoclonal antibodies against conformational epitopes. Monoclonal antibodies often recognise conformational rather than linear epitopes (Van Regenmortel et al., 1990a). A monoclonal antibody producing clone would be selected using an enzyme inhibition test.

It would be interesting to study the effect of a cocktail of anti-peptide antibodies produced in this study on C2/CP2 activity. Using antibodies raised against peptides corresponding to several different regions of the protein would be helpful because a single anti-peptide antibody may not recognise certain conformations of the peptide in the constrained regions of the protein molecule in its native state. When doing this test, it would be essential not to use antibody concentrations that are too high so as to avoid steric hindrance which may give false results. The concentration of the negative control antibody would have to be equal to the total concentration of the cocktail of anti-peptide antibodies. The effect of the anti-peptide antibodies on C2/CP2 activity using different substrates should be determined in future studies because congopain's *in vivo* substrate may be proteins which are much larger than the Z-Phe-Arg-AMC substrate. The antibodies might hinder access of protein to the active site and they would not be cleaved. Testing the effect of anti-peptide antibodies using different protein

substrates would help in coming to a conclusion as to whether these antibodies stimulate or inhibit enzyme activity.

This study has shown that the anti-peptide antibodies prepared were specific for the enzyme whether it is fully denatured (western blots), partially denatured (ELISA) or in its native form (enzyme fluorometric assay with the Z-Phe-Arg-AMC substrate). This means that antibodies were produced against various conformations which the peptides adopted in solution, some of which mimicked those found in the native protein and some of which mimicked those found in the denatured protein. This study proved that most of the peptides selected might be possible epitopes of congopain. Noticeably, antibodies recognised peptides around the active site best. However, antibodies produced against these peptides were not able to inhibit congopain activity. The possible explanation for this result might be that the peptides selected are partial epitopes of congopain. The epitope prediction program used by Cármenes et al. (1989) was useful in the analysis of both CP1 and CP2 amino acid sequences but this method may not be sufficient in determining the exact residues which make up optimum epitopes. Pepscan technology may be useful in determining residues which make up an optimum epitope because in this method, overlapping and progressively shorter, truncated versions of the peptides are synthesised and tested for recognition by antisera against the whole molecule (Sumar, 2001). The Pepscan method will be discussed more fully in Chapter 6.

The specificity of the anti-peptide antibodies raised in the present study, makes them useful reagents. Specific antibodies that bind to denatured protein in western blots are best suited for immunoscreening of expression libraries (Patrie, 1994). These antibodies can also be used in the immuno-affinity purification of the CP2 enzyme. The peptides representing the epitopes identified also have potential as diagnostic reagents. This possibility was tested as described in Chapter 4.

Chapter 4

Natural immunogenicity of congopain and vivapain peptides and their possible use as diagnostic tools for trypanosomosis

4.1 Introduction

Diagnosis of parasitic diseases is usually based on clinical signs and symptoms or by detecting either antibodies or parasite antigens. However, in trypanosomosis, early clinical signs are not specific (Rebeski et al., 1999a) and are not easy to use as a means of diagnosis. A diagnostic method which is sensitive, specific, simple and inexpensive is required.

Parasite detection depends on demonstrating the parasite in chancre fluid, lymph fluid, blood, bone marrow or cerebrospinal fluid. In wet blood film preparations, parasites can be detected but not identified by the disturbance they cause among red blood cells by their flagellar movement (Van Meirvenne and Le Ray, 1985). Examination of Giemsa-stained thick blood films is more sensitive. Parasites can also be localised using the dark ground buffy coat technique which involves the centrifugation of blood in a specialised tube, resulting in concentration of parasitised red blood cells in a layer because of their lower density. The centrifuged tube is observed directly using a fluorescence microscope. Since the contrast between stain and background is high, parasitised red blood cells are easily seen (Oaks et al., 1999). Parasites can also be localised by DEAE cellulose anion–exchange chromatography. Infected blood samples are subjected to anion exchange chromatography, where trypanosomes are selectively eluted and can be recovered by centrifugation or membrane filtration (Van Meirvenne and Le Ray, 1985). However, the diagnosis of trypanosomosis in animals with low parasitaemia is hampered by low sensitivity of traditional detection methods (Rebeski et al., 1999a). The diagnosis of trypanosomosis by direct parasitological techniques is feasible only in the acute state of the illness, when the blood is colonized by a large number of parasites. In the chronic state of the illness, when the level of parasiteamia is low, definitive diagnosis is difficult (Reyna-Bello et al., 1998).

The limitations of parasitological diagnosis have been the driving force for research into alternative techniques that give indirect evidence of infection. Serological techniques, such as indirect immunofluorescence and ELISA, detect antibodies directed at parasite components or

parasite antigens circulating in host blood (Masake et al., 2002). The availability of the ELISA technique has led to its use in veterinary diagnostic laboratories in at least 15 countries affected by trypanosomosis (Masake et al., 2002). There are various ELISA systems that have been developed for the detection of bovine trypanosomal antibodies (Rebeski et al., 2000) but there is still a need for better ELISA technologies for trypanosomosis detection. Serological assays can detect parasite antibodies but cannot determine whether the antibodies result from current or past infection. Comparison of sensitivities and specificities of the assay is difficult since protocols and antigen extracts have not been standardised (Oaks et al., 1999). One way of improving the ELISA technique could be by the use of epitopes, rather than whole antigen, since success of the latter greatly depends on the antigen being used (Sánchez et al., 2001). The antigen should be trypanosome specific and the immune response of the host against that particular antigen should be good. One of the advantages of using synthetic peptide epitopes as antigens in antibody detection ELISAs, is that the sensitivity of the assay improves because, when the infected host recognises the foreign protein molecule, antibodies against the epitopes are produced are specific for the epitopes. Peptide antigens provided a more sensitive and specific alternative to trypanosome lysates for detection of anti-*T. cruzi* antibodies as required for blood screening assays (Haughton et al., 2000).

In addition to serologic methods, molecular assays based on the polymerase chain reaction (PCR) have been proposed as alternative tools for parasite detection during the chronic stage of trypanosomosis (Gomes et al., 1999). This is a promising technique for the specific detection of different species of trypanosomes which is based on amplifying certain repetitive DNA sequences coding for trypanosome proteins (Masake et al., 2002). Among the few candidate proteins available is a 69 kDa invariant protein, present in *T. congolense*, *T. brucei* and *T. vivax*. The cloning of the gene of this protein revealed that it is a member of 70 kDa heat shock proteins (Boulangé et al., 2002). Kinetoplast DNA has been amplified by PCR for the detection of *T. cruzi* in human blood (Gomes et al., 1999). Sensitivity reported ranged from 96 to 100% compared with serologic diagnosis (Gomes et al., 1999). PCR takes about three to four hours, electric power is needed to run a thermal cycler and the reagents are relatively expensive. The PCR assay requires facilities that many laboratories do not have and it is not suitable for a large number of samples. A cheaper assay is thus required, but it would be difficult to get better sensitivity than that reported for the PCR technique.

There is a report of an ELISA type assay for PCR products which is under evaluation (Masake et al., 2002). In this method, DNA isolated from blood samples of infected cattle, is first amplified by PCR using oligonucleotide primers specific for *T. brucei* or *T. vivax*. The PCR products, which are exclusive to either *T. brucei* or *T. vivax,* are biotinylated. None of these primers are evident in the DNA from the blood samples of the cattle exposed to non-infected tsetse. The biotinylated PCR-products are applied to the wells of microtitre plates pre-coated with streptavidin. The unbound PCR-products are removed by washing. The bound PCR-products are incubated with a fluorescein-labelled oligonucleotide probe specific for the trypanosome species tested. Following the washing step, the fluorescein-labelled probe is revealed by incubation with anti-fluorescein antibody conjugated to alkaline phosphatase (Masake et al., 2002).

An objective of the present study was to investigate the use of congopain epitopes as diagnostic tools. This is based on the observation that congopain is the major antigen in *T. congolense* infected cattle (Authié, et al., 1992). Under natural tsetse challenge both trypanotolerant and trypanosusceptible cattle have high levels of anti-congopain IgG (Authié, 1994). These antibodies from infected cattle may be directed against discrete epitopes in congopain. In a previous study, chicken anti-sheep cathepsin L IgY recognised an active site associated cathepsin L peptide, suggesting that this peptide is a natural immunogenic epitope in chickens (Coetzer et al., 1992). Such a peptide could be used as a diagnostic tool in an ELISA rather than the whole cathepsin L enzyme.

In this study, peptides selected as described in Chapter 3 (Section 3.4.1, Tables 6 and 7) were immobilised on ELISA plates and indirect ELISAs were done to detect anti-congopain peptide antibodies in serum samples from cattle experimentally infected with *T. congolense* or immunised with C2, in comparison with non-immunised control cattle. The aim was to investigate whether the congopain peptides are naturally immunogenic in cattle, which could suggest that these sequences are partial or complete epitopes of congopain. The naturally immunogenic epitopes could be used as diagnostic tools by screening for the presence of antibodies that target the congopain epitopes in *T. congolense* infected sera using ELISAs. The ability of sera from *T. congolense* and *T. vivax* infected cattle to bind to peptides corresponding to sequences in the C-terminal extension of congopain and vivapain (Section 3.4.1, Table 7) was also tested in ELISAs. The aim was to find out whether these two peptides

could be used as tools to differentiate between *T. congolense* and *T. vivax* infections.

4.2 Detection of anti-congopain antibodies with indirect ELISAs using CP1 and CP2 peptides as antigens

In these assays levels of antibodies against congopain peptides were measured. This is based on the observation that *T. congolense* infected sera have high levels of anti-congopain antibodies (Authié et al., 1993a). These antibodies may be able to recognise specific congopain peptides if they are naturally immunogenic epitopes of congopain. Sera from cattle immunised with C2 were also tested since it has been hypothesised that epitopes of congopain are likely to be in the catalytic domain (Authié et al., 2001). An antigen may be immunogenic to one species and not immunogenic in another species (Harlow and Lane, 1988). Sera from rabbits immunised with native congopain or C2 were therefore tested to check whether peptides would be naturally more/less immunogenic in rabbits compared to cattle.

4.2.1 Reagents

ELISA reagents prepared as per Section 2.10.1

0.5% (w/v) gelatin-PBS. Gelatin (0.5 g) was dissolved in PBS (100 ml).

T. congolense infected sera. Sera from adult N'Dama and Boran cattle experimentally infected with *T. congolense* were a gift from Dr Edith Authié [ILRI, Nairobi, Kenya]. Sera were collected between 70 and 100 days post infection.

Sera from C2 immunised cattle, uninfected cattle. Sera from uninfected Boran cattle, immunised with *Pichia pastoris* expressed C2 were provided by Dr Edith Authié (ILRI, Nairobi, Kenya). Cattle were around 1 year old. Sera were collected 3 weeks after the second booster.

Sera from rabbits immunised with native congopain or C2. Serum from a rabbit immunised with monoclonal antibody affinity purified congopain from lysates of *T. congolense* bloodstream forms was used. Serum from a rabbit immunised with C2 was also used. Both

sera were a gift from Dr Edith Authié (ILRI, Nairobi, Kenya).

4.2.2 Procedure

Peptides (Section 3.4.1, Tables 6 and 7, 10 μg/ml) were coated to the wells of ELISA plates (16 h, 4°C). Non-specific binding of antibody to the wells was prevented by blocking with 0.5% gelatin-PBS (1 h, 37°C). Sera were diluted in serial two-fold dilutions using 0.5% gelatin-PBS from 1/100 to 1/1600 and incubated (2 h, 37°C). Goat anti-bovine IgG-HRPO (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) was used as detection antibody. Where sera from rabbits were used, goat anti-rabbit IgG-HRPO (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) was used as detection antibody. The remainder of the procedure was as described in Section 2.10.2.

4.3 Differentiation between *T. congolense* and *T. vivax* infection with indirect ELISA using C-terminal extension peptides.

Generally, methods for the detection of trypanosomal antigens in serum samples have proved to be unsatisfactory with respect to diagnostic sensitivity (Rebeski et al., 1999b). Therefore, more sensitive diagnostic methods, including the detection of *Trypanosoma* specific antibodies have been developed (Greiner et al., 1997). Peptides from C-terminal extension sequences of congopain and vivapain were coated as antigens in ELISA plates to investigate whether they would be useful in distinguishing between infections by *T. congolense* and *T. vivax*. A criterion for the selection of peptides was to choose a CP2 peptide sequence that does not match with the aligned sequence of vivapain and visa versa. This was done in order to avoid chances of cross-reactivity. Alignment of C-terminal extension sequences of vivapain and congopain, and the peptides selected, is shown in Figure 32.

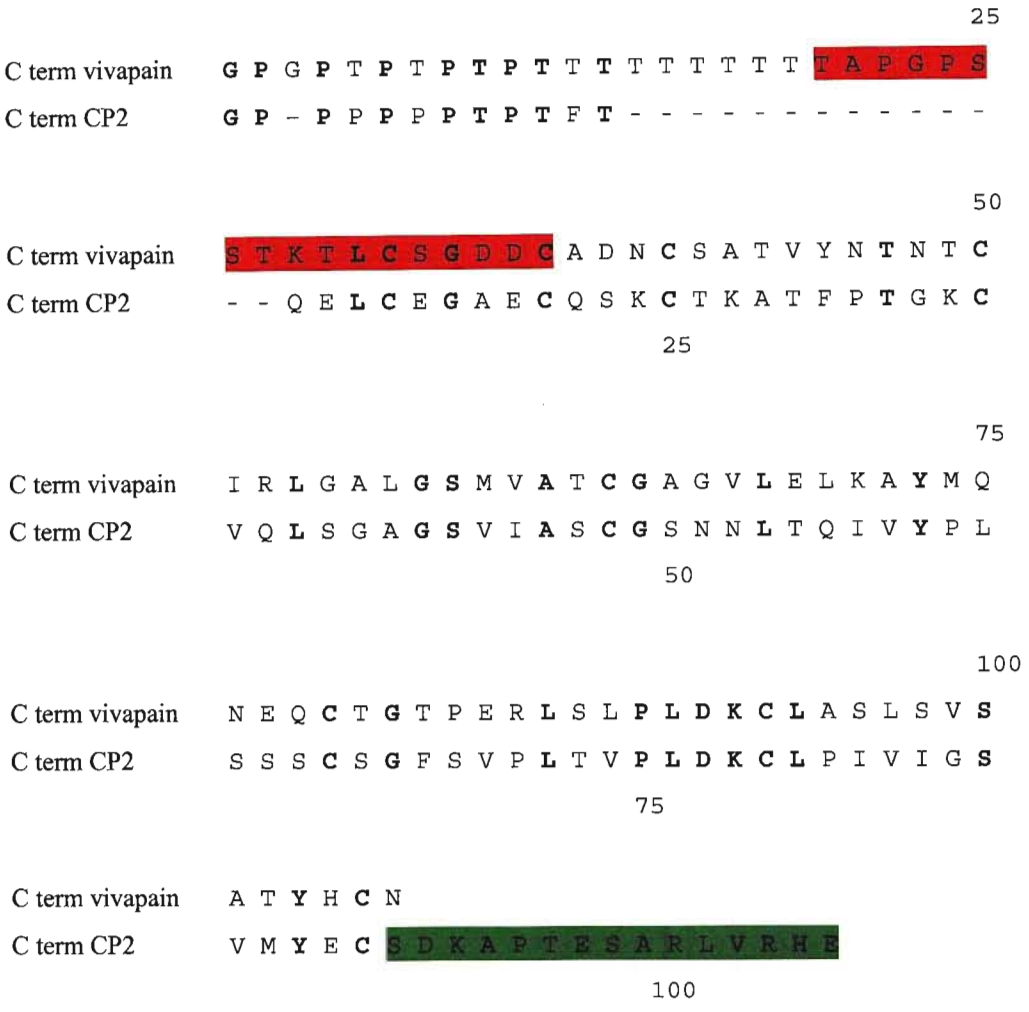

Figure 32. Alignment of the C-terminal extension sequences of vivapain and CP2. Identical residues are indicated in bold. The selected peptide from vivapain (amino acid sequence deduced by Prof. Theo Baltz, University of Bordeaux, France, personal communication) is highlighted in red, and the selected CP2 peptide (amino acid sequence deduced by Jaye et al., EMBL accession number L25130) is highlighted in green.

4.3.1 Reagents

ELISA reagents as per Section 2.10.1.

Sera from six crossbred Zebu × Baolés heifers, experimentally infected with *T. congolense* and *T. vivax* were a gift from Dr Marc Desquesnes [Centre International de Recherche-Développement sur l'Elevage en Zone Sub-humide (CIRDES), Bobo Dioulasso, Burkina Faso]. Cattle were around 2-3 years old. Sera from *T. congolense* infected cattle were collected between day 26 and day 58 post infection. Sera from *T. vivax* infected cattle were collected between day 55 and day 219 post infection.

4.3.2 Procedure

As per Section 2.10.2 and the modifications as per Section 4.2.2.

4.4 Results

4.4.1 Recognition of CP2 and CP1 peptides by sera from cattle experimentally infected with *T. congolense.*

Both N'Dama and Boran cattle experimentally infected with *T. congolense* elicited antibodies that recognised CP2 and CP1 peptides better in comparison to the recognition of peptides by the non-immune sera used as negative controls. On average, for CP2 peptides, the C-terminal extension peptide (# 4) was best recognised followed by the N-terminal peptide (# 1), the active site peptide (# 3) and the catalytic domain peptide (# 2). On average, for CP1 peptides, the N-terminal peptide (D) was best recognised followed by the active site peptide (F) and the catalytic domain peptide (E). Recognition of the peptides by the sera from trypanosusceptible Boran cattle, was not significantly different from the recognition of peptides by the sera from trypanotolerant N'Dama cattle. However, the poor recognition of peptides by the sera from the trypanosusceptible Boran heifer #407, is quite remarkable. This result suggests differences in the humoral responses to congopain between trypanosusceptible cattle and trypanotolerant cattle. The results are summarised in Table 10.

Table 10. Recognition of CP peptides by sera from cattle experimentally infected with *T. congolense*.

Sera	Peptides coated down in ELISA plates						
	D	E	F	# 1	# 2	# 3	# 4
ND 122 [a,d]	++	++++	+++	++++	+++	+++	++++
ND 139	+++	+++	++++	++++	+++	+++	++
ND 4	++++++	+++++	+++++	+++++	++++	+++	++++
BR 340 [b]	+++++	++++	+++++	+++++	++++	++++++	++++++
BR 723	++++++	+++++	++++++	++++	++++	++++	+++++
BR 407 [c]	++	+	-	+	-	+++	++
Average percentage recognition	97.2 ± 60.1	87.5 ± 55.8	93.9 ± 72.1	87.5 ± 44.1	64.3 ± 43.1	80.2 ± 41.6	91.4 ± 53.4

[a] ND, N'Dama cattle.
[b] BR, Boran cattle.
[c] A minus sign indicates less than 0% recognition when compared to recognition by non-immune sera.
[d] The number of plus signs indicate % recognition when compared to recognition by non-immune sera (one plus sign, 1-30%; two plus signs, 31-59%; three plus signs, 60-79%; four plus signs, 80-120%; five plus signs, 121-160% and six plus signs, 160-200%).

4.4.2 Recognition of CP2 and CP1 peptides by sera from C2 immunised, uninfected cattle.

All peptides (except the C-terminal extension peptide, # 4) were recognised by C2 immunised Boran cattle compared to the non-immune sera. This was expected because C2 does not have a C-terminal extension. This result is also an indication of the specificity of the response to the C2 immunogen. A representative result of recognition of peptides by C2 immunised sera is shown in Figure 33. The N-terminal extension peptide (# 1) and the catalytic domain peptide (# 2) were the main CP2 peptides that were targeted by antibodies in the sera. On the other hand, the active site peptide (F) was the main CP1 peptide targeted by the antibodies.

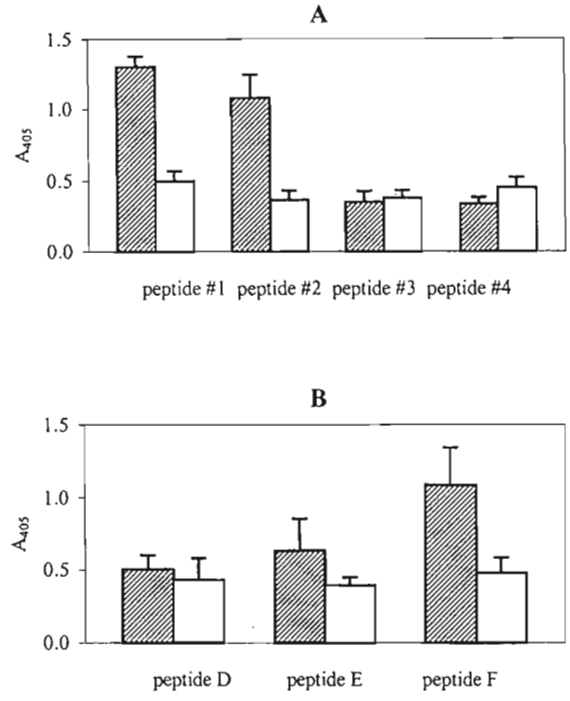

Figure 33. Recognition of CP2 and CP1 peptides by sera from C2 immunised, uninfected cattle. CP2 (Panel A) and CP1 (Panel B) peptides (10 µg/ml) were coated to the wells of ELISA plates. Sera of C2 immunised cattle (shaded bars) and non-immunised cattle (unshaded bars) were titrated between 1/100 and 1/1600 dilutions. Binding was detected using goat anti-bovine IgG-HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate. Representative data for the 1/100 dilution is shown. Bars represent SEM (n = 5).

4.4.3 Recognition of CP2 and CP1 peptides by sera from C2 and native congopain immunised rabbits

Antibodies in the sera produced against C2 in rabbits recognised mainly the active site peptide (# 3) and the N-terminal peptide (# 1) from CP2 and the active site peptide (F) from CP1 when compared to recognition by non-immune rabbit sera. All peptides were poorly recognised by antibodies in the sera produced against native congopain compared to recognition by non-immune rabbit sera (Table 11).

Table 11. Recognition of CP peptides by sera from C2 and native congopain immunised rabbits.

Immunogen	Peptides coated down in ELISA plates						
	D	E	F	# 1	# 2	# 3	# 4
C2[a,c]	+	++	+++	+++	++	+++	+
Native congopain[b]	+	-	-	-	-	-	-

[a]Representative data for the 1/100 dilution is shown.
[b]A minus sign indicates less than 0% recognition when compared to recognition by non-immune sera.
[c]The number of plus signs indicate % recognition when compared to recognition by non-immune sera (one plus sign, 1-30%; two plus signs, 31-59%; three plus signs, 60-79%)

4.4.4 Distinction between *T. congolense* and *T. vivax* infection in an ELISA by using C-terminal extension peptides as coating antigens.

The recognition of peptides by antibodies present in all the sera from the different crossbred Zebu × Baoulés cattle was consistently slightly higher for CP2 peptide (# 4) compared to vivapain peptide (# 5), when using *T. congolense* infected sera, and slightly higher for vivapain peptide compared to CP2 peptide when testing *T. vivax* infected sera (Figure 34). The results showed high levels of cross-reactivity.

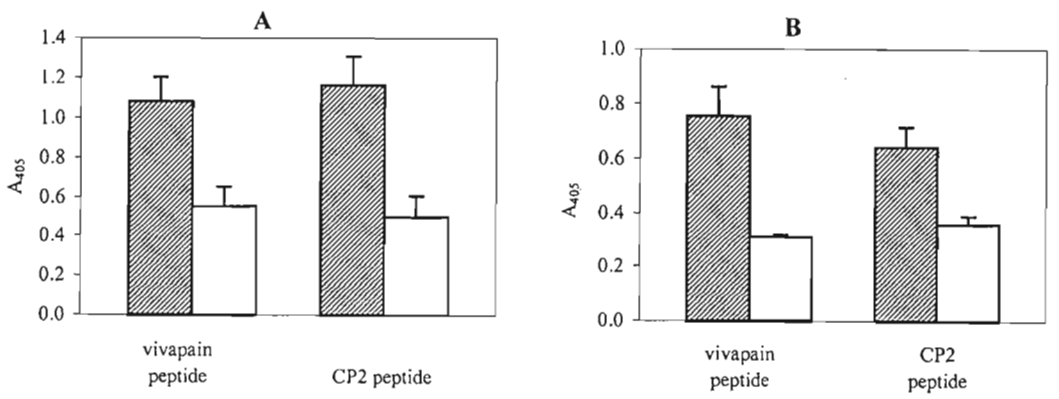

Figure 34. Recognition of CP2 and vivapain C-terminal extension peptides by *T. congolense* and *T. vivax* infected sera. CP2 and vivapain peptides (10 µg/ml) were coated to the wells of ELISA plates. *T. congolense* infected sera (Panel A, shaded bars), *T. vivax* infected sera (Panel B, shaded bars) and uninfected sera (unshaded bars) were titrated between 1/100 and 1/1600 dilutions. Binding was detected using goat anti-bovine IgG-HRPO-linked detection antibody (Jackson ImmunoResearch Laboratories inc, Pennsylvania, USA) and ABTS/H_2O_2 substrate. Representative data for the 1/100 dilution is shown. Bars represent SEM (n = 4).

4.5 Discussion

The work described in this Chapter determines whether cysteine proteinase peptides selected from the catalytic domain (*T. congolense*) and the C-terminal extension (*T. congolense* and *T. vivax*) described in Chapter 3 are natural immunogenic epitopes. This study took advantage of the fact that congopain is naturally immunogenic and in the course of an immune response to natural *T. congolense* infection, congopain elicits antibodies (Authié et al., 1993a). Paratopes on antibodies produced by the infected host against congopain interact with certain regions (epitopes) in the congopain molecule. These regions have not yet been identified, therefore the peptides selected were used to determine whether they are targeted by the antibodies in the infected sera or not. Should the main targets be identified, they could be used as diagnostic tools.

All congopain peptides, except the catalytic domain peptide (#2), were recognised by *T. congolense* infected sera compared to recognition by sera from uninfected cattle. This could either mean that the conformation adopted by peptide #2 coated in an ELISA plate did not mimic the conformation that occurs in the corresponding region of native congopain which is recognised by the antibodies or it could mean that peptide #2 is not an immunogenic epitope of congopain. On the other hand, sera from C2 immunised, uninfected cattle targeted mainly the N-terminal peptide #1, peptide #2 and the active site associated peptide F. Apparently peptide #2 becomes immunogenic when the truncated form of the enzyme is used as an immunogen. The region corresponding to peptide #2 possibly becomes more exposed and accessible for antibody binding in the truncated form of the enzyme. The poor recognition of the C-terminal extension peptide (#4) by the sera from C2 immunised cattle is not surprising because C2 is a truncated CP2 enzyme which does not include the C-terminal extension of congopain. Interestingly, the longer CP1 active site peptide (F) is recognised much better by sera (*T. congolense* infected and C2 immunised) compared to recognition of the shorter CP2 active site peptide (# 3). Longer peptides are better able to adopt the conformation in the mature, whole protein, thereby inducing protein reactive antibodies (Dr Stuart Rodda, Mimotopes, Clayton, Australia, personal communication). Peptide sequences may need to be modified to identify optimal peptide sequences which can result in a more optimal targeting of the peptide by antibody. However, C2 immunised rabbit sera targeted the shorter active site associated peptide #3. This suggests that the region corresponding to peptide #3 in C2 was more immunogenic in rabbits than in cattle. This result is in agreement with the suggestion by

Harlow and Lane (1988), that different species may respond differently to an immunogen. This is supported by the finding that antibodies raised in rabbits against cathepsin L did not recognise the cathepsin L active site His-containing peptide, whereas those produced in chickens against cathepsin L did recognise this peptide (Coetzer et al., 1992). Contrary to these findings, the epitopes identified in this study and shown to be immunogenic in chickens, were also immunogenic in cattle. The latter was evidenced by the recognition of all the peptides by the sera of *T. congolense* and/or *T. vivax* infected cattle.

None of the peptides were recognised by antibodies from the native CP2 immunised rabbit. It may be argued that antibodies made against native congopain may not have recognised the conformation adopted by the peptides coated in ELISA plates, which may not mimic the conformation that occurs in the corresponding regions of native congopain. Another possible explanation is that the immune response against native congopain was poor. These antibodies may be tested by determining whether they recognise native congopain coated on an ELISA plate. This control test could be done once native congopain is available from our collaborators.

Another objective of this study was to differentiate between *T. congolense* and *T. vivax* infection. There is a need for trypanosome antigens that would be species specific because diagnostic ELISAs for trypanosomes lack the desired specificity and sensitivity (Gomes et al., 1999). Although antibodies raised in chickens against the congopain and vivapain C-terminal extension peptides showed no cross-reactivity in an ELISA with the respective peptides coated, there was very high cross-reactivity by the *T. congolense* and *T. vivax* infected sera. However, *T. congolense* infected sera consistently recognised the congopain peptide slightly better than the vivapain peptide, while *T. vivax* infected sera recognised the vivapain peptide slightly better than the congopain peptide. Cross-reactivity may be due to polyreactive antibodies in *T. congolense* infected sera, as suggested by Buza and Naessens (1999). Another possibility is that the cattle tested had mixed infections of *T. congolense, T. brucei* and *T. vivax*. This was confirmed by indirect ELISA results from another laboratory provided by Dr Marc Desquesnes [CIRDES, Bobo Dioulasso, Burkina Faso] which showed that there was cross-reactivity by the sera. The antigens used (trypanosome lysates) were different from the peptides which were used in this study but the same sera were used. *T. congolense* infected sera and *T. vivax* infected sera recognised antigens specific for *T. congolense*, *T. brucei* and *T.*

vivax. *T. vivax* infected sera recognised the antigen specific for *T. congolense* by up to 16%, while the recognition of the antigen specific for *T. vivax* was 76%. On the other hand, *T. congolense* infected sera recognised the antigen specific for *T. vivax* by up to 32%, while the recognition of the antigen specific for *T. congolense* was 47%. The possibility of mixed infections in the sera provided was not expected since the cattle were bought seronegative. However, there are many reports of mixed infections in field investigations both in wildlife and domestic livestock (Mattioli et al., 1999), and mixed infections could occur between the time of testing prior to acquiring the cattle and the time of experimental infection. Further evidence for possible mixed infection is given by the results of parallel experiments conducted with the peptides selected in this study in a collaborator's laboratory in Belgium. Experimental infection of cattle and goats with *T. congolense* or *T. vivax* in a completely trypanosome free area (Belgium vs Burkina Faso), showed that the congopain C-terminal extension peptide is specific for *T. congolense* infection and is not recognised by sera from *T. vivax* infected cattle (Dr Phillippe Buscher, Institute of Tropical Medicine, Antwerp, Belgium). Optimal antibody binding sequences of these two peptides need to be identified by testing modified sequences of these two peptides and hopefully the sensitivity of this assay would improve.

This study has shown that most of the peptides, particularly the N-terminal peptide (# 1), the active site associated peptide (# 3), the C-terminal extension peptide (# 4) and the active site associated peptide from CP1 (F), are naturally immunogenic and possible epitopes of congopain because an immune response was induced against these regions (peptides) of CP2 during immunisation with CP2. These peptides seem to form a part of the essential regions of the enzyme since they were targeted partially by the tested sera. However, from each of these peptides, one would have to identify optimal peptide sequences corresponding to the regions of the enzyme by analysing a series of overlapping peptides. Identification of optimal antibody binding peptide can be done by window net analysis and replacement net analysis (Sumar, 2001). Window net analysis allows further optimisation of binding of antibodies by synthesising different lengths of overlapping peptides. Replacement net analysis allows the study of the effects of changes in amino acids within the predicted epitope to binding of antibodies (Sumar, 2001). These analysis methods will be discussed in more detail in Chapter 6.

After identifying optimal binding epitopes one would have to develop a standardised

diagnostic assay. Serum samples tend to give higher backgrounds compared to monoclonal and anti-peptide antibodies and the interpretation of data can be difficult in terms of what comprises an epitope and where the background cut off should lie (Sumar, 2001). For optimal interpretation of serodiagnostic results one would have to consider biological influencing factors, including sex, breed, age, health and nutritional status of the animals to be tested since these factors can greatly affect the immune system of the animals and hence their response to a trypanosomal proteinase during infection. This shows that there are some limitations and factors which can jeopardise the accuracy of the results when trying to diagnose trypanosomosis by using serological assays which detect antibodies. Furthermore, false positive results of antibody tests can be encountered as a result of antibodies that persist after successful therapy or self-cure (Greiner et al., 1997; Rebeski et al., 1999b), therefore antibody tests would be more useful when studying epidemiology of the disease rather than for diagnostic purposes.

It has been suggested that the most effective antigens in the diagnosis of *T. cruzi* are those with immunodominant, repeating B cell epitopes (Burns et al., 1992). In this study, synthetic peptides representing a single epitope of congopain were used as coating antigens to detect anti-congopain antibodies in *T. congolense* infected cattle. In contrast to this, a multiepitope synthetic peptide has been used successfully by Haughton et al. (2000) as an antigen. A tetrapeptide representing four immunodominant epitopes from several core antigens of *T. cruzi* was used to detect serum antibodies in an ELISA. Higher sensitivity and specificity were observed for the tetrapeptide/recombinant protein assay than for a lysate-based ELISA (Haughton et al., 2000). Multiepitope peptides could constitute reproducible, easily manufactured reagents that can give more sensitive and specific alternatives to trypanosome lysates in immunoassay development (Haughton et al., 2000). In future studies it would be interesting to apply the multiepitope synthetic peptide approach of Haughton et al. (2000) for the diagnosis of *T. congolense* when the optimal epitopes of congopain have been identified.

In conclusion, most congopain peptides identified and tested in this study seem to be essential regions of the enzyme. Further characterisation and modifications of these peptide sequences need to be done to identify optimal epitopes of congopain. The use of peptides as antigens in serum screening assays could provide a better alternative to trypanosome lysates, bearing in mind that there are some shortcomings when using this assay such as those mentioned above.

More tests need to be done before these potential diagnostic tools are used in detecting *T. congolense* infection and for use in differentiating between *T. congolense* and *T. vivax* infections.

Chapter 5

Interaction of CP2 with α_2-macroglobulin

5.1 Introduction

The implication of congopain in pathogenesis suggests that this enzyme interacts with host proteins. CP2 hydrolyses substrates over a wide pH range from pH 4.0 to 8.5 (Authié et al., 2001) and is therefore likely to be active at physiological pH. There are several endogenous host cysteine protease inhibitors which control cysteine proteinase activity. These include cystatins which are divided into three families: family one members which have no disulfide bonds, family two members which have two disulfide bonds and family three members (kininogens) which have nine disulfide bonds (Salvesen and Nagase, 1989). α_2-Macroglobulin (α_2M) is a natural inhibitor that fails to discriminate between proteinase classes and binds to and inhibits most proteinases (Salvesen and Nagase, 1989). CP2 interacts with natural inhibitors, for example, with cystatins (cystatin families 2 and 3) and kininogens (Lalmanach et al., 2002). The aim of this part of the present study was to determine whether CP2 interacts with a natural bovine protease inhibitor, α_2-macroglobulin. Other trypanosomal proteinases from *T. cruzi* (Ramos et al., 1997) and from *T. b. brucei* (Troeberg et al., 1996) interact with α_2M. This interaction could possibly be exploited to increase the immunogenicity of CP2 as was done previously for hen egg lysozyme (Chu and Pizzo, 1993; Chu et al., 1994), prostate-specific antigen (Otto et al., 1998), and hepatitis B virus surface antigen (Cianciolo et al., 2002).

α_2M is a member of a group of high molecular weight proteins collectively known as the α-macroglobulins (Salvesen and Nagase 1989). The α-macroglobulins are the only natural inhibitors that do not differentiate proteinase classes and are thought to play an important role in physiological control of extracellular proteolytic activity (Barrett et al., 1979). The α_2M molecule is a tetramer of four identical subunits that form two disulfide-bonded dimers which, in turn, are associated through non-covalent forces (van Jaarsveld et al., 1994) (Figure 35). The molecular weight of bovine α_2M is ~ 800 000 kDa and each subunit has a molecular weight of ~ 180 000 kDa (Nagasowa et al., 1970). In the middle of each subunit is a stretch of amino acid residues that are vulnerable to proteolysis. This region is called the 'bait region'. Cleavage of any peptide bonds in the 'bait region' triggers a conformational change in α_2M

resulting in the entrapment of the proteinase (Ramos et al., 2002) (Figure 36). The conformationally changed α_2M becomes more compact and migrates faster through native gels and is referred to as the fast form (F-α_2M), whereas the original form is known as the slow form (S-α_2M) (Chu and Pizzo, 1994).

Covalent linkage of a proteinase to α_2M does not occlude the enzyme's active site, so the sterically entrapped enzyme retains activity towards low molecular weight substrates but not substrates with a molecular mass higher than 30 kDa (van Jaarsveld et al., 1994). Inactive proteinases and zymogens are not bound by α_2M (Salvesen and Nagase, 1989). Since CP2 is likely to be active at physiological pH, it is possible that this enzyme is bound by α_2M.

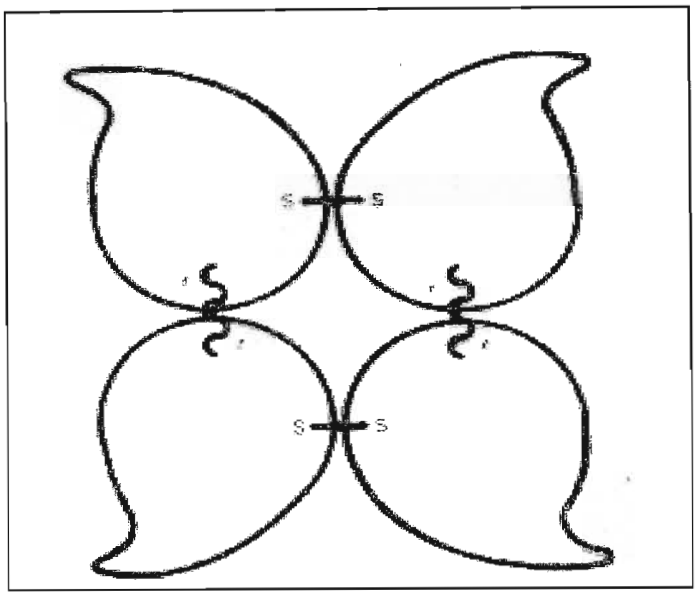

Figure 35. Diagrammatic representation of the α_2M molecule (Barrett et al., 1979). α_2M is a tetramer of four identical subunits formed by the noncovalent association (at the sites marked 'r') of two disulfide-bonded pairs of subunits.

The fact that α_2M bound proteinases retain activity against small substrates, suggests that the function of α_2M extends beyond simple proteinase inhibition (Chu and Pizzo, 1993). α_2M does not only bind proteolytic enzymes but other non-proteolytic proteins can be bound by α_2M (Chu and Pizzo, 1993). Non-proteolytic proteins such as insulin (Chu et al., 1991), cytokines (James, 1990), growth factors and hormones (Kratzsch et al., 1995) were shown to be bound by α_2M. The binding mechanisms of these molecules may vary and binding may be either covalent or non-covalent. In most cases covalent binding occurs via disulfide interchange

when hormones are present during proteolytic activation of α_2M and in some cases non-covalent entrapment may occur as in α_2M-protease interaction. The covalent-binding that occurs between these molecules and α_2M may be different from that accompanying protease binding (James, 1990).

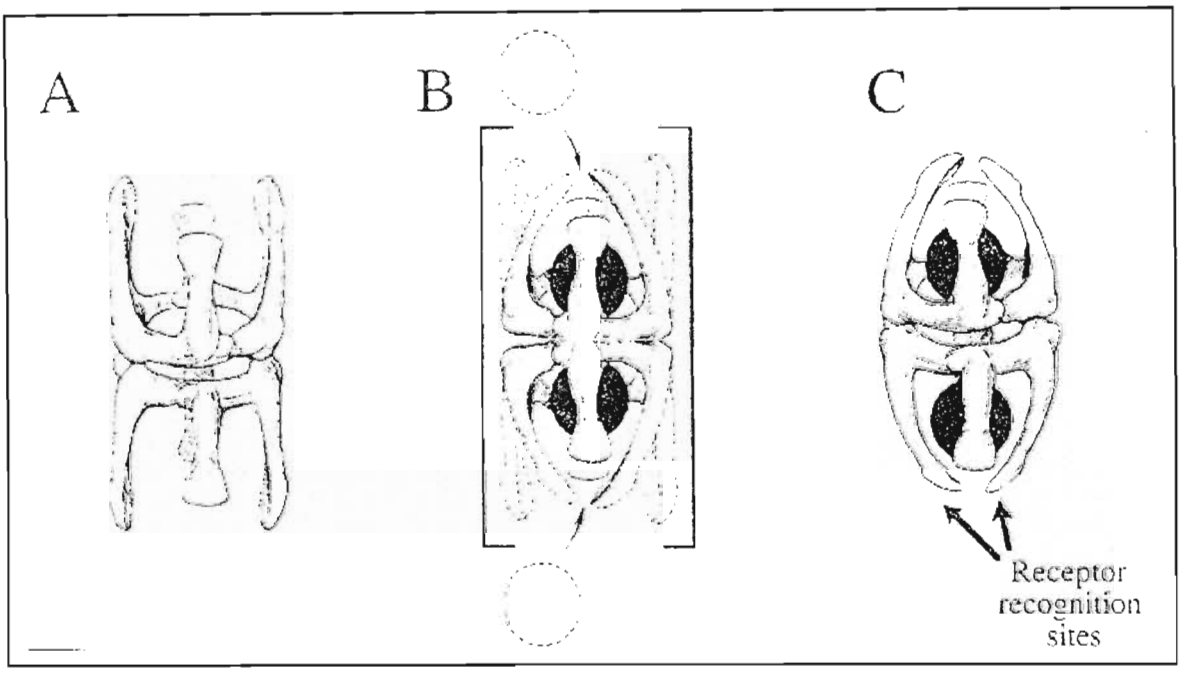

Figure 36. Schematic representation of the conformational change that α_2M undergoes upon proteolytic activation (Chu and Pizzo, 1994). A: Native α_2M which is able to inhibit proteinases but which is not receptor-recognised. B: Proteinase(s) (indicated by broken circles) enter the trap to get to the 'bait' region and when the proteinase cleaves the bait region, a conformational change of α_2M is initiated. C: α_2M with an entrapped proteinase(s) (indicated by shaded circles) looks more compact and the receptor recognition sites are exposed.

Studies have shown that conformational changes of α_2M-protein in complexes lead to the exposure of receptor recognition sites that result in clearance of α_2M-protein complexes from the circulation (van Jaarsveld et al., 1994). α_2M "senses" situations involving increased proteolysis, such as during inflammation or tissue destruction, where it inhibits potentially harmful proteinases, microbial or otherwise, and captures proteins for delivery to cells which have α_2M receptors, such as macrophages, hepatocytes and other cells involved in biological and chemical detoxification (Chu and Pizzo, 1994). The use of α_2M as an adjuvant takes advantage of its natural function. As in the natural mechanisim, antigens entrapped by α_2M with exposed receptor recognition sites are quickly delivered to macrophages, potentially

resulting in enhanced immunogenicity.

The possible enhancement of the immunogenicity of CP2 could be determined by complexing with α_2M and immunising a suitable animal with the complex, thereby enhancing delivery of CP2 to macrophages possessing α_2M receptors. However, this was beyond the scope of the present study. In this study α_2M was isolated from bovine plasma and characterised by western blotting (Section 5.2). The interaction between CP2 and α_2M was investigated as a preliminary study to the future use of α_2M to potentially increase CP2 immunogenicity.

5.2 Isolation and characterisation of α_2M from bovine plasma

The method used for the isolation of α_2M was that of Barrett et al. (1979) in conjunction with the modified method of Kurecki et al. (1979). The α_2M-containing fraction of bovine plasma was first precipitated with PEG 6000 (Barrett et al., 1979), followed by chromatography on a zinc chelate column (Kurecki et al., 1979). The isolation of the thiol-containing α_2M on a zinc-containing gel is based on the suggestion that imadozole and thiol groups on protein molecules are the most important binding sites for zinc- and copper- containing adsorbents (Porath and Olin, 1983). An additional molecular exclusion chromatography (MEC) step on Sephacryl-S-300 HR was done to further purify α_2M from other contaminating serum proteins which were also bound by the zinc chelate.

5.2.1. Reagents

Acid-citrate-dextrose. Citric acid.H_2O (4 g), $Na_3C_6H_5O_7.2H_2O$ (11 g) and glucose.H_2O (12.21 g) were dissolved in distilled H_2O and made up to 500 ml.

Bovine plasma. Blood was harvested from cattle at slaughter (Abakor abbatoir, Cato Ridge). Bovine blood (1 litre) was collected into two litre glass bottles containing acid-citrate-dextrose (140 ml). The collected blood was stored for 48 h at 4°C before the plasma was separated from the red blood cells by centrifugation (8000 x g, 30 min, 4°C). The plasma was re-centrifuged (8000 x g, 30 min, 4°C) and stored for 24 h at 4°C.

25% (w/v) PEG 6000. PEG 6000 (50 g) was dissolved in 200 ml of distilled H_2O.

50 mM Na_2EDTA buffer, 500 mM NaCl, pH 7.0. $Na_2EDTA.2H_2O$ (4.653 g) and NaCl (7.305 g) were dissolved in 200 ml of distilled H_2O, titrated to pH 7.0 with NaOH and made up to 250 ml with distilled H_2O.

3 mg/ml $ZnCl_2$, pH 6.0. $ZnCl_2$ (0.3 g) was dissolved in 90 ml of distilled H_2O, titrated to pH 6.0 with HCl and made up to 100 ml with distilled H_2O.

2 M Na_2CO_3. Na_2CO_3 (26.5 g) was dissolved in 125 ml of distilled H_2O.

250 mM Na-acetate buffer, 150 mM NaCl, pH 5.0. CH_3COOH (3.75 g) and NaCl (2.19 g) were dissolved in 200 ml of distilled H_2O, titrated to pH 5.0 with NaOH and made up to 250 ml with distilled H_2O.

20 mM Na-phosphate buffer, 150 mM NaCl, pH 6.0. $NaH_2PO_4.2H_2O$ (3.12 g) and NaCl (8.76 g) were dissolved in 950 ml of distilled H_2O, titrated to pH 6.0 with NaOH and made up to 1 litre with distilled H_2O.

20 mM Na-cacodylate buffer, 150 mM NaCl, pH 5.0. Na-cacodylate (1.07 g) and NaCl (8.76 g) were dissolved in 950 ml of distilled H_2O, titrated to pH 5.0 with NaOH and made up to 1 litre with distilled H_2O.

100 mM Na-phosphate buffer, 0.02% (w/v) NaN_3, pH 7.0. $NaH_2PO_4.2H_2O$ (31.202 g) and NaN_3 (0.4 g) were dissolved in 1.9 litres of distilled H_2O, titrated to pH 7.0 with NaOH and made up to 2 litres with distilled H_2O.

5.2.2 Procedure

Step 1. PEG precipitation. Plasma (250 ml) was mixed with 25% (w/v) PEG solution (70 ml). The mixture was stirred for 10 min, left for 20 min at RT and centrifuged (10 000 x g, 20 min, RT). 25% (w/v) PEG solution (180 ml) was added to the supernatant. The mixture was stirred for 10 min, left for 20 min at RT and centrifuged (10 000 x g, 20 min, RT). The precipitate was collected and dissolved in 20 mM Na-phosphate buffer, 150 mM NaCl, pH 6.0 (42 ml)

and dialysed against the same buffer overnight (2 x changes).

Step 2. Zinc chelate chromatography. Imidodiacetic acid-epoxy-activated Sepharose 4B was used. The gel was washed with 50 mM EDTA, 500 mM NaCl solution, pH 7.0 (1 column volume) before thoroughly washing with distilled H_2O. The gel bound chelate of zinc was obtained by passing a $ZnCl_2$ solution (3 mg/ml) through a column containing imidodiacetic acid-Sepharose 4B (0.75 × 9 cm, 0.67 ml/min, 4°C). Fractions (2 ml) were collected into tubes containing 2 M Na_2CO_3 (2 ml) and the presence of Zn^{2+} was assessed by the formation of an insoluble $ZnCO_3$ precipitate. The gel was washed with 250 mM Na-acetate buffer, 150 mM NaCl, pH 6.0 (1 column volume, 1.67 ml/min, 4°C). The gel was equilibrated with 20 mM Na-phosphate buffer, 150 mM NaCl, pH 6.0 (2 column volumes, 1.67 ml/min, 4°C). The dialysed α_2M-containing material was applied to the zinc chelate column. The column was washed with 20 mM Na-phosphate buffer, 150 mM NaCl, pH 6.0 and fractions were collected (5 ml fractions, 0.83 ml/min, 4°C). When the $A_{280\ nm}$ was less than 0.02, elution of α_2M was effected with 20 mM Na-cacodylate buffer, 150 mM NaCl, pH 5.0 (2 ml fractions, 0.83 ml/min, 4°C). Fractions which had high absorbance values were assumed to contain α_2M material because by lowering the pH from 6.0 to 5.0 the interaction between α_2M and zinc chelate matrix is destabilised (Kurecki et al., 1979). These fractions were therefore pooled.

Step 3. Chromatography on Sephacryl S-300 HR. The column (1.25 × 90 cm, 0.42 ml/min) was pre-calibrated with blue dextran (M_r 2000 000), bovine thyroglobulin (M_r 670 000), ferritin (M_r 440 000), ovalbumin (M_r 44 000) and horse myoglobin (M_r 17 000). Fractions from step 2 containing α_2M material were concentrated by dialysis against PEG 20 000 (Section 2.4.1.1) and applied to the Sephacryl S-300 HR column (0.42 ml/min, 4°C) pre-equilibrated with 100 mM Na-phosphate buffer, 0.02% NaN_3, pH 7.0. Protein was eluted with the same buffer and absorbance monitored at 280 nm. α_2M-containing fractions were evaluated by Laemmli SDS-PAGE (Section 2.5.2) and characterised by western blotting (Section 2.7.2) using rabbit anti-α_2M antibodies (a gift from Dr Edith Authié, ILRI, Nairobi, Kenya). α_2M fractions were stored at 4°C.

5.3 Inhibition of CP2 by α_2M

5.3.1 Azocasein assay

5.3.1.1 Reagents

As per Section 2.13.1

5.3.1.2 Procedure

Enzyme activity was assayed as described in Section 2.13.2, except that CP2 (0.068 nmoles in 100 µl Brij 35) was pre-incubated with α_2M (0.068 nmoles or 0.136 nmoles to give a 1:1 or a 1:2 molar ratio of CP2:α_2M in assay buffer, 1 h, 37°C) before adding azocasein/urea substrate. The experiment was repeated three times. Proteolytic activity was determined by measuring the hydrolysis of substrate at 366 nm and comparing to that of CP2 without α_2M (100% of activity) and expressed as % inhibition.

5.3.2. Bz-Pro-Phe-Arg-pNA assay

The procedure followed was according to Ramos et al. (2002).

5.3.2.1 Reagent

Substrate [30 mM Bz-Pro-Phe-Arg-pNA]. Bz-Pro-Phe-Arg-pNA (0.1 g) was dissolved in DMSO (1 ml) and diluted to 5 ml with distilled H_2O.

Phosphate buffered saline (PBS) pH 7.2. As per Section 2.10.1

5.3.2.2 Procedure

CP2 (44 pmoles) was incubated with different concentrations of α_2M at molar ratios of 1:0.25, 1:0.5, 1:1, and 1:2 for 60 min at 37°C. The enzyme activity of CP2 in the presence or absence of α_2M inhibitor was assayed with the synthetic chromogenic substrate Bz-Pro-Phe-Arg-pNA at pH 7.2 (100 µl, to give final concentration of 3 mM) and the reaction was followed spectrophotometrically at 405 nm. Proteolytic activity was measured and compared to that of a CP2 control without α_2M (100% activity) and expressed as percentage inhibition. To determine the time course of inhibition of CP2 activity by α_2M, CP2 was incubated with α_2M

(molar ratio 1:1) for 5, 10, 15, 20, 30, 40, 50 and 60 min at 37°C. Proteolytic activity was measured and expressed as above. Each of these experiments was repeated three times.

5.4 Results

5.4.1 Purification and characterisation of α_2M

α_2M precipitated by PEG 6000 was resolved from other plasma proteins by zinc chelate chromatography (Figure 37). The binding of proteins in metal chelate affinity chromatography depends on pH (Kurecki et al., 1979). α_2M and other plasma proteins which bind to the zinc chelate remained tightly bound to the zinc column at pH 6.0, allowing removal of other plasma proteins (unbound fraction). Lowering of pH to pH 5 allowed elution of bound protein in a sharp peak.

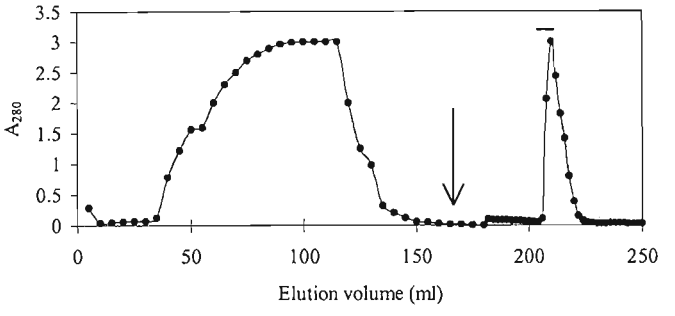

Figure 37. Chromatography of α_2M on a zinc chelate column. Zinc chelate column (0.75 × 9 cm, 0.83 ml/min, 4°C) was pre-equilibrated with 20 mM Na-phosphate buffer, 150 mM NaCl, pH 6.0. The PEG 6000 precipitate containing α_2M was loaded onto the column and 5 ml fractions collected. After elution of unbound protein with the equilibration buffer, bound protein was eluted with 20 mM Na-cacodylate buffer, 150 mM NaCl, pH 5.0, applied at point ↓ and 2 ml fractions collected. (—) fractions pooled.

Analysis of the zinc chelate bound α_2M-containing fraction on SDS-PAGE revealed protein contaminants smaller than 76 kDa (Figure 39, lane d). MEC on Sephacryl S-300 HR (Figure 38) succeeded in removing these protein contaminants from the zinc chelate bound fraction (Figure 39, lane e). The first peak was chosen because according to the calibration with standard proteins of known molecular weight, α_2M (M_r 720 00) would be eluted at this volume. Non-reducing Laemmli SDS-PAGE showed that the α_2M-containing material pooled after MEC consisted of an α_2M tetramer protein band at 720 kDa and an α_2M dimer band at

360 kDa (Figure 39, lane e). α_2M was thus purified to homogeneity using a combination of PEG precipitation, zinc chelate chromatography and Sephacryl S-300 MEC.

The purified α_2M sample was further characterised by western blotting (Figure 40). Purified α_2M was recognised by rabbit anti-α_2M antibodies. The α_2M dimer band at 360 kDa and the single quarter subunit band at 170 kDa were recognised in the reduced sample (Figure 40, lane b). The α_2M tetramer band at 720 kDa and dimer band at 360 kDa were recognised in the non-reduced sample (Figure 40 lane c).

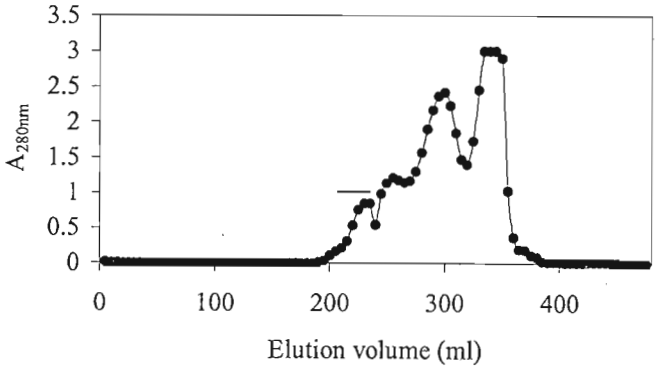

Figure 38. Molecular exclusion chromatography of α_2M on a Sephacryl S-300 HR. A Sephacryl S-300 HR column (1.25 × 90 cm, 0.42 ml/min, 4°C) was equilibrated with 100 mM Na-phosphate buffer, 0.02% NaN_3, pH 7.0. The concentrated sample (1.5 ml) from zinc chelate chromatography was loaded and protein was eluted with the equilibration buffer. (—) pooled fractions.

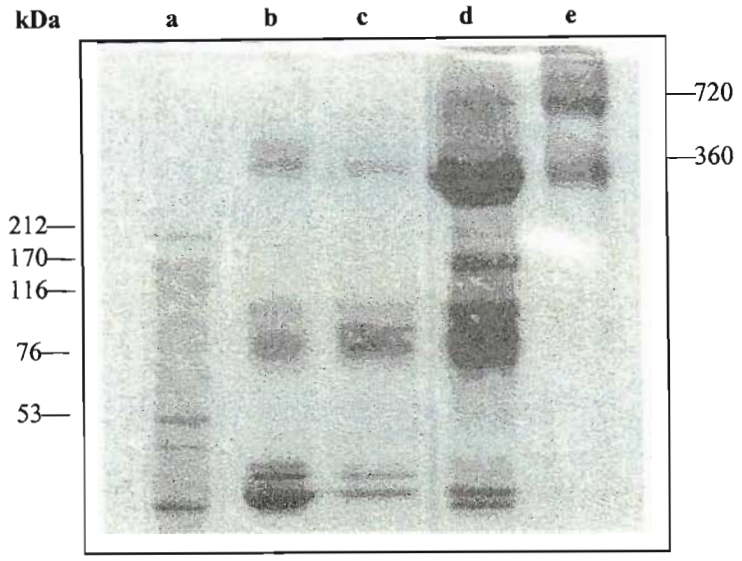

Figure 39. Non-reducing 5% Laemmli SDS-PAGE for evaluation of α_2M purification steps. Samples loaded on the gel were: molecular mass markers (myosin, 669 kDa; α_2M, 170 kDa; β-galactosidase, 116 kDa; transferrin, 76 kDa and glutamic dehydrogenase, 53 kDa; 5 µl, lane a), bovine plasma (15 µg, lane b), dissolved PEG 6000 precipitate (15 µg, lane c), zinc chelate bound fraction (5 µg, lane d) and α_2M fraction eluted from Sephacryl S-300 HR column (10 µg, lane e). The positions of the 720 kDa α_2M tetramer and 360 kDa dimer are shown on the right. The bands were visualised by Coomassie blue R-250 protein stain as described in Section 2.6.1.2.

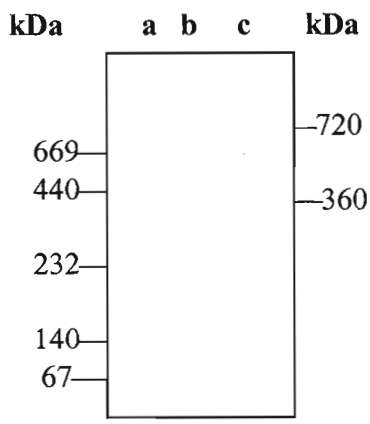

Figure 40. Recognition of purified α_2M by rabbit anti-α_2M antibodies on a western blot. Molecular mass markers (thyroglobulin, 669 kDa; ferritin, 440 kDa; catalase, 232 kDa; lactate dehydrogenase, 140 kDa and BSA, 67 kDa) (5 µl, lane a), reduced α_2M (10 µg, lane b) and non-reduced α_2M (10 µg, lane c) were electrophoresed on a 5% SDS-PAGE (Section 2.5.2), electroblotted onto nitrocellulose and probed with rabbit anti-α_2M antibodies. Immune reactions were detected using goat anti-rabbit alkaline phosphatase conjugate and the substrate used was NBT/BCIP (Section 2.7.2).

5.4.2 Inhibition of CP2 by α_2M.

CP2 activity was inhibited by α_2M. Inhibition tests using azocasein as substrate gave 26.6% inhibition and 59% inhibition at 1:1 and 1:2 molar ratios of CP2:α_2M, respectively (Table 12).

On the other hand, inhibition tests using Bz-Pro-Phe-Arg-pNA as substrate gave 40% inhibition at 1:0.25 molar ratio and at 1:0.5 molar ratio there was a slight decrease of inhibition and then the level of inhibition plateaued at higher molar concentrations of α_2M (Figure 41, panel A). Hydrolysis of Bz-Pro-Phe-Arg-pNA substrate by CP2 in the absence of α_2M expressed as a percentage was slightly higher after incubation for longer than 20 min than hydrolysis of the substrate in the presence of α_2M (1:1 molar ratio) (Figure 41, panel B). These results show that there is an interaction between CP2 and α_2M and the decreased hydrolytic activity of the active enzyme upon the Bz-Pro-Phe-Arg-pNA substrate can be attributed to its trapping by α_2M.

Table 12. Inhibition of CP2 by α_2M using azocasein as substrate.

Molar ratio of CP2: α_2M	Inhibition (%)[a]
1:0	0
1:1	26.6
1:2	59

[a]SEM less than 0.5 (n=2).

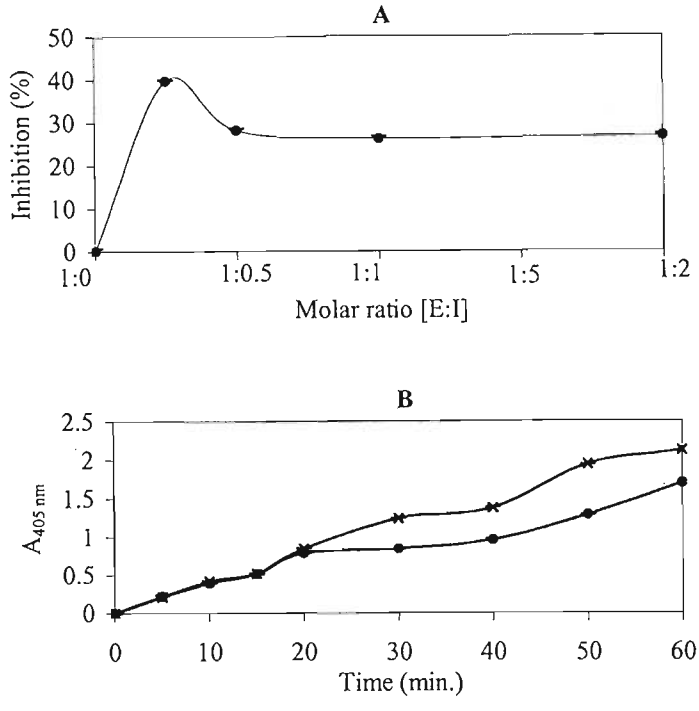

Figure 41. Inhibition of CP2 by α_2M using Bz-Pro-Phe-Arg-pNA as substrate. CP2 (44 pmoles) was incubated (60 min, 37°C) with various concentrations of α_2M (Panel A). Hydrolysis of the substrate by CP2 in the presence and absence (100% activity) of α_2M was measured. CP2 (44 pmoles) was incubated with α_2M at 1:1 molar concentration (•) and without α_2M (×) for different durations (Panel B). Hydrolytic activity was determined by taking absorbances at 405 nm. Bars represent SEM (n = 3).

5.5 Discussion

Long, complex purification procedures tend to result in the inactivation of α_2M (van Jaarsveld et al., 1994). An ideal purification method for α_2M is therefore one that balances obtaining pure α_2M with one that retains most of the α_2M in the active form (van Jaarsveld et al., 1994). In this study α_2M was purified in three major steps, namely, PEG precipitation, zinc chelate chromatography and MEC on Sephacryl S-300 HR. The method of Kurecki et al. (1979) was modified by employing PEG 6000 as precipitating agent instead of ammonium sulfate because fractionation with PEG is a mild procedure that removes the β-lipoprotein more efficiently than does preparative ultracentrifugation, and without conversion of the α_2M S-form into the F-form risked by the use of ammonium sulfate (Barrett et al., 1979). However, there has been some difficulty reported with the PEG precipitation procedure due to losses in α_2M inhibitory activity, consequently the precipitation step has been omitted and more milder techniques such as anion exchange chromatography have been used instead (van Jaarsveld et al., 1994).

In the present study, the precipitation of α_2M was complete at 12.5% (w/v) PEG but α_2M co-precipitated with other plasma proteins as revealed by SDS-PAGE. The affinity of α_2M for Zn^{2+} was used as a basis for its second purification step. Large amounts of α_2M remain tightly bound to the zinc column at pH 6.0 allowing removal of other plasma proteins (Kurecki et al., 1979). After this step, SDS-PAGE revealed that there were still contaminating small molecular weight proteins revealing the limited efficiency of the zinc chelate column. Porath and Olin (1983) reported that both imidazole and thiol groups on protein molecules are important zinc binding sites, therefore it is highly likely that there were other plasma protein molecules with these functional groups, resulting in their inevitable binding to the zinc chelate matrix. The MEC step on Sephacryl S-300 HR took advantage of the high molecular weight of α_2M and resulted in its successful resolution from other contaminating plasma proteins as shown by SDS-PAGE. Under non-reducing SDS-PAGE conditions, a 720 kDa and a 360 kDa band were observed. The expected molecular masses of α_2M under non-reducing conditions on SDS-PAGE is between 720 and 725 kDa for the tetrameric form of α_2M and at 360 kDa for the dimeric form of α_2M. This is because the minimal covalent subunit without reduction is the half molecule of α_2M of 360 kDa (Barrett et al., 1979). The result obtained in this study agrees favourably with the literature. The method used in this study for the purification of α_2M was successful in giving the purity of α_2M required for the purposes of this study and for further use as an adjuvant. The isolated α_2M was characterised immunologically by western blotting. Isolated α_2M was recognised specifically by anti-α_2M antibodies showing bands corresponding to the expected molecular mass of α_2M. This result confirmed that the protein isolated from bovine plasma was indeed α_2M.

This study has shown that α_2M interacts with CP2 and inhibits its activity. However, the activity of complexed CP2 enzyme remained when using azocasein and Bz-Pro-Phe-Arg-pNA as substrates. The proteolytic activity of complexed CP2 was higher when using Bz-Pro-Phe-Arg-pNA as substrate compared to azocasein. Salvesen and Nagase (1989), suggested that smaller substrates are more likely to be hydrolysed by α_2M-complexed proteinases. This is because proteinases entrapped within the large α_2M molecule are sterically hindered and only small substrates are able to access the entrapped proteinase.

Other trypanosomal cysteine proteinases which are thought to be pathogenic have been found

to be inhibited by host proteinase inhibitors. Examples include the inhibition of trypanopain-Tb from *T. b brucei* by α_2M, kininogens and various cystatins (Troeberg et al., 1996), cruzipain from *T. cruzi* by α_2M (Ramos et al., 1997; 2002) and congopain from *T. congolense* by kininogens (Lalmanach et al., 2002). However, these proteinases still remain pathogenic to the host *in vivo*. This suggests that the interaction of α_2M and other host inhibitors does not completely inhibit these trypanosomal proteinases. Studies of CP2 interaction with bovine kininogens indicated that there was not complete inhibition of CP2; moreover, the interaction triggered the release of kinins which may be harmful to the host (Lalmanach et al., 2002). This suggests that the interaction between trypanosomal proteinases and host proteinase inhibitors may increase rather than decrease the disease (Lalmanach et al., 2002). It has been demonstrated in this study that complexed enzyme retains activity against a small peptide substrate. α_2M-trypsin complexes retains activity against small substrates such as peptide hormones and cytokines (Borth and Teodorescu, 1986), thereby interfering with the normal regulatory system of the host animal. In a previous study by Ramos et al. (2002), α_2M-cruzipain complexes retained activity against a protein substrate since faint activity bands were visualised in gelatin SDS-PAGE. Their study indicated decreased proteolytic activity but not complete inhibition. These results show that the role of α_2M in inhibiting parasite cysteine proteinases may not be sufficient in hindering the pathogenic effects of these proteinases.

α_2M can also be used to "convert" a poorly immunogenic protein into a strongly immunogenic protein because the complexed α_2M becomes receptor recognised and can be efficiently internalised by macrophages and other cells that express α_2M receptors (Cianciolo et al., 2002). It has been suggested that immune responses to pathogenic CP2 may play an essential role in the mechanism of trypanotolerance (Authié et al., 2001). Therefore a more efficient immune response to this antigen may enhance disease resistance. The proof of interaction between CP2 and α_2M would help in studying the immunogenicity of CP2 complexed with α_2M in future studies.

In addition to studying the possible enhancement of the immunogenicity of CP2 by complexing with α_2M, one could also use the same approach to study the possibility of the enhancement of the immunogenicity of the epitopes of CP2 incorporated into α_2M. Incorporation of non-proteinase antigens into α_2M has been achieved (Cianciolo et al., 2002). α_2M is converted to receptor-recognised α_2M by incubation with ammonia, resulting in the

cleaving of the bait region of α_2M without any proteolysis step. α_2M-NH_3 is heated to approximately 50°C in the presence of a non-proteinase antigen. When cooling to RT the antigen gets incorporated (Cianciolo et al., 2002). α_2M also binds peptides *in vitro* by coincubation of peptides, proteins and α_2M at 50°C (Binder et al., 2001). It would be interesting for future studies to raise antibodies against critical epitopes of CP2 complexed with α_2M. Enhancement of the immunogenicity of CP2 or CP2 epitopes by complexing with α_2M needs to be determined in an optimised system because these antigens may be potentially viable vaccine candidates.

Chapter 6
General discussion

Trypanosomosis is a complex and often fatal disease caused by infection with one or more of the pathogenic tsetse-transmitted protozoan parasites of the genus *Trypanosoma*. The disease is known as nagana in livestock and is of major economic importance. *T. congolense*, *T. brucei* and *T. vivax* species are the major pathogenic trypanosomes in sub-Saharan Africa (Anene et al., 2001). The therapeutic and preventitive use of trypanocides has limitations which include toxicity and the development of resistance by the parasites (Aksoy et al., 2001). The effects on the environment, of insecticides used for vector control has been a subject of controversy (Grant, 2001).

An alternative approach to the control of the disease has been suggested. Several studies have confirmed the importance of parasite proteinases for parasite viability and as major pathogenic factors (for example, Mbawa et al., 1992; McKerrow et al., 1999; Troeberg et al., 1999; Cazzulo et al., 2001). In this and other studies (reviewed in Robertson et al., 1996; McKerrow et al., 1999; Klemba and Goldberg, 2002), the focus has been on the proteinases as targets for the development of therapeutic agents and vaccines, hence focusing on an anti-disease approach rather than an anti-parasite approach. One way of inhibiting proteinases is by specific antibodies. Trypanoresistant/tolerant cattle develop high IgG responses to congopain, a trypanosome cysteine proteinase, during primary infection with *T. congolense*, whereas susceptible cattle have only limited immune responses to congopain (Authié, 1994). Immunisation trials with congopain, aiming at enzyme inhibition through specific antibodies, have been carried out in cattle (Authié et al., 2001). Immunised trypanosusceptible cattle, unlike non-immunised cattle, maintained or gained weight during infection with *T. congolense* (Authié et al., 2001), suggesting that antibodies against congopain contribute to trypanotolerance. 'Protective' epitopes of congopain, which elicit antibodies with inhibitory effect over congopain activity, have not previously been identified, thus the main focus of this study was to map the immunogenic epitopes of congopain.

T. congolense possesses at least two families of closely related congopain enzymes named CP1 (Fish et al., 1995) and CP2 (Jaye et al., unpublished, EMBL accession no. L25130). CP1 and CP2 differ in their functional characteristics, suggesting two separate roles in host-parasite

interactions (Boulangé et al., 2001). Consequently peptides which have the potential of being immunogenic for the production of anti-catalytic antibodies were identified in the primary structures of both CP1 and CP2. The epitope prediction program, Predict 7 (Cármenes et al., 1989), was used to select the peptides. Using this program, numerical values which reflect hydrophilicity, flexibility, surface probability and antigenicity for overlapping sequences of amino acid residues in each enzyme were plotted. The features mentioned above are essential for predicting whether the sequence will be immunogenic or not, for example, hydrophilic residues are more likely to be located on the surface of the enzyme and would therefore be easily accessible to antibodies. Sequences which gave plots with local peaks of hydrophilicity, flexibility, surface probability and antigenicity were selected.

Peptides selected from the catalytic domain of CP2 include a peptide located at the N-terminus, a central catalytic domain peptide and an active site-associated peptide. Peptides were also selected from similar regions of the catalytic domain of CP1. CP2 peptides were longer than CP1 peptides (except for the active site associated peptide) but there was high sequence homology between the CP2 peptides and the CP1 peptides. The immunogenicity of the selected peptides, conjugated to rabbit albumin as a carrier protein, was determined by immunisation into chickens. Antibodies were produced against all peptides, as evidenced by their binding to the corresponding peptides in ELISAs. More importantly, the anti-peptide antibodies were able to recognise whole CP2 in ELISA and western blot tests. This suggests that there was similarity between the conformations adopted by peptides when exposed to the experimental animals' immune systems and the corresponding regions in the CP2 molecule. Even anti-CP1 peptide antibodies recognised sequences in the CP2 enzyme, reflecting the high degree of homology (90%) between CP1 and CP2 sequences. Recombinantly expressed, whole CP2 enzyme and the catalytic domain of CP2 (C2) were used in the tests because CP2 from *T. congolense* has been extensively studied and no native CP1 enzyme has been characterised from *T. congolense* bloodstream form lysates (Lalmanach et al., 2002).

Once it was established that the anti-catalytic domain peptide antibodies recognise the whole enzyme, their effects on the catalytic activity of CP2 was investigated. Studies have shown that anti-enzyme antibodies can have varying effects on enzymes, depending on the enzyme used, the region targeted and the substrate used (Richmond, 1977; Dennison and Pike, 1991; Coetzer et al., 1992; Troeberg et al., 1997). The epitopes which are able to produce inhibitory

antibodies are likely to be located in the catalytic domain of CP2, since IgG from C2 immunised cattle inhibited congopain (Authié et al., 2001). Anti-peptide antibodies produced in this study had either enzyme stimulating effects at lower antibody concentrations and enzyme inhibiting effects at higher antibody concentrations (biphasic) or they had an enhancing effect at all antibody concentrations used. The latter result suggests that some anti-peptide antibodies induced a more active CP2 conformation. Similar results were described by Richmond (1977) for β-lactamase and Troeberg et al. (1997) for trypanopain-Tb. Even though most of the anti-peptide antibodies did not have an inhibiting effect on the enzyme activity, they did recognise and bind to native CP2 but had a biphasic or stimulatory effect.

This study has been able to identify congopain peptides, using an epitope prediction program, which appear to be partial epitopes of congopain. This was deduced from the observation that antibodies against these peptides were able to recognise the whole CP2 enzyme in partially denatured (ELISA) and denatured (western blot) forms. However, the antibodies against these peptides did not have an enzyme activity inhibiting effect, but rather an enzyme activity enhancing effect or biphasic effect. Antibodies from cattle immunised with C2 inhibited congopain, suggesting that the essential epitopes of CP2 are located in the catalytic domain of CP2 (Authié et al., 2001). Inhibition tests in the latter study involved incubation of antibodies for 12 h on ice (to prevent hydrolysis of the antibodies by congopain). This was regarded as excessive since 15 min incubation at 37°C with the antibody before activation of the enzyme and assay against peptide substrate was found optimal for inhibition of cathepsin L and trypanosomal oligopeptidase B, respectively (Coetzer et al., 1991; Morty et al., 2000). Either the conformation adopted by the peptides presented to the immune system is not similar to that adopted by the corresponding regions in C2, or the peptides selected do not induce the production of neutralising antibodies. In addition to this, most epitopes are discontinuous, rather than continuous (Van Regenmortel, 1986), and the peptides selected may be integral parts of an epitopes. Binding of antibodies raised against these parts of the protease may have resulted in a more active enzyme conformation. The anti-peptide antibodies may have recognised the corresponding regions of the native CP2 enzyme, but because the Z-Phe-Arg-AMC substrate used in this study is small, the substrate might have gained access to the active site (especially in cases where the antibodies were not against active site associated sequences) where it was cleaved.

It would be interesting in future studies to look at the effect of a cocktail of the anti-peptide antibodies produced in this study on CP2 activity. It should also be taken into consideration that these antibodies may have different effects in the *in vivo* situation since the natural substrates are large protein molecules. Enzyme assays using different protein substrates in the presence of anti-congopain peptide antibodies need to be done. This will require more enzyme than was available in this study, since enzyme assays using protein substrates are less sensitive than those using synthetic peptide substrates. Nevertheless, the anti-peptide antibodies produced in this study react with full-length CP2 and can thus be useful in basic research. These antibodies would be useful in identifying the CP2 product of an open reading frame, localising the gene product to particular cells or subcellular organelles, identifying enzymatic functions, following the role of particular regions of enzyme through the enzyme maturation processes, analysing the expression of exons following DNA rearrangements and RNA splicing and purifying the enzyme by immunoaffinity chromatography techniques (Shinnick et al., 1983; Morty et al., 2000).

Another objective was to examine the natural antigenicity of the congopain peptides described above. This was done by testing the recognition of the peptides in an ELISA by sera from infected or immunised cattle. Sera from both trypanosusceptible Boran and trypanotolerant N'Dama cattle infected with *T. congolense* targeted all peptides compared to non-immune sera, but the CP2 catalytic domain peptide was the most weakly recognised peptide. Sera from uninfected cattle immunised with C2 were tested in an ELISA for their ability to target CP2 and CP1 peptides. CP2 peptides located at the N-terminus and in the catalytic domain were the primary targets of antibodies in these sera and the active site-associated peptide from CP1 was also the primary target. This suggests that these peptides form part of immunogenic epitopes of CP2. However, some peptides (especially the N-terminus peptide from CP1 and the active site associated peptide from CP2) cross-reacted only weakly with cattle IgG. It can be assumed that this weak cross-reactivity is due to the fact that the respective peptides represent only a portions of more complex epitopes and not able to adopt the correct conformation present in the folded enzyme that can be recognised fully by these antibodies. All peptides were poorly recognised by rabbit anti-native congopain antibodies. Apparently these peptides are naturally immunogenic in cattle but not in rabbits. A similar result was reported by Coetzer et al. (1992) where a peptide associated with the active site of cathepsin L was recognised by chicken anti-cathepsin L antibodies and not by rabbit anti-cathepsin L antibodies.

Only the C-terminal extension sequence for a *T. vivax* cysteine proteinase, called vivapain, had been determined when this study was undertaken (Prof. Theo Baltz, University of Bordeaux, France, personal communication). Therefore a peptide was selected from the C-terminal extension of vivapain using the same epitope prediction program that was used for selecting congopain peptides. The C-terminal extension sequences of congopain and vivapain were aligned and a peptide which has very little sequence homology between the two sequences was selected without compromising immunogenicity. The aim was to identify a peptide that could be used to distinguish between *T. vivax* and *T. congolense* infections. Sera from *T. congolense* infected cattle consistently targeted the congopain peptide slightly more than the vivapain peptide, while sera from *T. vivax* infected cattle consistently targeted the vivapain peptide slightly more than the congopain peptide. This result was not expected, since this assay failed to distinguish very clearly between *T. congolense* and *T. vivax* infections. Cross-reactivity might be due to trypanosome non-specific (polyreactive) antibodies. Trypanosome non-specific antibodies have been observed in cattle infected by *T. congolense* and *T. vivax* (Buza and Naessens, 1999). It is also possible that the sera used in this study had mixed infections of both *T. congolense* and *T. vivax*. This is supported by the results of the parallel experiments that were conducted by the collaborator in Beligium using peptides selected in this study and sera from experimentally infected cattle and goats in a trypanosome free area (Belgium). The congopain C-terminal extension peptide was specific for *T. congolense* infection and was not recognised by sera from *T. vivax* infected cattle (Dr Philippe Buscher, Institute of Tropical Medicine, Antwerp, Belgium). On the other hand, modification of the peptide sequences might improve the sensitivity of the assay, hence improving their potential use as diagnostic tools.

The congopain peptides selected cross-reacted with *T. congolense* infected sera but the assays were not very sensitive. This suggests that the peptides selected are effective but not optimal peptides. Optimal peptides can be identified by analysing a series of overlapping peptides. One way in which this can be done is by multiple pin peptide scanning ('Pepscan') technology (Sumar, 2001). In this method, a large number of peptides are synthesised and subsequently tested against a large number of antibody containing samples. Overlapping peptides, spanning the entire amino acid sequence of the enzyme, are built on polyethylene pins by the sequential addition of amino acids. The size of the peptides is usually between five to eight residues long.

The pins are mounted on a plastic holder with an 8 × 12 format similar to ELISA plates.

The epitopes of antibodies of interest are localised by ELISA using the pin bound peptides. In these tests, appropriate controls are included, for example, by including non-immune sera and by testing the binding of detection antibody-enzyme conjugates to pin bound peptides. Briefly, the peptide pins are blocked with blocking buffer to reduce non-specific binding to the pins. The pins are taken out of the blocking solution and placed in serum sample solutions in the plate. Following washing of the pins, the pins are incubated in enzyme-linked detection antibody. The pins are washed, incubated in the substrate after which the absorbance of the coloured product is read at the appropriare wavelength.

The ELISA is followed by further analysis of epitopes, which were recognised by the test sera, by window net analysis and replacement net analysis (Sumar, 2001). In window net analysis, peptides of different lengths representing one epitope are synthesised and tested with sera to identify the minimal antibody binding peptide. The replacement net analysis is done to identify residues which are essential for binding. This is done by replacing each amino acid residue of the epitope by the nineteen remaining amino acid residues and then testing each peptide with antisera of interest (Sumar, 2001).

The Pepscan technology provides an alternative approach for the synthesis, analysis and selection of candidate epitopes. Epitopes on the glycoprotein E of *Varicella zoster* virus that causes chicken pox have been identified by the Pepscan technology (Garcia-Valcarcel, 1997). These epitopes may be potentially viable candidates for a peptide-based vaccine. In conjunction with replacement net analysis, Pepscan can give well-defined epitopes, identifying the exact residues that are important for the binding of antibody to the epitope. Antigen antibody interactions for myoglobin were studied using Pepscan (Rodda et al., 1986). An epitope containing six residues was identified, followed by analysis of the peptide which showed that the N-terminal leucine was important to the binding of antibody to the epitope. For future studies one could use the Pepscan technology to identify optimal congopain peptides which could be used as diagnostic tools and possibly to raise CP2-inhibitory anti-peptide antibodies. The refined epitopes would probably include some of the peptides selected in this study, as they seem to have the potential of being partial CP2 epitopes.

In addition to attempting to define the epitopes of congopain, preliminary studies to increase the immunogenicity of congopain were also undertaken. It has been hypothesised that increasing the immunogenicity of congopain to elicit high titre anti-congopain antibodies would contribute to the modulation of the disease since trypanotolerant cattle have high titres of anti-congopain antibodies. α_2-Macroglobulin (α_2M) is a natural host inhibitor of proteinases. Inhibition occurs by entrapment of an active proteinase within the α_2M cage. In addition, it has been demonstrated that antigen complexed with α_2M becomes more immunogenic, resulting in an enhanced antigenic presentation of an entrapped antigen (Cianciolo et al., 2002). No reports have been published of congopain and α_2M interaction, only cruzipain-α_2M interaction has been reported (Ramos et al., 1997; 2002). The interaction between congopain and α_2M was investigated in order to assess the possibility of increasing the immunogenicity of congopain by immunising with CP2 complexed with α_2M.

α_2-Macroglobulin was isolated from bovine plasma and the inhibition of CP2 hydrolysis of azocasein and Bz-Pro-Phe-Arg-pNA by α_2M was determined. The results suggested that CP2 interacts with α_2M and inhibition tests using azocasein as substrate gave 26% inhibition of the activity of the entrapped enzyme. This means that α_2M may play an important role during *T. congolense* infection as a back-up inhibitor, reducing the effects of congopain. This may be especially relevant in the light of the observation by Lalmanach et al. (2002) that congopain interaction with bovine kininogens does not achieve complete enzyme inhibition and triggers release of kinins. Furthermore, Lonsdale-Eccles et al. (1995) reported that a kininogen-like molecule from rat serum enhances activity of the *T. b. brucei* cysteine proteinase, trypanopain-Tb, in fibrinogen zymograms of *T. b. brucei* lysates. The modulation of congopain activity by α_2M is evidently not sufficient by itself, however, since congopain has been shown to be one of the major pathogenic factors released by *T. congolense.* This is deduced from the observation that anti-congopain antibodies may contribute to the mechanism of trypanotolerance (Authié et al., 1993a).

The result obtained in this study, showing interaction of congopain with α_2M may be exploited to enhance the immunogenicity of congopain. By taking advantage of the adjuvanticity of the α_2M molecule, CP2 can be complexed with α_2M to raise antibodies against CP2, hence enhancing the immunogenicity of CP2 enzyme as was done for hen egg lysozyme (Chu and Pizzo, 1993), insulin (Chu et al., 1991) and a monomeric form of hepatitis B virus surface

antigen (Cianciolo et al., 2002). To do this, a study needs to be done to identify suitable molar ratio concentrations of CP2 and α_2M molecules, a suitable experimental animal system, the effect of the source of α_2M and the immune system challenged (for example, rabbit α_2M-CP2 complexes immunised into rabbits) and also to compare the antibody titres when using α_2M and other adjuvants. Freund's complete adjuvant, aluminium salts, muramyl dipeptide analogs and a variety of infectious agents have been used in various systems and have been shown to be effective adjuvants, although undesirable side effects have been reported (Muller, 1990b). These adjuvants, together with RWL (proprietary adjuvant from Smith Kline Beecham), which has been used in the successful production of antibodies against congopain (Authié et al., 2001), need to be tested and compared to antibody titres when using α_2M as adjuvant. Once optimal CP2 epitopes have been identified, these peptides could be complexed with α_2M by the chemistry described by Cianciolo et al. (2002) which involves incubation of α_2M with ammonia. The α_2M-NH_3 is heated in the presence of protein/peptide of interest resulting in substantial incorporation of protein/peptide when cooled to RT. For the incorporation of non-protease antigens, this approach avoids the chances of an antigen being cleaved by the proteinase (Cianciolo et al., 2002). Where congopain is the antigen, this could be evaluated as an alternative method for complexing congopain with α_2M. Human immunodeficiency virus-1 C4-V3 peptides complexed with α_2M have shown 100-1000 fold enhancement of both antibody and cytotoxic T lymphocyte response compared to peptides formulated in complete Freund's adjuvant (Cianciolo et al., 2002). The optimal peptides of CP2 could not only be valuable diagnostic tools, but may also be used for the development of an anti-disease vaccine.

In conclusion, anti-congopain peptide antibodies which interact with the whole enzyme were produced. These antibodies could be useful in basic research, such as immunoaffinity purification of congopain. Recombinant congopain is expensive and time consuming to generate. Purification of congopain from the parasites using these antibodies could provide a better alternative. The congopain peptides identified in this study also provide alternative tools that could be used in diagnostic assays rather than recombinant CP2. It was also shown in this study that congopain interacts with α_2M. This paves the way for the study of an alternative way of enhancing immunogenicity of congopain and congopain epitopes using α_2M as an alternative adjuvant. If α_2M shows superior adjuvanticity compared to other adjuvants, congopain peptides complexed with α_2M could be used to form a peptide based vaccine, once the optimal epitopes of congopain have been identified. This study has made contributions at

several fronts to the development of an anti-disease vaccine for trypanosomosis and sensitive diagnostic tools for the disease.

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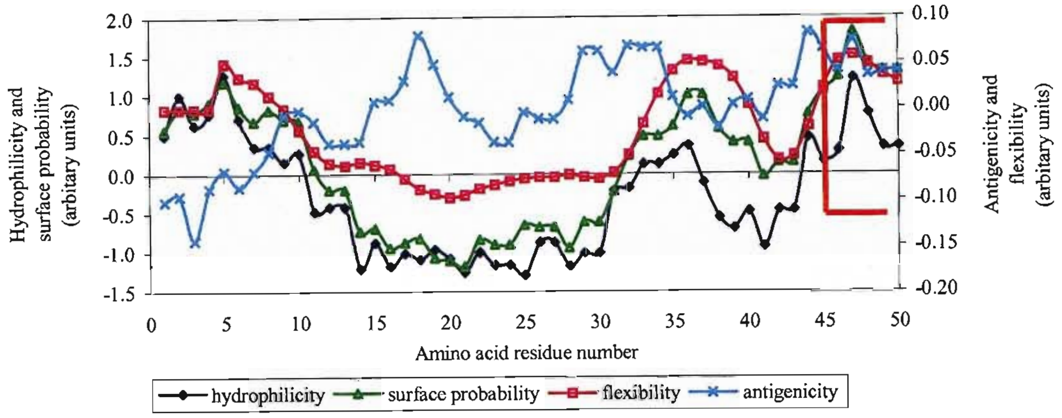
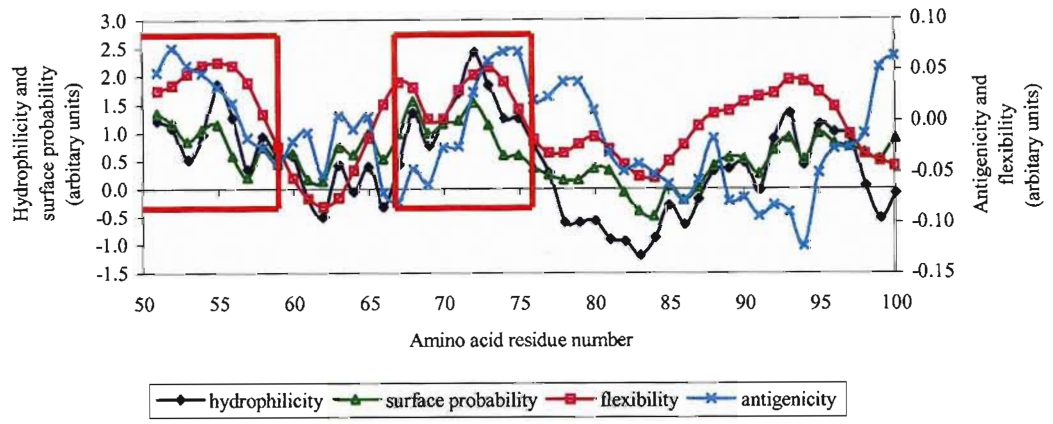
APPENDIX

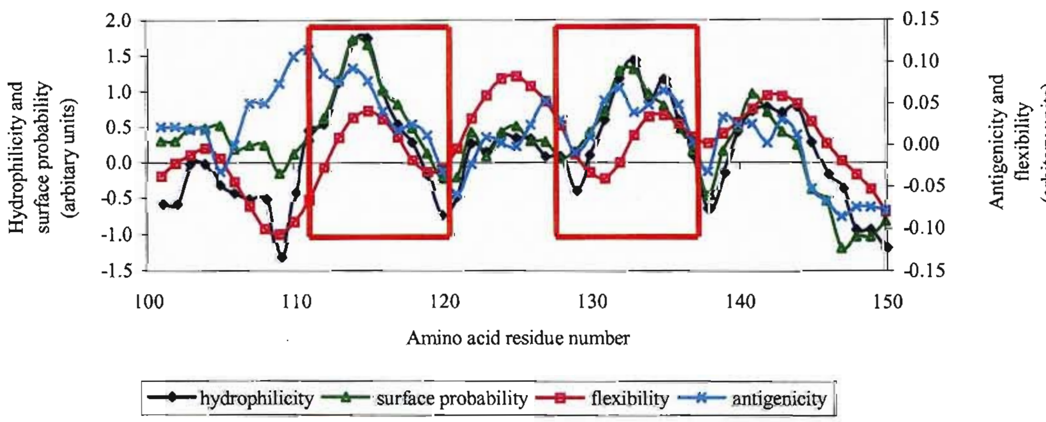
CONTENTS

Epitope prediction plots of trypanosomal cysteine proteinases amino acid sequences

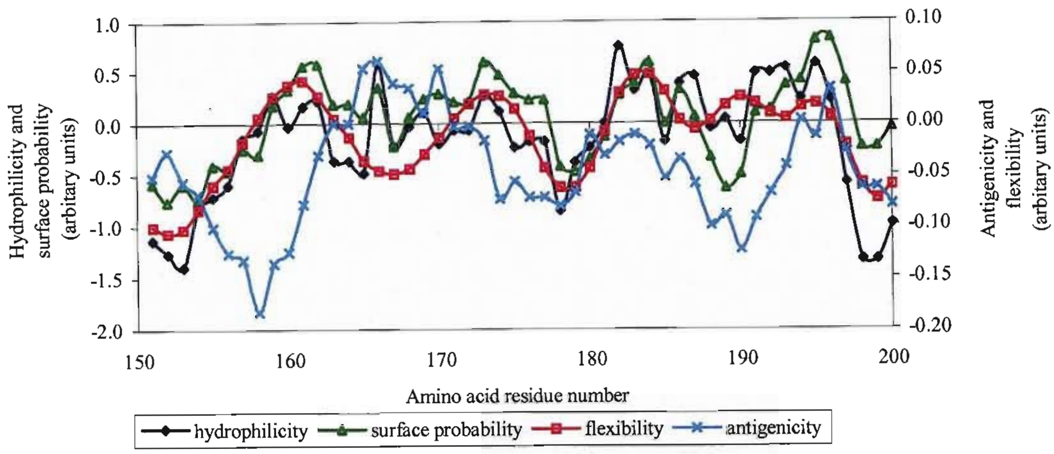
Epitope prediction plots of CP1 amino acid sequence[a]







[a]Boxes on the prediction plots indicate the amino acid sequences (peptides) that were selected for immunisation into chickens.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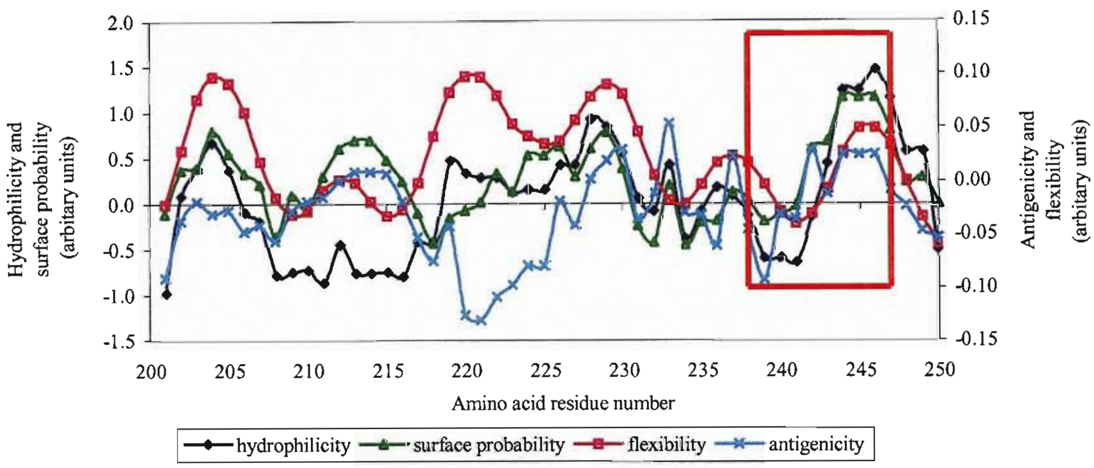
0.5
0.0
-0.5
-1.0
-1.5
-2.0
0.10
0.05
0.00
-0.05
-0.10
-0.15
-0.20
150
160
170
180
190
200
Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity


2.0
1.5
1.0
0.5
0.0
-0.5
-1.0
-1.5
0.15
0.10
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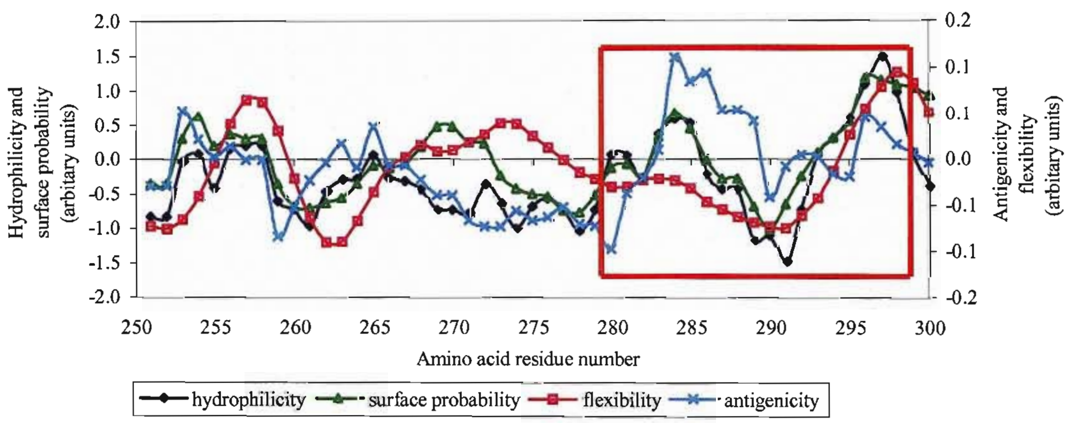
-0.05
-0.10
-0.15
200
205
210
215
220
225
230
235
240
245
250
Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity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-0.2
250
255
260
265
270
275
280
285
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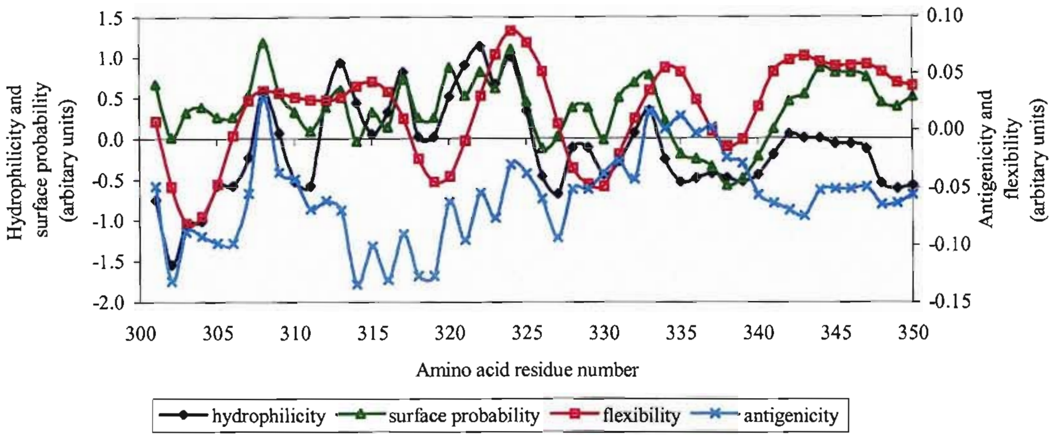
Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity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Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
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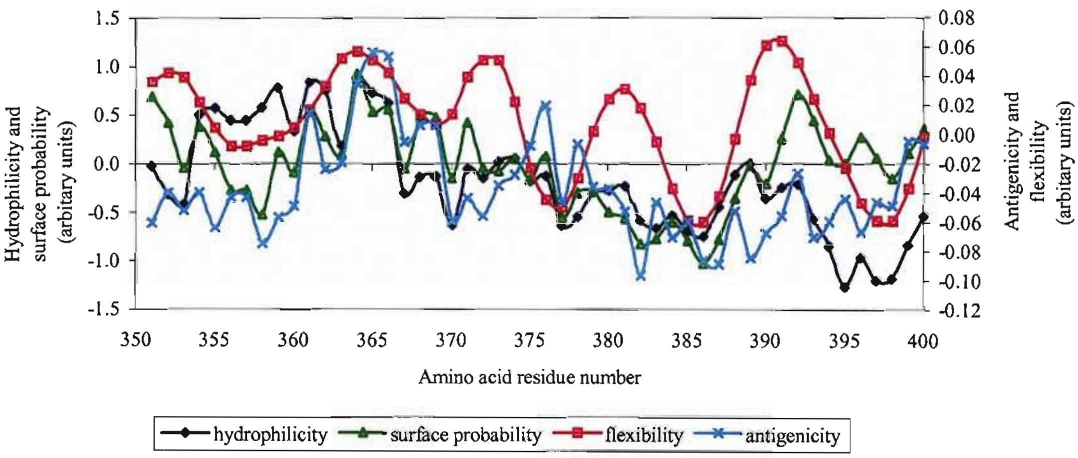
Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
surface probability
flexibility
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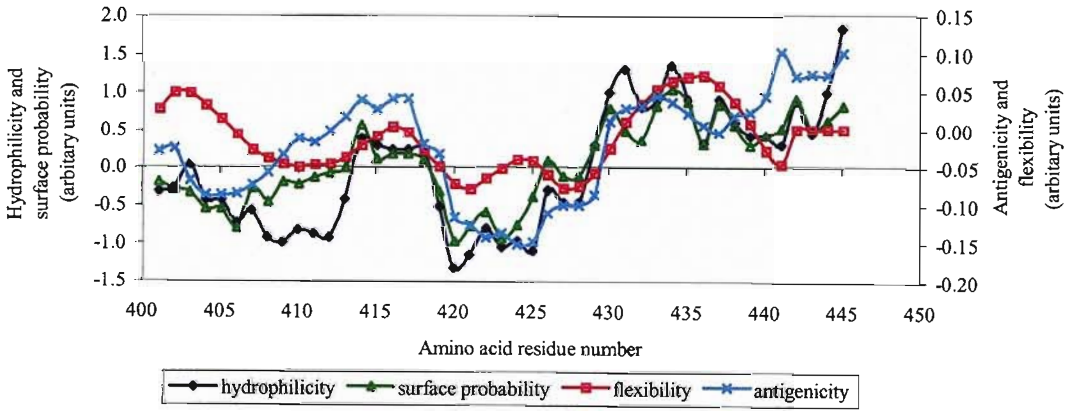
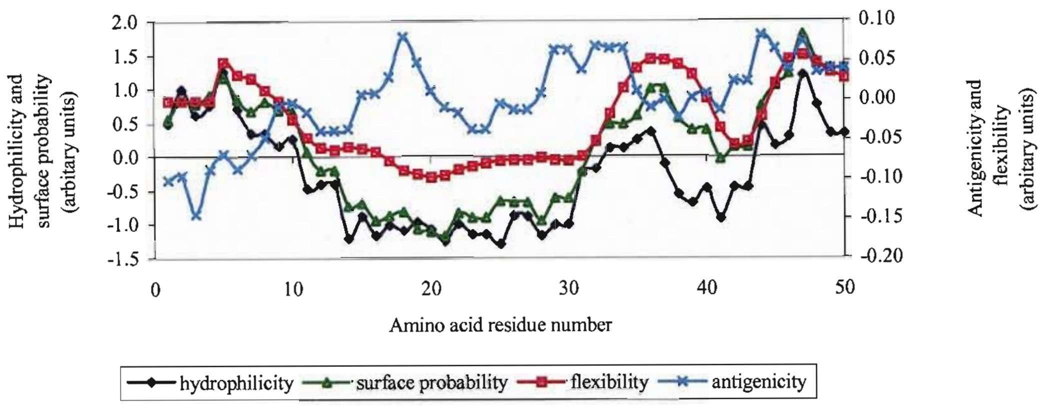
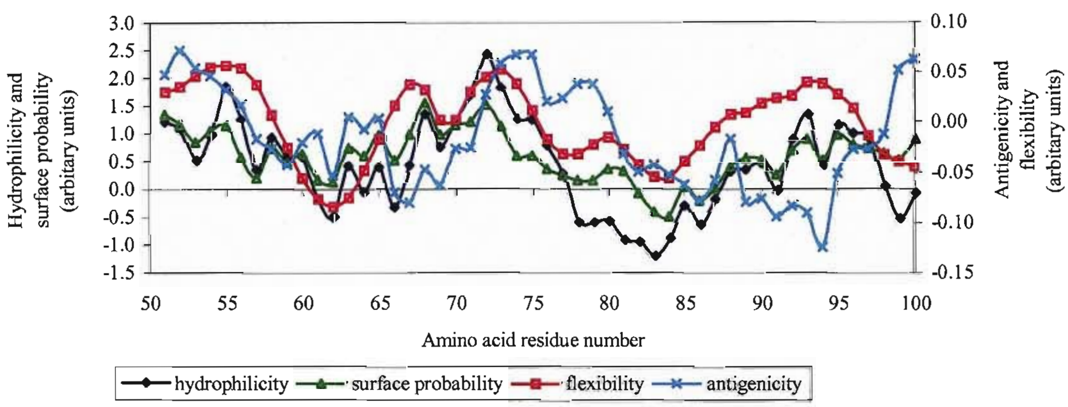
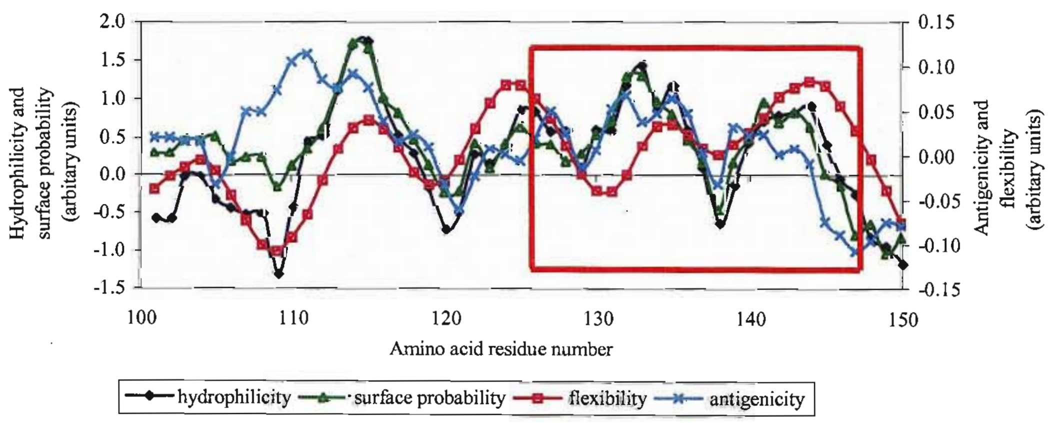
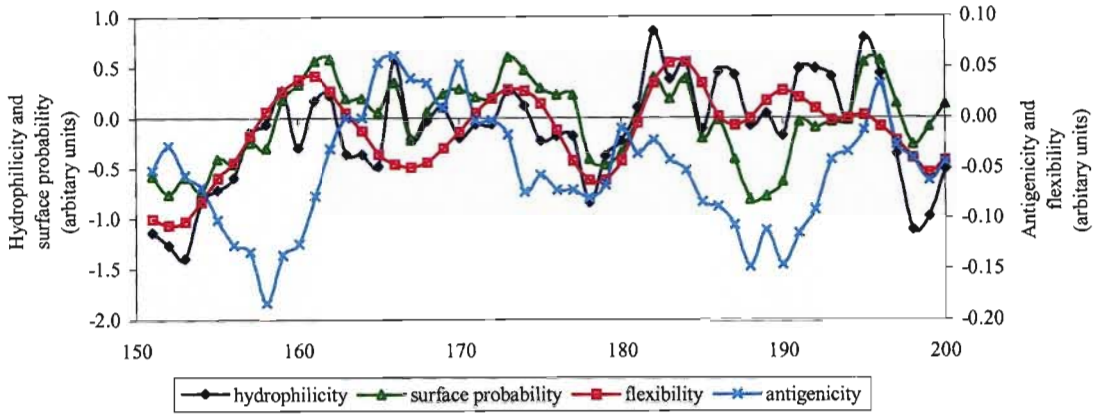

Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity

Epitope prediction plots of CP2 amino acid sequence








Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
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0.0
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0.10
0.05
0.00
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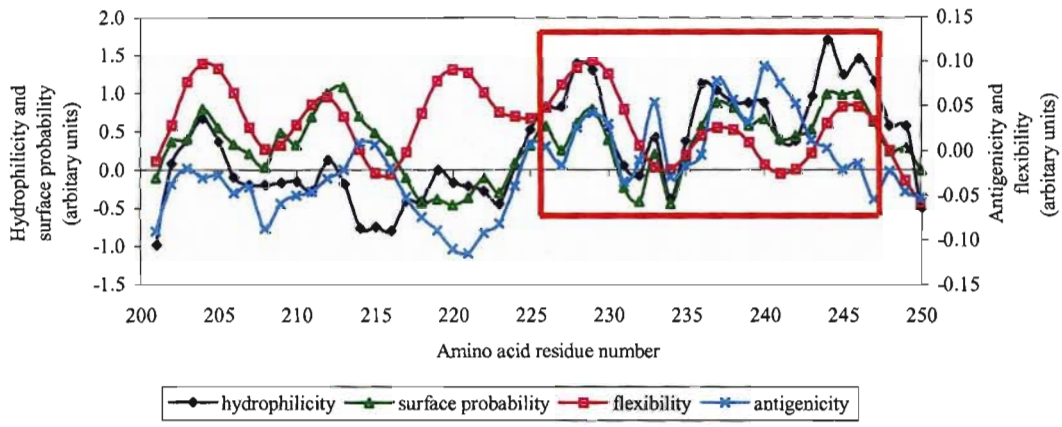
170
180
190
200
hydrophilicity
surface probability
flexibility
antigenicity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Antigenicity and flexibility (arbitary units)
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1.0
0.5
0.0
-0.5
-1.0
-1.5
0.15
0.10
0.05
0.00
-0.05
-0.10
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220
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230
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240
245
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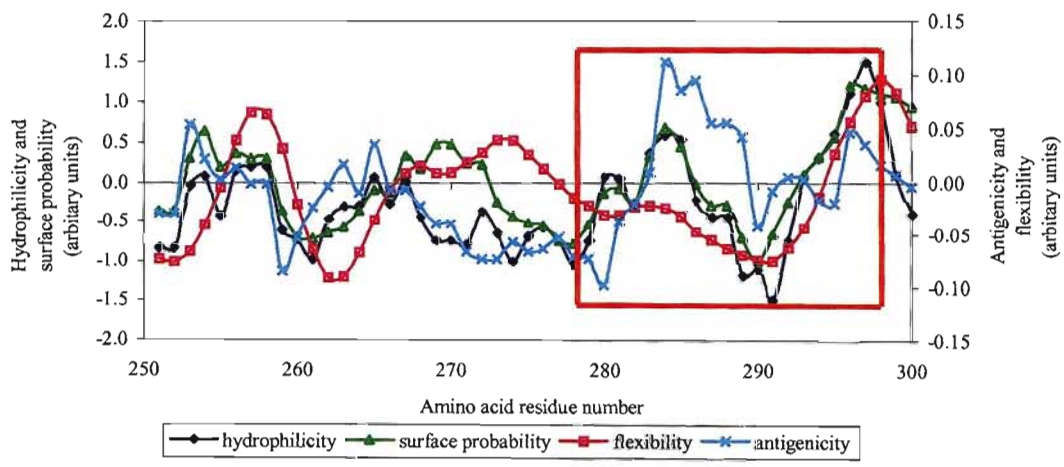
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity


Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
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1.0
0.5
0.0
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-1.5
-2.0
0.15
0.10
0.05
0.00
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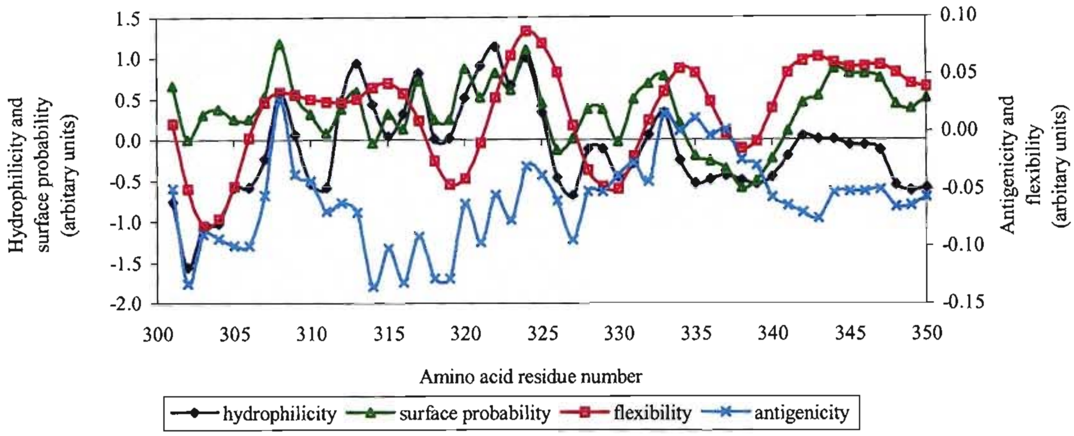
250
260
270
280
290
300
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity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-0.10
-0.15
300
305
310
315
320
325
330
335
340
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Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
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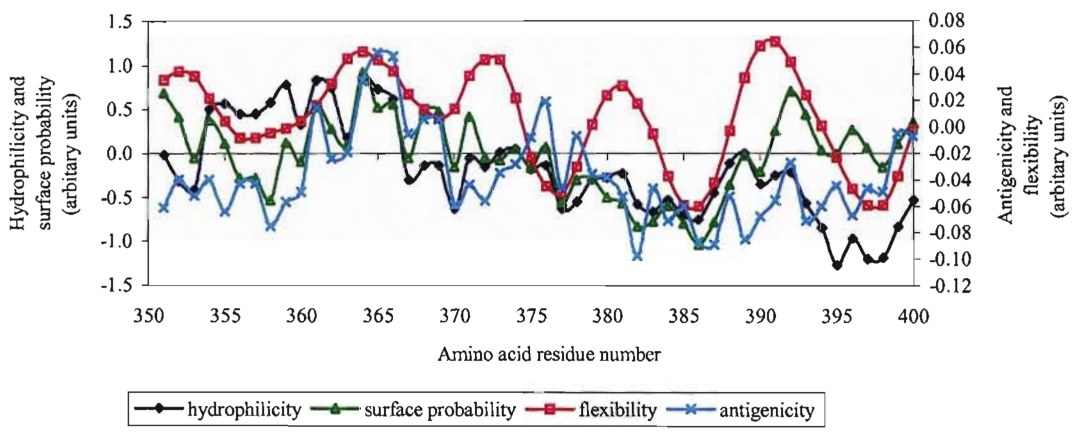
antigenicity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0.04
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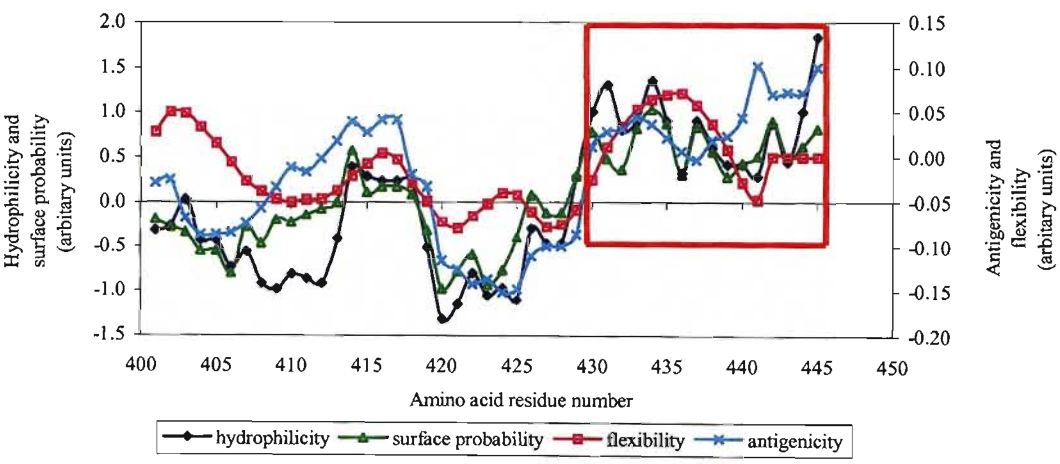
0.00
-0.02
-0.04
-0.06
-0.08
-0.10
-0.12
350
355
360
365
370
375
380
385
390
395
400
Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity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0.10
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0.00
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-0.10
-0.15
-0.20
400
405
410
415
420
425
430
435
440
445
450
Hydrophilicity and surface probability (arbitary units)
Antigenicity and flexibility (arbitary units)
Amino acid residue number
hydrophilicity
surface probability
flexibility
antigenicity

Epitope prediction plots of vivapain amino acid sequence