EVALUATION OF CONGOPAIN AND OLIGOPEPTIDASE B AS ANTI-DISEASE VACCINES FOR AFRICAN TRYPANOSOMIASIS.

LORELLE CLAIRE BIZAARÉ
BSc (Hons) Biochemistry

Submitted in fulfillment of the academic requirements for the degree of
Master of Science in the Discipline of Biochemistry,
School of Biochemistry, Genetics, Microbiology and Plant Pathology,
University of KwaZulu-Natal.

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PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, from January 2007 to November 2008,
under the supervision of Prof. Theresa H. T. Coetzer.

These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others it is duly acknowledged in the text.

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Lorelle Claire Bizaaré (candidate)

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Prof. Theresa H. T. Coetzer (supervisor)

ABSTRACT

The protozoan parasite *Trypanosoma congolense* is one of the aetiological agents of African animal trypanosomiasis that is transmitted by the tsetse fly. The parasite causes nagana in animals and affects livestock throughout sub-Saharan Africa. The toxicity of available drugs and the emergence of drug resistant parasites have affected the treatment of trypanosomiasis. Control of the disease has also been difficult due to ineffective vector control and the potential of trypanosomes to express hundreds of antigenetically distinct proteins on their surface. Vaccination against trypanosomiasis has been thought to be a possible control method. Since a vaccine based on variable surface proteins of the parasite is unlikely, research has been directed towards the identification of invariant pathogenic factors of the parasite as potential targets for therapy.

Congopain, the major cysteine protease of *T. congolense* has been implicated in the pathology of the disease. Antibodies against congopain are known to contribute to the mechanisms of natural resistance to trypanosomiasis known as trypanotolerance by neutralising the pathogenic effects of the enzyme.

Oligopeptidase B (OpdB), a trypanosomal serine protease has also been associated as a pathogenic factor of the disease. It is released into the host's circulation by dead or dying parasites and retains its catalytic activity since it is insensitive to host serum inhibitors.

In the present study, the catalytic domain of congopain (C2) and the use of alpha-2-macroglobulin (α_2M) as an adjuvant were investigated for their potential use in an anti-disease vaccine. α_2-Macroglobulin has been used to varying degrees to target different antigens to cells of the immune system and enhance their immunogenicity.

A previous study showed that antibodies raised in rabbits against C2 complexed to α_2M gave a higher percentage inhibition than antibodies made using C2 mixed with Freund's adjuvant. In the present study, goats were immunised with C2 complexed with α_2M to confirm the enhanced immunogenicity of C2 and the production of anti-C2 antibodies with superior inhibitory properties. Following immunisation, goats were challenged with *T. congolense* (strain IL 1180) and showed sustained antibody production during the two month infection period. Goat antibodies made using C2 in complex with α_2M inhibited the

hydrolysis of hide powder azure by C2 by 96%. Maximum inhibition of the hydrolysis of azocasein was observed to be 63% and hydrolysis of Z-Phe-Arg-AMC by C2 was inhibited by 73%.

In order to determine the vaccine potential of OpdB, protein was recombinantly expressed as a glutathione-S-transferase fusion protein in the pGEX expression system and purified by glutathione agarose affinity chromatography and molecular exclusion chromatography. Since a small yield of protein necessitated several rounds of expression and extensive purification, OpdB was subsequently expressed as a His-tagged fusion protein in the pET bacterial expression system. Recombinant protein was easily purified using nickel chelate affinity chromatography. Purified OpdB was used with alum for the immmunisation of mice to produce antibodies capable of inhibiting enzyme activity. Following immunisation, mice were challenged with *T. congolense* (strain IL 1180) and also showed sustained antibody production following two months infection. Since all mice died, the administration of OpdB conferred no protection; however, anti-OpdB mouse antibodies inhibited 86% of OpdB activity against the substrate Z-Arg-Arg-AMC. In addition immunised mice were observed to survive 40% longer than control mice as they had previously been immunised with OpdB and were able to mount a rapid immune response against this pathogenic factor during infection.

In general it could be concluded that immunisation of goats with C2 in complex with α_2M produced antibodies with superior inhibitory properties. The immunisation of mice with OpdB and alum also produced inhibitory antibodies and previous administration of OpdB enabled mice to mount a rapid immune response against OpdB during infection. Antibody mediated enzyme inhibition demonstrates the potential use of C2 and OpdB as vaccines that may contribute to the development of an effective anti-disease vaccine.

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Lastly, to my brain-thank you.

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ABBREVIATIONS

2XYT	2 x yeast tryptone medium
3D	three-dimentional
α_2M	α_2-macroglobulin
α_2MR	α_2-macroglobulin receptor
ABTS	2,2-azino-di-[3-ethylbenzthiazoline sulfonate]
AMC	7-amino-4-methylcoumarin
BCA	bicinchoninic acid
Bis-Tris	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol
BSA	bovine serum albumin
C2	catalytic domain of congopain
DMSO	dimethylsulfoxide
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(5-guanidino)butane
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
g	relative centrifugal force
GST	glutathione-S-transferase
HRPO	horse radish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-beta-D-thiogalactopyranoside
kDa	kiloDalton
LBTI	lima bean trypsin inhibitor
MEC	molecular exclusion chromatography
Min	minutes
OD_{600}	optical density at 600 nm
OpdB	oligopeptidase B
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
PSG	phosphate saline glucose

RT	room temperature
SAP	shrimp alkaline phosphate
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TCA	trichloroacetic acid
VAT	variable antigen type
VSG	variable surface glycoprotein
Z	benzyloxycarbonyl

CHAPTER ONE

LITERATURE REVIEW

1.1 AFRICAN TRYPANOSOMES

Trypanosomes are parasitic protozoa of the genus Trypanosoma that cause diseases in man and animals. From the mid-nineteenth century, these flagellated parasites were seen in fish, frogs, moles and mice and it was soon recognised that all vertebrates and invertebrates could be infected by these parasitic protozoa. The importance of these parasites as pathogens was first recognised by David Bruce in Africa (Haas, 2001). Bruce showed that these parasites found in the blood of sick animals were responsible for the disease referred to as nagana, meaning 'poorly'. African trypanosome parasites also cause sleeping sickness in man while South American trypanosomes cause Chagas disease. The African trypanosome parasites are transmitted by the tsetse fly (*Glossina* spp.) when a blood meal is taken from an infected animal and may sometimes be transmitted by biting insects. The distribution of African trypanosomiasis is analogous to that of the tsetse fly (Schmidt and Roberts, 1989). Tsetse flies are found exclusively in Africa in a belt that stretches south of the Sahara and north of the Kalahari Desert. In these vectors the parasites undergo important life cycle transformations involving genetic, morphological and proliferative changes (Matthews, 1999). Clinical manifestations of the disease are dependant on the species of infection. Trypanosomiasis is a wasting disease that affects land areas of up to 10 million km^2 with total annual losses amounting to $US 5 billion (Kristjanson *et al*., 1999).

1.1.1 Classification of African trypanosomes

The classification of the genus *Trypanosoma* is given in Fig. 1.1. Species of trypanosomes that are infective to mammals can be divided into two distinctive sections; the Stercoraria and Salivaria. This grouping is dependant on the region of the digestive tract of the vector in which the parasite develops into its infective form. Trypanosomes of the section Stercoraria are produced in the hindgut and are transmitted from the posterior, i.e. transmission via the faeces of infected insect vectors.

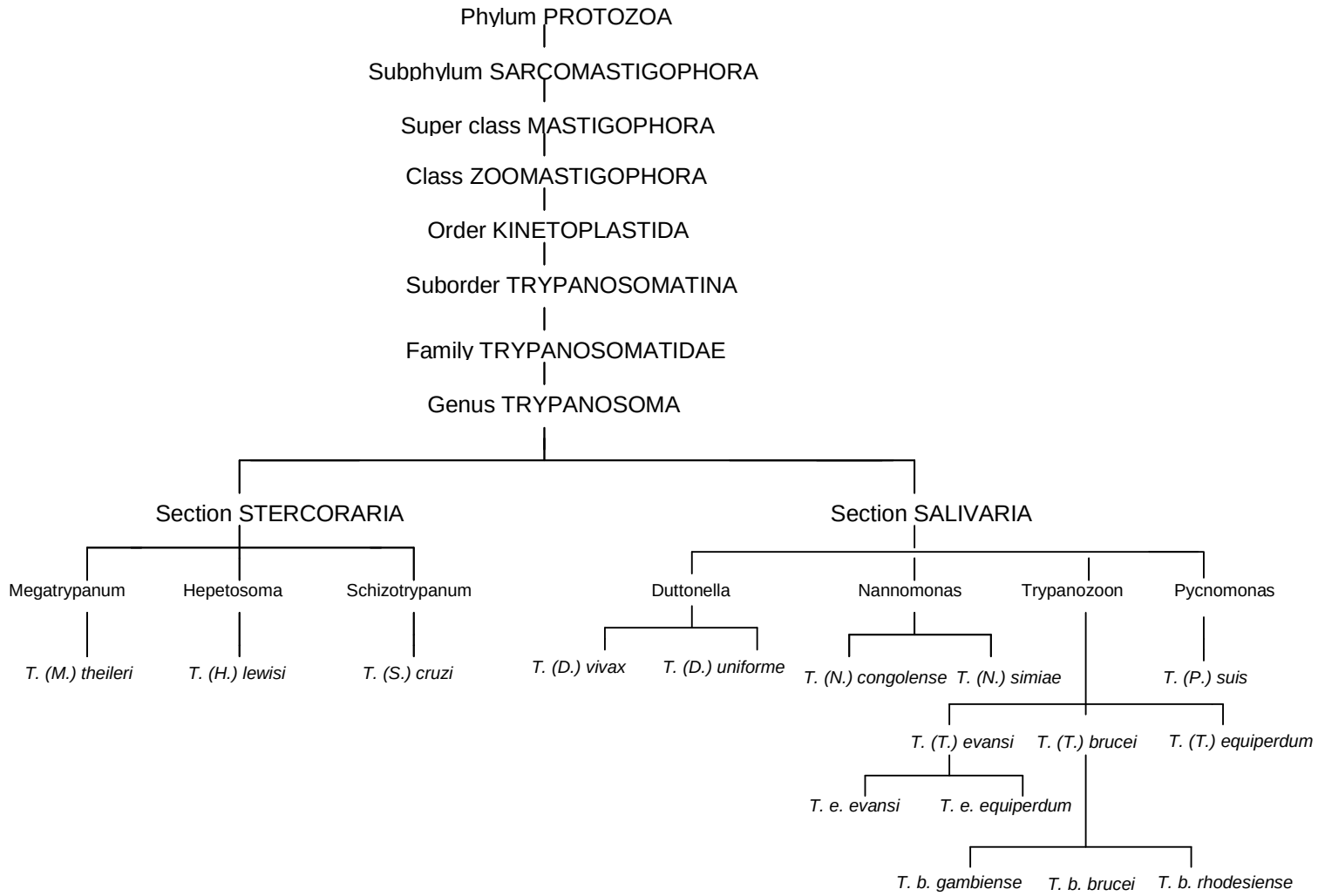


Figure 1.1 Classification of the genus *Trypanosoma* (Vickerman,1976).

Salivarian parasites are transmitted from the anterior, i.e. transmitted from the mouth parts of the infected insect vector (Bush *et al.,* 2001). The aetiological agent of Chagas disease is the Stercorarian parasite *T. cruzi* and that of African animal trypanosomiasis, the Salivarian parasites *T. congolense, T. vivax* and *T. brucei brucei*.

1.1.2 Morphology of trypanosomes

The different groups of trypanosome parasites can largely be defined by their morphological features. They are elongated cells with a single flagellum arising from a flagellar pocket at its posterior end (Vickerman, 1969) (Fig. 1.2). It extends along the body of the parasite to the anterior end. Trypanosomses have a single tubular mitochondrion that contains the mitochondrial DNA and is associated with the kinetoplast (Schmidt and Roberts, 1989). The parasite surface coat consists of variable surface glycoproteins (VSGs) which make up the major surface antigen of these parasites. Through antigenic variation this protective coat can be changed and other antigen types may be expressed (Borst, 2001). Each variant is known as a distinct variable antigen type (VAT) and by simply changing this outer coat some parasites evade the immune system and repopulate the host. In this way, the level of trypanosome parasites in the blood fluctuates with time. These fluctuations are commonly referred to as waves of parasitaemia (Barry and Carrington, 2004).

1.1.3 Life cycle of African trypanosomes

In an infected tsetse fly, trypanosome parasites are found in the salivary glands. These are non-dividing metacyclic forms and make up the infecting population which is introduced into the mammalian host during a blood meal. Once introduced into the host's bloodstream, the infecting population undergoes cell division by binary fission and morphological change (Vickerman, 1976). The bloodstream population is comprised of slender forms during the ascending phase of parasitaemia and stumpy forms at the peak of parasitaemia (Matthews *et al.,* 2004). Some of the short stumpy non-dividing forms go on to reinfect tsetse flies when a blood meal is taken. They enter the midgut of the fly and develop into procyclic forms. After further transformation to the epimastigote form they pass into the salivary glands to become metacyclic. The vast majority that is not taken up by the tsetse fly during a blood meal is killed by antibody responses of the host or

degenerates over time. Figure 1.3 shows the developmental changes of *T. brucei* in the tsetse fly and mammalian host during its life cycle.

Trypanosome parasites also undergo adaptive changes in their energy metabolism to ensure survival of the parasite in the vector and host. While in the midgut of the tsetse fly, the parasite relies on energy generated by the tricarboxylic acid (TCA) cycle, the electron transport chain and oxidative phosphorylation (Hill, 1976). Within the bloodstream of the mammalian host the parasites are reliant on the process of glycolysis for their principle source of energy (Bowman and Flynn, 1976).

1.2 AFRICAN TRYPANOSOMIASIS

Human African trypanosomiasis or sleeping sickness may be distinguished by two different causative *Trypanosoma* species that each induces its own unique clinical features; *Trypanosoma brucei rhodesiense* in East and Southern Africa and *T. b.* gambiense in West and Central Africa. The causative organisms of Rhodesian and Gambian sleeping sickness are morphologically indistinguishable. Rhodesian sleeping sickness is transmitted by the tsetse fly, *G. morsitans*, develops more rapidly and is far more virulent than Gambian sleeping sickness. Death may occur within several weeks of the onset of infection (Welburn *et al*., 2001). Gambian sleeping sickness is transmitted by *Glossina palpalis*. The disease is preceded by a long asymptotic stage followed later by a feverish illness and late stage chronic meningioencaphalitis. There may even be a possibility of death many years following the initial onset of the infection.

Animal African trypanosomiasis, also referred to as ‘nagana’, is transmitted by many *Glossina* species. *T. congolense, T. vivax* and to a lesser degree, *T. b brucei* are the major pathogenic species of animal trypanosomiasis (Stevens and Brisse, 2004). Nagana is typically divided into the initial acute phase and the late chronic phase that is characterized largely by anaemia, weight loss and poor productivity (Taylor and Authié, 2004).

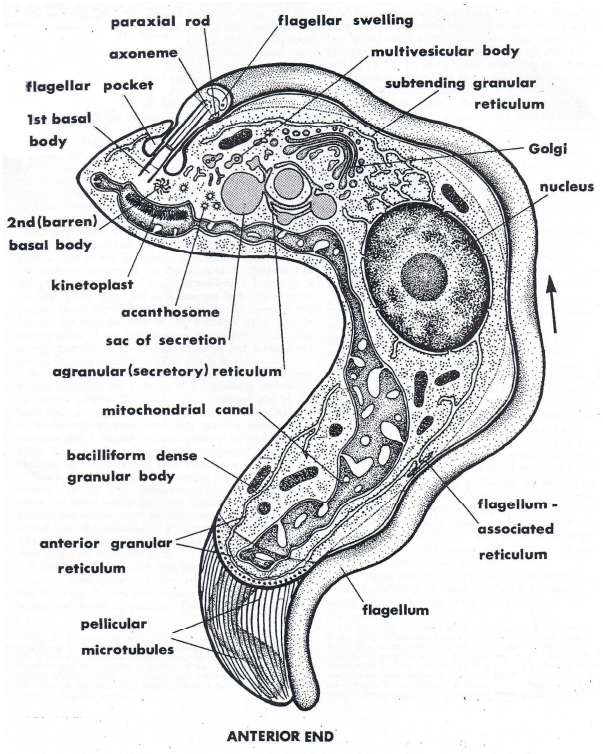


Figure 1.2 General morphology of *Trypanosoma congolense* (Vickerman, 1969)

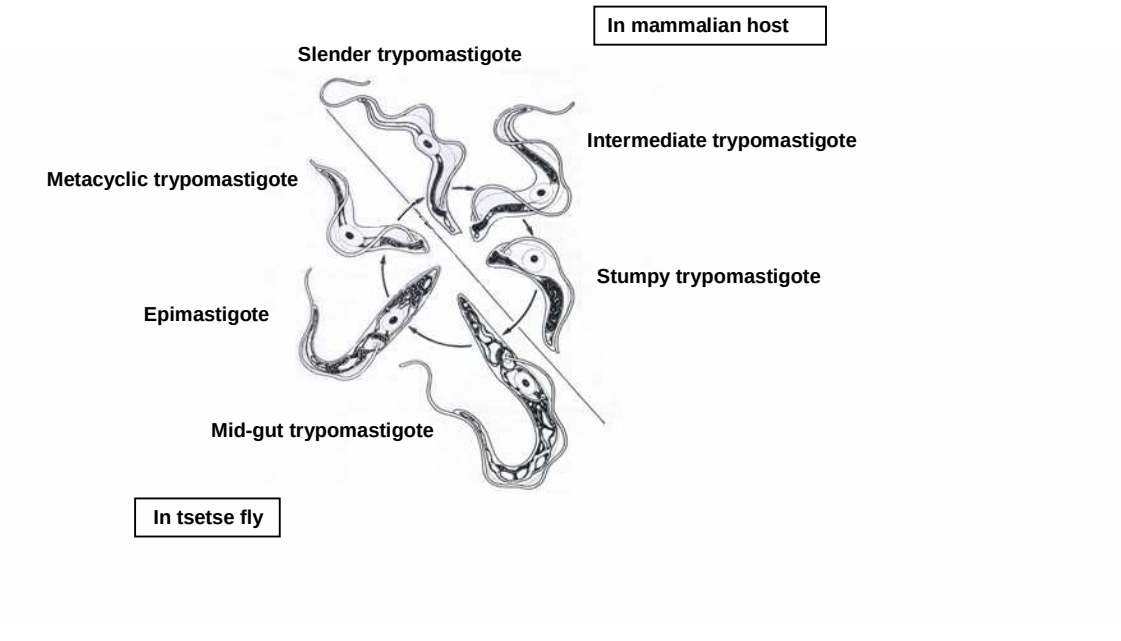


Figure 1.3 Life cycle of an African *T. brucei* trypanosome (Vickerman, 1976)

1.2.1 Pathogenesis of African trypanosomiasis

Infection with trypanosomes is initially characterized by the presence of parasites in the blood and lymphatic system. Trypanosomes are mechanically transmitted by the tsetse fly through the skin barrier of the host during a blood meal. Parasites undergo extensive multiplication in the skin and ultimately enter the lymphatic and blood system. This phase is also referred to as the haemolymphatic phase. Once in the blood the parasites gain access and are able to affect other organs of the host such as the spleen, liver and heart (Taylor and Authié, 2004).

Intermittent fever due to fluctuations in parasitaemia is common. The intensity and duration of these waves of parasitaemia affects the development and severity of anaemia. Anaemia is the most prominent characteristic of animal trypanosomiasis and the severity is also influenced by the virulence of the infecting population. With prolonged infection the role of the parasite in the maintenance of anaemia becomes less significant. Animals may be intermittently parasitaemic or aparasitaemic (Vickerman *et al*., 1993).

The presence of high levels of trypanosome antigens in the blood as a result of antigenic variation promotes high levels of circulating antibodies. Subsequent antibody and complement-mediated lysis of trypanosomes increases the concentration of circulating immune complexes and invariant pharmacologically active immunogens of the parasite. Invariant immunogens have been established as having a major role in pathogenesis (Tizard *et al*., 1978 ; Taylor and Authié, 2004).

1.2.2 Trypanotolerance

A degree of natural resistance to trypanosomiasis termed trypanotolerance has long been recognized in certain breeds of cattle. Trypanotolerance is the ability of certain animals to survive and remain productive in areas of tsetse challenge (Murray *et al*., 2004). Although most of the cattle found across Africa are of the Zebu type and are generally susceptible to trypanosome infection, other breeds such as *Bos taurus* N’ Dama, Muturu and Dohamey breeds, found mainly in west Africa are not susceptible (Taylor, 1998). Exposure of these cattle to tsetse challenge has over time, by natural selection, illustrated the accommodation between parasite and host and has emerged as a genetic trait (Authié, 1994). Trypanotolerant and trypanosusceptible breeds have distinct antibody responses (Taylor,

1998). The exact means by which trypanotolerance occurs is not fully understood but it is likely to involve both non-immunological as well as immunological mechanisms. This includes the host's capacity to control the initial parasetamia wave and limit the development of anaemia (Vickerman *et al.*, 1993; Naessens *et al.*, 2003).

1.3 DIAGNOSIS OF AFRICAN TRYPANOSOMIASIS

An accurate determination of the incidence and prevalence of African trypanosomiasis in humans and animals is difficult to establish as the available diagnostic tests are limited in their application. A direct method is the traditional microscopic means of diagnosis which relies on the presence of parasites in the blood. This is unreliable due to fluctuating levels of parasitaemia and the scarcity of parasites in the blood during the chronic stage of infection (Vickerman *et al*., 1993).

Indirect methods include tests for circulating antigens of the parasite but fall short as immune complex formation and removal during infection may eliminate much of the antigen present (Vickerman *et al.*, 1993). Indirect methods include the widely used indirect immunofluorescence antibody (IFA) test and direct and indirect enzyme-linked immunosorbent assays (ELISAs). In the IFA test the infected blood of the host serves as a source of antigen and the interaction with anti-trypanosomal antibodies is measured. Bound antibodies are visualised using an ultraviolet microscope by means of a fluorescent dye attached to the anti-host species immunoglobulin (Eisler *et al*., 2004). The ELISA test is widely used and involves immobilising antigens on to a microtitre plate and detection using a specific antibody conjugated to an enzyme that catalyses the conversion of a colourless substrate to a coloured product. The indirect ELISA is analogous to the IFA test. It also uses antigen immobilised to a microtitire plate and specific antibody interaction is detected by means of a labelled anti-species conjugate which catalyses the conversion of substrate to a coloured product (Eisler, *et al*., 2004). Molecular methods such as PCR are also available for detecting trypanosomes. This method is based on specific genetic characteristics of the parasite and allows species-specific detection (Desquesnes *et al*., 2001).

1.4 CONTROL OF AFRICAN TRYPANOSOMIASIS

Control of African animal trypanosomiasis is largely hindered by the ability of the parasites to change their outer surface coats. This phenomenon of antigenic variation enables them to establish chronic infections in their hosts. Efforts to control trypanosomiasis include elimination of the insect vector, the use of trypanotolerant cattle instead of trypanosusceptible cattle and treatment using trypanocidal drugs. These may be applied mutually or separately with varying success (McDermott and Coleman, 2001).

Tsetse control and eradication strategies traditionally included aerial and ground spraying of insecticides. Insecticides such as DTT and benzene hexachloride are highly effective for this purpose but this has become unsound practice due to the considerable harm which it causes to the environment (Schmidt and Roberts, 1989).

Drugs available for the treatment of animal trypanosomiasis include isometimidium, homidium and diminazene. Isometimidium can be used as a prophylactic drug and provides up to six months protection against tsetse challenge while homidium and diminazene are mainly used as therapeutic agents. The use of these drugs is mired by their toxicity but despite this they are still the most frequently used and well established veterinary drugs for the treatment of trypanosomiasis across Africa (Holmes *et al.*, 2004). The use of trypanocidal drugs has also been compromised by reports of drug resistant parasites in at least 13 countries in sub-Saharan Africa. (Geerts *et al.*, 2001). Less frequent and more strategic administration is necessary to prolong the efficacy of these drugs (McDermott and Coleman, 2001).

Trypanotolerant cattle may also be exploited in areas of high trypanosome challenge. Cattle of trypanotolerant breeds are generally smaller in size and although they do become infected they are able to control parasitaemia and resist anaemia and therefore remain just as productive as trypanosusceptible breeds (Vickerman *et al*., 1993).

With the combination of drug resistance and toxicity and the inability to completely eradicate the vector, vaccination against trypanosomiasis has been thought to be a potential control method despite the difficulty presented by antigenic variation.

1.4.1 Vaccine against trypanosomiasis

Cattle immunised with irradiated trypanosomes or purified VSG were protected against challenge with homologous, i.e expressing the same VSG, but not heterologous trypanosomes (Wellde *et al*., 1974; Morrison and Ulevitch, 1982). The potential of trypanosomes to express hundreds of antigenically distinct VSG genes (Borst, 2001) has meant that a VSG based vaccine is unlikely. As a result, research is now focussed on the identification of invariant trypanosome components as potential vaccine targets for therapy. Components of the trypanosome cytoskeleton, such as tubulin and microtubules, have been studied and shown to have potential as vaccines and confer protection from challenge with *T. b. brucei, T. congolense, T. evansi* and *T. rhodesiense* (Lubega *et al*., 2002; Rasooly and Balaban, 2004; Li *et al*., 2007). In addition, the proteolytic activity of pathogenic factors released by dead or dying parasites could be neutralised by antibodies produced through vaccination (Playfair *et al*., 1990; Authié, 1994; Authié *et al*., 2001). Central roles have been proposed for proteases of protozoan parasites such as host cell invasion and stimulation and evasion of the host immune system (Klemba and Goldberg, 2002). Cysteine proteases are suggested to be the most abundant and are best characterised among these proteases (North *et al*., 1990). They have been known to play a role in the development and differentiation of parasites such as *T. cruzi* (Martinez *et al*., 1991), *Plasmodium falciparum* (Rosenthal, 2004) and *Leishmania mexicana* (Mahmoudzadeh-Niknam and McKerrow, 2004).

1.4.1.1 Adjuvants

Other factors influencing the outcome of vaccines include the adjuvant carrier or delivery system. When adjuvants are administered with the antigen, a greater immune response is elicited than when using antigen alone (Singh and O'Hagan, 2003). Freund's complete and incomplete adjuvant is a common and standard adjuvant to which many other adjuvants are compared (Hunter, 2002). It constitutes a mineral oil that forms a water-in-mineral oil emulsion when triturated with antigen. It induces high antibody titres, a delayed release of antigen and slow antigen clearance (Johansson *et al*., 2004). However, the presence of reactogenic mycobacterial products in Freund's complete adjuvant, which costimulates cells of the immune system, has been known to cause chronic inflammatory responses and abscesses (Roitt, 2001). Aluminium hydroxide and aluminium phosphate salts, commonly

known as alum, have also been used in vaccinations and like Freund's adjuvant, alum provides an antigenic depot in the tissues (Wilson-Welder *et al.*, 2008). A study has shown that alum may induce dendritic cell maturation resulting in enhanced activation of CD4 T cells of the immune system but has also has been associated with tissue reactions like erythema, subcutaneous nodules and granulomas (Rimaniol *et al.*, 2004). Alpha-2-macroglobulin (α_2M), a high molecular weight glycoprotein has proven successful as an adjuvant option in some studies by entrapping the antigen and dramatically increasing its immunogenicity (Cianciolo *et al*., 2002). In the present study, parasite proteases have been combined with Freund's adjuvant, alum and/or α_2M as potentially useful components of an anti-disease vaccine.

1.5 PROTEOLYTIC ENZYMES OF TRYPANOSOME PARASITES

Proteolytic enzymes of parasites have a significant role in host-parasite interactions (North *et al*., 1990). Proteases are able to catalyse the hydrolysis of peptide bonds and have been classed according to their key active site residues (Barrett, 1994). Table 1.1 outlines the classification of the proteolytic enzymes. Proteases range from being highly specific to others that are active toward a range of different protein substrates. The specificity is determined by the substrate binding pockets of the enzyme as illustrated in Fig. 1.4. In a model set forward by Schechter and Berger (1967), the binding site on the proteinase for each of the amino acid residues of the substrate is known as the S pocket while the binding site on the protein or peptide substrate is referred to as the corresponding P pocket. Proteases may be further classified as either exopeptidases or endopeptidases depending on the site of cleavage in the substrate. Exopeptidases catalyse the cleavage of peptide bonds near the N- or C-terminus while endopeptidases cleave bonds within the protein (Barrett, 2002). Protease activity is strictly regulated in three ways. Some proteases are confined to membrane bound organelles such as lysozymes where they are active in a specialised environment. Others are synthesised as zymogens (inactive forms) that become active upon proteolysis or they may be regulated by other proteins that inhibit their activity (Khan and James, 1998; Barrett, 2002). Only the cysteine and serine proteases will be discussed further as they are of relevance to the current study.

Table 1.1 The classification of proteolytic enzymes (Barrett 1994, 2002).

Class/type:	Key active site residues :	Catalytic residues:	Example:
Cysteine	Thiol group of cysteine	Cys^{25}, His^{159}, Asp^{158}	Papain, congopain
Serine	Hydroxyl group of serine	Asp^{102}, Ser^{195}, His^{57}	Chymotrypsin, oligopeptidase B
Aspartic	Carboxyl groups of aspartic residues	Asp^{33}, Asp^{213}	Pepsin, HIV protease
Metallo	Metal atoms (zinc)	Glu^{270}, Trp^{248}	Thermolysin, Matrix metalloproteases (MMPs)

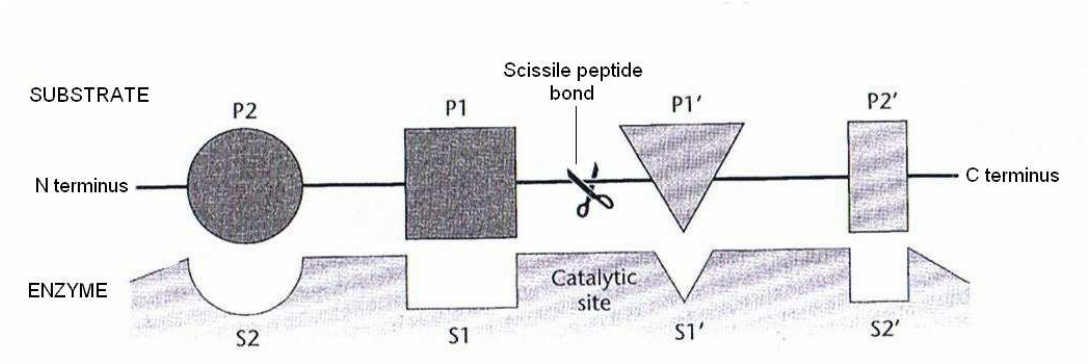


Figure 1.4 Subsite specificity of proteolytic enzymes (Barrett, 2002). The pockets of the enzyme which bind the substrate residues on the N terminal side are designated S_1, S_2, S_3…, while the pockets binding the substrate residues on the C terminal side are designated S_1', S_2', S_3'… The pockets of the substrate residues on the N terminal side are designated P_1, P_2, P_3…, while the pockets of the substrate residues on the C terminal side are designated P_1', P_2', P_3'…

1.5.1 Cysteine proteases

Cysteine proteases have been found to exist in many groups of parasitic protozoa and typically have high activity in at least one stage of the parasite's life cycle (North *et al.*, 1990). In most cases these cysteine proteases are lysosomal. Lysosomes provide the ideal acidic environment for these enzymes. Cysteine proteases are inhibited by the cystatin

family of proteinase inhibitors (Barrett, 2002). Trypanosomal cysteine proteases share a likeness with the C1 family of cysteine proteases (Rawlings and Barrett, 1994b). Papain is a common cysteine protease from this group. Its active site is composed of a catalytic triad; Cys^{25}, His^{159} and Asn^{175} and this arrangement is conserved in all members of the same family. Papain and most other family members show a preference for a bulky hydrophobic residue in the S_2 subsite and are inhibited by L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64). Whether secreted or lysosomal enzymes, members of the papain family are synthesised with a signal peptide and pro-peptide at the N-terminus. In addition, some members of the family also have long C-terminal extensions (Rawlings and Barrett, 1994b). Congopain, a major cysteine protease from *T. congolense* has been recognised as one of the major pathogenic factors of trypanosomiasis (Authié *et al*., 2001) and as a result is potentially useful as a vaccine candidate.

1.5.1.1 Congopain

Cysteine proteases are amongst the most abundant and fully characterised proteinases of Trypanosomatids. Congopain from *T. congolense* has been fully characterised (Mbawa *et al*., 1992) and was initially observed as a 33 kDa protease localised in lysosomes. It was also observed that the protease was susceptible to inhibition by E-64 and had the ability to hydrolyse the synthetic substrate Z-Phe-Arg-AMC (Mbawa *et al*., 1991a).

Congopain consists of four distinctive domains (Fig. 1.5). These include a hydrophobic signal (pre) sequence, a hydrophilic pro-region, a central domain encompassing all highly conserved residues including the active site triad, and a C-terminal extension (Authié, 1994). The proposed 3D structure has been modelled on the crystal structure of cruzain and is shown in Fig. 1.6 (Lecaille *et al*., 2001). Congopain and cruzain share 68% sequence identity in their active sites and have similar enzymatic specificities (Chagas *et al*., 1997). Congopain consists of 2 major domains; an L domain consisting mainly of α helices and an R domain consisting mostly of anti-parallel β sheets. The residues defining the catalytic triad and substrate binding site are located in a cleft between the two domains (Lecaille *et al*., 2001).

Congopain is synthesized as a zymogen together with its pre- and pro-forms. The pro-region plays a role in protein folding and regulation of the protein activity by blocking

the active site (Lalmanach *et al*., 1998). Removal of the pre and pro regions by proteolytic cleavage at low pH gives rise to the mature form of the enzyme (Serveau *et al*., 2003). The mature form of the enzyme consists of the conserved catalytic domain and a highly immunogenic C-terminal extension (Authié *et al*., 2001).

Congopain shows a preference for dipeptide substrates with arginine in the P_1 position and bulky hydrophobic residues in the P_2 position. Congopain is activated by thiol compounds and is inhibited by specific inhibitors of cysteine proteases such as cystatins, E-64 and leupeptin (Chagas *et al*., 1997).

Congopain has been established as a circulating antigen in the serum from cattle infected with *T. congolense* (Authié, 1994). Immunisation with congopain has been shown to elicit a prominent immune response in trypanosusceptible cattle. Although immunisation did not prevent the onset of acute anaemia, cattle showed a tendency to recover their leukocyte counts 2-3 months following infection (Authié *et al.*, 2001). This suggests that congopain contributes to the mechanisms of anaemia. Despite immunisation, the development of infection in these cattle was observed to be no different from cattle that were not immunised. This indicates that immunisation with congopain alone is not sufficient. Immunisation together with other pathogenic factors may be considered or alternatively a more efficient response to congopain may be required. In the present study, α_2M was used in complex with congopain to enhance antibody production against the protease. The properties of α_2M and its use as an adjuvant will be discussed in further detail in Section 1.6.

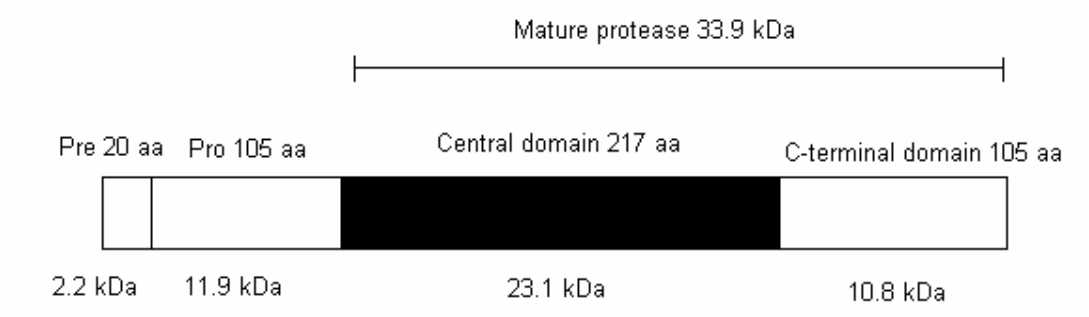


Figure 1.5 Diagrammatic presentation of the four domains of the cysteine protease congopain.

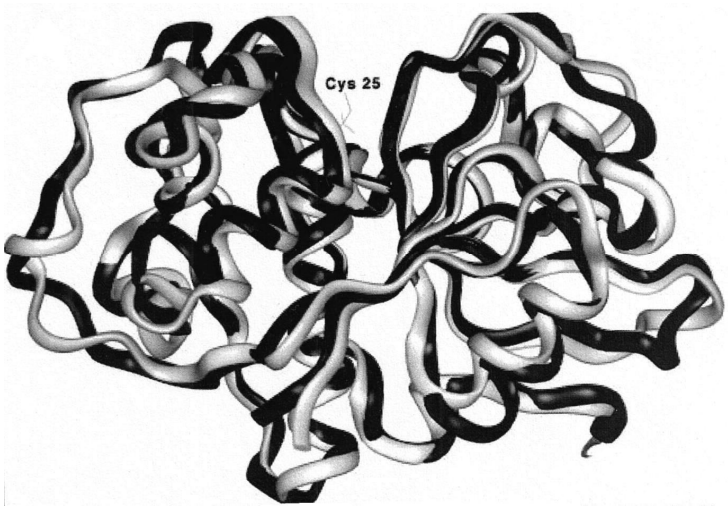


Figure 1.6 The proposed 3D structure of congopain (Lecaille *et al*., 2001). The X-ray crystallographic structure of cruzain is represented in black and the proposed structure of congopain is shown in grey. The cysteine residue of the catalytic triad (with papain numbering) is also shown.

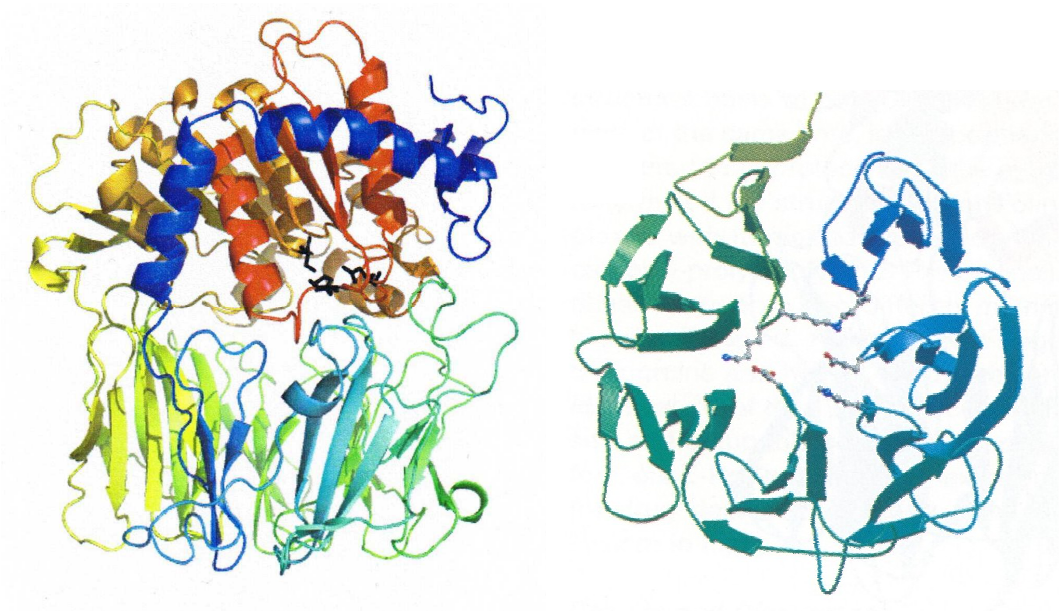
1.5.2 Serine proteases

Serine proteases are the most diverse group of proteolytic enzymes. Using the criteria to distinguish between families of peptidases, over 20 families are recognised. On the basis of their 3D structures they can be classed into five or six clans. General classification is based on the amino acid sequences surrounding the catalytic serine residue (Rawlings and Barrett, 1994a). The catalytic triad of these proteases is made complete by histidine and aspartate residues. Serine proteases are most active at neutral or alkaline pH. Of particular significance to the current study is the protease of the family prolyl oligopeptidase, oligopeptidase B (OpdB, S9 family of clan SC). The S9 family contains soluble and membrane bound peptidases. OpdB is a cytosolic enzyme and cleaves peptide bonds C-terminal to dibasic amino acid residues with a preference for Arg in both P_1 and P_2 (Coetzer *et al.*, 2008). All members of this family share significant amino acid sequence conservation within their catalytic regions and do not need co-factors for activity. The chemistry of the hydroxyl group of the active site serine is such that it requires neutral or slightly alkaline pH (Barrett, 2002). Members of the prolyl oligopeptidase family differ from the classical serine proteases in that they all display limited activity towards peptides and not entire proteins (Morty *et al.*, 1999a).

1.5.2.1 Oligopeptidase B

Oligopeptidase B was first isolated from *Escherichia coli* cells and was shown to have trypsin like specificity (Pacaud and Richaud, 1975; Tsuru and Yoshimoto, 1994). Trypanosomal OpdB was first isolated in eukaryotes in *T. cruzi* (Cazzulo, 2002) and is expressed at all life stages. It has since also been isolated from the African trypanosomes, *T. b. brucei* and *T. congolense* (Troeberg *et al*., 1996 ; Morty *et al*., 1999b) and from another kinetoplastid of the same family, *Leishmania amazonensis* (de Matos Guedes *et al*., 2007).

OpdB has been crystallised from *T. b. brucei* but a 3D structure of the protease has not yet been determined (Rea *et al*., 2006). However, oligopeptidase B is homologous to prolyl oligopeptidase, the structure of which has been determined (Fülöp *et al*., 1998), allowing the construction of the 3D structure for OpdB (Fig. 1.8) by homology modelling (Gérczei *et al*., 2000). The enzyme consists of two domains. The N-terminal regulatory domain consists of a seven-bladed β-propeller which excludes large peptides and proteins from the active site. This domain is covalently attached to the C-terminal or peptidase domain that contains the catalytic triad and substrate binding residues.



A **B**

Figure 1.7 A Representation of the structure of oligopeptidase B as modelled by Gérczei *et al.* (2000). The N-terminus is indicated in blue and the C-terminus is indicated in red. The catalytic triad residues, Ser^{554}, Asp^{641} and His^{680} are shown in black. **B Non-catalytic β-propeller domain of oligopeptidase B (Fülöp *et al*., 1998).** The residues (Lys^{82}, Glu^{134}, His^{180}, Asp^{242}, Lys^{389} and Lys^{390}) are shown in ball-and-stick representation at the entrance to the propeller.

The *OpdB* gene occurs as a single copy gene in kinetoplastidiae (Burleigh *et al*., 1997; Morty *et al*., 1999b; de Matos Guedes *et al*., 2007) and is expressed as an 80 kDa peptidase (Morty *et al*., 1999b, 2002). OpdB is released as an active enzyme as no evidence of signal- or pro-peptides exists (Burleigh *et al*., 1997; de Matos Guedes *et al*., 2007).

OpdB has been shown to have a preference for substrates with basic amino acid residues in the P_1 and P_2 positions (Kanatani *et al*., 1991; Morty *et al*., 1999b; de Matos Guedes *et al*., 2007). Peptides with Arg residues present in both positions are hydrolysed at a much faster rate (Morty *et al*., 2002) and hence the activity of OpdB is assayed using the synthetic substrate Z-Arg-Arg-NHMec. Molecular modelling and mutation studies have shown that carboxyl dyads form both the S_1 and S_2 binding pockets that interact with basic residues in the P_1 and P_2 positions (Gerczei *et al*., 2000; Morty *et al*., 2002). The S_1 and S_2 acidic dyads of trypanosomal OpdB are conserved except for *T. congolense* OpdB that has a Gln residue in place of the second Glu residue in the S_2 dyad, which is similar to that of *Leishmania* (Coetzer *et al*., 2008). Since OpdB from *T. congolense* differs in its catalytic specificity as a result of the S_2 dyad (Huson, 2006), further elucidation regarding its use in a vaccine is important.

The activity of OpdB is enhanced by reducing agents such as dithiothreitol and heparin, spermine and spermidine (Morty *et al*., 1999b). OpdB is rapidly inhibited by the serine peptidase inhibitors 3,4-dichloroisocoumarin, chloromethyl ketone and diisopropylfluorophosphate (DFP). Inhibition by thiol reactive agents such as iodoacetate is also made possible by interaction with the cysteine residue (Cys^{256}) which subsequently interferes with the P_1 substrate binding site and prevents substrate binding (Morty *et al*., 2005b).

The physiological role of OpdB has not yet been established. The enzyme has been thought to play a role in host cell invasion particularly in the case of *T. cruzi*. Studies suggest that OpdB is involved in intracellular calcium transients which have been shown to be vital for mammalian host cell invasion (Burleigh *et al*., 1997). During infection, OpdB is released into the bloodstream of the host following lysis of dead or dying trypanosomes where it is free to cleave peptides as it is insensitive to serum protease inhibitors. Peptides such as atrial natriuretic factor, which are rich in basic residues, are particulary susceptible to cleavage by OpdB (Troeberg *et al*., 1996). Degradation of host regulatory pepides such as

atrial natriuretic factor has the potential to severely disrupt control mechanisms of the host and thus contributes to the development of trypanosomiasis (Troeberg *et al*., 1996; Morty *et al*., 2001, 2005a). OpdB therefore acts as another possible pathogenic factor. Just as immune responses have been found against the lysosomal cysteine protease of *T. congolense*, immune recognition of OpdB may also suggest that the enzyme contributes to the pathological effects of trypanosome infection.

1.6 ALPHA-2-MACROGLOBULIN (α_2M) AS A POSSIBLE ADJUVANT

Alpha-2-macroglobulin (α_2M) is a high molecular weight glycoprotein consisting of four identical subunits of approximately 180 kDa each (Swenson and Howard, 1979). Two of these subunits which are non-covalently bound together, are joined to the other two subunits by disulfide bonds to form a double sided molecular "trap" (Feldman *et al*., 1985). The molecular weight of the entire α_2M tetramer is 720 kDa (Swenson and Howard, 1979). The primary biological role of α_2M is uncertain. It is commonly considered to be a broad spectrum protease inhibitor (Barrett and Starkey, 1973). However, it is not only able to entrap proteases but also has the capacity to form complexes with non-proteolytic proteins upon thiolester activation by a protease (Van Leuven *et al*., 1982). In the present study the ability of α_2M to act as an antigen delivery system for use in vaccine development was studied. This feature of α_2M has previously been proposed and has had varied success. Chu *et al*. (1994) tested the ability of human and rabbit α_2M to stimulate specific *in vivo* antibody responses to hen egg lysozyme and porcine pancreatic elastase complexes and found that both human and rabbit α_2M were capable of antigen binding and enhanced adaptive immune responses. In addition, Liao *et al*. (2002) showed that an HIV envelope subunit peptide covalently complexed with α_2M was required in doses up to 100-fold less than those needed with Freund's adjuvant to produce anti-HIV envelope antibodies.

1.6.1 Mechanism of proteinase complex formation

Each of the four subunits of α_2M contains three major sites involved in protein complex formation. These include the bait region, the internal thioester and the receptor recognition site (Van Leuven *et al*., 1986).

The bait region is a stretch of exposed amino acids located within the middle of each subunit and is easily cleaved by any active protease (Barrett and Starkey, 1973). Bait

region cleavage results in an intermediate and distinct conformational change of the α_2M molecule that is also linked to the cleavage of the internal thiol ester. Lysine residues are able to covalently bind the α_2M molecule at a thiol ester on each of the four subunits. When the bait region is cleaved, the α_2M molecule undergoes a conformational change resulting in irreversible binding of the protease. In this state the active site is not blocked. The bound protease can still maintain activity towards small peptide substrates whereas activity towards large protein substrates is restricted. Cleavage of both the bait region and internal thiol ester of α_2M exposes recognition sites for α_2M receptors (CD 91) on cells such as macrophages and hepatocytes (Van Leuven *et al*., 1986). The conformational change also results in an overall electrophoretic shift from the native or slow α_2M form (s-α_2M) to a more compact receptor-recognised or fast α_2-M form (f-α_2M) in native PAGE (Barrett *et al*., 1979). Fig. 1.8 shows a model illustrating the trapping of a protease and the subsequent conformational changes that occur.

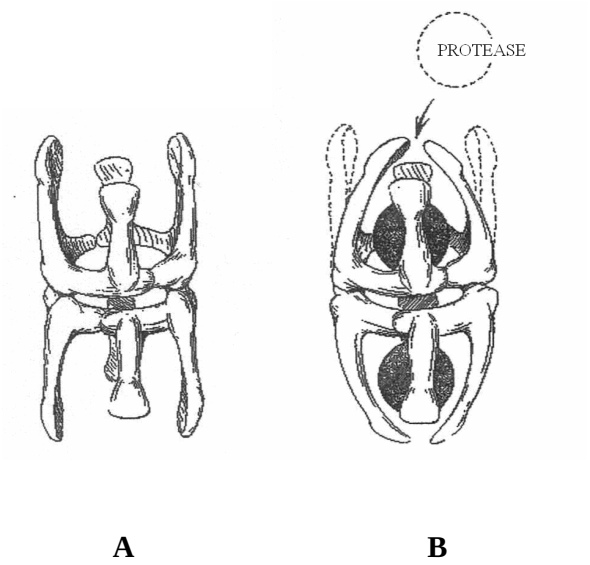


Figure 1.8 Protease-induced α_2M transformation. Protease trapping by α_2M results in a conformational change from its slow or native form (A) to a more compact, receptor-recognised, fast form (B) (Feldman *et al.*, 1985).

1.6.2 α_2M-receptor interaction

Following binding, α_2M-proteinase complexes are rapidly cleared from the circulation by hepatocytes, monocytes, macrophages and dermal dendritic cells (Moestrup and Gliemann, 1991). All these cells express a specific receptor for recognising the proteinase-activated

form of α_2M (Van Leuven *et al*., 1986). Conformational change of α_2M can be induced *in vitro* by treatment with methylamine (Barrett *et al*., 1979). The methylene activated form of α_2M is recognised in the same way as protease activated α_2M (Van Leuven *et al*., 1981).

The α_2M receptor (α_2MR), also referred to as the low-density lipoprotein receptor-related protein (LPR) or CD 91 (Strickland *et al*., 1990), is a 600 kDa glycoprotein that undergoes proteolysis to form two subunits of 515 kDa and 85 kDa that are non-covalently associated. The α_2MR subunits have multiple domains composed of ligand binding and growth factor domain repeats that undergo conformational changes under the acidic conditions of endocytosis. At pH 6.8 α_2M dissociates from the receptor early in the pathway of intracellular ligand sorting (Borth *et al*., 1990). The 85 kDa subunit contains a cytoplasmic tail which is believed to direct the receptor to pits on the cell surface (Chen *et al*., 1990). A third protein of 39 kDa with a heparin binding domain may be associated with the receptor to regulate the affinity of the receptor subunits for its ligands (Herz *et al*., 1991).

Following processing of the α_2M complexes, the antigen presenting cells such as dendritic cells and macrophages, that posses the α_2MR, present the antigen to the immune system resulting in the subsequent production of antibody molecules. In this way, α_2M interacts directly with the cells of the immune system that stimulate antibody production.

1.6.3 α_2M as an adjuvant

α_2M's ability to form covalent complexes with proteins allows it to act as a carrier molecule. Through receptor-mediated endocytosis α_2M is able to enhance antigen processing by targetting the antigen to the cells of the immune system (Chu and Pizzo, 1993). Studies have also shown that α_2M is not only capable of antigen delivery but decreases the minimal antigen concentration required for presentation to these cells. Chu and Pizzo (1993) showed that 200-250 times less α_2M complexed antigen was required to achieve effective presentation to T cells. Evidence of other biological roles of α_2M has been suggested. Hoffman *et al*., (1987) report regulation of immune cell development and function by α_2M. It has also been associated with enzyme inhibition (Ikari *et al*., 2001), regulation of growth factors and cytokines (Liu *et al*., 2001) and protection against demyelination (Gunnarsson and Jensen, 1998).

For the purposes of this study the potential of α_2M as an adjuvant was studied. Huson (2006) successfully used α_2M as an adjuvant. This was the first study to investigate immunisation of α_2M complexed with trypanosome proteases. The present study further evaluated the effect of immunisation with an α_2M-protease complex on the production of antibodies following trypanosome infection.

1.7 OBJECTIVES OF THE PRESENT STUDY

Current control strategies for trypanosomiasis are largely limited due to the parasite's ability to alter the antigenic make up of its surface proteins and evade the host's immune system. An alternative approach may be an anti-disease vaccine directed at antigens of the parasite that play a role in the pathogenesis of the disease. Congopain and OpdB have both been established as pathogenic factors of trypanosomiasis. The immunogenicity of these pathogenic factors may be enhanced by complexing with α_2M. α_2-Macroglobulin has already been shown to bind proteases to form complexes that preserve antigen conformation and undergo rapid endocytosis by antigen presenting cells.

The aim of this investigation was to study the antibody-mediated inhibition of the cysteine protease, congopain and the serine protease, oligopeptidase B following immunisation of goats with α_2M-congopain complexes and mice with OpdB using alum. Studying the potential of α_2M as an adjuvant for immunisation with congopain in goats is reported in Chapter 2. This includes the analysis of the inhibitory properties of antibodies made against congopain in complex with α_2M using fluorogenic and chromogenic peptide and protein substrates. Chapter 3 describes the results obtained for the recombinant expression of OpdB in a bacterial system for immunisation of mice. Chapter 2 and 3 also include a description of antibody production during immunisation and infection monitored by ELISA. The results are discussed in the final chapter.

CHAPTER TWO

EVALUATION OF THE ADJUVANT POTENTIAL OF ALPHA-2-MACROGLOBULIN BY IMMUNISATION WITH CONGOPAIN

L. Bizaaré[1], A. F. V. Boulangé[1, 2] and T. H. T. Coetzer[1].

[1]School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa
[2]UMR-IRD/CIRAD 117 BIOS, 34398 Montpellier, France

ABSTRACT

Congopain, the major cysteine protease of *T. congolense* acts as a pathogenic factor during trypanosomiasis infection. Together with alpha-2-macroglobulin (α_2M), a general serum protease inhibitor, immunisation with congopain produces antibodies with enhanced inhibitory properties. Alpha-2-macroglobulin acts as an adjuvant by binding active proteases and these complexes are rapidly endocytosed by antigen presenting cells. A previous study showed that antibodies raised in rabbits against α_2M complexed with the recombinant catalytic domain of congopain (C2) gave a higher percentage inhibition of enzyme activity than antibodies produced using C2 mixed with Freund's adjuvant. The present study aimed to confirm these findings in small livestock (goats), in addition to studying the immune response following trypanosome challenge. Levels of anti-C2 antibodies produced with Freund's adjuvant and with C2 complexed with α_2M were comparable while the latter showed greater inhibition of congopain. Following challenge a booster effect was observed for all immunised groups of goats. Overall, the findings of the present study indicated that antibodies produced using α_2M were able to inhibit C2 to a far greater extent than those produced using Freund's adjuvant or no adjuvant at all. Alpha-2-macroglobulin was thus shown to be an effective adjuvant for immunisation with C2.

2.1 INTRODUCTION

Widespread vaccination of animals is still considered the most successful method to protect livestock against infectious diseases. Conventional vaccines commonly make use of live attenuated or killed pathogens. Attenuated pathogens aim to mimic the natural pathogen without being infective. However, this is expensive to do and certain pathogens have been known to revert to their live virulent forms (Wilson-Welder *et al*., 2008). Killed vaccines constitute pathogens that have been treated with heat or chemicals. In this way the

cellular integrity and hence the antigenic constituents of the organism are retained. The pathogen is however, unable to induce persistent infection or revert back to a virulent form. A vaccine for trypanosomiasis would prove to be difficult to develop based on conventional strategies using killed or live attenuated parasites. Protozoa are difficult to grow in bulk culture (Roitt, 2001) and trypanosomes have a complex life cycle. A vaccine using killed parasites would have to target and be effective against all life cycle stages. Trypanosome parasites also have the ability to change their outer surface coat which is made up of variable surface glycoproteins (VSGs) (Taylor, 1998). This behaviour referred to as antigenic variation implies that a vaccine using attenuated forms of the parasite would have to include the identification and production of parasites expressing a large series of VSGs. Due to these limitations, efforts have been geared towards developing a vaccine that targets the invariant immunogens of the parasite (Playfair, 1991; Authié, 1994). In a study by Mkunza *et al*. (1995) partial protection against infection with *T. congolense* and *T. vivax* in cattle was induced by vaccination with flagellar pocket antigens derived from *T. b. rhodesiense*. Antigenic constituents of the parasite that are not exposed to the host include proteases released by dead or dying parasites (Tizard *et al*., 1978). This change from an anti-parasite vaccine strategy to an anti-disease vaccine aims to neutralise the pathogenic factors of the parasite rather than target the parasite itself (Authié, *et al*., 2001).

Congopain acts as a major antigen in trypanosomiasis infection (Authié *et al.,* 1992) and has been associated with resistance to the disease, a phenomenon known as trypanotolerance (Authié, 1994). It is therefore a fitting vaccine candidate. In addition to a catalytic domain, congopain has a highly immunogenic C-terminal domain that is unique to trypanosomal cysteine proteases. Like for cruzipain, the major cysteine protease of *T. cruzi*, this highly immunogenic C-terminus is likely to induce the production of antibodies that do not affect the enzyme's catalytic site and therefore its proteolytic function (Cazzulo and Frasch, 1992). A truncated form of congopain, devoid of this C-terminal extension (C2) can be used to produce antibodies that would inhibit the enzyme. This would avoid any mis-direction of the immune system by the highly immunogenic C-terminus. C2 has previously been determined to contribute to the pathogenicity of the parasite in an immunisation trial (Authié *et al.,* 2001). Immunisation of cattle with truncated forms of congopain had no effect on the establishment and development of infection and anaemia. Immunised cattle however, recovered their

haematocrit and leukocyte counts following 2-3 months infection, suggesting a probable role of C2 in the development of the disease.

In order to enhance the immune response during immunisation an adjuvant that is co-administered with the immunogen provides a long-lasting reserve of immunogen. This prevents the rapid dispersal of the antigen from the site of injection (Wilson-Welder *et al*., 2008). Many experimental adjuvants demonstrate high potency but are too toxic for common use (Singh and O' Hagan, 2003). A commonly used adjuvant is Freund's complete and incomplete adjuvant. The incomplete adjuvant consists of a mineral-in-oil emulsion with the emulsifying agent, mannide mono-oleate (Johansson *et al*, 2004). Freund's complete adjuvant additionally contains heat killed *M. tuberculosis* and this causes chronic inflammatory responses and abscesses (Roitt, 2001). For this reason an alternative adjuvant, α_2M, a naturally occurring protease inhibitor, has been used with varying success as an adjuvant (Chu *et al.,* 1994; Cianciolo *et al.,* 2002). Alpha-2-macroglobulin is able to bind to active proteases and undergoes a conformational change that exposes recognition sites on its surface. This receptor recognised form of α_2M allows targeting of the complex to cells of the immune system (Chu and Pizzo, 1993). These complexes are rapidly and efficiently internalised by antigen presenting cells via their CD 91 receptors (Hart *et al*., 2004).

A previous investigation showed that rabbits immunised with C2 emulsified in Freund's adjuvant mounted a greater immune response overall than rabbits immunised with C2 complexed with α_2M or free C2 (Huson, 2006). However, the antibodies produced against C2 using α_2M as an adjuvant were more inhibitory towards C2 activity than antibodies produced against C2 mixed with Freund's adjuvant. Antibodies produced against C2 either complexed to α_2M or mixed with Freund's adjuvant were also assessed for their ability to inhibit C2 *in vitro.* It was found that the antibodies produced in rabbits against C2-α_2M complexes generally showed slightly higher inhibition. In the present investigation, C2 complexed with α_2M, C2 emulsified in Freund's adjuvant and free C2 were used for the immunisation of goats to test the reproducibility of these results in small livestock. Furthermore, the animals were challenged with *T. congolense* to determine the sustainability of the immune response and to asses whether immunisation with C2-α_2M complexes confer protection against trypanosome challenge.

2.2 MATERIALS AND METHODS

2.2.1 Materials

Buffer salts and other common chemicals were obtained from Saarchem (South Africa), Roche Diagnostics (Germany), BDH (England), Fermentas (Lithuania), Merck (Germany) and ICN Biomedicals (USA) and were of the highest purity available.

Sephacryl S-300 HR, Coomassie blue R-250, 4-chloro-1-naphthol, hide powder azure, azocasein, Z-Phe-Arg-AMC, lima bean trypsin inhibitor (LBTI), Freund's complete and incomplete adjuvant and L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) were obtained from Sigma (USA). Horse anti-goat IgG horse radish peroxidase (HRPO) conjugate was obtained from Jackson Immunochemicals (USA). High range molecular mass markers were from BioRad. Recombinant C2 was provided by Hlumani Ndlovu (University of KwaZulu-Natal). Vacutainer^{TM} tubes were from BD Vacutainer Systems (UK). Nunc-Immuno^{TM} 96-well plates and FluorNunc® fluorometry plates were from Nunc Intermed (Denmark). ELISA and fluorometry plates were read in a FLUORStar Optima Spectrophotometer (BMG Labtech, Germany). Procedures carried out at the University of KwaZulu-Natal (UKZN) using animals received approval from the UKZN animal ethics committee (Reference number 002/07/Animal and 025/08/Animal).

2.2.2 Isolation of α_2-Macroglobulin (α_2M)

A variety of isolation procedures are available for the isolation of α_2M. The procedure used for the isolation of goat α_2M is a combination of procedures used previously for the isolation of human α_2M as described by Barrett (1981), Kurecki *et al*. (1979) and Salvesen and Enghild (1993). The first step involves the isolation of α_2M from blood using polyethylene glycol (PEG), Mr 6 000. PEG 6000 is particularly useful for the isolation of large proteins (Polson, 1977). This was followed by affinity purification of α_2M from the redissolved plasma precipitate by zinc chelate chromatography. Alpha-2-macroglobulin is capable of binding to zinc by the interaction between its electron donor groups on the surface of acidic amino acid side chains and the immobilised electron acceptor zinc metal group (Giroux, 1975). Alpha-2-macroglobulin was further purified by molecular exclusion chromatography (MEC) using Sephacryl S-300 HR (Sigma) as some contaminating proteins were present following zinc chelate chromatography (Imber and Pizzo, 1981).

2.2.2.1 PEG precipitation

Goat blood was collected in K3E Vacutainer ™ tubes and centrifuged to remove the cells (10 000 *g*, 20 min, 4ºC). The resulting plasma was combined with 0.28 volumes aqueous 25% (w/v) PEG 6 000, stirred (10 min, RT) and left to stand (20 min, RT). Following centrifugation (10 000 *g*, 20 min, 4°C), the supernatant was mixed with 0.72 volumes aqueous 25% (w/v) PEG 6 000 and left to stand (30 min, RT). The mixture was centrifuged (10 000 *g*, 20 min, 4°C) and the resulting precipitate dissolved in a minimum volume in 20 mM sodium phosphate buffer, pH 6 containing 150 mM NaCl. Residual PEG was removed by dialysis against two changes of 20 mM sodium phosphate buffer (4°C).

2.2.2.2 Zinc chelate chromatography of α_2-Macroglobulin

The redissolved PEG 6 000 precipitate was loaded onto a prepared zinc affinity chelate column (15 x 95 mm, 50 ml. h^{-1}, RT). Unbound protein was eluted with 20 mM sodium phosphate buffer, pH 6 containing 150 mM NaCl while bound protein was eluted with 20 mM sodium cacodylate buffer, pH 5 containing 150 mM NaCl. The eluted protein was titrated to pH 7 with 1 M sodium phosphate buffer, pH 7 and concentrated by dialysis against PEG 20 000.

2.2.2.3 MEC purification of α_2-Macroglobulin

The concentrated sample from zinc chelate chromatography was applied to a Sephacryl S-300 HR MEC column (fractionation range 10–1 500 kDa, 840 x 25 mm, 25 ml. h^{-1}). For calibration the column was equilibrated in MES buffer (0.5 M, pH 6.5) and calibrated with blue dextran (2 000 kDa, 2 mg/ml), bovine serum albumin (66 kDa, 5 mg/ml), ovalbumin (45 kDa, 5 mg/ml), soybean trypsin inhibitor (21 kDa, 5 mg/ml) and lysozyme (14 kDa, 5 mg/ml). Fractions were eluted with MES buffer (0.5 M, pH 6.5) and the absorbance at 280 nm of each sample monitored to construct an elution profile. The concentrated sample from zinc chelate chromatography was loaded onto the column equilibrated with 100 mM sodium phosphate buffer, pH 7 containing 0.02% (w/v) NaN_3. Fractions of 5 ml were eluted with the same buffer.

2.2.2.4 SDS-PAGE and western blot analysis of isolated α_2-macroglobulin

In order to confirm the isolation of α_2M, the pooled fractions eluted from the Sephacryl S-300 HR MEC column were analysed by reducing SDS-PAGE (7.5%). SDS-PAGE analysis

of proteins was carried out using the discontinuous Tris-glycine buffer and gel system described by Laemmli (1970) using the BioRad Mini-PROTEAN 3 gel system. Protein bands were visualized by staining with Coomassie blue R-250 or blotted onto nitrocellulose membrane as described by Towbin *et al*. (1979) using a wet blotter (BioRad, Hercules, CA, USA). Following blotting, the nitrocellulose membrane was stained with Ponceau S to identify the positions of the molecular mass markers. The membrane was destained by washing in distilled water. Following blocking with 5% (w/v) low fat milk in Tris-buffered saline, pH 7.4 (TBS; 20 mM Tris, 200 mM NaCl) for 1 h, the membrane was washed with TBS and incubated for 2 h with chicken anti-bovine α_2M [25 mg/ml diluted in 0.5% (w/v) BSA-TBS]. Following washing with TBS detection was made possible by incubation with rabbit anti-chicken IgG coupled to horse radish peroxidase made up in 0.5% (w/v) BSA-PBS (1: 12 000). After washing with TBS, substrate solution [0.06 % (w/v) 4-chloro-1-naphthol, 0.1 % (v/v) methanol, 0.0015 % (v/v) H_2O_2 made up in TBS] was added and the membrane was left to react in the dark until dark bands appeared. SDS-PAGE and western blot images were captured using a VersaDoc imaging system using Quantity One software from BioRad (USA).

2.2.3 Complexing C2 with α_2-Macroglobulin

C2 was combined in excess with α_2M in 100 mM Bis-Tris buffer, pH 6 containing 4 mM Na_2EDTA, 0.02% NaN_3 and 40 mM cysteine and incubated at 37ºC for 1 h. The mixture was loaded onto a calibrated Sephacryl S-300 HR column and eluted with 100 mM sodium phosphate buffer, pH 7 containing 0.02% NaN_3. Fractions of 5 ml were collected (results not shown).

2.2.4 Production of antibodies in goats (immunisation)

An immunisation/infection trial was set up using goats at the Faculty of Veterinary Science, University of Eduardo Mondlane, Maputo, Mozambique. Three groups of goats were used to produce antibodies against the catalytic domain of congopain from *T. congolense* (C2). Each group of five goats was immunised with C2 (50 µg) and a different adjuvant system or with no adjuvant. The first group was immunised with C2 complexed with goat α_2M, the second group with C2 emulsified in Freund's adjuvant and the third group was immunised with C2 alone (no adjuvant). All goats were immunised subcutaneously on the inside of the thigh. Boosters were administered every ten days with the last booster given on day 40. All times referred to relate to the time following the

initial immunisation on day 0. Goats were bled from the jugular vein prior to immunisation to collect non-immune sera. Blood samples were collected weekly in the same way and sera were analysed by enzyme-linked immunosorbent assay (ELISA). During the immunisation period some goats contracted rickettsiosis. Rickettsiosis is a tick-borne disease caused by obligate intracellular bacteria that infect the ticks' salivary glands. The disease is transmitted to vertebrate hosts during feeding (Raoult and Roux, 1997). As a result of this disease, numbers were reduced to four goats for the group immunised with C2 with no adjuvant and three goats for the group immunised with C2 mixed with Freund's adjuvant. No goats in the group immunised with C2 complexed with α_2M died (Table 2.1).

Table 2.2 Description of the different adjuvants used with C2 for immunisation of goats.

Name	Immunogen and adjuvant
C1; C2; C3; C4	C2 + no adjuvant
C5; C6; C7	C2 + Freund's adjuvant
C8; C9; C10; C11; C12	C2 + α_2M

2.2.5 ELISA evaluation of immune kinetics

ELISAs were carried out to evaluate the antibody response against C2 using different (Freund's or α_2M) or no adjuvant in goats. Microtitre plates (NUNC-Immuno^{TM}) were coated with C2 (100 ng) in PBS, pH 7.2 overnight at 4ºC. Unoccupied sites in wells were blocked with BSA-PBS [0.5% (w/v), 150 µl/well, 1 h, RT]. Wells were washed with 0.1% (v/v) PBS-Tween 20 and incubated with the respective anti-sera diluted in BSA-PBS (100 µl/well, 2 h, RT). Following washing with 0.1% (v/v) PBS-Tween 20, HRPO -conjugated anti-goat IgG (1:10 000, 100 µl/well, 1 h, RT) diluted in BSA-PBS was used as the detection system. Plates were washed again with 0.1% (v/v) PBS-Tween 20 and the enzyme reaction was developed with ABTS-H_2O_2 substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2] in 150 mM citrate phosphate buffer, pH 5.0. The optical density was measured using an ELISA microplate reader (FLUOStar OPTIMA, BMG Labtech) at 405 nm.

2.2.6 Isolation of antibodies

Goat IgG was isolated from serum by PEG precipitation (Polson *et al*., 1964). Following collection of serum into glass vacutainers, serum was clarified by centrifugation (10 000 *g*, 10 min, 4ºC). Clarified serum was combined with double the volume of borate buffered saline, pH 8.6 and PEG 6 000 added to 14% (w/v). After the PEG was dissolved the solution was centrifuged (12 000 *g*, 10 min, 4ºC) and the pellet resuspended in the original serum volume using 100 mM sodium phosphate buffer, pH 7.6. PEG was added to 14% (w/v), dissolved and the solution was centrifuged (12 000 *g*, 10 min, 4ºC). The pellet was dissolved in half the serum volume using 100 mM sodium phosphate buffer, pH 7.6 containing 60% (v/v) glycerol and 0.02% (w/v) NaN_3. The resulting solution was stored at -20ºC.

2.2.7 Challenge of goats with *T. congolense* (strain IL 1180)

All goats were challenged with *T. congolense* (strain IL 1180). Infected mouse blood collected at the peak of parasitaemia was used to infect the 3 groups of goats at a dose of 10^4 parasites per animal on day 98. Goats were not bled from day 50-98 as the animals were rested prior to infection. Following challenge, blood samples were collected weekly and analysed by ELISA for anti-C2 antibody production as described in Section 2.2.5.

2.2.8 Active site titration of C2 with E-64

The concentration of active C2 was determined by active site titration using the inhibitor E-64 and the fluorogenic substrate Z-Phe-Arg-AMC. E-64 is an irreversible cysteine protease inhibitor that binds rapidly with the active site of cysteine proteases (Barrett *et al*., 1982). When activity is plotted against the molarity of E-64, the active molarity of the enzyme being studied can be determined at the point where the plot reaches zero activity (Barrett and Kirschke, 1981).

Enzyme (0.1 - 1 µM) diluted with 0.1% (w/v) Brij was added to E-64 (0.1 – 1 µM) also diluted in 0.1% (w/v) Brij and incubated with 100 mM Bis-Tris buffer, pH 6.4 containing 4 mM Na_2EDTA, 0.02% (w/v) NaN_3 and 8 mM DTT for 30 min at 37ºC. Aliquots were combined with substrate Z-Phe-Arg-AMC (20 µM) and following incubation for 10 min at 37ºC, the fluorescence was read (excitation at 360 nm and emission at 460 nm) using a fluorescence FLUOStar OPTIMA microplate reader.

2.2.9 Inhibition of congopain activity by goat IgGs

2.2.9.1 Inhibition of congopain hydrolysis of Z-Phe-Arg-AMC

The inhibition of C2 enzyme activity by specific goat IgGs was assayed over time with serial two-fold dilutions starting from 2 mg/ml. C2 (10 ng active enzyme) was diluted with 0.1% (w/v) Brij and combined with an equal volume of IgG diluted in 200 mM sodium phosphate assay buffer, pH 7.2 containing 4 mM Na_2EDTA, 0.1% (v/v) Tween 20 and 40 µg/ml lima bean trypsin inhibitor to give final IgG concentrations of 1000, 500, 250 and 125 µg/ml. Lima bean trypsin inhibitor was added to inhibit any residual Z-Phe-Arg-AMC hydrolysing serum protease activity. Samples were incubated at 37ºC for 15 min. Aliquots were combined with 200 mM sodium phosphate assay buffer, pH 7.2 containing 4 mM Na_2EDTA, 0.1% (v/v) Tween 20 and 8 mM DTT and activated at 37ºC for 1 min. Substrate Z-Phe-Arg-AMC (20 µM diluted from a 1 mM stock made up in DMSO) was added and the microplate was incubated for 5 min at 37ºC. Fluorescence was read over time (excitation at 360 nm and emission at 460 nm) using a FLUOStar OPTIMA microplate reader and inhibition was expressed as a percentage of the activity of non-immune antibody at the same concentrations.

2.2.9.2 Inhibition of congopain hydrolysis of hide powder azure

C2 (200 pmol active enzyme) was diluted in 100 mM Bis-Tris assay buffer, pH 6.4 containing 4 mM Na_2EDTA and 0.02% (w/v) NaN_3 and combined with 100 mM cysteine (100 µl) and activated for 5 min (RT). Antibody (0-400 pmol) diluted in assay buffer was added and incubated for 20 min at 37ºC. Substrate solution (12.5 mg hide powder azure, 600 mM sucrose) made up in assay buffer was added and incubated for 1 h at 37ºC with agitation. Undigested substrate was removed by centrifugation (13 700 *g*, 5 min, RT) and the absorbance read at 595 nm using a FLUOStar OPTIMA microplate reader. Inhibition was expressed as a percentage of the activity compared to non-immune antibody at the same concentrations. This assay was performed in triplicate.

2.2.9.3 Inhibition of congopain hyrolysis of azocasein

C2 (100 pmol active enzyme) diluted with 0.1% (w/v) Brij and activated with 100 mM Tris-HCl assay buffer, pH 8.4 containing 4 mM Na_2EDTA and 0.02% (w/v) NaN_3, for 5 min at RT. Antibody (0-200 pmol) diluted in 0.1 % (w/v) Brij was added and incubated for 20 min at 37ºC. Substrate solution [3 M urea made up in 6% (w/v) azocasein] was

added and incubated in a water bath for 1 h at 37ºC. The reaction was stopped with the addition of 5% (w/v) trichloroacetic acid and centrifuged (13 700 *g*, 5 min, RT). The absorbance of the supernatant was read at 366 nm using a FLUOStar OPTIMA microplate reader and inhibition expressed as a percentage of the activity compared to non-immune antibody at the same concentrations.

2.3 RESULTS

2.3.1 Isolation of alpha-2-macroglobulin

Alpha-2-macroglobulin was isolated from goat plasma. The elution profile for PEG precipitated plasma purified by zinc chelate chromatography showed a sharp single peak after application of the elution buffer for goat α_2M (Fig. 2.1).

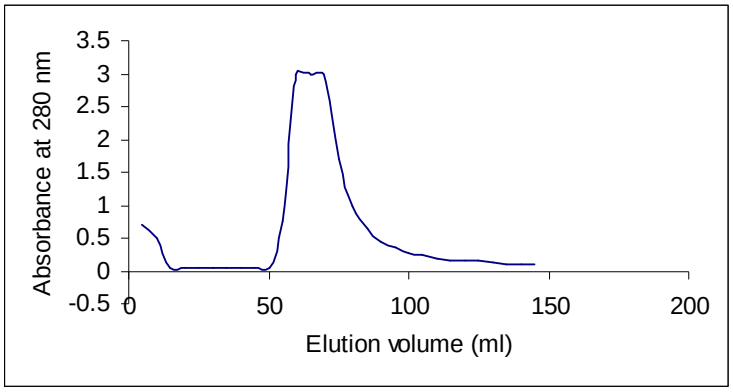


Figure 2.1 Afffinity chromatography of goat α_2M on a zinc chelate column. Zinc chelate affinity column (95 x 15 mm, 50 ml. h^{-1}) was equilibrated with 200 mM sodium phosphate buffer, pH 6 containing 150 mM NaCl. Bound protein was eluted with 20 mM sodium cacodylate buffer, pH 5 containing 150 mM NaCl. Absorbance at 280 nm was determined for all fractions eluted.

Subsequent MEC on a Sephacryl S-300 HR column showed two major peaks in the elution profile (Fig. 2.2). The high molecular weight peak corresponds to α_2M at an elution volume of 195 ml and approximate size of 720 kDa. The lower molecular weight peak corresponds to an approximate size of 66 kDa.

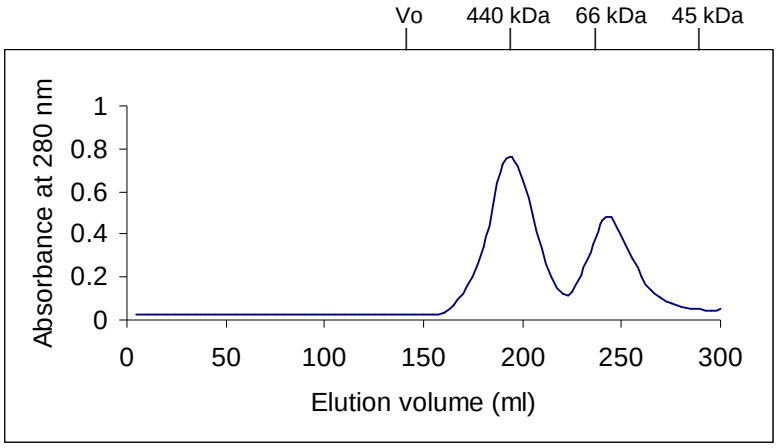


Figure 2.2 Molecular exclusion chromatography of goat α_2M on Sephacryl S-300 HR. Sephacryl S-300 HR (840 x 215 mm, 25 ml. h^{-1}) was equilibrated and eluted with 100 mM sodium phosphate buffer, pH 7 containing 0.02% NaN_3. Absorbance at 280 nm was determined for all fractions eluted.

Fractions containing the high molecular weight protein were pooled and analysed by reducing SDS-PAGE and western blotting. A protein band at 170 kDa is indicated by the arrow and corresponds to the α_2M monomer that is expected under reducing conditions (Fig. 2.3). A band at 120 kDa was observed in addition to the 170 kDa monomer in western blot analysis and corresponds to fragments of the α_2M monomer that result from reducing conditions (Fig. 2.4).

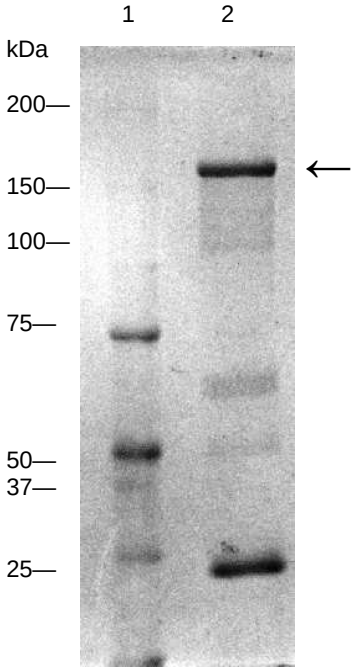


Figure 2.3 Reducing SDS-PAGE (7.5%) gel analysis of pooled high molecular weight fractions from Sephacryl S-300 HR. Lane 1, BioRad Precision Plus high range molecular mass marker; lane 2, pooled fractions eluted from the MEC column (20 µl). Proteins were stained with Coomassie blue R-250.

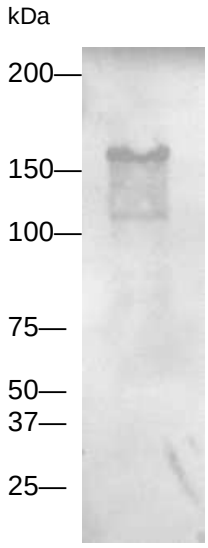


Figure 2.4 Western blot analysis of pooled high molecular weight fractions from Sephacryl S-300 HR. Protein was electrophoresed on a 7.5% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with anti-goat α_2M. Secondary antibody was HRPO-conjugated rabbit anti-chicken IgG. Reaction was developed with 4-chloro-1-naphthol/H_2O_2. BioRad Precision Plus high range molecular mass marker is shown alongside pooled fractions eluted from MEC column.

2.3.2 Evaluation of antibody response against C2 in goats using ELISA

The production of antibodies in goats immunised with C2 without adjuvant or with either Freund's adjuvant or complexed with α_2M was monitored by ELISA over a 50-day immunisation period (Fig. 2.5). C2 specific antibodies were produced and detected after the primary immunisation in each of the goats immunised as shown in Fig. 2.5. The antibody response appeared to peak after the second boost, i.e. from day 21 to the third boost at day 35 (i.e. week 3-5).

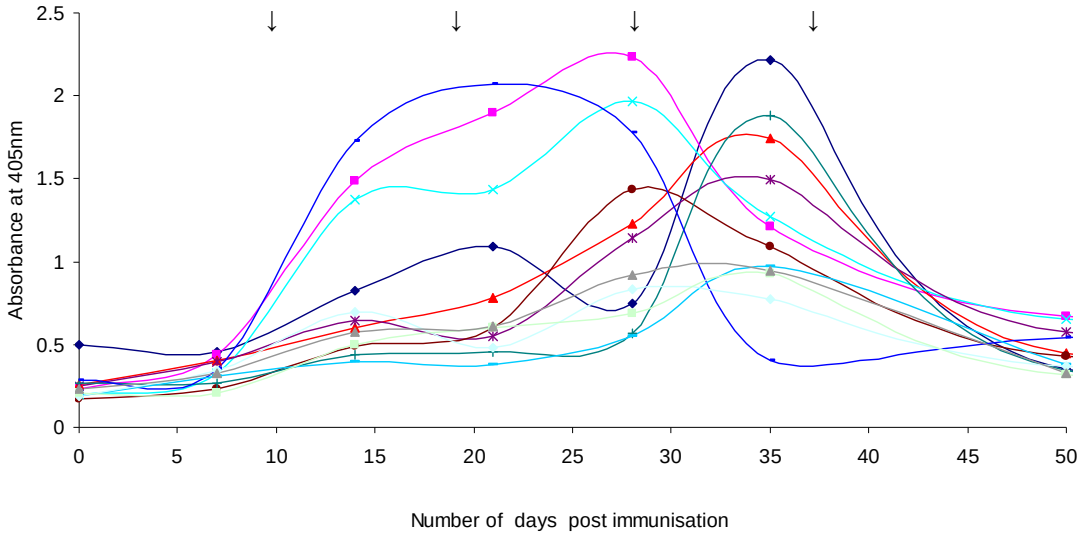


Figure 2.5 ELISA showing immune response against C2 in goats immunised with C2 in the presence or absence of different adjuvants. Antibody production was monitored for goats immunised with C2 and Freund's adjuvant (■, x, -), C2 and alpha-2-macroglobulin (*, ●, ◆, ■, ▲) and C2 with no adjuvant (◆,▲,', -). C2 was coated onto ELISA plates (100 ng/well) and incubated with the sera collected weekly following the first immunisation at a dilution of 1:250 (in BSA-PBS, 100 µl/well, 2 h, RT). Horse anti-goat IgG HRPO-conjugate at a dilution of 1: 10 000 (in BSA-PBS, 100 µl/well, 1 h, RT) and ABTS/H_2O_2 (100 µl/well) was used as the detection system. Absorbance readings at 405 nm represent the average of duplicate experiments. Arrows (↓) indicate when booster immunisations were given.

The data shown in Fig. 2.5 was averaged for each adjuvant and the control (no adjuvant) and plotted (Fig. 2.6). Antibodies against C2 mixed with Freund's adjuvant generally showed a strong initial response peaking at weeks 2-4 while those antibodies produced against C2 complexed with α_2M or those produced with no adjuvant peaked between weeks 4-5.

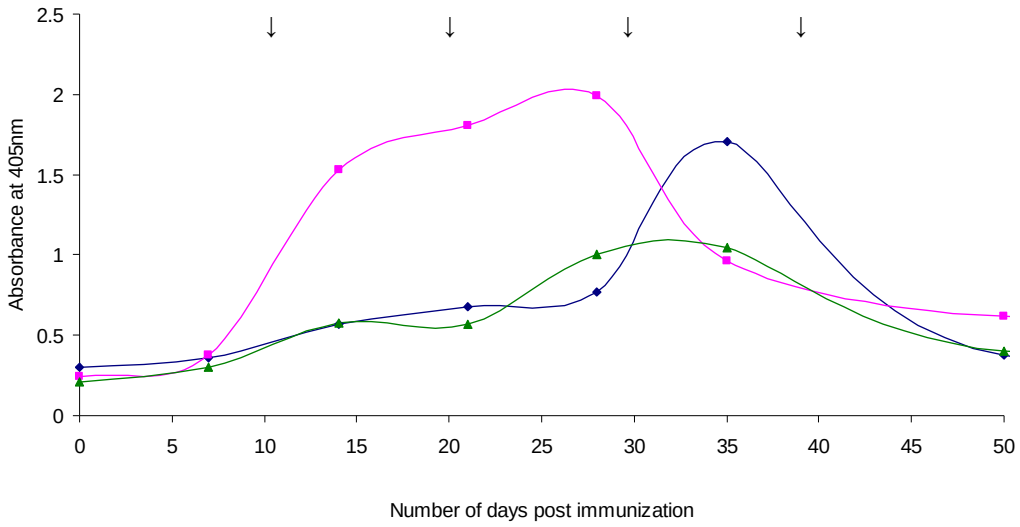


Figure 2.6 ELISA showing the antibody response against C2 by measuring the recognition of C2 by pooled serum samples from goats immunised with C2 using different adjuvants. Antibody production was monitored for goats immunised with C2 and Freund's adjuvant (■), C2 and alpha-2-macroglobulin (▲) and C2 with no adjuvant (◆). C2 was coated onto ELISA plates (100 ng/well) and incubated with sera collected weekly following the first immunisation at a dilution of 1:250 (in BSA-PBS, 100 µl/well, 2h, RT). Horse anti-goat IgG HRPO-conjugate at a dilution of 1: 10 000 (in BSA-PBS, 100 µl/well, 1 h, RT) and ABTS/H_2O_2 (100 µl/well) was used as the detection system. Absorbance readings at 405 nm represent the average of duplicate experiments. Arrows (↓) indicate when booster immunisations were given.

In summary, goats immunised with C2 mixed with Freund's adjuvant consistently showed a high antibody response throughout the immunisation schedule. Goats immunised with C2 alone or complexed with α_2M showed far lower antibody responses except at day 35 where the antibody response for C2 with no adjuvant showed a significant increase.

Following challenge with *T. congolense* (IL 1180) infected mouse blood, a booster effect in the immune response of all goats was observed (Fig. 2.7). Antibody production following infection appeared to peak between days 112 and 126 (weeks 16-17).

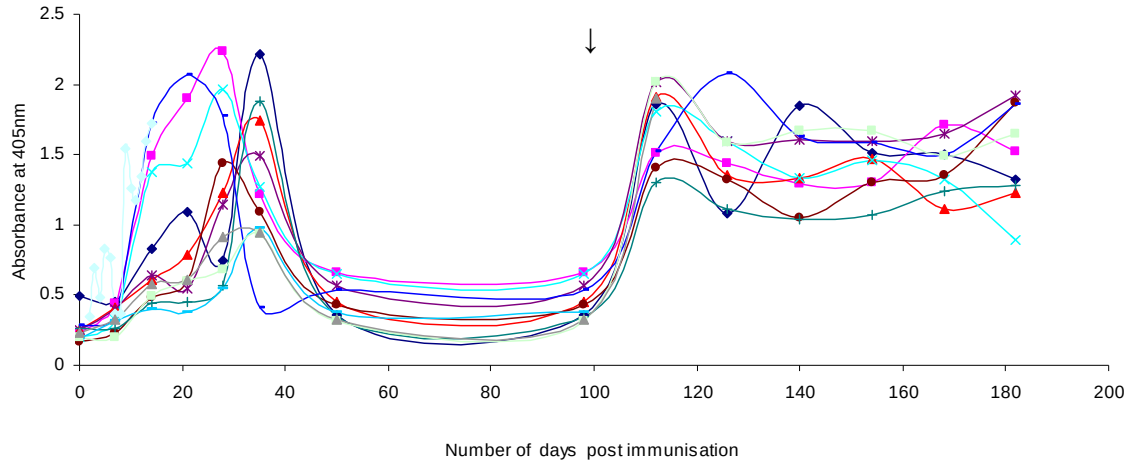


Figure 2.7 ELISA showing immune response against C2 in goats immunised with C2 in the presence or absence of different adjuvants after trypanosome challenge. Antibody production was monitored for goats immunisation with C2 and Freund's adjuvant (■, x, -), C2 and alpha-2macroglobulin (*, ●, ◆, ■, ▲) and C2 with no adjuvant (◆, ▲, ', -). C2 was coated onto ELISA plates (100 ng/well) and incubated with sera collected weekly following the first immunisation at a dilution of 1:250 (BSA-PBS, 100 µl/well, 2h, RT). Horse anti-goat IgG HRPO-conjugate at a dilution of 1: 10 000 (in BSA-PBS, 100 µl/well, 1 h, RT) and ABTS/H_2O_2 (100 µl/well) was used as the detection system. Absorbance readings at 405 nm represent the average of duplicate experiments. Arrow (↓) indicates the time of trypanosome challenge.

ELISAs conducted with pools of antibodies collected after trypanosome challenge again showed that antibodies produced against C2 mixed with Freund's adjuvant had a consistent and high immune response overall as was the case during the immunisation schedule (Fig. 2.8). However, antibodies produced against C2 complexed with α_2M showed a significant boost after trypanosome infection. Anti-C2-α_2M antibody levels peaked at day 112 and exceeded antibody levels produced using C2 with Freund's adjuvant and C2 in the absence of adjuvant.

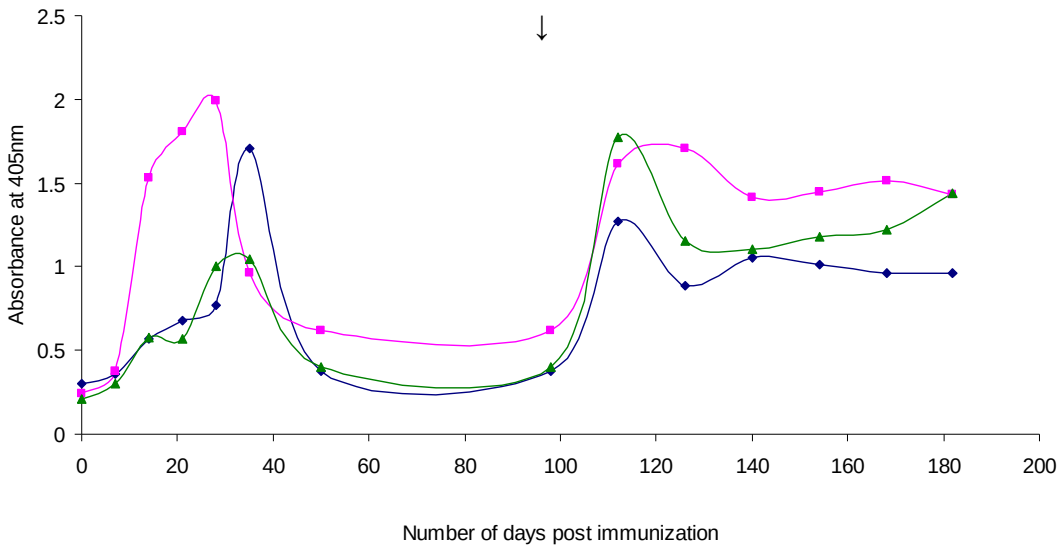


Figure 2.8 ELISA showing the antibody response against C2 by measuring the recognition of pooled serum samples from goats immunised with C2 using different adjuvants after trypanosome challenge. Antibody production was monitored for goats immunisation with C2 and Freund's adjuvant (■), C2 and alpha-2-macroglobulin (▲) and C2 with no adjuvant (◆). C2 was coated onto ELISA plates (100 ng/well) and incubated with sera collected weekly following the first immunisation at a dilution of 1:250 (in BSA-PBS, 100 µl/well, 2h, RT). Horse anti-goat IgG HRPO-conjugate at a dilution of 1: 10 000 (in BSA-PBS, 100 µl/well, 1 h, RT) and ABTS/H_2O_2 (100 µl/well) was used as the detection system. Absorbance readings at 405 nm represent the average of duplicate experiments. Arrow (↓) indicates the time of trypanosome challenge .

2.3.2 Inhibition of C2 activity by antibodies raised in goats against C2

2.3.2.1 Inhibition assays using Z-Phe-Arg-AMC as a substrate

Total IgGs from day 50 (week 7) were purified as described in Section 2.2.6 and assessed for their capacity to inhibit the hydrolysis of Z-Phe-Arg-AMC by C2 (Table 2.2). Fluorescence was read continuously over a 20 min period. The resulting initial rates of the enzymatic reaction were used to determine the inhibitory activity of the antibodies. The average inhibition of the antibodies against C2 is illustrated in Fig. 2.9

Table 3.2 Inhibition of C2 activity by anti-C2 antibodies

Animal:	**Adjuvant:**	**Inhibition (%)**	**Average inhibition (%)***
C2; C5; C13; C15	No adjuvant	38; 30; 27; 37	33
C3; C7; C14	Freunds	31; 18; 57	36
C8; C10; C16; C17; C18	α_2-Macroglobulin	35; 39; 54; 50; 73	50

*for IgG at 1 mg/ml final concentration

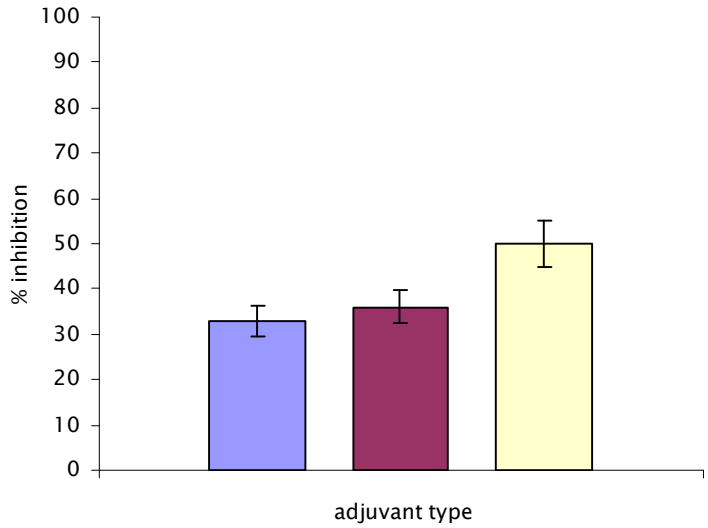


Figure 2.9 Inhibition of C2 activity against Z-Phe-Arg-AMC by goat IgGs at 1 mg/ml. Inhibition of C2 hydrolysis of Z-Phe-Arg-AMC by antibodies against C2 complexed to α_2M ( ), C2 mixed with Freund's adjuvant ( ) or C2 with no adjuvant ( ) was expressed as percentage inhibition relative to C2 activity in the presence of non-immune IgG control (0% inhibition). Each bar represents the average % inhibition of each group and is the mean of duplicate experiments.

All IgGs isolated from day 50 (week 7) inhibited C2 activity. The most efficient inhibition was observed for those antibodies produced against C2 complexed with α_2M. The greatest inhibition was determined at a final antibody concentration of 1 mg/ml, giving an average 50% inhibition. More specifically, IgGs isolated from an individual experimental animal in the α_2M group was found to give up to 73% inhibition at the same concentration. At day 50, antibodies produced with Freund's adjuvant showed the highest antibody levels, but despite this they did not show the greatest inhibition. In some cases a titration effect may be observed when antibody concentration was decreased in a serial manner but this was not consistent for any of the groups or IgGs isolated from individual goats of the same group. Antibodies produced with no adjuvant showed the least inhibition, i.e. 33% at 1 mg/ml final antibody concentration. Overall, antibodies produced against C2 complexed with α_2M showed 14% higher inhibition than those produced by mixing with Freund's adjuvant and 16% higher inhibition than those produced with no adjuvant. This is a significant difference as there is only a 2% difference in inhibition by antibodies produced using no adjuvant and those produced by using the conventionally used Freund's adjuvant.

2.3.2.2 Inhibition assays using hide powder azure as a substrate

Total IgGs from day 50 (week 7) was purified and inhibition of the hydrolysis of hide powder azure by C2 assessed. Hydrolysed substrate can be quantified by absorption at 595 nm. Proteolytic activity was determined by the extent of the hydrolysis of the hide powder azure substrate compared to a C2 control with pre-immune control antibody (0% inhibition) and is expressed as percentage inhibition (Fig. 2.10).

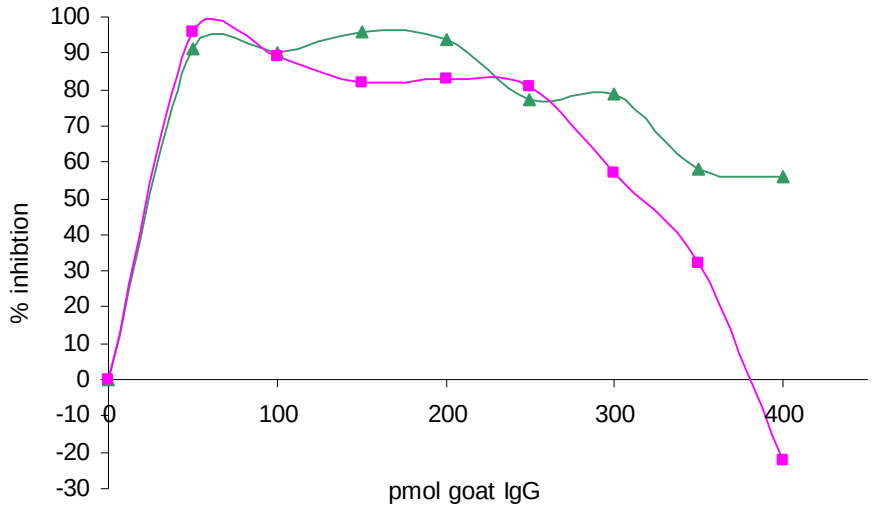


Figure 2.10 The effect of anti-C2 antibodies on the activity of C2 against hide powder azure. Different concentrations of antibody (50, 100, 150, 200, 250, 300, 350 and 400 pmol) produced against C2 complexed with α_2M (▲) or mixed with Freund's adjuvant (■) was combined with C2. The extent of inhibition of hydrolysis of hide powder was measured by reading the absorbance at 595 nm in comparison with C2 in the presence of non-immune control antibody (0% inhibition).

Anti-C2 antibodies produced by complexing with α_2M were able to inhibit up to 96% of C2 activity at increased amounts of IgG (150 pmol) in the presence of 200 pmol C2. As the amount of specific antibody was increased, inhibition was seen to decrease slightly but remained fairly high. The lowest inhibition was seen at 400 pmol IgG but good inhibition (58%) was still observed even at this high level of antibody. Anti-C2 antibodies produced with Freund's adjuvant were able to inhibit 96% of C2 activity at low levels of antibody (25 pmol). The least inhibition was seen at 400 pmol antibody and at this high level antibodies seemed to activate, rather than inhibit C2.

2.3.2.3 Inhibition assay using azocasein as a substrate

Total IgGs from day 50 (week 7) were purified and the inhibition of the hydrolysis of azocasein by C2 assessed. Proteolytic degradation of azocasein results in the release of peptides that are soluble in trichloroacetic acid whilst undigested fragments precipitate.

The soluble peptides have an intense yellow colour that can be quantified by measuring the absorption at 366 nm (Fig. 2.11).

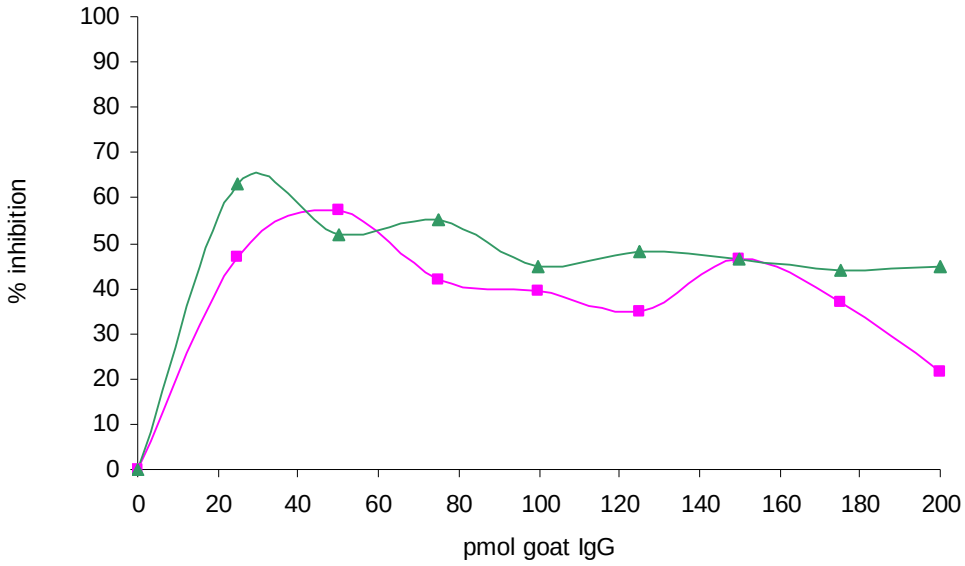


Figure 2.11 The effect of anti-C2 antibodies on the activity of C2 against azocasein. Different concentrations of antibody (0, 25, 50, 75, 100, 125, 150, 175 and 200 pmol) produced by complexing C2 with α_2M (▲) or by mixing with Freund's adjuvant (■) was combined with C2. The effect on azocasein hydrolysis was measured by reading the absorbance at 366 nm and comparison with hydrolysis of azocasein in the presence of non-immune control antibody (0% inhibition).

Anti-C2 antibodies produced by complexing with α_2M inhibited up to 63% of C2 activity at low levels of IgG (25 pmol) in the presence of 100 pmol C2. As the amount of specific antibody is increased, inhibition was seen to decrease slightly but remained fairly consistent and reached a plateau with an average inhibition of 49%. Anti-C2 antibodies produced with Freund's adjuvant inhibited C2 activity with the greatest inhibition of 57% at 50 pmol antibody. The least inhibition was seen at 200 pmol antibody (i.e., double the amount of antibody compared to that of enzyme) giving 21% inhibition. Similar results were seen for antibodies produced against C2-α_2M complexes, although the latter still showed good inhibition of 44% at this high level of antibody.

2.4 DISCUSSION

Since antigenic variation is an obstacle to the development of a vaccine against trypanosomiasis, the concept of an anti-disease vaccine that targets pathogenic factors of the parasite has been proposed (Playfair, 1991; Authié, 1994). Congopain has been implicated as a pathogenic factor necessary for the development of anaemia in the host as shown by Authié *et al*. (2001). Prominent IgG responses were observed in cattle

immunised with congopain in RWL, a proprietary adjuvant from Smith Kline Beecham. This adjuvant induced higher and more sustained antibody levels. Immunised cattle recovered and maintained leukocyte counts following 2-3 months infection, indicating the role of congopain during infection. However immunised cattle did not show any difference in the development of infection compared to non-immunsed cattle. In addition, the production of RWL (Smith Kline and Beecham) was discontinued. In the present study, the use of α_2M to enhance the immunogenicity of C2 to produce antibodies with the potential capacity to inhibit the enzyme was investigated.

The results presented show that antibodies against recombinantly expressed trypanosomal C2 were produced in all goats immunised. This verifies the use of congopain as a vaccine candidate (Authié, 1994) as high amounts of specific antibodies that inhibit C2 were produced in the experimental animals.

Alpha-2-macroglobulin was successfully isolated by zinc chelate and molecular exclusion chromatography. The method used in the isolation was modelled on the technique described by Salvesen and Enghild (1993) for the isolation of human α_2M. C2 was complexed with α_2M and any unbound C2 was separated from complexed C2 by MEC. The C2-α_2M complexes were used for the immunisation of goats in the present study.

The highest titres of antibody produced during immunisation were apparent for those goats immunised with C2 mixed with Freund's adjuvant. Freund's adjuvant has a depot effect and as a result the antigen is retained in the tissues at the site of injection (Wilson-Welder *et al.*, 2008). Upon completion of protease binding and conformational change, α_2M complexes are rapidly cleared from circulation (Imber and Pizzo, 1981; Enghild *et al.*, 1989). Since the antigen is removed from the tissues there is no continuous stimulation of the immune system to produce antibodies. An unusually high antibody titre was observed for antibodies against C2 alone. The titre however is not analogous to the level which would be observed if it were administered together with an adjuvant, but since C2 is immunogenic by itself it is able to elicit an immune response. Antibodies with the greatest inhibitory capacity were produced with C2 complexed with α_2M. The superior inhibitory properties of these antibodies may be attributed to the preservation of the three-dimensional conformation of C2 when bound to α_2M. The shielding properties of α_2M have been used to protect bound proteins from plasma proteases during presentation

to cells of the immune system (Chu *et al*., 1994). Freund's adjuvant offers no such protection to the enzyme and results in antibody production against linear epitopes rather than conformational epitopes. Conformational epitopes are able to inhibit the enzyme to a greater extent. Complexing antigen with α_2M and using the unique binding capacity of α_2M to CD 91 on antigen presenting cells enhances the rate and efficiency of antigen uptake and presentation to the cells of the immune system (Hart *et al*., 2004).

Binding of antibody to enzyme is thought to affect the enzyme in two ways. Antibodies may induce conformational changes at the active site of the enzyme resulting in a less active or more active conformation of the enzyme. The antibody may also physically occlude the active site of the enzyme and prevent any access of the substrate. In addition, occlusion of the active site by the antibodies may not always be evident depending on the size of the substrate used (Richmond, 1977). In the present study inhibition of C2 activity was tested with the substrates Z-Phe-Arg-AMC, hide powder azure and azocasein.

Inhibition using Z-Phe-Arg-AMC showed fairly good inhibition at an antibody concentration of 1 mg/ml. The effect of different antibody concentrations was investigated with a fixed amount of enzyme but it was difficult to obtain reproducible results at these lower concentrations of enzyme. IgGs produced using C2-α_2M isolated from serum of individual goats showed up to 73% inhibition. An overall 50% inhibition of C2 was observed against Z-Phe-Arg-AMC by antibodies produced against the C2-α_2M complexes. These antibodies also showed approximately 16% greater inhibition of C2 activity compared to antibodies against C2 produced with Freund's adjuvant and 14% greater inhibition compared to antibodies produced against free C2. A maximum inhibition of 86% has been reported for the inhibition of the cysteine protease trypanopain from *T. b. brucei* against Z-Phe-Arg-AMC by anti-trypanopain antibodies made in chickens (Troeberg *et al*, 1997).

Inhibition assays for C2 tested with hide powder azure also showed very high levels of inhibition, particularly at the lower amounts of antibody used. Of all the substrates used to assay for inhibition by anti-C2 antibodies, hide powder azure showed the greatest inhibition. Maximum inhibition was observed to be 95% at low levels of antibody (50 pmol antibody in the presence of 200 pmol C2). This was apparent for antibodies made using C2 mixed with Freund's adjuvant and C2-α_2M complexes. It is important to note the

difference between the inhibition capacity of antibodies produced using C2-α_2M complexes and C2 mixed with Freund's adjuvant as the amount of antibody is increased. For antibodies produced using C2-α_2M complexes inhibition decreases notably from antibody amounts in excess of 200 pmol while for antibodies produced using C2 mixed with Freund's adjuvant, inhibition begins to decrease steadily from levels in excess of 100 pmol. Antibodies produced with C2-α_2M complexes inhibited C2 activity to a greater extent (i.e., higher % inhibition overall) and had more sustained inhibition than antibodies produced with C2 mixed with Freund's adjuvant.

Inhibition of the hydrolysis of azocasein by C2 showed fairly high levels of inhibition. Maximum inhibition of C2 activity in this assay was approximately 60% for antibodies produced with C2-α_2M complexes (25 pmol antibody to 100 pmol C2) and 50% for antibodies produced using C2 mixed with Freund's adjuvant (50 pmol antibody in the presence of 100 pmol C2). Anti-C2-α_2M antibodies were observed to have a higher maximum inhibition at lower levels of antibody. Increasing the amount of antibody in solution resulted in a steady decrease in inhibition. Overall, antibodies produced with C2-α_2M complexes were observed to generally have greater inhibition than antibodies produced using C2 mixed with Freund's adjuvant at all levels of antibodies.

Hide powder azure is a very large substrate and may be too large to enter the active site of C2. Azocasein and Z-Phe-Arg-AMC are smaller substrates and can interact with the active site of C2 more effectively. In a study on the effects of anti-trypanopain antibodies on the enzyme activity against Z-Phe-Arg-AMC, Troeberg *et al.*, (1997) suggested that since Z-Phe-Arg-AMC is a small substrate, inhibition in this case is mediated through antibody binding that induces a less active conformation of the enzyme rather than sterically hindering substrate binding. This was also apparent for the present study. Using substrates of differing sizes, it was determined that enzyme inhibition was mediated through the binding and induction of a less active form of the enzyme rather than occlusion of the active site by antibodies. The marked inhibition of the hydrolysis of hide powder azure by C2 was due to a less active enzyme induced by the anti-C2 antibodies in addition to ineffective substrate binding due to the large size of the substrate. In the same way, a less active form of the enzyme interacted with azocasein and Z-Phe-Arg-AMC, but because these are smaller substrates they were still able to interact with the active site of C2 to some extent. Immunoenhancement of enzyme activity is also possible due to the binding

and induction of a more active enzyme conformation by antibodies (Richmond, 1977). At higher levels of antibodies (in excess of 200 pmol), significant enzyme activation was observed against both hide powder azure and azocasein. Higher levels of antibody may be responsible for binding to C2 in such a way to induce a more active form of the enzyme.

The inhibition assays were carried out under conditions of pH similar to physiological conditions. These assays determined the ability of the antibodies to inhibit the proteolytic activity of C2 *in vitro*. In an attempt to understand what would happen *in vivo* where parasites, upon lysis, would release congopain into the host's bloodstream, conditions close to physiological pH were used.

Following challenge a booster effect was observed for all immunised groups. Twelve days after infection (day 110) the immune response was observed to decrease but began to increase slightly ten days thereafter (day 120) and remained stable over the remainder of the test period. This phenomenon where the immune response was observed to decrease for a short time may be due to the process of "mopping up" or parasite clearance by the immune system. The dominant immune response of the host to infection is antibody mediated and targets the exposed surface epitopes of the VSGs on the parasite (Taylor, 1998). Antibody and complement-mediated immune responses may expose previously concealed pathogenic factors upon parasite lysis. This has been confirmed by the superior IgG responses of trypanotolerant cattle to buried VSG epitopes (Taylor *et al.*, 1996; Williams *et al.*, 1996) and congopain (Authié *et al.*, 1992, 1993). Antibodies specific for these buried epitopes and pathogenic factors may aid in the clearance of opsonised trypanosomes (Taylor, 1998). The binding of pathogenic factors makes it easier for phagocytes to latch on to the particles and engulf them and hence remove the immune complexes from the blood (Roitt, 2001). However, since antibodies are absorbed and cleared from circulation, serum antibody titres do not accurately indicate the production of antibodies at the cellular level (Nielsen *et al.*, 1978; Taylor *et al.*, 1996) and this may be observed as a lower immune response in an ELISA as was observed in this study.

None of the goats challenged with *T. congolense* IL1180 developed any noteworthy symptoms of trypanosomosis. They had very intermittent parasitaemia, no significant anaemia, and finally all but self-cured. As a result, it is difficult to asses whether immunisation with C2 in complex with α_2M conferred any protection against trypanosome

challenge. No formal studies of the pathogenicity of challenge by *T. congolense* IL 1180 of goats has been published, but it is reported to induce severe anaemia, weight loss and immunosuppression in infected cattle (Wellde *et al*, 1974). In general, small ruminants such as goats do generally exhibit fewer clinical manifestations of the disease than those shown by cattle (Taylor and Authié, 2004). The behaviour of this local breed of goats from Mozambique may also be attributed to a natural resistance to trypanosomosis. This phenomenon has previously been described for African cattle (Authié, 1994). It is possible that the strain of *T. congolense* IL1180, which is extremely pathogenic in cattle, may not be as pathogenic in goats. It may also be possible that the goats had previously been exposed to trypanosome parasites prior to immunisation and challenge. This may even explain the inhibitory activity of antibodies isolated from goats immunised with C2 alone. It is therefore imperative to conduct a preliminary pathogenicity experiment with several *T. congolense* strains on goats. Goats should also be selected based on a low anti trypanosome antigen antibody levels.

The general objective of this study was to determine the adjuvant effect of α_2M on the inhibitory capacity of the antibodies produced by a natural trypanosome host immune system for use in an anti-disease vaccine with congopain (C2) as an immunogen. Goats were immunised with C2 in complex with α_2M or Freund's adjuvant or with no adjuvant and the humoral response to immunisation was determined by ELISA. All goats immunised showed the production of antibodies specific to C2 and the highest titres of antibody produced during immunisation was observed for goats immunised with C2 mixed with Freund's adjuvant. The immune response for all immunised goats was maintained following challenge and a booster effect was observed. Enhanced enzyme inhibiting antibodies were isolated and analysed using various substrates. Antibodies with the greatest inhibitory capacity were produced with C2 complexed with α_2M. In general therefore it was found that immunisation using Freund's adjuvant resulted in quantitively better antibody production. Complexing antigen with α_2M and using the unique binding capacity of α_2M in the present study, led to qualitatively superior antibody production that is capable of inhibiting C2.

CHAPTER THREE

RECOMBINANT EXPRESSION AND PURIFICATION OF OLIGOPEPTIDASE B AND ITS USE AS AN ANTI-DISEASE VACCINE IN A MOUSE MODEL.

L. Bizaaré[1], A. F. V. Boulangé[1,2] and T. H. T. Coetzer[1].

[1]School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Private Bag X01, Scottsville 3209, South Africa
[2]UMR-IRD/CIRAD 117 BIOS, 34398 Montpellier, France

ABSTRACT

Trypanosome parasites are surrounded by an outer coat consisting of variable surface antigens. Due to antigenic variation, efforts have been focussed on using the invariant antigens of the parasites for vaccine targets. Among such is the serine protease, oligopeptidase B (OpdB). Oligopeptidase B is a pathogenic factor of trypanosomes. It is released into the host bloodstream following parasite death and retains catalytic activity as it is insensitive to host serum protease inhibitors. In the bloodstream it hydrolyses host peptides and hormones such as atrial natriuretic factor and vasopressin thus contributing to disease pathology. The focus of this study was to test the use of OpdB by vaccination and trypanosome challenge experiments in a mouse model. Oligopeptidase B from *Trypanosoma congolense* was recombinantly expressed in an *Escherichia coli* bacterial system. The resulting fusion protein was purified by affinity and molecular exclusion chromatography. The presence of purified protein was confirmed by SDS-PAGE and western blot analysis using anti-OpdB antibodies produced in-house. The mice were challenged with *T. congolense* and the parasitaemia was monitored throughout the infection period. The immune response in mice to OpdB was monitored before and after challenge by enzyme-linked immunosorbent assay (ELISA) and anti-OpdB antibodies showed 83% inhibition of enzyme activity. In general OpdB was observed to be highly immunogenic in mice and elicited an immune response that produced antibodies capable of inhibiting OpdB activity.

3.1 INTRODUCTION

Vaccination against trypanosomiasis has long been thought to be the most desirable control method to combat the disease. Conventional control strategies have largely been hampered due to the toxicity of drugs used to treat the disease and the emergence of drug resistant parasites (Taylor, 1998). Despite the difficulty presented by antigenic variation, many of

the parasite's components are pathogenic factors and are potentially useful as vaccine candidates. Pathogenic factors may be actively secreted or released by the parasite upon death (Playfair *et al*., 1991). The concept of an anti-disease vaccine has been proposed and entails antibody-mediated inhibition of these pathogenic factors (Authié, 1994). It is likely that an anti-disease vaccine would be most effective if it encompassed a variety of factors that contribute to pathogenesis, resulting in a multi-component vaccine (Price and Kieny, 2001). Identification of the various pathogenic factors is therefore essential in the development of a vaccine. Among the pathogenic factors are proteolytic enzymes of the parasite, some of which have already been implicated in the pathogenesis of the disease (Sajid and McKerrow, 2002).

Oligopeptidase B, a serine protease of *Trypanosoma congolense*, has been established as a virulence factor in trypanosomiasis. It is released into the host's bloodstream during infection by dead or dying parasites and maintains catalytic activity as it is resistant to host serum inhibitors (Troeberg *et al*., 1996, Morty *et al*., 2001, 2005a). OpdB is involved in the degradation of host regulatory peptides such as neurotensin and atrial natriuretic factor which have a significant role in the maintenance of blood volume. This is particularly important as elevated blood volume is typical of infection with bloodstream trypanosomes (Troeberg *et al*., 1996; Morty *et al*., 2001).

Recombinant expression of proteins is a more favourable way to produce proteins, particularly when using them for immunisation trials, as a large amount of protein can be produced in this way. Isolating native protein directly from the parasite is far more time consuming and tedious and the yield of protein is not as substantial. The recombinant production of OpdB in the *Escherichia coli* (*E. coli*) bacterial expression system for its use in immunisation trials to study the antigenicity of OpdB and its use as a vaccine against trypanosomiasis is detailed in this chapter.

Potential endotoxin contamination originating from the bacterial expression system was removed from the recombinantly expressed and purified OpdB by EndoTrap® affinity chromatography prior to immunisation. Endotoxins are highly immunogenic and would mis-direct the immune system resulting in antibodies produced against endotoxins rather than the protein that was injected. Endotoxins also activate complement and inflammatory responses (Morrison and Ulevitch, 1982).

In the current study OpdB from *T. congolense* was expressed in the pGEX4T1 and pET28a bacterial systems. OpdB was expressed as a glutathione-S-transferase (GST) fusion protein and was purified by on column cleavage as well as cleavage outside the column using thrombin. Further purification was facilitated by molecular exclusion chromatography (MEC). In the pET28a expression system, OpdB was expressed as a fusion protein with a histidine tag and subsequently purified by nickel chelate affinity chromatography. Purified OpdB with alum was used for the immunisation of mice. It was previously found that the type of adjuvant appeared to have no impact on the levels of anti-OpdB antibodies in rabbits but greatly affected the inhibitory capacity of the antibodies towards OpdB. Immunisation with alum was previously determined to be the best as it promoted the production of antibodies in rabbits that inhibited up to 100% of the activity of OpdB (Huson, 2006). In the present study mice were challenged with *T. congolense* (Strain IL 1180) following immunisation to determine the sustainability of the immune response. The inhibitory capacity of anti-OpdB antibodies was also determined before and after challenge with trypanosomes.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Buffer salts and other common chemicals were obtained from Saarchem (South Africa), Roche Diagnostics (Germany), BDH (England), ICN Biomedicals (USA), Merck (Germany) and Fermentas (Lithuania) and were of the highest purity available.

Sephacryl S-200 HR, Sephacryl S-300 HR, Glutathione agarose, Coomassie blue R-250, 4-chloro-1-naphthol, MES [2-(N-morpholino)ethanesulfonic acid], Z-Arg-Arg-AMC, Ampicillin, and Antithrombin III were from Sigma (USA). *Eco*RI, *Not*I, shrimp alkaline phosphatase (SAP), T4 DNA ligase, DNA dilution buffer, 10 mM dNTP mix, DNA high and middle range molecular marker mix, GeneJet^{TM} Plasmid Miniprep Kit and TranformAid^{TM} Bacterial Transformation Kit were obtained from Fermentas (Lithuania). *Taq* polymerase, 10 x PCR reaction buffer and $MgCl_2$ were from Solis Biodyne (Tartu, Estonia). The Ni-NTA agarose was obtained from Invitrogen (USA). Horse radish peroxidase (HRPO) labelled horse anti-mouse IgG was obtained from Southern Cross Biotechnology, Vector Labs. 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) was

obtained from Roche (Germany). EndoTrap® columns were from Profos AG (Germany). High range molecular mass markers were from Pharmacia. Low mass molecular markers and Poly-Prep® chromatography columns were from BioRad Laboratories (USA). Thrombin, thrombin dilution buffer and thrombin cleavage buffer were from Novagen (USA). Nunc-Immuno^{TM} 96-well plates were from Nunc Intermed (Denmark). Trypanosomal lysates for SDS-PAGE and western blotting were provided by Richard Kangethe (University of KwaZulu-Natal). Ethical approval for experimental immunisation studies was obtained from the University of KwaZulu-Natal animal ethics committee (Reference number 002/07/Animal and 025/08/Animal)

3.2.2 Protein analysis

3.2.2.1 Determination of protein concentration

Protein concentration was determined by the Bicinchoninic Acid (BCA^{TM}) Protein Assay Kit (Pierce, Rockford, IL, USA). Protein sample solution was combined with a working reagent (sample to working reagent ratio is 1: 20) in a microtitre plate and incubated for 30 min at 37ºC. The BCA-protein complex exhibits a strong absorbance at 562 nm and protein concentrations can be determined with reference to standards of bovine serum albumin (BSA) from 60-2000 µg/ml. This assay is not an end point method as the final colour continues to develop and as a result a standard curve (Fig. 3.1) is constructed each time the assay is performed.

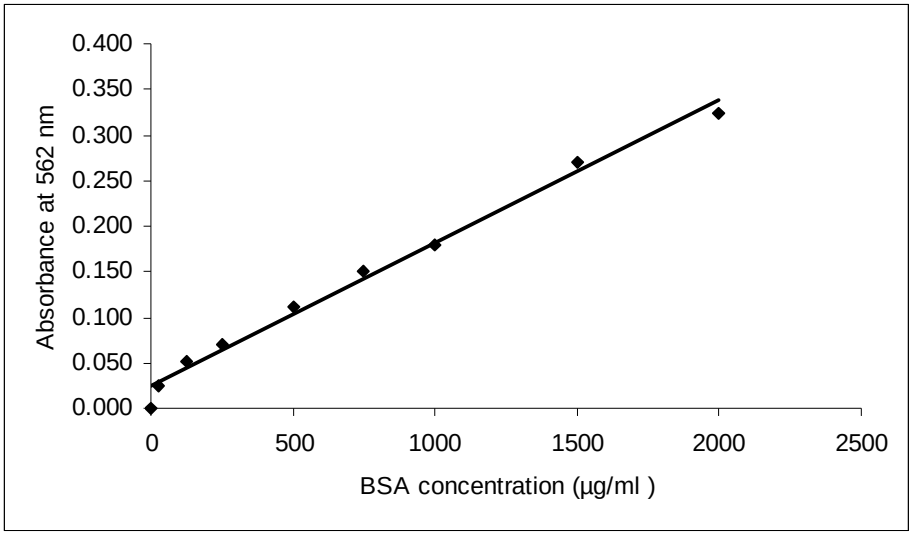


Figure 3.1 Standard curve used for protein quantitation using the BCA™ Protein Assay Kit. Protein (0-2000 µg/ml) was related to absorbance at 595 nm. The equation of the trend line was y = 0.0002x + 0.0242, with a correlation coefficient of 0.9876.

Protein quantitation was further verified by SDS-PAGE. Equal volumes of protein and standard BSA samples (125-2000 µg/ml) were run on SDS-PAGE and compared (Fig. 3.2).

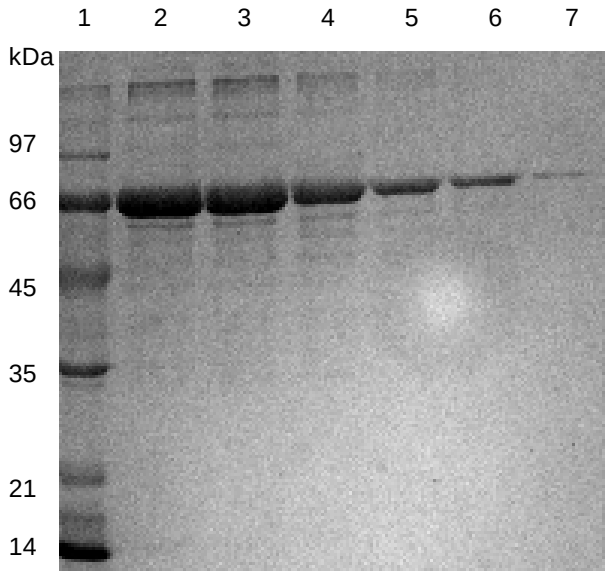


Figure 3.2 SDS-PAGE gel (10 %) of BSA of known concentrations to verify protein quantitation. Equal volumes of protein samples were run alongside known concentrations of BSA on an SDS-PAGE gel allowing quantitation of the unknown concentrations. Lane 1, BioRad low molecular mass markers; lanes 2-7, BSA at 2, 1, 0.75, 0.5, 0.25, 0.125 mg/ml. The gel was stained with Coomassie blue R-250.

3.2.2.2 Concentration of dilute protein samples

Dilute protein samples were routinely concentrated by dialysis against polyethylene glycol (PEG) 20 000. Protein sample was placed in dialysis tubing (molecular weight cut-off 10 kDa) and covered with PEG 20 000. After concentrating the sample to the desired volume the dialysis tubing was washed in distilled water and the sample removed and stored.

Protein was also concentrated using the Amicon concentration cell (model 202) using an X-M 50 membrane and very low pressure.

When protein samples required for analysis, by reducing SDS-PAGE, were too large in volume, SDS-KCl precipitation was used. 5 % (w/v) SDS (10 µl) was added to protein sample (100 µl) and mixed by inverting the tube. 3M KCl (10 µl) was added and the mixture was inverted and centrifuged (12 000 *g*, 2 min, RT). The supernatant was discarded and the precipitate was resuspended in reducing treatment buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8, 10µl].

3.2.2.3 SDS-PAGE and western blot analysis of protein

SDS-PAGE analysis of proteins was carried out using the discontinuous Tris-glycine buffer and gel system described by Laemmli (1970) using the BioRad Mini-PROTEAN 3 gel system. BioRad or Pharmacia molecular mass markers were run on each gel and used to determine the size of sample proteins as the relative migration of proteins in a gel is inversely proportional to its molecular mass (Dennison, 1999). A standard curve was constructed relating the relative migration of proteins to their known molecular sizes to determine the sizes of sample proteins (Fig. 3.3). SDS-PAGE gels were run at 18 mA per gel.

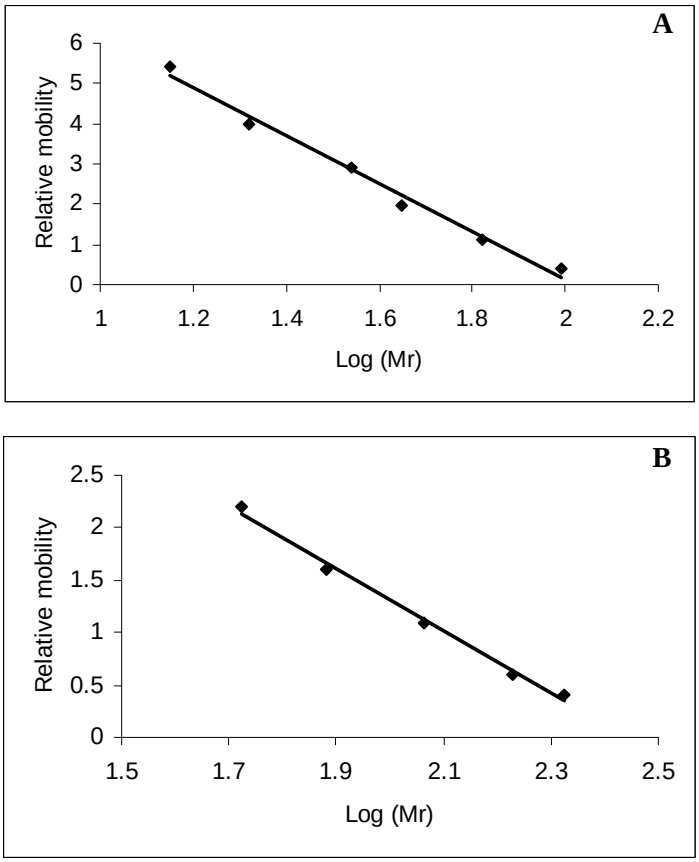


Figure 3.3 Standard curves relating relative mobility of proteins of known size to log (Mr) on a 10 % SDS-PAGE gel. A. BioRad low range molecular mass markers include phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), chicken egg ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trysin inhibitor (21 kDa) and chicken egg white lysozyme (14 kDa). The equation of the trend line was y = -5.9511 x + 12.024 with a correlation coefficient of 0.9896. **B. Pharmacia high range molecular mass markers** include myosin (212 kDa), α_2-Macroglobulin (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa) and glutamic dehydrogenase (53 kDa). The equation of the trend line was y = -2.9747 x + 7.2633 with a correlation coefficient of 0.9938.

Following electrophoresis protein bands were detected by staining with Coomassie blue R-250. For more sensitive staining, silver staining was used (Blum *et al.*, 1987).

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes for western blotting as described by Towbin *et al.* (1979) using a wet blotter (BioRad, Hercules, CA, USA). Following blotting, the nitrocellulose membrane was stained with Ponceau S to identify the positions of the molecular mass markers. The membrane was destained by washing in distilled water. Following blocking with 5% (w/v) low fat milk in Tris-buffered saline, pH 7.4 (TBS; 20 mM Tris, 200 mM NaCl) for 1 h, the membrane was washed with TBS and incubated for 2 h with the appropriate dilution of primary antibody in 0.5 % (w/v) BSA-TBS. Following washing with TBS, detection was made possible by

incubation with the secondary HRPO-linked antibody in 0.5 % (w/v) BSA-TBS for 1 h. After washing with TBS, substrate solution [0.06 % (w/v) 4-chloro-1-naphthol, 0.1 % (v/v) methanol, 0.0015 % (v/v) H_2O_2 made up in TBS] was added and the membrane was left to react in the dark until dark bands appeared. SDS-PAGE and western blot images were captured using a VersaDoc imaging system using Quantity One software from BioRad (USA).

3.2.3 Enzyme activity assays

The activity of OpdB was measured using the fluorogenic substrate Z-Arg-Arg-AMC. OpdB, diluted with 0.1% (w/v) Brij 35 was added to 200 mM Tris-HCl buffer, pH 8 containing 10 mM DTT and 0.02% (w/v) NaN_3 and was allowed to incubate for 5 min at 37ºC. Substrate (20 µM diluted from a 1 mM stock made up in DMSO) was added and fluorescence (excitation at 360 nm and emission at 460 nm) was read using the FLUOStar OPTIMA spectrophotometer plate reader (BMG Labtech, Germany) following 5 min incubation at RT.

3.2.4 Expression of recombinant oligopeptidase B as a GST-fusion protein in E. coli

2YT medium (100ml) with ampicillin (50µg/ml) was inoculated with a single colony of recombinant pGEX4T1 (Fig. 3.4) containing OpdB transformed into *E. coli* JM109 cells previously prepared by Huson (2006) and incubated overnight at 37°C. The overnight culture was transferred into 2YT medium (900 ml) containing ampicillin (50 µg/ml). The culture was incubated at 37°C until an OD_{600} of one was reached. Isopropyl-beata-D-thiogalactopyranoside (IPTG) (0.1 M; 3 ml) was added to induce expression and the culture was incubated for 4 h at 37°C. Following expression, cells were collected by centrifugation (5 000 *g*, 10 min, RT). The pellet was resuspended in 0.1 % (v/v) PBS-Triton X-100 containing lysozyme (1 mg/ml) and stored at -20ºC.

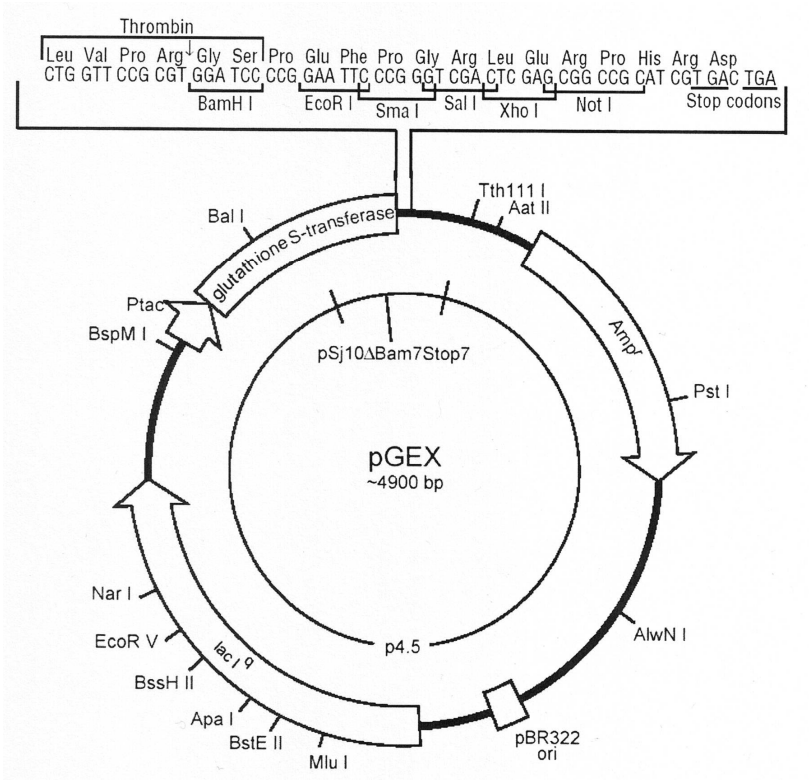


Figure 3.4 Schematic map of the pGEX4T1 expression vector (Amersham Biosciences).

3.2.5 Purification of recombinant oligopeptidase B

3.2.5.1 'On-column' cleavage using thrombin

Before purification of OpdB from the bacterial lysate, the lysate was thawed, sonicated (VirSonic 60, Virtis, USA) and clarified by centrifugation (5000 *g*, 10 min). Additional clarification of the lysate was carried out by filtering the supernatant through Whatman No. 1 filter paper. The lysate was mixed with glutathione agarose resin (Sigma) overnight at 4°C by end-over-end mixing. The matrix was allowed to settle in the column and was washed with 0.1 % (v/v) PBS-Triton X-100 (10 column volumes) and equilibrated with 20 mM Tris-HCl thrombin cleavage buffer, pH 8.4 containing 150 mM NaCl and 2.5 mM $CaCl_2$ (Novagen) and resuspended to a 50% slurry to which thrombin [4U from a 1 U/µl stock made up in 50 mM citrate dilution buffer, pH 6.5 containing 200 mM NaCl, 1 mg/ml PEG 6000 and 50% (v/v) glycerol (Novagen)] was added. The column was incubated overnight at RT with gentle rocking. Cleaved recombinant OpdB was directly eluted from the column. Any thrombin present in the sample was inactivated by the addition of an equimolar amount of antithrombin III (Sigma). Any bound GST and uncleaved fusion protein was eluted from the resin with reduced glutathione (10 mM) dissolved in 50 mM Tris-HCl, pH 8 containing 0.02% (w/v) NaN_3. The column was regenerated by washing

with 100 mM sodium borate buffer, pH 8.0 containing 500 mM NaCl followed by dH_2O, 100 mM sodium acetate buffer, pH 4.0 containing 500 mM NaCl and again with dH_2O. The resin was stored in PBS with 0.02% (w/v) NaN_3 at 4°C.

3.2.5.2 Cleavage outside the column using thrombin

This method was carried out exactly as for the 'on column' cleavage except after washing with 0.1 % (v/v) PBS-Triton X-100 (10 column volumes) the GST-OpdB fusion protein was directly eluted from the resin with reduced glutathione (10 mM) dissolved in 50 mM Tris-HCl, pH 8 containing 0.02% (w/v) NaN_3. The column was regenerated and stored as described in Section 3.2.5.1. Thrombin (1U per 1 mg protein) was added to the eluted fusion protein sample, together with the appropriate amount of 10 x thrombin cleavage buffer consisting of 20 mM Tris-HCl buffer, pH 8.4 containing 150 mM NaCl and 2.5 mM $CaCl_2$ (Novagen). The solution was left overnight at room temperature. Following overnight incubation the thrombin present in solution was inactivated by the addition of an equimolar amount of antithrombin III (Sigma) and analysed by SDS-PAGE to check the extent of cleavage of the fusion protein.

3.2.5.3 Purification of oligopeptidase B by molecular exclusion chromatography (MEC)

Oligopeptidase B eluted from the glutathione agarose column using the on-column cleavage method was loaded onto a Sephacryl S-300 HR MEC column (fractionation range 10–1 500 kDa, 840 x 25 mm, 25 ml. h^{-1}) to eliminate the numerous proteins that co-eluted with OpdB. Since these proteins were of similar molecular weight, a resin with a large fractionation range was used. Oligopeptidase B eluted from the glutathione agarose column using the cleavage method outside the column was loaded onto a Sephacryl S-200 HR MEC column (fractionation range 5-250 kDa, 1.5 x 40 cm, 20 ml. h^{-1}). GST was the only contaminating band and is much smaller in size than OpdB and was therefore easily separated using a resin with a smaller fractionation range. Both columns were calibrated and equilibrated using MES buffer (0.5 M, pH 6.5) and calibrated with blue dextran (2 000 kDa, 2 mg/ml), bovine serum albumin (66 kDa, 5 mg/ml), ovalbumin (45 kDa, 5 mg/ml), soybean trypsin inhibitor (21 kDa, 5 mg/ml) and lysozyme (14 kDa, 5 mg/ml). Fractions were eluted with MES buffer (0.5 M, pH 6.5) and the absorbance at 280 nm of each sample monitored to construct an elution profile.

3.2.6 Sub-cloning and expression of recombinant oligopeptidase B as a His-tag fusion protein in *E. coli*

3.2.6.1 Sub-cloning of oligopeptidase B into pET28a

Glycerol stocks of the OpdB clone inserted into pGEX4T1, prepared by Huson (2006) were used to streak a 2X yeast tryptone (2X YT) agar plate containing ampicillin (50 µg/ml) and grown overnight at 37ºC. A single colony was picked and grown overnight in liquid 2X YT medium (5 ml, 50 µg/ml ampicillin). Plasmid DNA was isolated by the miniprep method according to the manufacturer's instructions (Section 3.2.6.3) and digested using the restriction enzymes *Eco*RI and *Not*I (Section 3.2.6.4). The resulting 2.1 kb insert was purified from the agarose gel (Section 3.2.6.5) and ligated into the prepared pET28a vector (Fig. 3.5) that had previously been isolated and restricted in the same way. The vector was dephosphorylated using SAP according to manufacturer's instructions (Fermentas). The ligated vector and insert were transformed into competent JM 109 *E. coli* cells (Section 3.2.6.7) and plated onto 2X YT agar plates using kanamycin (30 µg/ml) as a marker for transformants. Colonies were screened for recombinants by colony PCR using specific OpdB primers (Table 3.1). Recombinant colonies (2) were grown in 2X YT media containing kanamycin (30 µg/ml) and plasmid DNA was extracted by miniprep and transformed into competent BL21 (DE3) cells and plated onto 2X YT agar plates containing kanamycin (30 µg/ml). Colonies were considered recombinant by digestion with *Eco*RI and *Not*I and the subsequent release of a 2.1 kb OpdB insert.

Table 3.1 Sequences of the primers used in colony PCR.

Primer name	Direction	Sequence (5'-3')
Oligopeptidase B	Forward	CGT TCT AGA ATG TAC GCC ATT GCA GTG CGC
Oligopeptidase B	Reverse	CAC TCT AGA TTA GTC CGT CTT GAG CTC CCG

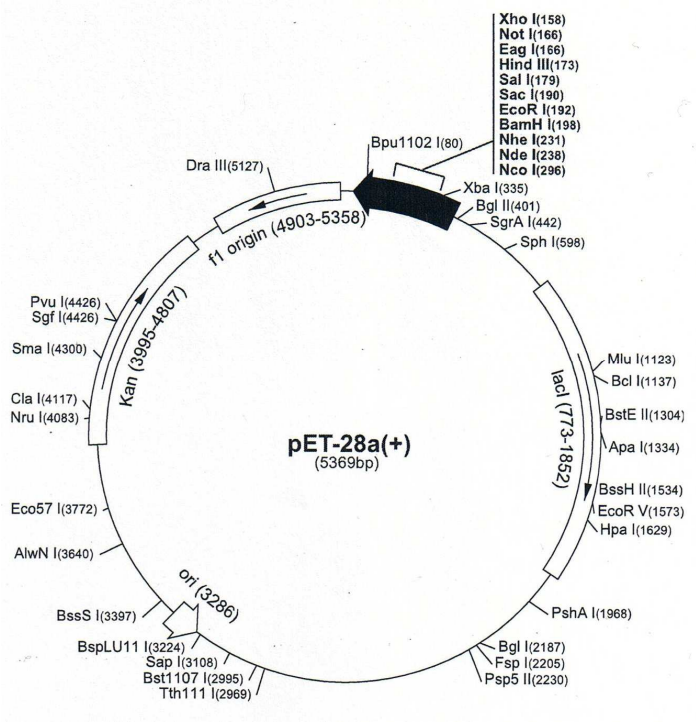


Figure 3.5 Schematic map of pET28a expression vector (Novagen).

3.2.6.2 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate and visualise DNA by staining with ethidium bromide. Agarose (0.3 g) was dissolved in 30 ml 1 x TAE buffer (40 mM Tris acetate buffer, pH 8 containing 1 mM Na_2EDTA) by heating in a microwave oven. A small amount of ethidium bromide (1µl) was added to the solution and once cooled, was poured into a Perspex casting tray. Once set, the gel was submerged in a tank filled with 1 x TAE buffer. Loading dye containing 20% (v/v) glycerol was added to DNA samples and samples were loaded into the gel wells. Electrophoresis was carried out at 80 V until the tracking dye had migrated sufficiently through the gel (about half way). The gel was viewed under ultraviolet light and captured using the BioRad VersaDoc system.

3.2.6.3 Isolation of DNA (GeneJET™ Miniprep Kit)

DNA is usually isolated on a small scale from an overnight culture by a method called miniprep. The GeneJET™ Miniprep Kit was used for all isolations of plasmid and insert DNA. Cells from an overnight culture of plasmid (5 ml) grown at 37ºC were harvested by centrifugation (12 000 *g*, 2 min, RT). The supernatant was discarded and the pellet was resuspended in resuspension solution (250 µl) and vortexed. Lysis solution (250 µl) was

added to the resuspended solution and the tube was inverted 5 times. Neutralisation solution was added (350 µl) and the tube was inverted 5 times. The resulting solution was centrifuged (12 000 *g*, 5min, RT). The supernatant was transferred to a GeneJET™ spin column and centrifuged (12 000 *g*, 1 min, RT). The pellet (cell debris and chromosomal DNA) was discarded. Wash solution was added (500 µl) and the solution was centrifuged (12 000 *g*, 30-60 s, RT). The flow through was discarded and the column was centrifuged (12 000 *g*, 1 min, RT). This wash step was repeated. The GeneJET™ column was transferred to a new sterile microfuge tube and the DNA was incubated with elution buffer (50 µl, 2 min). DNA was collected in the flow through from the column following centrifugation (12 000 *g*, 2 min, RT) and stored at -20ºC.

3.2.6.4 Restriction digestion of DNA

When two DNA samples are digested with the same restriction enzymes that result in overhangs or 'sticky ends,' once mixed together, these samples combine due to base pairing. *Eco*RI (G↓AATTC) and *Not*I (GC↓GGCCGC) were used for restriction digestion of plasmid and insert DNA. The use of restriction enzymes was carried out as outlined by the manufacturer (Fermentas) and the amount used was dependent on the amount of sample DNA in the solution. The restriction solution consisted of sample DNA, restriction buffer (10 x), sterile distilled water and restriction enzyme. Solutions were mixed by gentle finger tapping and incubated (16 h at RT or 2 h at 37ºC).

3.2.6.5 Purification of DNA from agarose gels

Digestions are often analysed by agarose gel electrophoresis. DNA must therefore be purified from the agarose gel following separation. In this study plasmid and insert DNA were purified using a peqGOLD Gel Extraction Kit (Classic Line) from peqLab Biotechnologie. Following separation by agarose gel electrophoresis, the DNA band was excised from the gel using a sterile scalpel blade. The DNA was suspended in an equal volume of XP2 buffer (0.2 g gel is equivalent to 0.2 ml). The solution was incubated at 60ºC for 7 min with vortexing every 2-3 min until all agarose was dissolved. A maximum of 750 µl of sample was loaded onto a HiBind® DNA spin column for adsorption of the DNA to the membrane. The membrane was washed with buffer XP2 (300 µl) and centrifuged (10 000 *g*, 1 min, RT). This was followed by two incubations and washes with SPW buffer containing ethanol (2-3 min) followed by centrifugation (10 000 *g*, 1 min, RT). The column was dried by centrifugation (10 000 *g*, 1 min) and the DNA eluted with

elution buffer (40 µl) and collected in the flow through after centrifugation (10 000 *g*, 1 min, RT).

3.2.6.6 Colony PCR

Recombinant clones of bacterial colonies were screened by colony PCR. Primers (Table 3.1) were used to amplify a 400 bp product from the coding sequence of OpdB. PCR products were analysed by gel electrophoresis. The PCR conditions for 25 cycles were as follows; denaturation (95ºC, 1 min), annealing (55ºC, 1 min), and extension (72ºC, 2 min). Components of the PCR reaction are outlined in Table 3.2.

Table 3.2 PCR reaction components used to amplify a 400 bp product from the coding sequence of OpdB.

PCR reaction component	Volume (µl)	Final concentration
Forward primer (10 µM)	0.5	0.25 µM
Reverse primer (10 µM)	0.5	0.25 µM
10 x PCR reaction buffer	2.0	1 x
$MgCl_2$ (25 mM)	2.0	2.5 mM
Taq	0.25	1U
dNTP mixture	2.0	250 µM
Sterile distilled water	11.75	-
Total	20	

3.2.6.7 Transformation of pET28a into *E. coli* cells

For transformation into *E. coli* JM 109 cells, DNA was combined with electrocompetent cells using the TransformAid^{TM} Bacterial Transformation Kit according to manufacturer's instructions (Fermentas). For transformation into BL21 (DE3) cells, DNA (5 µl) was combined with electrocompetent BL21 (DE3) cells (50 µl) by electroporation in 1.5 mm gap vials (BioRad, Hercules, CA, USA, 1.5 kV, 25 µF, 200 Ω). Immediately following electroporation, cells were resuspended in 2X YT medium without antibiotic and collected by centrifugation (13 000 *g*, 30 s). Most of the supernatant was removed and the cells were resuspended in the remaining supernatant before being plated onto 2X YT agar plates containing kanamycin (30 µg/ml) which were grown overnight at 37ºC.

3.2.6.8 Expression of recombinant oligopeptidase B as a His-tag fusion protein in *E. coli*

2YT medium (100 ml) with kanamycin (30 μg/ml) was inoculated with a single colony of recombinant *E. coli* BL21 (DE3) pET28a-OpdB from *T. congolense* and incubated overnight at 37°C. The overnight cultured cells were collected by centrifugation (5000 *g*, 10 min, RT) and the pellet was resuspended in 50 mM NaH_2PO_4 binding buffer, pH 8 containing 0.5 M NaCl, 10 mM imidazole and 1 mg/ml lysozyme and stored at -20ºC. Prior to purification, the lysate was thawed and sonicated (4 x 10 s) and centrifuged (3000 *g*, 15 min, 4ºC) to pellet the cell debris. The supernatant was transferred to a clean tube. Due to the effects of leaky promoters, the expression of His-tagged OpdB in this system did not make use of IPTG and expression was confirmed by comparison with non-transformed BL21 (DE3) cells.

3.2.7 Purification of recombinant oligopeptidase B by nickel chelate chromatography

The Ni-NTA Purification System consists of Ni-NTA Agarose (Qiagen®). Ni-NTA agarose uses the tetradentate chelating ligand, nitrilotriacetic acid (NTA) that binds Ni^{2+} ion by four coordination sites. The system is designed around the affinity and selectivity of Ni-NTA Agarose for recombinant fusion proteins that are tagged with histidine residues. Nickel is a transition metal and is able to bind electron rich molecules such as histidine which consists of an imidazole ring sructure. This resin exhibits low non-specific binding of other proteins. Bound proteins are eluted by competition with excess imidazole. Proteins may be purified under native or denaturing conditions depending on the solublility of the recombinant protein. In this case, because OpdB was soluble, it was purified under native conditions.

3.2.7.1 Preparation of the Ni-NTA agarose column

Ni-NTA Agarose (1 ml) was pipetted into a conical centrifuge tube (15 ml). The resin was allowed to settle by centrifugation (800 *g*, 1 min, RT) and the supernatant removed. The resin was resuspended in sterile, distilled water (6 ml) with gentle tapping and invertion of the tube. The resin was allowed to settle by centrifugation as done previously and the supernatant removed and discarded. The resin was resuspended in 50 mM NaH_2PO_4 native binding buffer, pH 8 containing 10 mM imidazole (6 ml) and was once again settled by centrifugation. This step was repeated and the supernatant discarded after each step.

3.2.7.2 Purification under native conditions

Lysate (8 ml) was added to a prepared Ni-NTA column (1 ml) and allowed to bind for 1 h at 4ºC by end-over-end mixing. The resin was allowed to settle by centrifugation (800 *g*, 1 min, RT) and the unbound lysate removed and kept for analysis by SDS-PAGE. The resin was washed with 50 mM NaH_2PO_4 native wash buffer, pH 6.5 containing 20 mM imidazole (8 ml) and settled by centrifugation as done previously. The supernatant was removed and kept for SDS-PAGE analysis. This wash step was repeated three more times. The resin was resuspended in 50 mM NaH_2PO_4 native elution buffer, pH 8 containing 250 mM imidazole (10 ml) and transferred to a BioRad Purification Column. Fractions were collected (1 ml) and analysed by SDS-PAGE. The resin was washed with 0.5 M NaOH (10 column volumes) and equilibrated in 50 mM NaH_2PO_4 native binding buffer, pH 8 containing 10 mM imidazole and stored at 4ºC.

3.2.8 Production of anti-oligopeptidase B antibodies in mice

3.2.8.1 Endotoxin removal by EndoTrap®

In the present study, endotoxins were removed from recombinantly expressed proteins using an endotoxin affinity resin called EndoTrap® (Profos AG, Germany), according to manufacturer's specifications. Since the ELISA test for testing the successful removal of endotoxin was prohibitively expensive, an irrelevant antigen, VP2, recombinantly expressed in the same expression system as OpdB, was included as a control antigen.

3.2.8.2 ELISA evaluation of immune kinetics

Mice were used to produce anti-OpdB antibodies. Each group of 10 mice was immunised with OpdB (20 µg) using alum. A control group was immunised with recombinantly expressed viral protease 2 (VP2) (20 µg) also using alum. All mice were immunised intraperitoneally. Boosters were administered monthly. Mice were bled via the tail vein prior to immunisation to collect pre-immune sera and blood samples were collected weekly for analysis by enzyme-linked immunosorbant assay (ELISA). Microtitre plates (NUNC-Immuno™) were coated with 100 ng OpdB in PBS, pH 7.2 (100 µl/well) and incubated overnight at 4ºC. Wells were blocked with 5% (w/v) low fat milk in PBS (150 µl/well, 1 h, RT) and incubated with the respective anti-sera diluted in 0.5% (w/v) BSA-PBS (100 µl/well, 2 h, RT). Horse radish peroxidase-conjugated anti-mouse IgG diluted in 0.5% (w/v) BSA-PBS (1: 10 000; 100 µl/well, 1 h, RT) was used as the detection system. Plates were washed with 0.1% (v/v) PBS-Tween between incubations. The enzyme

reaction was developed with ABTS-H_2O_2 substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2] in 150 mM citrate phosphate buffer, pH 5.0. The optical density at 405 nm was measured using an ELISA microplate reader (FLUOStar OPTIMA, BMG Labtech).

3.2.8.3 Challenge of mice with *T. congolense* (strain IL 1180)

The sustainability of the immune response to OpdB was studied by challenge with *T. congolense* IL 1180. Infected mouse blood collected at the peak of parasitaemia was used to infect both groups of mice at a dose of 10^3 parasites per animal. Mice were bled prior to infection to collect pre-infection sera. Blood samples were collected weekly and analysed by ELISA as described in Section 3.2.8.2. Packed cell volume (PCV). i.e. the proportion of blood volume that is occupied by red blood cells, was monitored following challenge using the capillary microhaematocrit centrifugation to determine the extent of anaemia. Parasitaemia was also monitored during infection. Blood withdrawn from the tail vein was diluted with 1% (w/v) phosphate saline glucose (PSG) and the number of parasites was determined using a Neubauer haemocytometer (Weber, England). This was done weekly. After approximately 1 month infection parasitaemia was only determined fortnightly. Parasites were considered viable when flagellar movement was observed. The latent period, (number of days post-challenge when parasites were first detected in the blood) and persistence span (number of days post-challenge when the infected mice died) were monitored.

3.2.9 Inhibition of oligopeptidase B activity by mouse IgGs

Anti-OpdB IgG in mouse serum collected before and after infection was assayed over time to analyse the inhibition of enzyme activity. Recombinant OpdB (20 ng) was diluted with 0.1% (w/v) Brij and combined with an equal volume of IgG (present in serum) diluted in 200 mM sodium phosphate assay buffer, pH 7.2 containing 4 mM Na_2EDTA, 0.1% (v/v) Tween 20 and 40 µg/ml lima bean trypsin inhibitor to give final concentrations of 1000, 500, 250 and 125 µg/ml. The concentration of IgGs in serum was taken to be approximately 15 mg/ml. Lima bean trypsin inhibitor was added to inhibit any residual Z-Arg-Arg-AMC hydrolysing serum protease activity. Samples were incubated at 37ºC for 15 min. Aliquots were combined with 200 mM sodium phosphate assay buffer, pH 7.2 containing 4 mM Na_2EDTA, 0.1% (v/v) Tween 20 and 8 mM DTT and activated at 37ºC for 1 min. Substrate Z-Arg-Arg-AMC (20 µM diluted from a 1 mM stock made up in DMSO) was added and the microplate was incubated for 5 min at 37ºC. Fluorescence was

read over time (excitation at 360 nm and emission at 460 nm) using a FLUOStar OPTIMA microplate reader and inhibition was expressed as a percentage of the activity of non-immune antibody at the same concentrations.

3.3 RESULTS

3.3.1 Expression and purification of recombinant oligopeptidase B as a GST-fusion protein in *E. coli*

3.3.1.1 Expresion of recombinant oligopeptidase B

Expression of the recombinant OpdB as a fusion protein with glutathione-S-transferase in *E. coli* was successful as shown by SDS-PAGE and western blot (Fig. 3.6). The fusion protein produced by induction with IPTG is seen at the expected size of approximately 106 kDa.

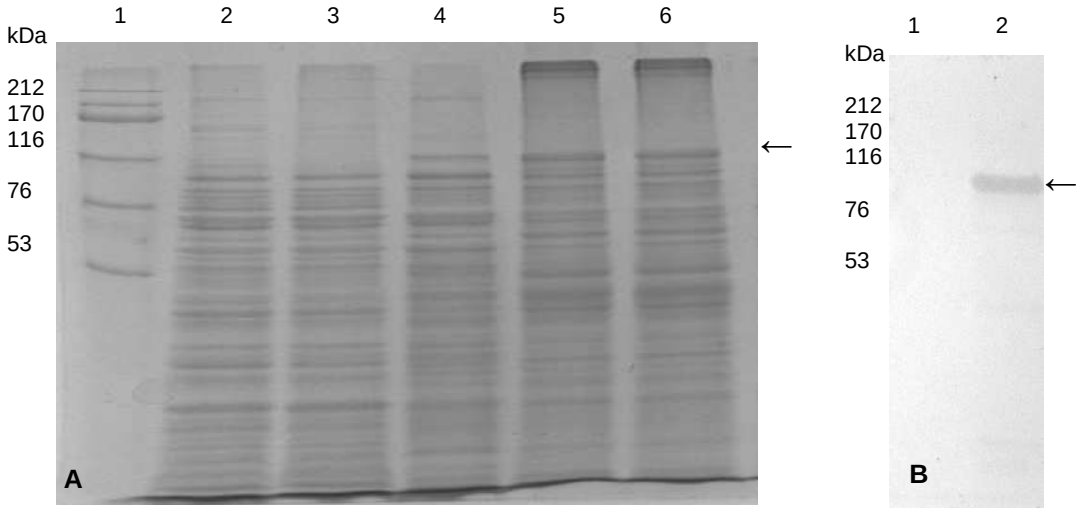


Figure 3.6 Analysis of recombinant OpdB fusion protein in bacterial lysate.
A. SDS-PAGE gel analysis (10%) of recombinant OpdB fusion protein in bacterial lysate. Lane 1, Pharmacia high molecular mass markers; lanes 2 & 3, pGEX4T1-Opd B uninduced (10 µl); lanes 4-6, pGEX4T1-Opd B induced with IPTG (10 µl). Proteins were stained with Coomassie R-250. Arrow indicates position of OpdB-GST fusion protein at 106 kDa.
B. Western blot analysis of recombinant OpdB fusion protein in bacterial lysate Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with mouse anti-Opd B serum (1:500 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, pEX4T1-Opd B uninduced; lane 2, pGEX4T1-Opd B induced with IPTG.

3.3.1.2 Purification of recombinant oligopeptidase B by 'on-column' cleavage

The 106 kDa OpdB-GST fusion protein was purified by affinity chromatography using a glutathione agarose column. The OpdB-GST fusion protein was cleaved with thrombin while still immobilised on the resin to facilitate separation of the recombinant protein from the GST carrier. Oligopeptidase B has an apparent molecular weight of 80 kDa while GST has a molecular weight of 26 kDa. Purification of recombinant OpdB by cleavage of the fusion protein while adsorbed to the glutathione agarose was found to be successful as shown by SDS-PAGE and western blot (Fig. 3.7).

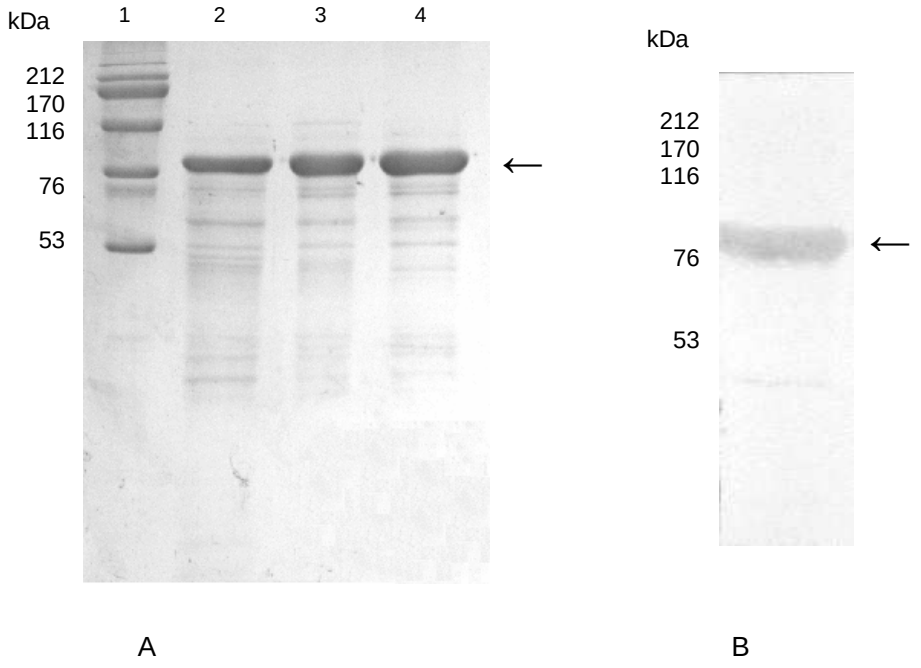


Figure 3.7 Analysis of OpdB purified by glutathione affinity chromatography ('on-column' cleavage).
A. SDS-PAGE analysis (10%) of OpdB purification by glutathione affinity chromatography. Lane 1, Pharmacia high molecular mass markers; lanes 2-4, purified OpdB eluted from the glutathione column (10 µl). Proteins were stained with Coomassie blue R-250.
B. Western blot analysis of OpdB purification by glutathione affinity chromatography. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with mouse anti-Opd B serum (1:500 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Arrows indicate the position of OpdB at 80 kDa.

Oligopeptidase B required further purification as small amounts of contaminating proteins were eluted along with the cleaved protein. Since anti-OpdB antibodies did not recognise the contaminating proteins in the eluted fractions and it was concluded that these proteins were bound to the affinity matrix by non-specific interaction.

3.3.1.3 Purification of recombinant oligopeptidase B by cleavage outside the column

Cleavage of the recombinant oligopeptidase B from GST outside the column was successful as shown by SDS-PAGE and western blot (Fig. 3.8). The purified OpdB is seen at the expected size of approximately 80 kDa. Oligopeptidase B however, required further purification as GST, seen at 26 kDa, remained in solution with the cleaved protein.

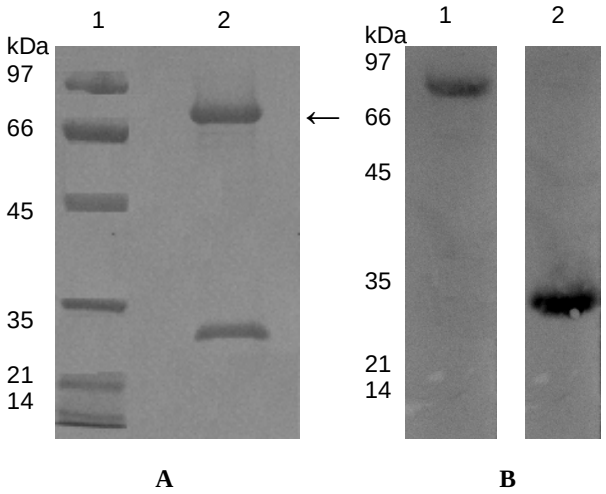


Figure 3.8 Analysis of cleavage of OpdB-GST fusion protein outside the column.
A. SDS-PAGE gel (10%) analysis of OpdB-GST cleavage outside the column Lane 1, BioRad low molecular mass markers; lane 2, cleaved OpdB-GST fusion protein (10 µl). Arrow indicates position of OpdB at 80 kDa. Proteins were stained with Coomassie blue R-250.
B. Western blot analysis of OpdB-GST cleavage outside the column. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with; lane 1, anti-Opd B serum (1:500 dilution) and lane 2, anti-GST monoclonal antibody (1: 10 000 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. Reaction was developed with 4-chloro-1-naphthol/ H_2O_2.

3.3.1.4 Purification of oligopeptidase B by molecular exclusion chromatography (MEC)

Some contaminating proteins were eliminated during the on-column cleavage method by extensive washing of the column. However, this was not sufficiently stringent and MEC was used to further purify OpdB. Oligopeptidase B purified by 'on-column' cleavage was separated from contaminating lower molecular weight proteins on a Sephacryl S-300 HR resin. The elution profile (Fig. 3.9) shows a peak eluting at 245 ml correlating with a size of 80 kDa which is analogous with the expected size for OpdB. Fractions eluted corresponding to this peak that showed activity against the substrate Z-Arg-Arg-AMC were analysed by SDS-PAGE and silver stained. A band at 80 kDa was observed for these fractions (Fig. 3.10).

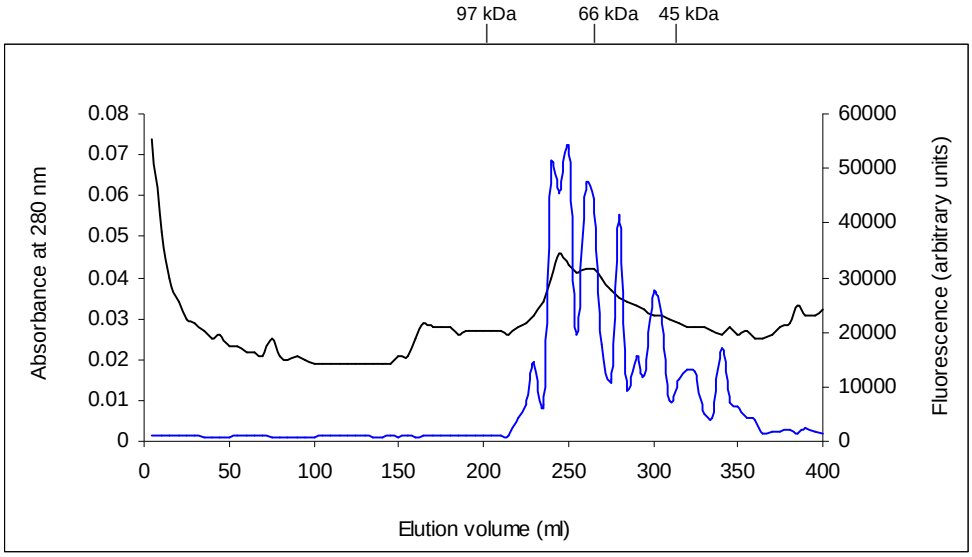


Figure 3.9 Elution profile of molecular exclusion chromatography of OpdB following 'on-column' cleavage on Sephacryl S-300 HR resin. The column was equilibrated with 200 mM Tris-HCl buffer, pH 8 containing 10 mM DTT and 0.02% NaN_3. Sample (3ml) was loaded onto the column and eluted with Tris-HCl buffer. Absorbance at 280 nm (—) and Z-Arg-Arg-AMC activity (fluorescence; arbitrary units, —) were determined for all fractions eluted. The molecular weights of the calibration proteins are indicated at the top.

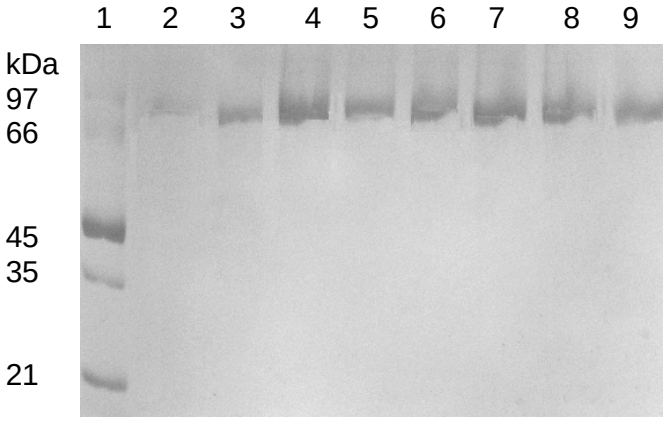


Figure 3.10 SDS-PAGE gel analysis (10%) of OpdB fractions eluted by Sephacryl S-300 HR MEC. Lane 1, BioRad low molecular mass markers; lanes 2-9 Fractions active against Z-Arg-Arg-AMC corresponding to the elution peak from Sephacryl S-300 MEC column (10 µl). Proteins were visualised by silver staining.

MEC was also used to separate the contaminating GST from OpdB on a Sephacryl S-200 resin following OpdB-GST fusion protein cleavage outside the GST column. The elution profile (Fig. 3.11) shows two peaks eluting at 56 ml and 112 ml corresponding to sizes of approximately 85 kDa and 22 kDa respectively which are analogous with the expected

sizes of 80 kDa for OpdB and 26 kDa for GST. Fractions from the first elution peak showed activity against the substrate Z-Arg-Arg-AMC and were analysed by SDS-PAGE and visualised by silver staining. A band at 80 kDa was observed for fractions eluted corresponding to the first peak (Fig. 3.12).

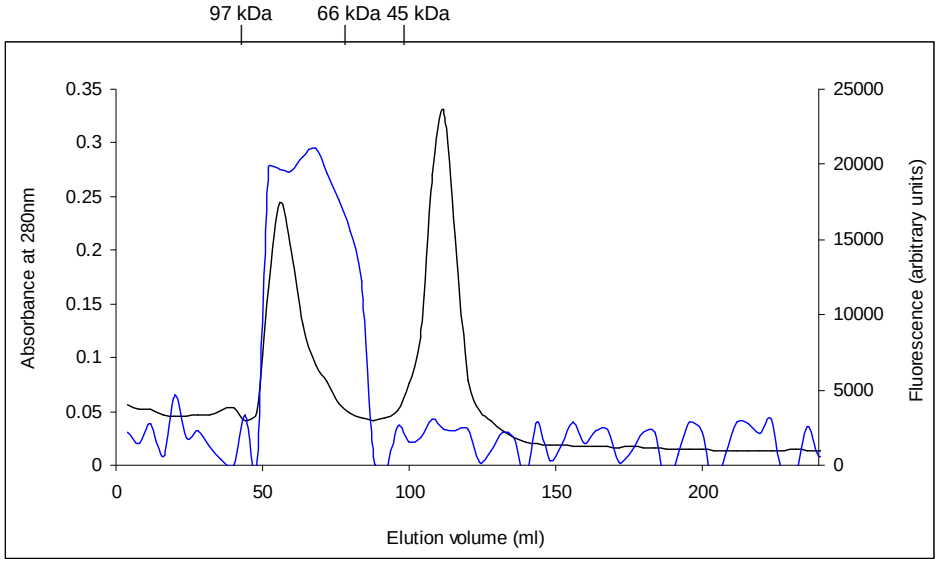


Figure 3.11 Elution profile of molecular exclusion chromatography of OpdB following cleavage outside the column on Sephacryl S-200 HR resin. The column was equilibrated with 200 mM Tris-HCl buffer, pH 8 containing 10 mM DTT and 0.02% NaN_3. Sample (4ml) was loaded onto the column and eluted with Tris-HCl buffer. Absorbance at 280 nm (—) and Z-Arg-Arg-AMC activity (fluorescence; arbitrary units, —) were determined for all fractions eluted. The molecular weights of the calibration proteins are indicated at the top.

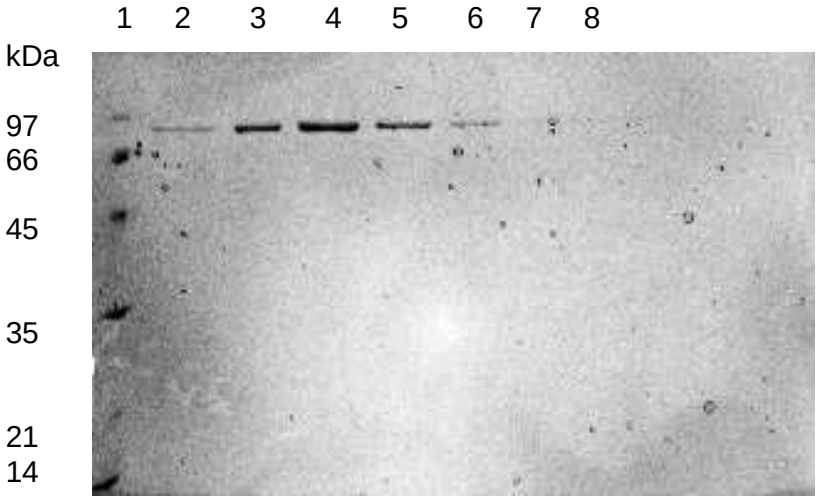


Figure 3.12 SDS-PAGE gel analysis (10%) of OpdB fractions eluted by Sephacryl S-200 HR MEC. Lane 1, BioRad low molecular mass markers; lanes 2-8, Fractions active against Z-Arg-Arg-AMC corresponding to the first elution peak from Sephacryl S-200 MEC column (10 µl). Proteins were visualised by silver staining.

3.3.1.5 Concentration of oligopeptidase B

All fractions containing OpdB eluted from MEC were pooled and concentrated using the Amicon concentration cell (Fig. 3.13).

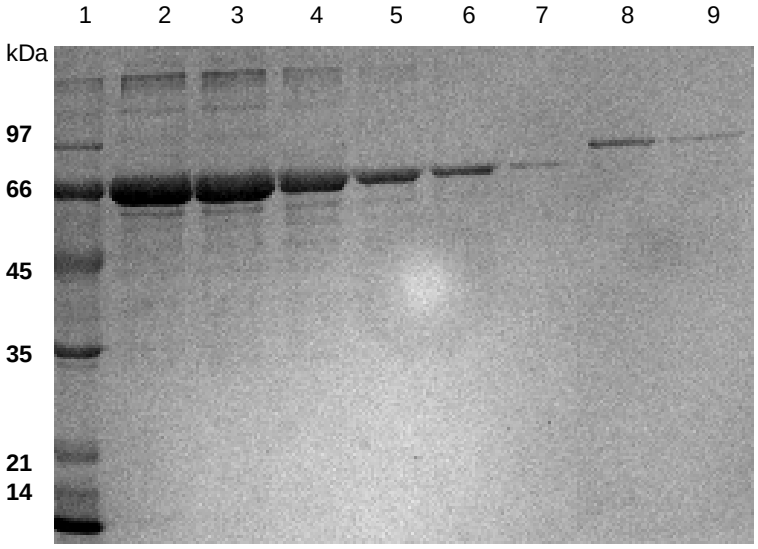


Figure 3.13 SDS-PAGE gel analysis (10%) of OpdB fractions concentrated by the Amicon concentration cell. Lane 1, BioRad low molecular mass markers; lanes 2-7 BSA at 2, 1, 0.75, 0.5, 0.25, 0.125 mg/ml; lane 8, concentrated OpdB eluted from Sephacryl S-200 HR resin (10 µl); lane 9, concentrated OpdB from Sephacryl S-300 HR resin (10 µl). Proteins were stained with Coomassie blue R- 250.

Fractions pooled from the Sephacryl S-300 HR resin were concentrated to 0.125 mg/ml (Fig. 3.13, lane 9). Before MEC, the concentration of OpdB loaded onto the column was approximately 1 mg/ml. Fractions pooled from MEC (Sephacryl S-200 HR resin) were concentrated to approximately 0.25 mg/ml (Fig. 3.13, lane 8) and prior to loading onto the column, the concentration of OpdB was 0.54 mg/ml. Due to the tedious manner in which OpdB was required to be purified from the pGEX4T1 bacterial system and the low yield of recombinant protein obtained following MEC, expression in another vector which could be purified by a simpler method was proposed. Hence, OpdB was sub-cloned into the pET28a expression vector for expression as a His-tag fusion protein that could be purified by nickel chelate chromatography.

3.3.2 Expression and purification of recombinant oligopeptidase B as a His-tag fusion protein in *E. coli*

3.3.2.1 Sub-cloning of the oligopeptidase B gene into pET28a

The pET28a plasmid (cut with *Eco*RI and *Not*I) and OpdB insert DNA (also cut with *Eco*RI and *Not*I) were analysed by agarose gel electrophoresis (Fig. 3.14) to determine the relative quantities of vector and insert DNA. The vector can be seen at size of 5 kb (lane 2) and the OpdB insert DNA can be seen at approximately 2.1 kb (lane 3).

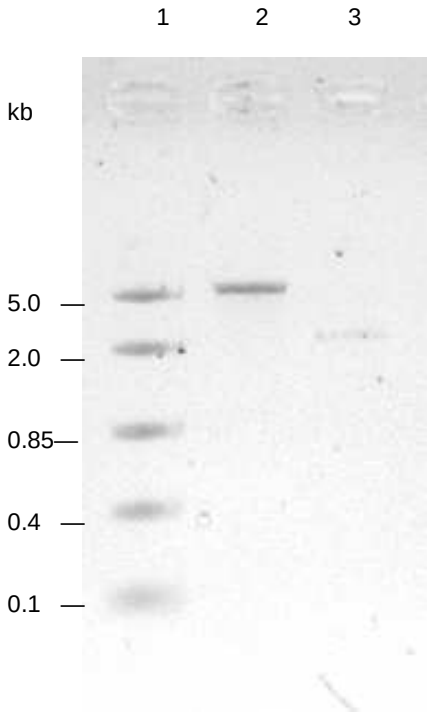


Figure 3.14 Agarose gel (1%) electrophoresis analysis of *Eco*RI and *Not*I digestion of pET28a and OpdB insert DNA for the determination of the relative quantities of DNA to be used for ligation. Lane 1, Middle Range DNA markers; lane 2, pET28a (1 µl); lane 3, OpdB insert DNA (1 µl). Vector and insert DNA were digested with *Eco*RI and *Not*I. DNA was visualised with ethidium bromide.

Vector and insert DNA were added in a 1:3 ratio and combined with DNA dilution buffer and T4 DNA ligase. The ligated vector and insert was transformed into *E. coli* JM 109 cells and colonies were screened by colony PCR for recombinants using specific OpdB primers (Table 3.1). All ten colonies tested were shown to be recombinant as shown by the presence of a 400 kb PCR product upon analysis by agarose gel electrophoresis (Fig. 3.15).

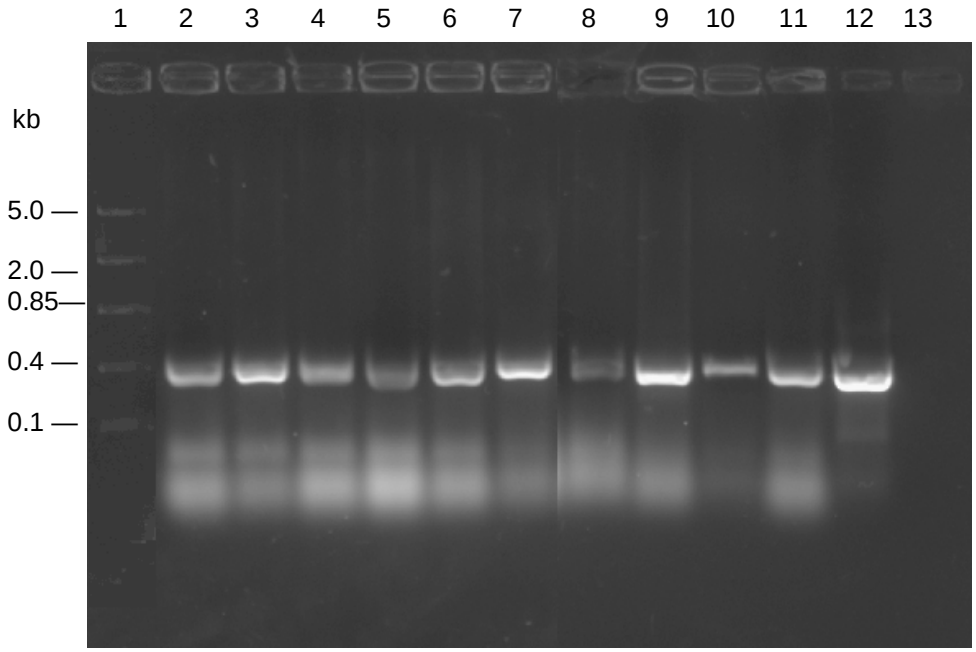


Figure 3.15 Agarose gel (1%) electrophoresis analysis of screening for recombinant OpdB-pET28a clones after transformation into *E. coli* JM 109 cells. After colony PCR, samples were analysed by agarose gel electrophoresis. Lane 1, Middle range DNA markers; lanes 2-11, PCR of 10 individual colonies; lane 12, positive PCR control; lane 13, negative PCR control (excludes template DNA).

Two of the positive clones were assessed for the presence of the insert by miniprep and restriction digestion with *Eco*RI and *Not*I. Recombinant plasmid was then transformed into *E. coli* BL21 (DE3) cells for expression.

3.3.2.2 Expression of recombinant oligopeptidase B as a His-tag fusion protein in *E. coli*

Following expression of the recombinant OpdB as a His-tagged fusion protein in *E. coli*, cells were harvested, lysed and analysed by SDS-PAGE. The fusion protein (shown by the arrow) is seen at the expected size of approximately 80 kDa (Fig. 3.16).

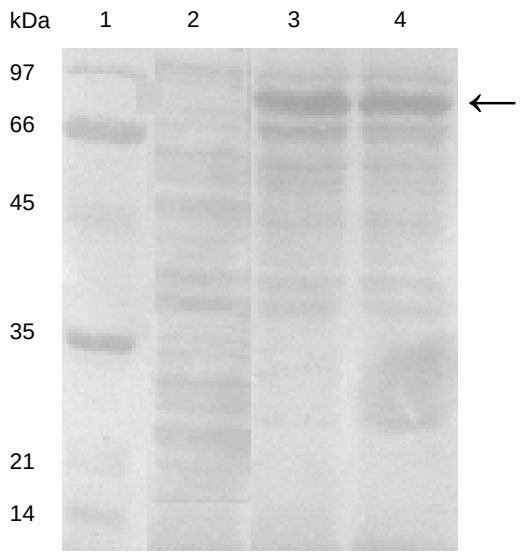


Figure 3.16 SDS-PAGE gel analysis (10%) of recombinant OpdB-His-tag fusion protein expression in bacterial lysate. Lane 1, BioRad low molecular mass markers; lane 2, non-transformed BL21 (DE3) cells; lanes 2-3, OpdB-His-tag fusion protein. Proteins were stained with Coomassie R-250. Arrow indicates the fusion protein at 80 kDa.

Western blotting of the bacterial lysate with anti-His tag monoclonal antibody confirmed the expression of OpdB at the expected size of 80 kDa (Fig. 3.17).

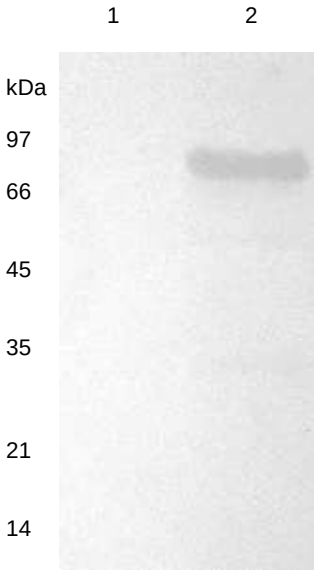


Figure 3.17 Western blot analysis of recombinant OpdB-His-tag fusion protein in bacterial lysate. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with anti-His monoclonal antibody (1:1000 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG (1:12 000 dilution). Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, non-transformed BL21 (DE3) cells; lane 2, OpdB His-tag fusion protein.

3.3.2.3 Purification of recombinant oligopeptidase B by nickel chelate chromatography

The recombinant OpdB His-tagged fusion protein was purified by nickel chelate chromatography under native conditions. The purified protein was analysed by SDS-PAGE with silver staining and protein corresponding to purified OpdB could be seen at an expected size of 80 kDa (Figure 3.18).

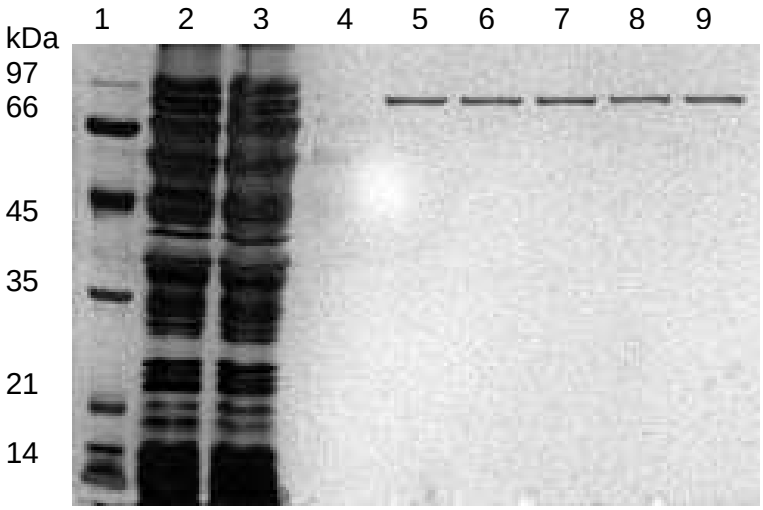


Figure 3.18 SDS-PAGE gel analysis (10%) of recombinant OpdB-His-tagged fusion protein purification from bacterial lysate by nickel chelate chromatography. Lane 1, BioRad low molecular mass markers; lane 2, bacterial lysate before cycling over Ni-NTA resin; lane 3, bacterial lysate after cycling over Ni-NTA resin; lane 4, native wash; lanes 5-9, fractions eluted from Ni-NTA resin.

3.3.3 ELISA evaluation of antibody production in mice

Purified recombinant OpdB (20 μg) in alum was used to immunise mice over a 12 week period. Mice received three booster immunisations at 1 month intervals and were bled weekly. Non-immune control serum was collected before immunisation. Specific antibodies produced against recombinant OpdB in mice was monitored by ELISA (Fig. 3.19). High levels of antibodies against OpdB were detected approximately 30 days following the first immunisation and increased substantially over the immunisation period. Antibody production appeared to peak initially between days 40-60 (weeks 5 and 6) of the immunisation protocol and continued to increase thereafter.

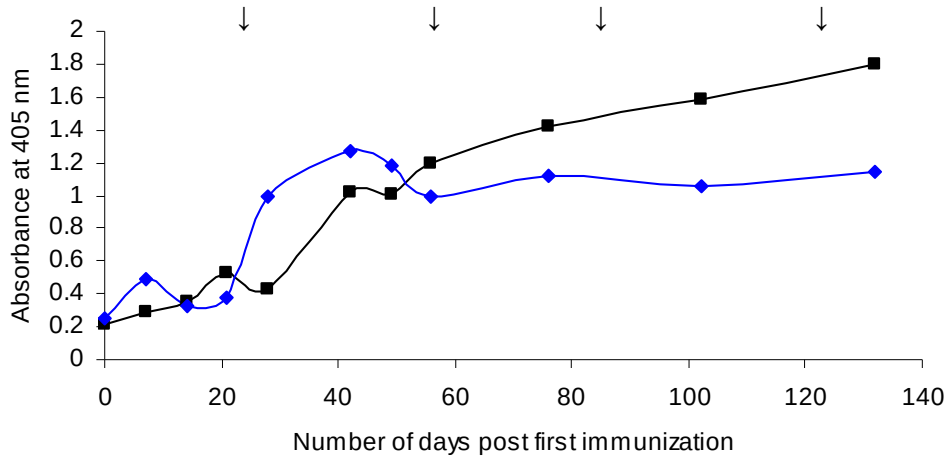


Figure 3.19 ELISA showing immune response in mice. Antibody production was monitored in mice immunised with OpdB (■), and VP2 as a control (◆). OpdB or VP2 was coated onto ELISA plates (100 ng/well), incubated with sera collected weekly following the first immunisation at a dilution of 1:500 (inBSA-PBS, 100 µl/well, 2 h, RT) and detected by horse anti-mouse IgG HRPO-conjugate and ABTS/H_2O_2 (100 µl/well). Absorbance readings at 405 nm represent the average of duplicate experiments. Arrows (↓) indicate when booster immunisations were given.

Following challenge with *T. congolense* (IL 1180) infected mouse blood, a booster effect in the immune response was observed for all mice immunised against OpdB (Fig. 3.20). Mice immunised against VP2 did not show a booster effect. A slight decline in the VP2 immune response was observed following trypanosome challenge.

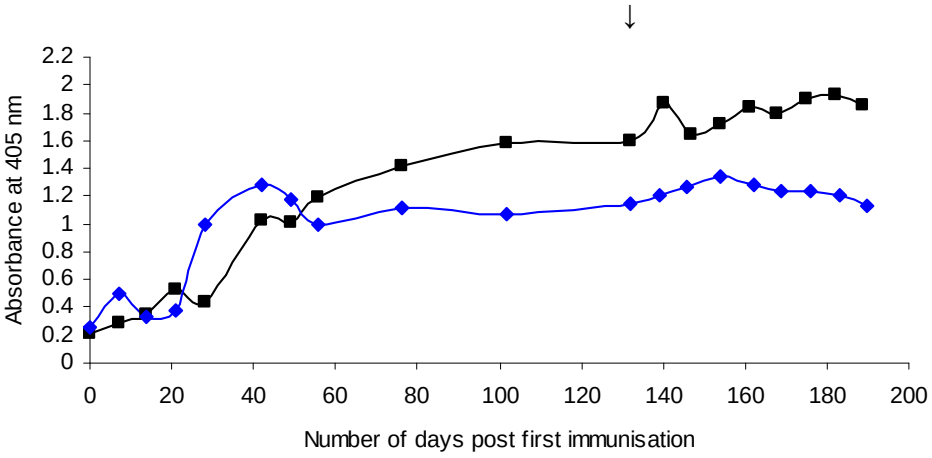


Figure 3.20 ELISA showing immune response in mice following challenge with *T. congolense* (IL 1180). Antibody production was monitored for mice immunised with OpdB (■), and VP2 as a control (◆). OpdB or VP2 was coated onto ELISA plates (100 ng/well), incubated with sera collected weekly following the first immunisation at a dilution of 1:500 (in BSA-PBS, 100 µl/well, 2h, RT) and detected by horse anti-mouse IgG HRPO-conjugate and ABTS/H_2O_2 (100 µl/well) was used as the detection system. Absorbance readings at 405 nm represent the average of duplicate experiments. Arrow (↓) indicates the time of trypanosome challenge.

3.3.4 Western blotting evaluation of anti-OpdB antibodies

Anti-OpdB antibodies in the pooled serum collected weekly during the immunisation period was used to probe recombinant bacterial lysate to determine the specificity of the antibodies. A single band at 80 kDa was observed (Fig. 3.21).

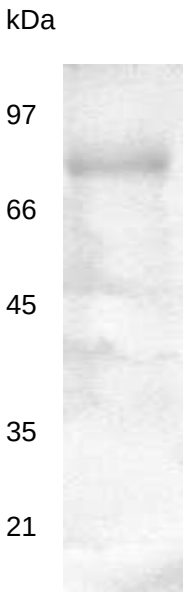


Figure 3.21 Western blot analysis to determine recognition of OpdB in bacterial lysate by pooled serum. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with anti-OpdB serum (1:500 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, BioRad low molecular mass markers; lane 2, pET28a-OpdB bacterial lysate.

Anti-OpdB sera from individual mice were used to probe recombinant OpdB in bacterial lysate samples (Fig. 3.22) and native OpdB in *T. congolense* parasite lysate samples (Fig. 3.23). Lanes 1-10 represented sera obtained from mice 1-10 while lane 11 was a control using pooled non-immune serum. A band at 80 kDa, corresponding to OpdB was observed for eight out of ten blots for recombinant and native lysate samples. Some sera appeared to have recognised additional bands in both the recombinant bacterial lysate and in the *T. congolense* parasite lysate. Serum used to probe recombinant bacterial lysate (Fig. 3.22) and parasite lysate (Fig. 3.23) in lane 7 showed no recognition of OpdB. Similarly, no recognition of OpdB was observed for serum used to probe recombinant bacterial lysate in lane 3 (Fig. 3.22) and parasite lysate in lane 5 (Fig. 3.23).

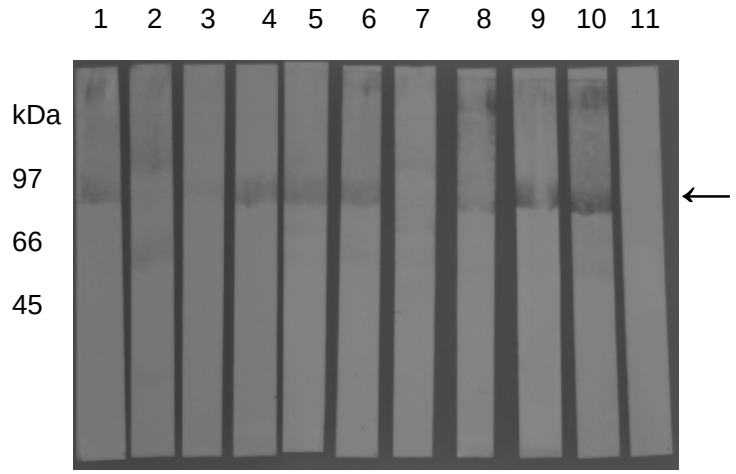


Figure 3.22 Western blot analysis to determine recognition of recombinant OpdB in bacterial lysate by sera from individual mice. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with individual anti-Opd B sera (lanes 1-10) and non-immune serum (lane 11) (1:500 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. The reaction was developed with 4-chloro-1-naphthol/ H_2O_2. Arrow indicates OpdB at 80 kDa.

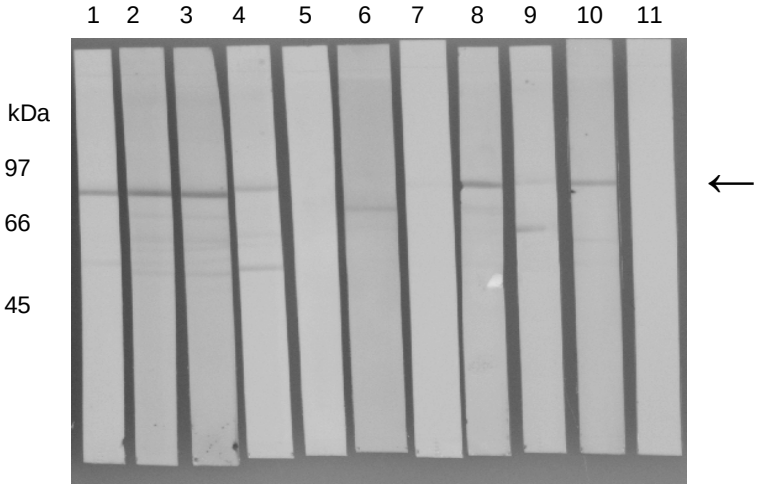


Figure 3.23 Western blot analysis to determine recognition of native OpdB in *T. congolense* parasite lysate by individual mouse serum. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with individual anti-OpdB sera (lanes 1-10) and non-immune serum (lane 11) (1:500 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. The reaction was developed with 4-chloro-1-naphthol/ H_2O_2. Arrow indicates OpdB at 80 kDa.

Anti-OpdB sera from individual mice following challenge with *T. congolonse* were used to probe recombinant OpdB in bacterial lysate samples and *T. congolense* parasite lysate samples to determine recognition of recombinant and native OpdB. A band at 80 kDa, corresponding to OpdB was observed for all blots. All sera recognised recombinant OpdB in the bacterial lysate (Fig. 3.24) and native OpdB in the *T. congolense* parasite lysate (Fig. 3.25).

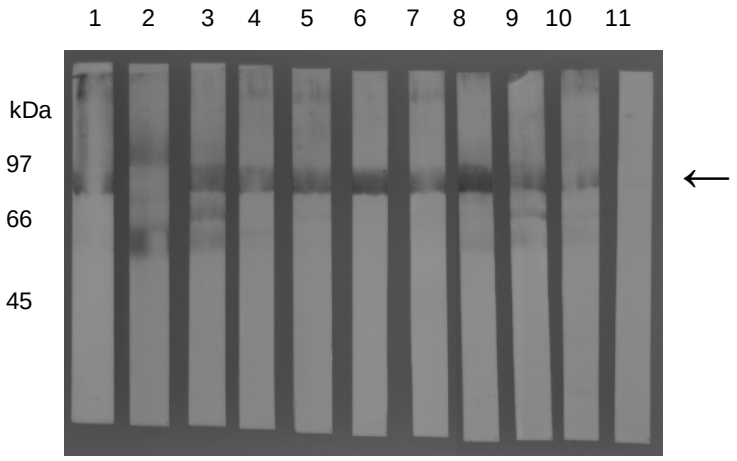


Figure 3.24 Western blot analysis to determine recognition of recombinant OpdB in bacterial lysate by sera from individual mice following trypanosome challenge. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with individual anti-Opd B sera (lanes 1-10) and non-immune serum (lane 11) (1:500 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. The reaction was developed with 4-chloro-1-naphthol/ H_2O_2. Arrow indicates OpdB at 80 kDa.

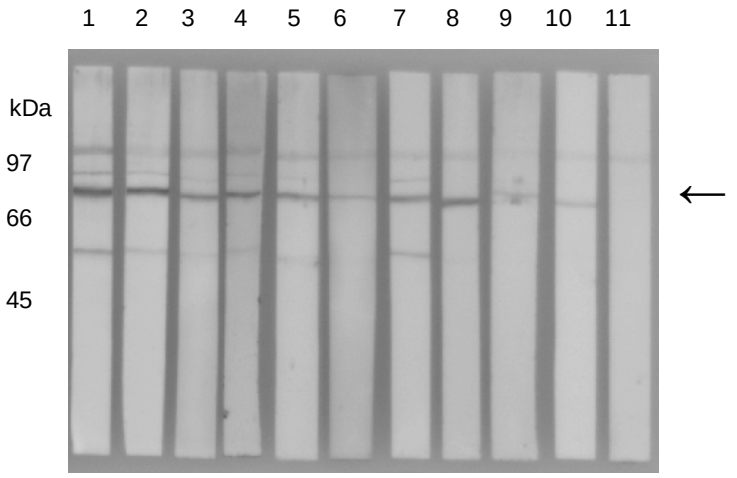


Figure 3.25 Western blot analysis to determine recognition of native OpdB in *T. congolense* parasite lysate by sera from individual mice following trypanosome challenge. Protein was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with individual anti-OpdB sera (lanes 1-10) and non-immune serum (lane 11) (1:500 dilution). Secondary antibody was HRPO-conjugated horse anti-mouse IgG. The reaction was developed with 4-chloro-1-naphthol/ H_2O_2. Arrow indicates OpdB at 80 kDa.

The recognition of OpdB was observed to be more intense following challenge and numerous other bands were recognised by most serum samples. Following trypanosome challenge and the subsequent increase in circulating immune complexes in sera, various other proteins apart from OpdB were also recognised in the parasite lysate.

3.3.5 Inhibition of OpdB activity by mouse IgGs present in serum

Pooled serum collected before and after infection was assessed for the capacity of IgGs present in the serum to inhibit the hydrolysis of Z-Arg-Arg-AMC by OpdB. Fluorescence was read continuously over a 20 min period. The resulting initial rates of the enzymatic reaction were used to determine the inhibitory activity of the antibodies. The inhibition of OpdB by antibodies present in sera is illustrated in Fig. 3.26.

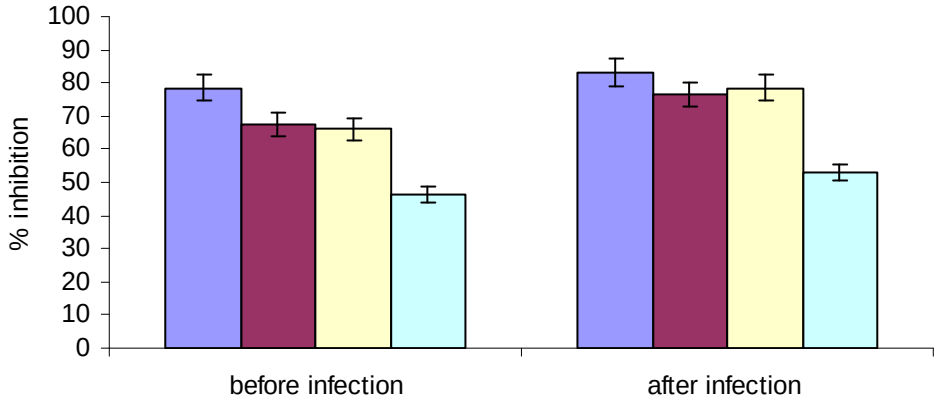


Figure 3.26 Inhibition of OpdB activity against Z-Arg -Arg-AMC by mouse IgGs present in sera collected before and after infection. Inhibition of OpdB hydrolysis of Z-Arg-Arg-AMC by anti-OpdB sera was determined at 1000 µg/ml (■), 500 µg/ml (■), 250 µg/ml (■) and 125 µg/ml (■) and was expressed as a percentage inhibition relative to OpdB activity in the presence of non-immune sera control (0% inhibition). Each bar represents the average % inhibition of each group and is the mean of duplicate experiments.

IgGs present in sera collected before and after infection were all observed to inhibit OpdB activity to some extent. A maximum inhibition of 83% was observed at 1000 µg/ml for IgGs present in sera after challenge with trypanosomes while the least inhibition was 49% at 125 µg/ml for IgGs present in sera before challenge. In general, greater inhibition was observed at the higher concentrations of serum IgG and sera following challenge showed greater inhibition of OpdB activity than sera prior to challenge.

3.3.6 Challenge of mice with *T. congolense*: Parasitaemia and survival rate

During infection, blood withdrawn from the tail vein was diluted with 1% (w/v) phosphate saline glucose (PSG) and the number of parasites was determined using a Neubauer haemocytometer (Weber, England). Parasites were considered viable when flagellar movement was observed. No substantial difference in parasitaemia was noted between mice immunised with OpdB and mice immunised with VP2 (Fig. 3.27). Parasitaemia

increased over the infection period and remained relatively stable from 30 days post infection onwards.

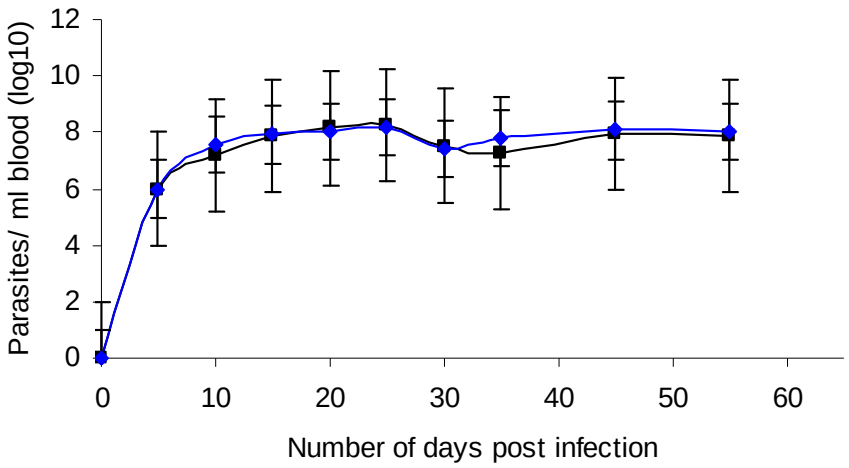


Figure 3.27 Parasitaemia during *T. congolense* infection. Mice were infected with 10^3 parasites/mouse and the parasitaemia was monitored for mice immunised with OpdB (■) and VP2 (◆) using a Neubauer haemocytometer.

Since all mice became infected no protection was conferred by immunisation with OpdB. However, mice immunised with OpdB had an increased latent period and persistence span. On average, mice immunised with OpdB became latent nine days post infection, while mice immunised with VP2 became infected seven days post infection. Mice immunised with OpdB survived on average 15 days longer than control mice immunised with VP2. The survival of mice immunised with OpdB was observed to be approximately 40 % greater (Fig. 3.28). At the time point when all control mice had died, four out of a total of ten mice immunised with OpdB had still survived. After challenge with *T. congolense*, PCV dropped from 55-60% to 40% for mice immunised with OpdB and VP2. Following two months infection, PCV for mice immunised with OpdB increased to 45%.

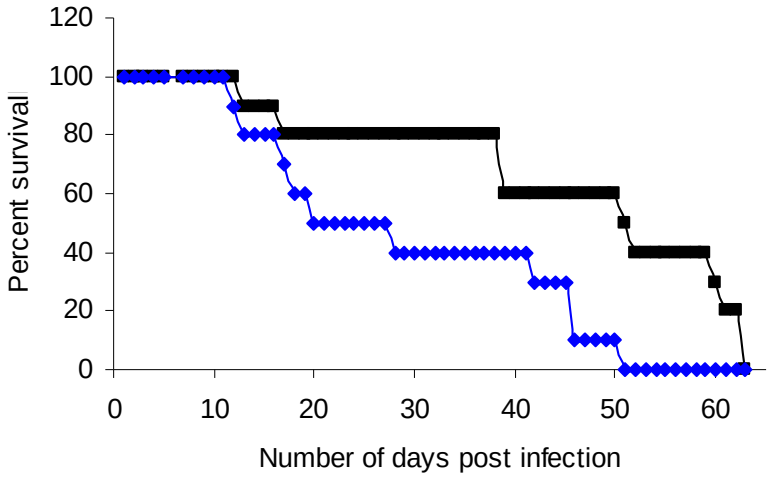


Figure 3.28 Survival curves for mice immunised with OpdB and VP2. The survival of mice immunised with OpdB (■) and VP2 (◆) was recorded during the infection period.

3.4 DISCUSSION

The impact of trypanosomiasis on livestock in sub-Saharan Africa has led to the need for improved disease control methods. It is well accepted that pathogenic factors of the parasite contribute to the development of infection (Tizard *et al*., 1978; Taylor and Authié, 2004) and it is imperative that this relationship between the trypanosome parasite and its natural host is understood for the development of a vaccine. Parasite components such as proteolytic enzymes have long been known to play significant roles in host-parasite interactions (North *et al*., 1990). Oligopeptidase B, a serine protease of African trypanosomes has been implicated as a pathogenic factor (Morty *et al*., 2001) and a therapeutic agent (Morty *et al*., 1998, 2000) of the disease. It is known to degrade regulatory peptides of the host and contribute to the development of the infection (Troeberg *et al*., 1996; Morty *et al*., 2001, 2005a). For this reason the antigenicity of OpdB was studied as a potentially useful candidate in the development of a vaccine to combat trypanosomiasis.

In the present study OpdB was successfully expressed in the pGEX4T1 bacterial system. Expression is under the control of the *tac* promoter, which is induced by the lactose analog isopropyl β-D thiogalactoside (IPTG). The *lac* gene product is a repressor protein that binds to the operator region of the *tac* promoter, preventing expression until induction by IPTG. In this way, control over expression of the fusion protein is maintained (De Boer *et al*., 1983).

SDS-PAGE analysis confirmed the expression of OpdB as a fusion protein with glutathione-S-tranferase (GST) at a size of 106 kDa. Carrier proteins are often introduced in the expression vector to act as tags for the gene product to assist in the purification of the expressed protein (Murby *et al*., 1996). pGEX vectors express fusion proteins with a glutathione-S-transferase-tag which can be easily purified on an affinity column consisting of a glutathione agarose matrix. GST was readily cleaved from OpdB by thrombin while adsorbed onto the glutathione agarose column as described by Smith and Johnson (1988). In the pGEX4T1 vector, the inclusion of a cleavage site for a specific protease allows separation of the two components of the fusion protein. The proteolytic cleavage site in this vector is specific for thrombin and hydrolyses the bond between the Arg and Gly residues in the sequence Leu-Val-Pro-Arg-Gly-Ser (Smith and Johnson, 1988). Other proteases that may be used include Factor X_a that is used in other pGEX vectors which has its own specific proteolytic cleavage site (Eaton *et al*., 1986).

Cleavage using thrombin is a relatively rapid and efficient technique that requires no further purification. However, some contaminating proteins were co-eluted with OpdB. During the expression of a foreign protein some degradation of the protein by host proteases may be expected to occur. In the current study the presence of contaminating proteins was not a result of OpdB degradation by host proteases as analysis by western blotting using antibodies specific to OpdB showed only one band at approximately 80 kDa. The contaminating bands may be due to over-sonication of the lysate and the subsequent co-purification of host proteins with the fusion protein (Smith and Johnson, 1998). Efforts were made to use shorter bursts of sonication and the lysate was kept on ice during this process. Despite this, contaminating bands were still observed. As a result an additional purification step was used. Purification by MEC using the Sephacryl S-300 HR matrix resulted in electrophoretically pure OpdB. The Sephacryl S-300 HR resin has a fractionation range of 10-1500 kDa. Due to the numerous contaminating bands, many of which were of similar lower molecular weight, a resin with a large fractionation range and high resolving power (Dennison, 1999) was used.

The GST-OpdB fusion protein was also directly eluted from the glutathione column to asses the efficiency of cleavage outside the column. Cleavage outside the column resulted in significantly purer protein that showed no contamination with host proteins. Oligopeptidase B and the GST tag were easily separated by MEC using the Sephacryl

S-200 HR matrix. The S-200 HR resin has a fractionation range of 5-250 kDa. Unlike the 'on-column' cleavage method, where OpdB was eluted with many contaminating proteins, cleavage outside the column showed only two major proteins that were easily separated on a column with a smaller fractionation range. The components of the fusion protein following cleavage were confirmed as GST and OpdB by western blot analysis using anti-GST and anti-OpdB antibodies.

Expression in the pGEX4T1 system was shown to be efficient, however SDS-PAGE analysis following concentration showed OpdB to be present in very low amounts when compared to the OpdB generated from 'on-column' cleavage or from cleavage outside the column prior to MEC. The tedious manner in which this protein was required to be purified to yield only small amounts of protein led to consideration of the expression of OpdB in another system which required simpler purification methods.

OpdB was sub-cloned into the pET28a expression vector in the hope that larger amounts of protein would be purified more easily. OpdB was successfully sub-cloned into the pET28a system as confirmed by restriction digestion and was also successfully expressed in culture. Expression in the pET system is under the under the control of the bacteriophage T7 promoter. Unlike systems based on *E. coli* promoters (e.g: *lac* gene in pGEX4T1), the pET system uses the bacteriophage T7 promoter to direct the expression of target genes. Since *E. coli* RNA polymerase does not recognise the T7 promoter, there is no transcription of the target gene in the absence of a source of T7 RNA polymerase. The construct is therefore transformed into BL21 (DE3) cells which have the T7 RNA polymerase necessary for transcription (Mierendorf *et al*., 1994). Expression in the pET28a system was confirmed by western blotting of bacterial lysate and probing with an anti-histidine tag monoclonal antibody. Using a Ni-NTA agarose column, OpdB was successfully purified under native conditions due to the soluble nature of the protein and required no further purification as determined by SDS-PAGE and silver staining. Larger amounts of protein were not necessarily obtained when using the pET28a expression system and purification method, but the additional step of further purification by MEC was eliminated. In this way, a comparable amount of OpdB was produced in a shorter time frame. Since the histidine tag is very small in size and does not affect the size of protein, it was not necessary to remove it from the purified protein. The histidine tag is too small to be immunogenic and therefore would not interfere with the immune response of animals

immunsed with OpdB. In addition, Morty *et al.* (1999, 2005a) showed unaffected enzyme activity for recombinant *T. b. brucei* and *T. evansi* OpdB expressed with an N-terminal histidine tag. Expression in the pET system and the generation of a histidine-tagged fusion protein that does not require removal, for the purposes of this study, also eliminated the overnight incubation with enzyme to remove the fusion tag.

The ELISA results presented showed the production of anti-OpdB antibodies. High titres of antibody were apparent for both immunised groups from 5-6 weeks during the immunisation period. Mice immunised with the control protein, VP2 showed consistently high levels of antibody production that appeared to reach a constant titre from week 6 onwards. Antibody production against OpdB showed a constant increase following week 6. OpdB elicited a suitable immune response since high levels of specific antibodies were produced in all immunised mice.

Serum collected from individual mice was analysed by western blot to determine whether antibodies present in the sera were able to recognise recombinant OpdB from bacterial lysate as well as native OpdB from parasite lysate. Oligopeptidase B has previously been isolated from *T. b. brucei* (Troeberg *et al.*, 1996), *T. cruzi* (Burleigh *et al.*, 1997), *T. congolense* (Morthy *et al*., 1999) *and T. evansi* (Morty *et al*., 2005a) where it is expressed in all life cycle stages. However, in the present study whole *T. congolense* lysate was used. Prior to infection, eight out of a total of ten samples of mouse serum were able to recognise both recombinant and native protein. The two samples showing no recognition of recombinant and native OpdB were not from the same mouse. The inability to recognise OpdB may be due to individual serum samples containing lower levels of anti-OpdB antibodies that may take longer to interact with denatured OpdB.

Following infection, all mice sera showed recognition of both recombinant and native OpdB. Other protein bands were detected by individual mouse sera in the parasite lysate. This is expected since sera from infected animals contain antibodies and immune components against circulating antigens present during the infection (Taylor, 1998). However, the most prominent response to be expected is against OpdB as the mice had a pre-existing immune response against this antigen prior to trypanosome challenge. This was also observed by Katzenback *et al*., (2008) for immunisation with recombinant

β-tubulin. Immunised animals showed a more effective immune response during following challenge due to previous administration of β-tubulin.

A booster effect from trypanosome challenge was observed for mice immunised against OpdB (Fig. 3.20). OpdB has been shown to be released into the host system by dying trypanosomes where it retains its full catalytic activity as it is resistant to host serum inhibitors (Troeberg *et al.*, 1996; Morty *et al.*, 2001, 2005a). In the present study OpdB would be released into the system of infected mice by dead or dying parasites. No booster effect was observed for the VP2 control group. Viral protease 2 is unrelated to trypanosomiasis infection. For this reason we did not expect to see a boost in the immune response for this protein.

During the infection period, parasitaemia was maintained in similar ways for mice immunised with VP2 and OpdB. No significant difference was observed. A key factor to be monitored during infection is the ability of animals to control the initial parasitaemia and the resistance to the development of anaemia (Vickerman *et al.*, 1993). Although parasitaemia was maintained in a similar way for both groups, the severity of the infection cannot be determined by measuring parasitaemia. *T. congolense* is not able to invade tissues as it is an extracellular parasite but has been reported to attach to the endothelial cells lining the blood vessels (Banks, 1978). Parasites circulating in the blood are therefore not a true representative of the entire population. Anaemia is the most prominent characteristic of animal trypanosomiasis and with prolonged infection the role of the parasite in the maintenance of anaemia becomes less significant (Vickerman *et al.*, 1993). Animals may be intermittently parasitaemic or aparasitaemic.

Packed cell volume (PCV) is the proportion of blood volume that is occupied by red blood cells and in mammals is dependent on body size (Purves *et al.*, 2004). In bovine trypanosomiasis, PCV may fall from a normal 30% or above to 15% following 2-3 months of infection with *T. congolense*, indicating the development of anaemia (Taylor and Authié, 2004). Prior to infection PCV for mice immunised against OpdB and VP2 ranged from 55-60%. Following infection a general PCV of 40% was observed for both groups. Over the period of infection this level remained relatively stable at 40% for mice immunised with VP2 while the packed cell volume for mice immunised with OpdB was observed to increase to 45% following two months of infection.

Although mice immunised with OpdB were not able to resist the development of the infection, leukocyte counts were partially recovered. In addition, mice immunised with OpdB had a greater persistence span and were observed to survive 40% longer than control mice. Mice that were not immunised with OpdB were unable to build up a rapid protective immune response and this resulted in their higher mortality (Katzenback *et al*., 2008). These combined effects suggest a delay in the onset and development of anaemia which was induced by anti-OpdB antibodies. It is well known that OpdB degrades atrial natriuretic factor which causes elevated blood volume, a key characteristic of trypanosome infection (Troeberg *et al*., 1996; Morty *et al*., 2001).

Inhibition of recombinant OpdB by anti-OpdB antibodies, present in sera collected prior to and after infection, using Z-Arg-Arg-AMC showed good inhibition at antibody concentrations of 1000, 500, 250 and 125 µg/ml. Anti-OpdB IgGs present in sera after infection showed maximum inhibition of 83%. A maximum inhibition of 92% has been reported for the inhibition of OpdB from *T. b. brucei* against Z-Arg-Arg-NHMec by anti-OP-Tb antibodies made in chickens (Morty *et al*, 2001). Huson (2006) also reports 100% inhibition of *T. congolense* OpdB against Z-Arg-Arg-AMC by antibodies produced in rabbits.

The antigenicity of other intracellular parasite antigens has also been described. Lubega *et al*. (2002) showed that mice immunised with native tubulin, a cytoskeletal protein of the parasite, were able to produce anti-tubulin specific antibodies and could be protected after *T. brucei* and *T. congolense* challenge. The same response was also observed when Li *et al*. (2007) used recombinant beta-tubulin of *T. evansi* for immunisation of mice and rabbits. Significant amounts of anti-tubulin antibodies were produced and inhibited the growth of trypanosomes in *in vitro* culture. In addition, the paraflagellar rod protein of trypanosomes has also been shown to have potential as a common vaccine antigen that could be effective against different strains within the same species as well as different species of the same genus since the gene is highly conserved (Abdille *et al*, 2008). Microtubule associated proteins (MAP), such as MAP p15, which enhance tubulin polymerisation, have also been shown to be effective as vaccines and show up to 100% protection from challenge with *T. brucei* (Rasooly and Balaban, 2003).

The focus of this study was to determine whether OpdB would be a suitable vaccine candidate. Specific antibodies against OpdB capable of inhibiting enzyme activity were produced. Immunised mice were able to mount a greater immune response upon infection since they had an already established response to a pathogenic factor of the disease. Since mice immunised with OpdB outlived non-OpdB-immunised mice, it may be suggested that OpdB plays a role in the onset and development of anaemia. Although no protection was conferred by immunisation with OpdB, the enzyme may potentially be used in a multi-component vaccine which would target multiple pathogenic factors of the trypanosome parasite.

CHAPTER FOUR

GENERAL DISCUSSION

African trypanosomiasis has severely affected ruminant livestock across Africa. Trypanosome parasites cause nagana in cattle and sleeping sickness in humans. They are single celled flagellates that are transmitted by insect vectors and evade the host-cell immunity to establish infection (Vickerman *et al.*, 1993). Symptoms of trypanosomiasis include fever, progressive anaemia and ultimately death of the infected host. It is estimated that 45-50 million cattle live under trypanosomiasis risk (Shaw, 2004). This debilitating disease is responsible for up to US $ 5 billion in economic losses per annum in Africa (Kristjanson *et al.*, 1999).

Surrounding the trypanosome is a dense coat of 12-15 nm made of variant surface glycoproteins (Vickerman, 1969). This coat protects the parasite from the host. The ability of the parasite to change or replace this surface antigen, a phenomenon known as antigenic variation, allows the parasite to evade antibody-mediated killing by the host. In this way the parasite can repopulate the host resulting in a long-lasting chronic infection which manifests itself as waves of parasitaemia (Borst 2001).

Current control and treatment strategies for African animal trypanosomiasis include control of the vector (tsetse fly), trypanocidal drugs and the exploitation of trypanotolerant cattle (McDermott and Coleman, 2001). However, since the emergence of drug resistant parasites, toxicity of trypanocidal drugs and the inability to completely eradicate the vector, vaccination against trypanosomiasis has been proposed as a potential control method. Due to the antigenic variation of the parasite the approach in the development of a vaccine must be carefully thought out.

Vaccines are typically based on killed or live attenuated forms of the pathogen (Roitt, 2001). For trypanosomes this is difficult as large amounts of the pathogen are required. Protozoans are generally difficult to grow in culture and trypanosomes in particular have very complex life cycle transformations involving genetic, morphological and proliferative changes (Matthews, 1999). A vaccine based on live attenuated or killed forms of the parasite would have to be effective at all the life cycle stages. In addition, the ability of the parasites to undergo antigenic variation implies the identification and production of

parasites expressing a large series of VSGs. As a result, the concept of an anti-disease vaccine based on pathogenic factors of the parasite has been proposed (Authié, 1994; Authié *et* al., 2001). Invariant immunogens, which are not normally exposed to the host, are released into circulation upon antibody and complement-mediated lysis of trypanosomes during infection. These immunogens have been established as having a major role in pathogenesis (Tizard *et al*., 1978; Taylor and Authié, 2004). Instead of targeting the parasite, pathogenic factors of the parasite are targeted (Authié, 1994; Authié *et al*., 2001).

Proteases of the trypanosome parasite are particularly interesting vaccine candidates. Proteases play various roles in parasite-host interactions and many are involved in cell invasion, catabolism of host cell proteins as well as the stimulation and evasion of host immune responses (Klemba and Goldberg, 2002). By understanding the structural design and determining the enzymatic characteristics of these proteases, specific inhibitors or chemotherapeutic strategies could be used to inhibit their activities.

Congopain, the major cysteine protease of *Trypanosoma congolense* has been identified as having a probable role in trypanosomiasis. Antibodies against congopain have been suggested to contribute to mechanisms of trypanotolerance (Authié *et al.,* 1992, 1993; Authié 1994). Trypanotolerance is the genetic capacity of some cattle to limit parasitaemia and anaemia during the development of infection (Authié *et al.,* 2001). Congopain consists of two major domains; a catalytic domain and a C-terminal extension. As found in *T. cruzi*, the C-terminus is highly immunogenic and elicits an immune response that results in antibodies targeting the C-terminus (Cazzulo and Frasch, 1992). Antibodies directed against the C-terminal extension would have no effect on the activity of the enzyme. Instead a truncated version of the protease, with this C-terminus removed, would ensure an immune response that would result in the production of antibodies specifically against the catalytic domain.

The initial part of the present study included the production and study of inhibitory antibodies against the truncated form of congopain, viz., C2. C2 has already been used in immunisation trials in cattle (Authié *et al.,* 2001). Although immunised cattle were not able to prevent the onset of infection, they mounted an early and prominent IgG response to congopain and leukocyte counts were recovered 2-3 months after infection. This suggested

a probable role for congopain in the development of infection. If this response could be enhanced, antibodies against congopain may contribute to an overall state of trypanotolerance. In order to enhance the production of inhibitory antibodies in the present study, C2 was complexed to alpha-2-macroglobulin (α_2M). Alpha-2-macroglobulin is a high molecular weight plasma glycoprotein that is able to inhibit proteases by trapping it in a cage-like structure that only allows access to small peptide substrates. It has been used with varying success as an antigen delivery system since antigen-α_2M complexes are rapidly endocytosed by antigen presenting cells of the immune system (Chu and Pizzo, 1993). Once the antigen is bound, the α_2M molecule undergoes a conformational change that exposes receptors on its surface which specifically targets it to the cells of the immune system that express the CD 91/ α_2M receptor (Cianciolo *et al.*, 2001).

Alpha-2-macroglobulin has several advantages as an adjuvant system in comparison to other commonly used adjuvants. In general, commonly used adjuvants are potent but are often toxic to the animal being immunised (Singh and O' Hagan, 2002). A frequently used adjuvant is Freund's complete and incomplete adjuvant. This adjuvant works well as it has a depot effect. The antigen is slowly released into circulation, enhancing the immune response and preventing rapid dispersal from the site of injection (Wilson-Welder *et al.*, 2008). The disadvantage of using this adjuvant is that it contains mycobacterial cells which cause inflammatory responses in the animals being immunised. Alpha-2-macroglobulin is a natural glycoprotein and therefore does not pose any threat on the immune system of animals being immunised. However, because α_2M is internalised by the cells of the immune system, it does not have a depot effect as that of Freund's adjuvant. Studies have also shown that α_2M is not only capable of antigen delivery but decreases the minimal antigen concentration for presentation and internalisation to cells of the immune system (Chu *et al.*, 1993). In the present study, the amount of C2 injected per immunisation was 50 µg, which is on the lower end of the recommended dosage of 50-1000 µg for enzymes (Harlow and Lane, 1988). Trypanopain, a cysteine protease from *T. b. brucei*, has previously been used to immunise chickens using a total amount of 30 µg with Freund's adjuvant (Troeberg *et al.*, 1997) and OpdB from *T. b. brucei* has also been used to immunise chickens using a total amount of 50 µg with Freund's adjuvant (Morty *et al.*, 1999b).

All goats immunised with C2 produced antibodies specific to C2 as determined by ELISA. Goats immunised with C2 and Freund's adjuvant showed the highest immune response. This is likely to be due to the depot effect that this adjuvant has. Goats immunised with C2 complexed to α_2M did not show as high an immune response and this may be attributed to the internalisation of α_2M following endocytosis. No prolonged release of antigen would be expected in this case. Goats immunised with C2 alone also produced antibodies specific to C2. The immune response for goats immunised with free antigen was noted to be unusually high.

A booster effect by challenge with trypanosomes was observed in all immunised groups. During infection immune responses manifest as antibody and complement-mediated lysis of trypanosomes (Taylor, 1998). As a result, free antigen would circulate in the bloodstream and cause a boost in the immune response, particularly against C2 as the goats were immunised with this antigen. Following the booster effect, a drop in the immune response can be seen. This may be attributed to mopping up or clearance of circulating congopain antigens by antibodies against its catalytic domain. It would be interesting to determine in future studies whether antibody affinity and avidity increased as a result of antibody maturation particularly following infection.

None of the goats infected developed any notable symptoms of trypanosomiasis. They had very irregular parasitaemia, no significant anaemia and in due course recovered from infection. No formal studies have been published regarding the pathogenicity of infection by *T. congolense* IL 1180 of goats but it is reported to induce severe anaemia, weight loss and immunosuppression in infected cattle (Wellde *et al*., 1974). However, it has been observed that local breeds of small ruminants such as goats do generally exhibit fewer clinical manifestations of the disease than those shown by cattle (Taylor and Authié, 2004).

The high immune response observed for goats immunised with C2 alone prior to and after infection may be attributed to the exposure of the goats to trypanosome parasites prior to immunisation. As a result antibodies against free C2 also showed unusual inhibitory activity. In future, in order to evaluate the efficacy of immunisation of C2 complexed with α_2M for an anti-disease vaccine, animals should be selected with the knowledge that they have not been exposed to trypanosome parasites. Different strains of trypanosomes used for infection should be compared to ensure that a strain that is pathogenic to the test animal

is used in the challenge phase of the study. Although the *T. congolense* IL 1180 strain has been shown to be very pathogenic in cattle, its virulence in goats was not known.

Another invariant antigen, the protease oligopeptidase B (OpdB), has been established as a pathogenic factor in trypanosomiasis (Morty *et al*., 1999a). Oligopeptidase B is a serine protease that is involved in the degradation of host regulatory peptides (Morty *et al*., 2001). Oligopeptidase B is released into the blood stream by dead or dying parasites where it remains active due to its resistance to host serum protease inhibitors (Troeberg *et al.,* 1996; Morty *et al*., 2001, 2005a). In a previous study the interaction of OpdB with α_2M and the use of α_2M as an adjuvant system for immunisation with OpdB was investigated (Huson, 2006). OpdB does not interact with the bait region of α_2M and another protease, C2, was used to induce the conformational change to allow interaction between OpdB and α_2M and subsequent presentation to the immune system. Although antibodies were produced against OpdB, alum was determined to be the best adjuvant to use. Immunisation of OpdB with alum produced anti-OpdB antibodies that showed up to 100% inhibition of the protease *in vitro*.

In the second part of the present study, OpdB was recombinantly expressed in two bacterial systems. Following purification, OpdB was used for immunisation of mice with alum. All mice were challenged with *T. congolense* (IL 1180).

Large amounts of protein are required for immunisation. Due to the laborious isolation of protein from native trypanosome lysates, OpdB was expressed using the bacterial expression systems pGEX4T1 and pET28a. Successful expression in these systems allowed for the purification of OpdB for use in immunisation trials. OpdB was expressed as a 106 kDa GST-tagged fusion protein in the pGEX4T1 system and an 80 kDa histidine-tagged fusion protein in the pET28a system. GST fusion protein was directly eluted from the column and cleaved using thrombin. OpdB was also eluted from the GST column as an already cleaved product by incubation with thrombin while the fusion protein was still adsorbed onto the glutathione agarose resin. The inclusion of a cleavage site for a specific protease allows separation of the two components of the fusion protein. The proteolytic cleavage site in this vector is specific for thrombin and hydrolyses the bond between the Arg and Gly residues in the sequence Leu-Val-Pro-Arg-Gly-Ser (Smith and Johnson, 1998). In both

cases, i.e., cleavage outside the column and on the column, eluted fractions needed further purification due to contaminating host proteins or contaminating GST.

Cleavage outside the column resulted in an 80 kDa band corresponding to OpdB and a 26 kDa band corresponding to GST which were separated by Sephacryl S-200 HR MEC. Analysis of cleavage of the fusion protein while still adsorbed to the resin resulted in a protein band of 80 kDa corresponding to OpdB and the co-elution of multiple contaminating host proteins which were separated and eliminated by Sephacryl S-300 HR MEC. The Sephacryl S-300 HR resin has a larger fractionation range (10-1500 kDa) than the Sephacryl S-200 HR resin (5-250 kDa) and was used to separate the numerous contaminating host proteins from OpdB. All fractions were observed to be pure following purification by MEC as determined by silver stained SDS-PAGE analysis. These fractions were pooled and concentrated using the Amicon concentration cell under low pressure.

Following concentration it was observed that the yield of protein obtained compared to the initial amount observed before MEC was very small. The cost of thrombin is also substantial especially when a small yield of protein necessitates several rounds of expression and purification. OpdB was subsequently sub-cloned into the pET28a expression vector in the hope that larger amounts of His-tagged OpdB could be purified by the simpler method of nickel-chelate chromatography. OpdB was successfully cloned and expressed in pET28a and was purified by nickel-chelate chromatography under native conditions due to the soluble nature of the protein. This method of expression and purification allowed for the production of OpdB in a smaller time frame and also involved fewer purification steps. Further purification of the protein by MEC was eliminated as protein eluted from the Ni-NTA column was determined to be substantially pure by silver stained SDS-PAGE analysis. Since the histidine tag does not interfere with the activity of the protein (Morty *et al.*, 1999, 2005a) and is too small to elicit an immune response against histidine, it was not necessary to remove it from OpdB.

Purified recombinantly expressed OpdB was mixed with alum for the immunisation of mice. The immune response was monitored by ELISA during the immunisation period. Specific antibodies were produced against OpdB in all mice. Following challenge with trypanosomes (*T. congolense* strain IL 1180) a slight booster effect was observed for mice immunised with OpdB. No booster effect by infection was observed for the control group immunised with the irrelevant protein VP2, which was expressed in the same bacterial system as OpdB. Free antigen circulating in the bloodstream possibly causes a boost in the immune response,

more specifically against OpdB as the mice were immunised with this antigen. Serum collected from individual mice showed recognition of both recombinantly expressed and native OpdB. In western blots multiple bands showing the recognition of various proteins in parasite lysate samples were observed by mouse sera collected following infection. Since sera from infected animals contain antibodies and immune components against many circulating antigens present during the infection, the recognition of other proteins apart from OpdB is expected (Taylor, 1998). Due to the already prominent and established IgG response against OpdB from immunisation, the most significantly recognised antigen was OpdB. As suggested for C2, it would also be interesting to determine in future studies if there is an increase in antibody affinity due to antibody maturation during the course of immunisation and post trypanosome challenge.

Parasitaemia was monitored during infection and all mice controlled parasitaemia in a similar way. Following the initial increase in parasitaemia from infection, parasiteamia remained fairly constant. However, the role of parasitaemia is not a true indicator of infection (Vickerman *et al*., 1993). Through antigenic variation many antigen types may be expressed by the parasite (Borst, 2001) resulting in fluctuations in parasite numbers which is seen as waves of parasitaemia (Barry and Carrington, 2004). During infection animals may be intermittently parasitaemic or aparasitaemic (Vickerman, *et al*., 1993).

The resistance of the mice to the development of anaemia was also monitored. A drop in PCV from an initial 55% and above to 40% was evident for both immunised groups and is indicative of the development of anaemia (Taylor and Authié, 2004). Although the mice immunised with OpdB did not resist the development of infection, leukocytes did show some recovery after two months. Mice immunised with OpdB also showed 40% greater survival. Non-immunised mice were unable to mount a rapid immune response following infection and had a higher mortality than those with an already established immune response to OpdB. It may be suggested that as with congopain, OpdB is likely to play a role in the development of anaemia during infection. OpdB has already been implicated in elevated blood levels during infection as it degrades host proteins responsible for the maintenance of blood volume (Troeberg *et al*., 1996; Morty *et al*., 2005a). Elevated blood volume is typical of infection of bloodstream forms of trypanosomes.

The antigenicity and protective potential of various other intracellular antigens of the parasite cytoskeleton have also been described by Lubega *et al*., (2002) and Li *et al*., (2007) who showed that mice immunised with native or recombinant tubulin were able to produce anti-tubulin specific antibodies. The paraflagellar rod protein of trypanosomes may also be used as a potential vaccine antigen that could be effective against different trypanosome strains since it is conserved (Abdille *et al*, 2008). The protective ability of immunisation with various components of the cytoskeleton may be used in conjunction with proteases of the trypanosome in an attempt to induce a state of trypanotolerance.

The use of rodent models in this study is convenient as they are an economical alternative for studying the effects of specific antibodies on the pathogenic factors of the parasite. Studies are currently underway in Uganda, Mozambique and Burkina Faso where Ankole cattle have been immunised with C2 and OpdB.

This study shows that immunisation using α_2M complexed to the catalytic domain of the protease congopain, which is involved in the pathogenesis of the disease, has the ability to produce antibodies that have inhibitory properties against the enzyme. In addition, OpdB, also involved in pathogenesis, was shown to be immunogenic and elicited a good immune response. Anti-oligopeptidase B antibodies collected before and after challenge with *T. congolense* also inhibited OpdB activity. The study of these proteases and the production of inhibitory antibodies, forms part of a collaboration that investigates the feasibility of an alternative vaccine for trypanosomiasis. This approach focuses on creating an immune response against antigens of the parasite that are involved in the pathogenesis of the disease. Future work may include immunisation trials and infection with a highly virulent strain of *T. congolense* in goats to asses a more accurate protective effect of immunisation with proteases complexed to α_2M. Immunising with C2 in complex with α_2M may also be studied in larger ruminants such as cattle. The antigenicity of OpdB has been previously established in rabbits (Huson, 2006) and anti-OpdB antibodies showed 100% inhibition of OpdB activity. The antigenicity of OpdB was confirmed in the present study and trypanosome challenge following immunisation of mice showed the production of anti-OpdB antibodies that inhibited OpdB activity up to 83%. These results should be confirmed in other small and larger livestock. Immunisation using both congopain complexed to α_2M and OpdB in alum may also be investigated to produce superior inhibitory antibodies which may be the start of a multi-component vaccine.

In the present study, immunisation of goats with C2 complexed with α_2M and mice with OpdB in alum elicited immune responses that induced the production of antibodies capable of inhibiting the activity of the respective enzymes. In both cases, a booster effect induced by challenge with *T. congolense* (strain IL 1180) was observed and antibody production was sustained over the infection period. Antibodies made using C2 in complex with α_2M showed superior inhibitory properties with a maximum inhibition of 95% of C2 activity. Anti-OpdB antibodies showed up to 86% inhibition of OpdB activity and although immunisation with OpdB had no effect on the establishment of infection and the development of anaemia, mice immunised with OpdB were observed to live 40% longer. The results of the present study showed that immunisation with proteases of the trypanosome parasite may be used in the development of an anti-disease vaccine.

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