Antibody-mediated inhibition of proteases of African trypanosomes

by

Laura Huson
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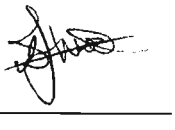
Preface

The experimental work described in this thesis was carried out in the School of Biochemistry, Genetics, Microbiology and Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, from January 2004 to November 2006, under the supervision of Professor Theresa H. T. Coetzer. The studies represent original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others it is duly acknowledged in the text.



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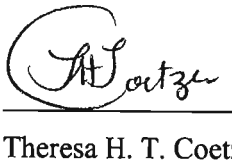

Theresa H. T. Coetzer

Abstract

The protozoan parasites *Trypanosoma congolense* and *T. vivax* cause trypanosomosis in cattle. The major lysosomal cysteine proteinase of *T. congolense*, congopain, may contribute to pathogenesis of the disease, and antibody-mediated inhibition of this enzyme may contribute to mechanisms of trypanotolerance. Oligopeptidase B, a trypanosomal serine peptidase, is also a potential virulence factor in African trypanosomes because it is released into the host circulation by dead or dying parasites, where it retains catalytic activity due to the enzyme's insensitivity to serum protease inhibitors. The vaccine potential of the catalytic domain of congopain, C2, and oligopeptidase B complexed with α_2-macroglobulin (α_2M) was evaluated by producing antibodies in rabbits. Inhibition of congopain and oligopeptidase B activity by these antibodies was assessed.

The oligopeptidase B open reading frame from *T. congolense* and *T. vivax* was cloned and expressed in *Escherichia coli*, from which active recombinant enzymes were purified. These recombinant enzymes exhibited trypsin-like specificity for peptide substrates, cleaving on the carboxy side of basic amino acid residues such as arginine and lysine. Enzymes were found to be optimally active between pH 8 and 10, optimally stable at pH 6, and showed activation by reducing agents and sensitivity to ionic strength. The enzymes showed typical oligopeptidase B-like inhibitor profiles, except that they were not inhibited by thiol sensitive inhibitors such as iodoacetamide and *N*-ethylmaleimide.

High yields of bovine and rabbit α_2M were isolated by a three-step procedure of fractionation by PEG 6000, and zinc chelate and Sephacryl S-300 HR chromatography. Congopain, its catalytic domain C2, papain and cathepsin L all cleaved the bait region of bovine α_2M and became trapped inside the α_2M molecule, where their activity against large molecular weight substrates was inhibited. C2 could thus be complexed with α_2M directly or used to form C2-α_2M-oligopeptidase B complexes for immunisation purposes.

The catalytic domain of congopain, C2, was used to immunise rabbits either without adjuvant, as a water-in-oil emulsion with Freund's adjuvant, or in a complex with either bovine or rabbit α_2M. Freund's adjuvant elicited the highest anti-C2 antibody response. However, the greatest inhibition, 65%, of C2 activity against Z-Phe-Arg-AMC was obtained with antibodies produced by rabbits receiving C2-α_2M complexes.

In a second study, C2 and oligopeptidase B were used to immunise rabbits, either in alum, or complexed to bovine α_2M. Anti-C2 antibody levels were highest in rabbits immunised with the free proteins in alum, whereas anti-oligopeptidase B antibody levels were comparable for each adjuvant system. Anti-oligopeptidase antibodies produced with alum gave 100% inhibition of oligopeptidase B activity. In contrast, antibodies produced against C2-α_2M-oligopeptidase B complexes had little effect on oligopeptidase B activity. However, these antibodies inhibited 55% of C2 activity. Alum was a slightly less efficient adjuvant for C2 and 50% inhibition of C2 activity was observed.

It appeared that immunisation of rabbits with C2 complexed to α_2M resulted in the production of antibodies that were better able to neutralise the proteolytic activity of C2 and congopain *in vitro* than that with conventional adjuvants. The immunisation of C2 complexed to bovine α_2-macroglobulin therefore has the potential to neutralise parasite congopain *in vivo*, and may contribute to an anti-disease vaccine against African trypanosomosis. Complexation of oligopeptidase B to α_2M offers no benefit, since antibodies produced against this complex are not able to inhibit the activity of oligopeptidase B. Immunisation with oligopeptidase B in alum is sufficient to produce efficient enzyme-inhibiting antibodies in the context of an anti-disease vaccine against African trypanosomosis.

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Abbreviations

2YT	2 × yeast extract, tryptone
3D	three-dimensional
α_1M	α_1-macroglobulin
α_2M	α_2-macroglobulin
α_2MR	α_2-macroglobulin receptor
A_x	absorbance at x nm
ABTS	2,2-azino-di-[3-ethylbenzthiazoline sulfonate]
ACD	acid citrate dextrose
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
AMC	7-amino-4-methylcoumarin
AMT	acetate-MES-Tris
APC	antigen presenting cell
ATP	adenosine triphosphate
Bis	N,N'-methylenebisacrylamide
Bis-Tris	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol
BMGY	buffered glycerol complex medium
BMM	buffered minimal medium
BLAST	basic local alignment search tool
Brij	polyoxyethylenlaurylether
BSA	bovine serum albumin
Bz	benzoyl
c	concentration
C2	congopain catalytic domain
cDNA	copy deoxyribonucleic acid
C-terminal	carboxy terminal
DAF	decay accelerating factor
DCI	3,4-dichloroisocoumarin
dH_2O	distilled water
DMSO	dimethylsulfoxide

DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
ε	extinction coefficient
$[E]_0$	active enzyme concentration
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
FCS	fetal calf serum
g	relative centrifugal force
GST	glutathione S-transferase
GTP	guanosine triphosphate
HRPO	horseradish peroxidase
I	ionic strength
[I]	inhibitor concentration
ICP	inhibitor of cysteine peptidases
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
IPTG	isopropyl-beta-D-thiogalactopyranoside
ISG	invariant surface glycoprotein
ISP	inhibitor of serine peptidases
k_{ass}	rate of complex association
K_{av}	availability constant
k_{cat}	turnover number
kDa	kiloDalton
k_{diss}	rate of complex dissociation
K_i	inhibition constant
$K_{i(app)}$	apparent inhibition constant
K_m	Michaelis-Menten constant
k_{obs}	pseudo first-order inhibition rate constant

L	length of the light path
LB	Luria Bertini
LBTI	lima bean trypsin inhibitor
LMP	low melting point
LPS	lipopolysaccharide
MBS	M-maleimidobenzoyl acid N-hydroxy succinimide ester
MCP	membrane cofactor protein
MD	minimal dextrose
MEC	molecular exclusion chromatography
MES	acetate-2(N-morpholino)ethanesulphonic acid
MHC	major histocompatibility complex
MMP	matrix metalloproteinase
MU	4-methylumbelliferone
MUGB	4-methylumbelliferyl-*p*-guanidobenzoate
N-terminal	amino terminal
OPC	oligopeptidase B open reading frame (*T. congolense*)
OPV	oligopeptidase B open reading frame (*T. vivax*)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PEG	polyethylene glycol
PBM	peripheral blood monocyte
PBS	phosphate buffered saline
PEG	polyethylene glycol
pI	isoelectric point
PMSF	phenylmethylsulfonylfluoride
pNA	para-nitroanilide
PVDF	polyvinylidene difluoride
RBD	receptor binding domain
rOPC	recombinant oligopeptidase B from *T. congolense*
rOPV	recombinant oligopeptidase B from *T. vivax*

RT	room temperature
[S]	substrate concentration
SBTI	soy bean trypsin inhibitor
SEM	standard error of mean
SOB	super optimal broth
SDS	sodium dodecyl sulfate
$t_{½}$	half-life
TBS	tris buffered saline
TCA	trichloroacetic acid
TEMED	N,N,N',N'-tetramethyl ethylene diamine
TLCK	N-tosyl-L-lysyl chloromethylketone
TPCK	N-tosyl-L-phenylalanyl chloromethylketone
Tris	2-amino-2-(hydroxymethyl)-1,3-propandiol
VAT	variant antigen type
V_e	elution volume
V_{max}	maximum velocity
V_o	void volume
v_0	initial velocity
VSG	variant surface glycoprotein
V_t	total column volume
YNB	yeast nitrogen base
YP	yeast extract, peptone
YPD	yeast extract, peptone, dextrose
Z	benzyloxycarbonyl

CHAPTER 1
INTRODUCTION

1.1 African trypanosomosis

African bovine trypanosomosis, caused by the tsetse-transmitted *Trypanosoma congolense*, *T. vivax* and *T. brucei brucei*, is a severe constraint to livestock production. The disease occurs in 37 sub-Saharan African countries (Swallow, 2000), impacting negatively on food production and economic growth. Estimates of the affected land area range from 8 to 11 million km^2, inhabited by 260-300 million people and 45-50 million cattle. Estimates of total annual losses range from 1.3 to 5 billion US$ (Kristjanson *et al.*, 1999; McDermott and Coleman, 2001), while the disease is directly responsible for the deaths of three million animals (Hursey and Slingenbergh, 1995).

The geographic distribution of bovine trypanosomosis, also called nagana, coincides with that of their tsetse fly vectors, *Glossina* spp., and includes large expanses of sub-Saharan Africa. Trypanosomes have been found in many species of wild herbivores as well as some carnivores, so the wildlife found naturally in Africa serve as a reservoir for infection. Trypanosomes are generally not pathogenic for wild animals, probably as a result of their co-evolution (Taylor, 1998).

Cattle trypanosomosis has historical associations with South Africa, specifically the Zululand area of KwaZulu-Natal, the earliest reference of which dates back to 1870. In 1894, Sir David Bruce was ordered to Zululand to investigate the cause of death of hundreds of cattle. This investigation resulted in the first demonstration of the transmission of a protozoan parasite by an insect bite. The parasite was later named *T. b. brucei* (Taylor, 1998). The most recent outbreak of trypanosomosis in this region occurred in 1990 between the Umfolozi river and Mozambique border, where the prevalence of trypanosomosis was determined to be between 10 and 15% (Kappmeier *et al.*, 1998).

1.1.1 Classification of trypanosomes

The classification of the genus *Trypanosoma* is given in Fig. 1.1. Members of the class *Zoomastigophora* are classified on the basis of their number of flagella. Members of the order *Kinetoplastida* possess one or two flagella, typically with a paraxial rod. They have, at some stage in their life cycle, a single mitochondrion which contains up to 20% of the cell's DNA. Representative genera of this order are *Trypanosoma* and *Leishmania*. The genus *Trypanosoma* is split into two sections, stercoraria or salivaria, depending on the location in the digestive tract of the vector within which the parasite develops to an infective metacyclic stage in the vector. South American trypanosomes, such as *T. cruzi*, are stercorarian parasites, being transmitted in the faeces of infected insect vectors. *T. congolense*, as well as most species of African trypanosomes, is a salivarian parasite, with development of metacyclics occurring in the anterior part of the digestive system (i.e. mouthparts) of their vector (Bush *et al.*, 2001). *T. congolense*, *T. vivax* and *T. b. brucei* are the type species of the subgenera *Nannomonas*, *Duttonella* and *Trypanozoon* respectively (Vickerman, 1982).

1.1.2 Morphology of trypanosomes

The general morphology of *T. congolense* is illustrated in Fig. 1.2. *T. congolense* is a short flagellate of an average size of 13 μm. A single flagellum arises from the basal body at the floor of the flagellar pocket, which occurs at the posterior end of the body. The basal body is a cylinder composed of nine equally spaced fibrils, and averages 1.5 μm in diameter. The fibrils are connected to those of the axoneme within the flagellum (Noble and Noble, 1982). The medium-sized kinetoplast is a spherical rod-shaped mass of up to 20% of the cell's DNA, associated with the single tubular mitochondrion (Bush *et al.*, 2001). A ring of microtubules is located just under the cell membrane, extending the length of the cell. The microtubules are connected by microfibrils, creating a ladder-like appearance. This microtubule-microfibril complex presumably gives structural support to the trypanosome's cell membrane (Sasse and Gull, 1988). The morphology of *T. vivax* differs in that the kinetoplast is distinctively larger than that of *T. congolense*. Also, the posterior end of *T. vivax* is typically club-shaped, tapering towards the anterior, whereas the posterior end of *T. congolense* is round (in smaller forms) to pointed (in

larger forms) (Stevens and Brisse, 2004). *T. vivax* has a distinctive mobility, whereby its rapid vibrational movement can be used as a diagnostic feature (Gardiner, 1989).

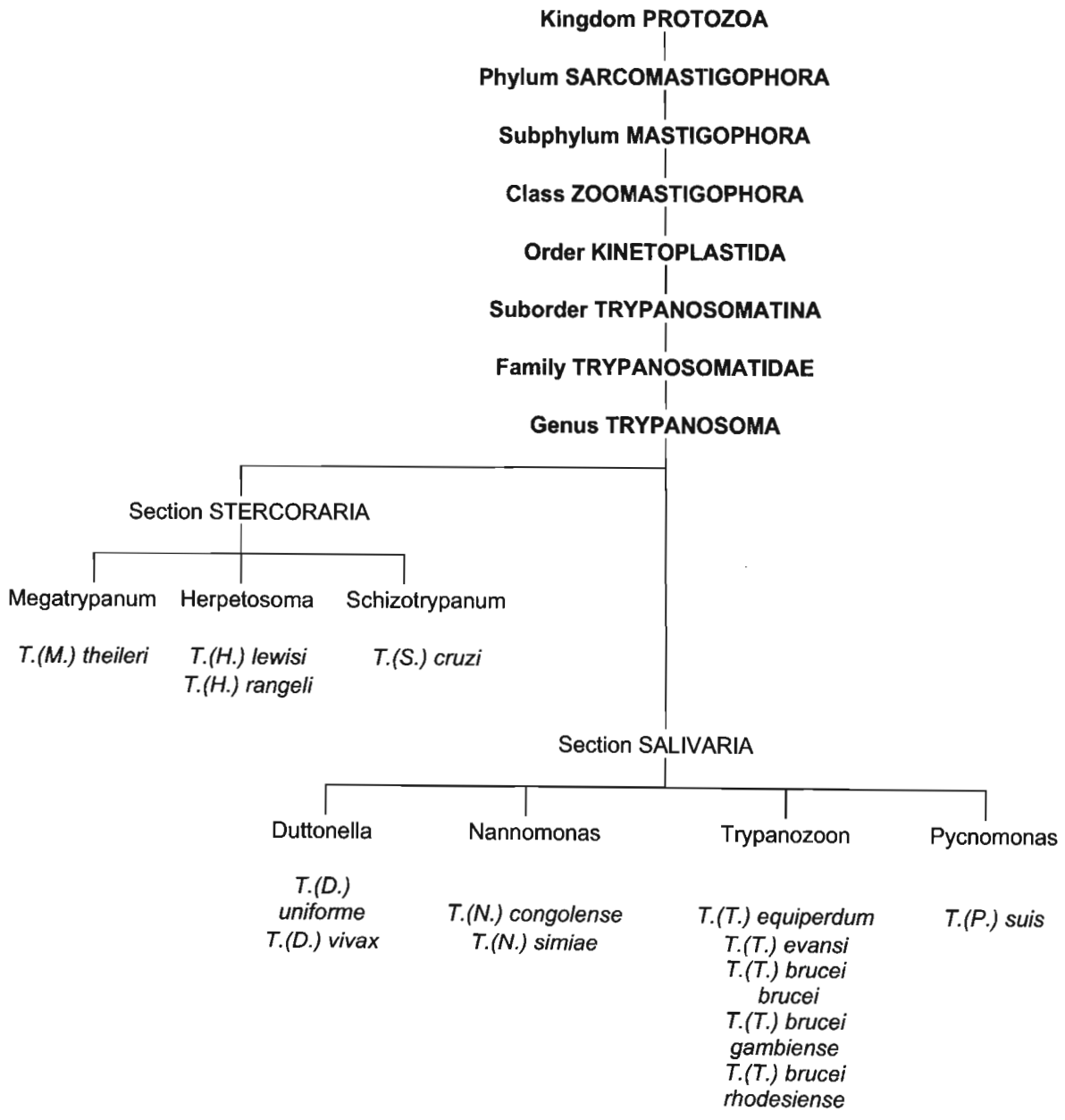

Figure 1.1. Classification of the genus *Trypanosoma* (Vickerman, 1982).

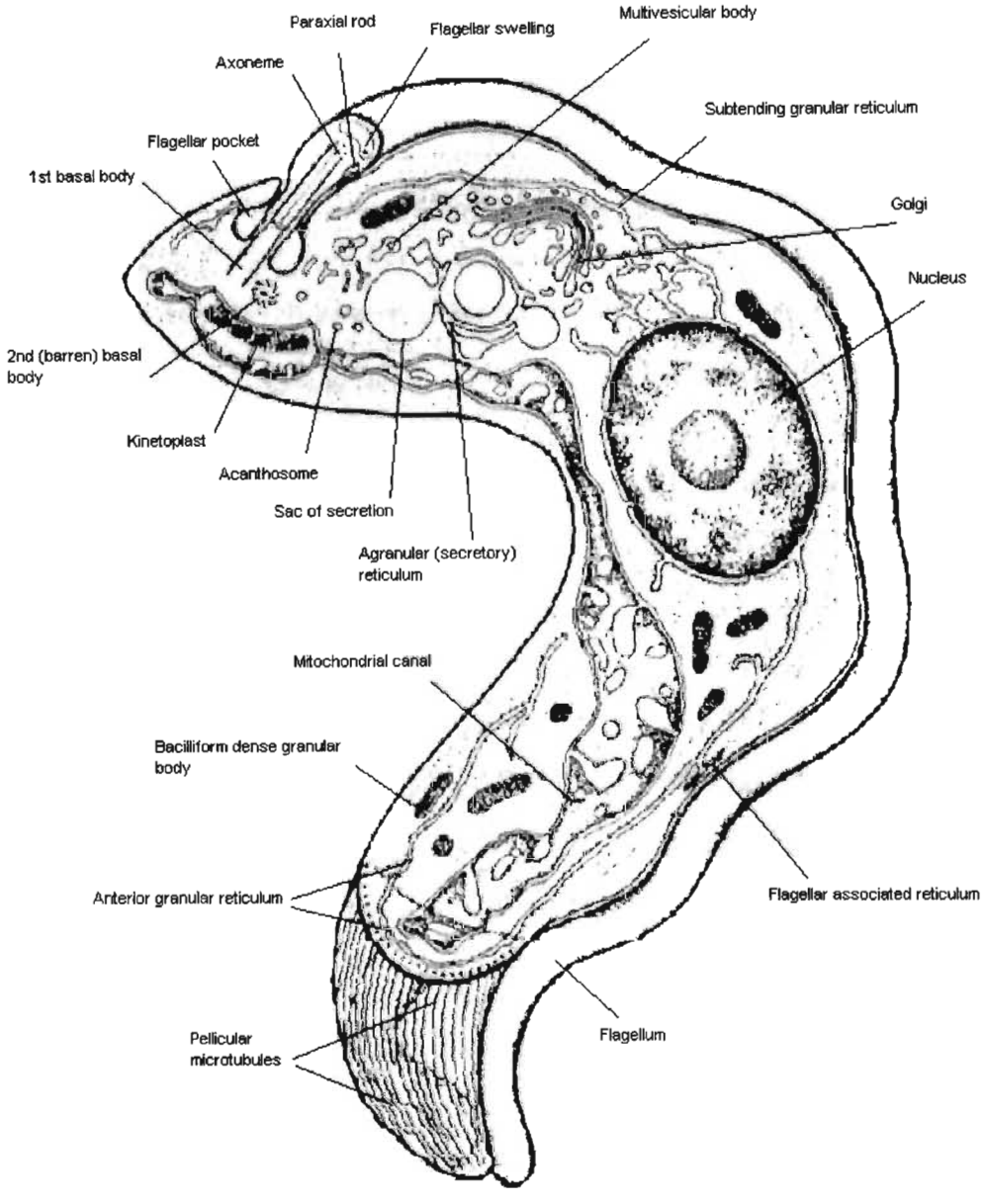

Figure 1.2. The general morphology of *Trypanosoma congolense* in its bloodstream stage (Noble and Noble, 1982).

1.1.3 Trypanosome life cycle

The life cycle of African trypanosomes is totally extracellular, and that of *T. b. brucei* has been extensively studied. The parasites are transmitted during feeding by the tsetse fly vector, *Glossina* (Schmidt, 1992). The bloodstream forms of *T. b. brucei* are represented by three forms: long slender, intermediate, and short, stumpy forms (Fig. 1.3). The short, stumpy, non-dividing trypomastigote stage of the parasite is acquired in the tsetse's blood meal. It travels to the midgut and subsequently to the hindgut as a procyclic form that maintains the trypomastigote morphology. After a period of development, the procyclics migrate anteriorly to a region of the midgut where they divide and multiply. Between 14 and 21 days later, the parasites enter the proventricular area of the gut. From there they migrate anteriorly into the mouthparts where they transform into epimastigotes and again multiply. The epimastigotes attach themselves by their flagella to microvilli that line the epithelium of the mouth parts. In this site the epimastigotes transform into non-dividing, infective, metacyclic forms that are released from their attachment to microvilli (Bush *et al.*, 2001).

The entire cycle from the time of the first blood meal containing short, stumpy trypanosomes to infective metacyclics takes approximately 3 to 4 weeks. The metacyclics are injected with the saliva into a new host during a blood meal (Bush *et al.*, 2001). The fly's bite deposits the metacyclic trypanosomes into the host's dermal connective tissue and a local inflammatory reaction, called the chancre, develops within 7-11 days of the bite (Vickerman *et al.*, 1993). Dividing trypanosomes are found extensively throughout the chancre, among collagen fibres, and in dermal lymph spaces, from which they spread via the dermal lymphatics to the circulation. Once in the mammalian host, the metacyclics transform to a long, slender stage (trypomastigote), which divide rapidly by binary fission. As the parasitaemia reaches its peak, a percentage of the long slender forms transforms into a short, stumpy, non-dividing, trypomastigote stage that is infective for the vector (Bush *et al.*, 2001).

The life cycle of *T. congolense* and *T. vivax* parasites differ from that of *T. b. brucei* in that they are characterised by a single bloodstream form. Also, the development of *T. vivax* in *Glossina* spp. is confined entirely to the proboscis (Gardiner, 1989).

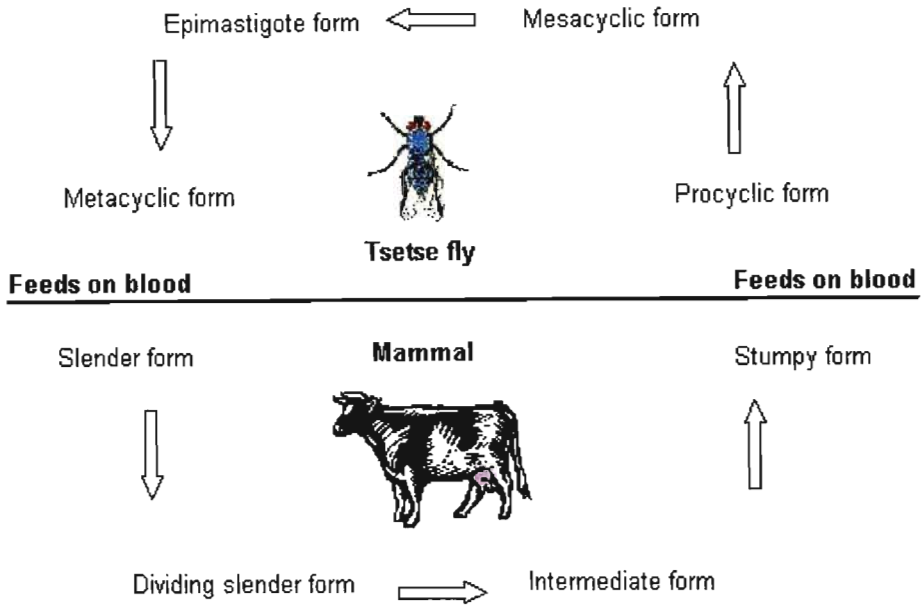

Figure 1.3. Life cycle of *T. b. brucei* showing the morphological stages present in tsetse fly and mammalian hosts.

1.1.4 Antigens of African trypanosomes

The antigens of African trypanosomes are classified as either invariant or variable. Stable antigens are parasite components, such as structural proteins and enzymes, which are usually present at every stage of the life cycle of the parasite. Variable antigens are confined to the metacyclic stage and trypanosome populations in the mammalian host.

Most of the trypanosomes' invariant antigens are not exposed to the host's immune response since they are not located at the surface of the parasite. They may however be highly immunogenic and play a role in immunopathology of the infection. Two such invariant antigens in *T. congolense* infection are congopain and hsp70 (Authié *et al.*, 1993b). Several invariant surface glycoproteins (ISGs), ISG64, ISG65, ISG100 and ISG75 (according to their apparent molecular weight in kDa), have been identified in the

bloodstream stage of *T. b. brucei* (Ziegelbauer and Overath, 1992; Jackson *et al.*, 1993; Nolan *et al.*, 1997). ISG75 has been targeted as a possible diagnostic feature since it is distributed over the surface of bloodstream forms of trypanosomes (Ziegelbauer and Overath, 1992).

The variable antigens are components of the surface coat of the trypanosome and are responsible for differences between serologic variants or variant antigen types (VATs) of the trypanosome. The 12-15 nm thick coat is made up of 5-10 × 10^6 molecules of a single variant surface glycoprotein (VSG), and changes in its composition are associated with antigenic variation (Cross, 1990). The structure of the VSG determines the parasites' VAT, and antibodies produced against the VSG only react with parasites of the same VAT. Antigenic variation occurs in infections initiated even by a single trypanosome. *T. b. brucei* has around 1000 alternative VSG genes. The total number of VATs in a parasite's genetic repertoire is yet to be determined for any trypanosome species (Vickerman *et al.*, 1993), even with the current information from the trypanosome genome sequencing project (Barry *et al.*, 2005). The VSG is a major cell product of the parasite and provokes a strong antibody response. This antibody response successfully kills most of the parasites, but a small proportion survives because they express a different antigenic form of the VSG. The surviving trypanosomes can continue to divide at a rapid rate so that within a few days they can again produce a full scale infection. The glycoprotein coat of metacyclic and bloodstream forms of trypanosomes seem to render the parasites refractory to the host's innate immunity, i.e. complement activation and opsonisation for uptake by macrophages (Behnke, 1990).

1.1.5 Immunopathology of African trypanosomosis

The most important pathologic effects of African trypanosome infections are lymphoid cell proliferation, anaemia, weight loss, circulatory disturbances, tissue lesions, and immunosuppression (Vickerman *et al.*, 1993). Of the trypanosome species that are pathogenic in cattle, *T. b. brucei* is generally less pathogenic than *T. congolense* and *T. vivax*. Parasitaemias during *T. congolense* and *T. b. brucei* infections are generally lower than during *T. vivax* infection. The most common form of disease in endemic areas is

chronic, but hyperacute syndrome may occur, leading to death in 3-4 weeks (Taylor and Authié, 2004).

Immune complex formation and the consequent release of pharmacologically active substances, such as amines, peptides and lipids, have been attributed a major role in the pathology of trypanosomosis. It probably results in the widespread complement activation leading to hypocomplementemia (Taylor and Authié, 2004). Activation of complement by immune complexes on the surface of various host cell types, such as red blood cells, is believed to result in lysis or opsonisation of these cells. Red blood cell destruction by such means is believed to be important in the generation of anaemia, which is reportedly the primary cause of death of infected cattle (Taylor, 1998). However, complement-mediated lysis of red blood cells is usually regulated by a protein called decay accelerating factor (DAF) (Roitt, 1997).

1.1.6 Trypanotolerance

When animals are infected with trypanosomes but show few clinical signs of disease, it is termed trypanotolerance. In cattle, trypanotolerance is a genetic trait possessed by *Bos taurus* breeds of West and Central Africa, notably the N'Dama longhorn and the West African shorthorn. Cattle of *Bos indicus* breeds, such as the Zebu, do not exhibit this genetic trait and are thus referred to as trypanosusceptible (Vickerman *et al.*, 1993).

The key factors of the trypanotolerant trait are the ability to control parasitaemia and resist anaemia (Vickerman *et al.*, 1993). Differences in pathology, immune responses and cytokine levels are also apparent between tolerant and susceptible cattle, but despite extensive research, the exact mechanisms underlying trypanotolerance have not been identified. A recent study suggests that the trypanotolerance trait involves two distinct mechanisms: one that improves the host's capacity to control parasitaemia and is likely to be an innate mechanism; and another which limits anaemia and loss of white blood cells (Naessens *et al.*, 2003).

Trypanosusceptible cattle's peripheral blood monocytes (PBMs) express more CR1 (receptor for the third component of complement), and more major histocompatibility complex II molecules than trypanotolerant cattle's PBMs. This means that trypanotolerant PBMs will be more proficient in processing and presenting antigen to T and B cells for a more antigen-specific cell- and antibody-mediated response. Trypanosusceptible PBMs have more phagocytic potential, leading to destruction of more erythrocytes and thus more severe anaemia. The lesser ability of trypanosusceptible cattle to produce a specific anti-parasite immune response results in higher parasitaemia than tolerant cattle (Taiwo *et al.*, 2000).

Trypanotolerant cattle respond faster and produce a quantitatively and qualitatively different humoral immune response than susceptible cattle. There are more circulating B cells before infection and a higher level of circulating B cells is maintained throughout infection (Taylor *et al.*, 1996). Trypanosusceptible cattle sera contain high titres of polyreactive IgM that are not specific to trypanosome antigens (Buza and Naessens, 1999). Trypanotolerant cattle sera display higher levels of IgG, specifically IgG1, to the buried epitopes of VSG, and the two invariant trypanosome antigens, hsp70 and congopain (Authié *et al.*, 1993b; Taylor *et al.*, 1996). An association has been observed between the level of specific IgG and the resistance to trypanosomosis in both tolerant and susceptible cattle. It has been hypothesised that the switch from the production of IgM to IgG may be impaired in susceptible cattle (Authié *et al.*, 1993b). Because of the involvement of T cells and T cell-derived interleukins in B cell activation and antibody isotype regulation, the different antibody responses between tolerant and susceptible cattle may be the result of a differential stimulation of T-helper cell subsets (Lutje *et al.*, 1995b).

Antibody responses to congopain appear to be associated with higher resistance to disease. An anti-congopain IgG response may be important in either inhibiting the enzyme activity or in removal of circulating protease via immune complexes. Thus, animals that fail to mount a prominent IgG response may be exposed to more of the potential harmful effects of the protease (Authié *et al.*, 1993a).

In experimental infections of mice with *T. congolense,* changing from a predominantly type I cytokine environment, with classical activation of macrophages in the early stage of infection, to a type II cytokine environment, with alternative macrophage activation and enhanced membrane cofactor protein (MCP)-1 secretion in the late stage of infection, correlates with resistance. A type I cytokine environment enables the host to control the first peak of parasitaemia, but is detrimental to the host if sustained. Thereafter, a switch to a type II cytokine environment is required to induce an IgG1 antibody response to parasite antigens and to prevent excessive production of inflammatory mediators (De Baetselier *et al.*, 2001; Noël *et al.*, 2002).

1.1.7 Control of African trypanosomosis

Control of trypanosomosis is hindered considerably by the fact that trypanosomes are able to establish chronic infections in their mammalian hosts because of their highly developed system of antigenic variation (Barry *et al.*, 2005). At present, there are three principle control strategies for tsetse-transmitted trypanosomosis: trypanocidal drugs, trypanotolerant cattle, and tsetse control/eradication. Each of the control methods has its own advantages and disadvantages, and can be applied singly or in combination, with variable success (McDermott and Coleman, 2001).

Trypanocides play a key role in the control of trypanosomosis in Africa. The total expenditure on trypanocides in sub-Saharan African has been estimated to be between 12 and 35 million US$/year. They are the only widely used treatment and control measures applied by individual farmers (McDermott and Coleman, 2001). There are currently only three trypanocides available for controlling trypanosomosis in domestic ruminants: isometamidium (Samorin®, Trypamidium® and Veridium®) and homidium (Novidium® and Ethidium®), which have both prophylactic and therapeutic effects, and diminazene (Berenil®), which has only therapeutic properties. All three drugs have been on the market for over 50 years, and resistance of trypanosomes to these drugs has emerged (Geerts *et al.*, 2001). Although the problem of drug resistance has yet to be quantified, it has led to consensus that to prolong their efficacy, trypanocidal drugs need to be

administered less frequently and more strategically within integrated control programmes (McDermott and Coleman, 2001).

The trypanotolerance of various cattle breeds, namely the N'Dama longhorn and the West African short horn, has been widely exploited in the humid and subhumid zones of West and Central Africa. In the highest trypanosome challenge areas of these zones, almost all of the cattle are of trypanotolerant breeds. Tolerant cattle will become infected and can succumb to high challenge. Thus tolerant cattle are treated, although relatively less frequently than susceptible cattle. These cattle are also considered to be tolerant to dermatophilosis and other diseases of the humid tropics (McDermott and Coleman, 2001). Cattle of the trypanotolerant breeds are small in size but can be just as productive as the susceptible Boran (Vickerman *et al.*, 1993).

The method of choice for controlling trypanosomosis in areas with low or moderate tsetse fly density is the eradication of the vector (Mehlhorn, 1988). This is achieved by spraying of insecticides, although this practice has essentially been replaced by the placing of stationary odour-baited traps, pesticide-treated targets or cattle treated with pour-on insecticide (McDermott and Coleman, 2001). Another method is the sterile insect technique which is the liberation of sterilised flies to compete with wild flies (Schofield and Maudlin, 2001). Advances in recombinant DNA technologies have recently fuelled the development of molecular genetic approaches for the control of vector-borne diseases. Characterisation of fly gut molecules responsible for parasite killing early in infection and their constitutive expression in the tsetse gut may render flies refractory to parasite transmission. Subsequent population replacement of susceptible flies with their engineered counterparts might provide an alternative control strategy in the long run (Hao *et al.*, 2001).

One control strategy with great potential impact, if it could be developed, is vaccination. There are, however, many difficulties facing development of a vaccine against trypanosomosis. In general, development of a vaccine against parasitic organisms is challenging. Protozoa are difficult to grow up in sufficient numbers to manufacture

killed vaccines, and there is a possibility of destroying epitopes during the inactivation process (Roitt, 1997). Therefore, subunit vaccines are more suitable for parasite vaccines. However, due to parasites' complex life cycles, the target antigen would need to be present in all of the life cycle stages in the host. VSG is the most abundant, exposed and immunologically dominant parasite antigen in the mammalian host. However, immunisation based on VSG would only induce protection against the immunising antigenic types. Successful immunisation would therefore require the identification and production of the seemingly inexhaustible repertoire of VSGs. This bleak prospect is forcing scientists to turn to invariant antigens exposed to the host immune system as possible immunogens.

Congopain, a cysteine proteinase of *T. congolense*, is an invariant antigen that is currently of interest. As discussed in Section 1.2.1.1, there is accumulating evidence that congopain plays a role in the pathology of trypanosomosis. Antibodies against congopain are not likely to affect the survival of the parasite, and would therefore not be considered in a traditional anti-parasite vaccine. However, antibodies against congopain may contribute to the mechanisms of trypanotolerance by neutralising the pathogenic effects of the enzyme. This type of vaccine has been termed "anti-disease" and will be discussed in Section 8.1. The same consideration is also currently being given to a number of other parasite antigens that play a role in pathogenicity, such as oligopeptidase B, a serine oligopeptidase found in all three species of trypanosomes that cause African trypanosomosis (discussed in Section 1.2.2.1).

Another consideration for vaccines is the type of adjuvant used. Broadly speaking, adjuvants are substances used in combination with an antigen that produce a more vigorous immune response than the antigen alone (Singh and O'Hagan, 2003). A key issue in adjuvant use is toxicity, and the benefits of using adjuvants must be weighed against the risk of the adjuvant causing undesirable reactions. Whilst new, safer adjuvants are becoming available, Freund's adjuvant, introduced over 50 years ago, remains the most commonly used, despite its potential hazards (discussed in Chapter 8). α_2-Macroglobulin (α_2M) has recently become an adjuvant option, with its success proven

in a few studies (Chu *et al.*, 1994; Cianciolo *et al.*, 2002; Liao *et al.*, 2002). In the present study, the efficiency of Freund's, aluminium salts, and α_2M as adjuvants was compared (Chapter 8).

1.2 Structure and classification of proteolytic enzymes

Proteases are catalytically active proteins that hydrolyse proteins and protein fragments. They are biological catalysts that carry out the disassembly of protein molecules to create the pool of free amino acids required for protein synthesis. Positional selectivity distinguishes between proteases that are restricted to acting near the N- or C- terminus of the polypeptide chain, called exopeptidases, and those that can act on the internal bonds of a polypeptide, called endopeptidases. Both exopeptidases and endopeptidases show varying preferences for particular amino acids near the peptide bonds to be cleaved. The active site of a protease is commonly located in a groove on the surface of the molecule, and the substrate specificity is dictated by the properties of binding sites arranged along the groove on one or both sides of the catalytic site. The specificity is described by use of a model in which the sites are numbered from the catalytic site, S1, S2 and so on towards the N-terminus, and S1′, S2′ and so on towards the C-terminus. The substrate amino acids they accommodate are numbered P1, P2, etc., and P1′, P2′, etc., respectively (Schechter and Berger, 1967) (Fig. 1.4).

Six groups of proteases are recognised according to the chemistry of the catalytic site: serine, threonine, cysteine, aspartic acid, glutamic acid, and metalloproteases (Table 1.1). There are a few proteases for which the catalytic mechanism has not yet been identified.

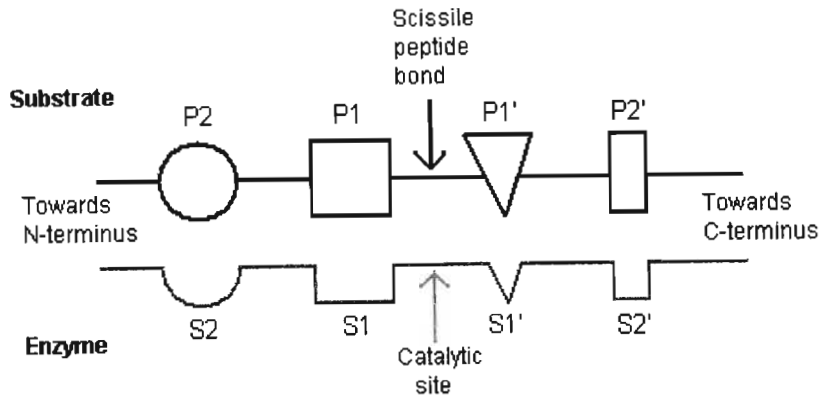

Figure 1.4. Scheme for the specificity subsites of proteases (adapted from Schechter and Berger, 1967).

Table 1.1 The six catalytic types of proteases (Barrett, 2002; Fujinaga *et al.*, 2004).

Catalytic type	Primary catalytic group	Secondary catalytic group	Example
Serine	Hydroxyl group of serine	Imidazole nitrogen of histidine (sometimes ε-amino group of lysine)	Chymotrypsin, oligopeptidase B
Threonine	Hydroxyl group of threonine	N-terminal α-amino group	Proteasome
Cysteine	Thiol group of cysteine	Imidazole nitrogen of histidine	Papain, cathepsin L, congopain
Aspartic acid	Carboxyl groups of two aspartic acid residues	-	Pepsin, HIV protease
Glutamic acid	Carboxyl group of glutamic acid residue	Carbonyl oxygen group of glutamine	Scytalidoglutamic peptidase
Metallo	Zinc atom (sometimes another metal)	Carboxyl group of glutamate	Thermolysin

Since living cells are largely constructed of proteins, enzymes must be strictly regulated to prevent them from doing damage to these cells in which they are synthesised and function. This is commonly managed by the synthesis of proteases as inactive proenzymes that require activation. Some proteases are confined to membrane-limited organelles such as lysosomes, where they act in a specialised environment. A third form of regulation is mediated by proteins that inhibit activity of these enzymes, e.g. α_2M,

cystatins, and pepstatin. A new group of inhibitors of cysteine peptidases (ICPs), occurring in parasitic protozoa (*T. cruzi* and *T. brucei*), bacteria and archaea, has been identified (Smith *et al.*, 2006). It remains uncertain, however, if the inhibitors' main function is modulation of the parasite's own enzymes, as suggested for *T. cruzi* (Santos *et al.*, 2005), or those of the host. Similarly, there exists a group of inhibitors of serine peptidases (ISPs), of which ecotin from *Escherichia coli* is an example (Eggers *et al.*, 2004). Two genes with homology to ecotin have been identified in *T. b. brucei*. They are expressed preferentially in the infectious, bloodstream forms of the parasite and encode proteins that appear to be located in the endosomal lysosomal compartment (Prof Jeremy Mottram, University of Glasgow, Scotland, personal communication).

1.2.1 Cysteine proteases

The best known cysteine proteases are those in family C1, of which papain is the prime example. The active site of papain consists of Cys^{25}, His^{159} and Asn^{175}. The same arrangement is found in all the relatives of papain. Papain-like enzymes, including cathepsins B and L, are found in the lysosomes of mammalian cells. Lysosomes contain an acidified environment suited to the activity of these proteases. Lysosomal cysteine proteases sometimes escape from lysosomes, and may also be secreted from cells, so mechanisms are required to control their activities. Generally they become irreversibly inactivated under conditions of neutral or alkaline pH, but there is also a family of proteins called cystatins, present in the cytoplasm or in the bloodstream, that inhibit these enzymes (Barrett, 2002). Trypanosomal cysteine proteinases that have been identified and studied to various extents include congopain from *T. congolense* (Authié *et al.*, 1992), trypanopain from *T. b. brucei* (Troeberg *et al.*, 1996), cruzipain from *T. cruzi* (Eakin *et al.*, 1992), vivapain from *T. vivax* (Prof. Theo Baltz, University of Bordeaux, France, personal communication) and evansain from *T. evansi* (Prof. Marisa Gonzatti, Universitad Simón Bolivar, Venezuela, personal communication).

1.2.1.1 Congopain

Congopain is a cysteine proteinase of *T. congolense*. Two cysteine protease genes have so far been identified in *T. congolense*, leading to the conclusion that there are at least

two closely related enzymes named CP1 (Fish *et al.*, 1995) (EMBL accession number Z25813) and CP2 (Jaye *et al.*, 1993) (EMBL accession number L25130). Based on N-terminal sequencing, congopain has been identified as CP2. Despite a high degree of sequence similarity between CP1 and CP2 (86% in the catalytic domain; Boulangé *et al.*, 2001), there are distinct differences between the enzymes. Their predicted pI values are 8.35 and 4.74 for the catalytic domains of CP1 and CP2 respectively, and CP2 shows activity over a much broader pH range, whereas CP1 is only active at acidic pH (Boulangé *et al.*, 2001). CP1 and CP2 also show some differences in their substrate specificities.

Congopain is a 33 kDa cysteine protease that represents at least 1% of the total protein of *T. congolense* (Authié *et al.*, 1992). The enzyme is preferentially expressed in the life cycle stages that are infective for the mammalian host (Authié, 1994). Congopain is located within the lysosomal system of the parasite and high amounts are likely to be released in plasma following complement mediated lysis of the parasites. Congopain may also be actively secreted (Mbawa *et al.*, 1991). The enzyme is synthesised as an inactive zymogen, consisting of a signal sequence, a proregion, a central domain including the catalytic residues of the active site (called the catalytic domain in the present study), and a 130-amino acid long C-terminal extension linked to the catalytic domain by a proline-rich hinge region. The C-terminal extension, of unknown 3D structure and function, similarly occurs in other trypanosomal cysteine proteases, but not mammalian cysteine proteases (Cazzulo and Frasch, 1992).

Congopain is a cathepsin L-like enzyme that shares 68% sequence homology with cruzipain in its central catalytic domain, and similar enzymatic specificity (Chagas *et al.*, 1997). Like mammalian cysteine proteases, congopain and cruzipain are synthesised as prepro-enzymes, which are further converted to the mature form by proteolytic cleavage. The identification of a short consensus inhibitory sequence in the propeptide of trypanosomal enzymes suggests that their prosegment may specifically bind to, and block the active site of the enzyme (Lalmanach *et al.*, 1998).

The 3D model for congopain, proposed by Lecaille *et al.* (2001), has a framework common to most papain-like cysteine proteases, which folds into two domains, an α-helical L-domain and an R-domain mostly composed of antiparallel sheets. The catalytic triad, i.e. Cys^{25}, His^{159}, and Asn^{175} (papain numbering), together with the residues defining the extended substrate-binding site, are located in a cleft between the R-domain and the L-domain (Turk *et al.*, 1998). Invariant residues include those involved in the catalytic function of cysteine proteases, as well as the charged buried residues that form electrostatic interactions at the interface of the two domains and are conserved in all known sequences of trypanosomal cysteine proteases and in cathepsins B and L (Lecaille *et al.*, 2001).

Congopain, like cruzipain and trypanopain, is activated by reducing agents (DTT, L-cysteine, 2-mercaptoethanol), and shows a marked preference for aromatic residues, such as Phe, at P2, and hydrolyses Z-Phe-Arg-AMC, but not Z-Arg-Arg-AMC (Troeberg *et al.*, 1996; Chagas *et al.*, 1997). Congopain is inhibited by low molecular weight inhibitors such as E-64 and leupeptin. It is strongly inhibited by cystatins and kininogens (Chagas *et al.*, 1997), and by ICPs (Prof Jeremy Mottram, University of Glasgow, Scotland, personal communication). The pH profile of congopain differs from that of cruzipain and trypanopain and its pH optimum is 6.4, compared to optima of pH 5 for cruzipain (Chagas *et al.*, 1997) and pH 5.5 for trypanopain (Troeberg *et al.*, 1996).

Congopain is a dominant antigen of *T. congolense* parasites, eliciting both humoral (Authié *et al.*, 1993a) and cellular responses (Lutje *et al.*, 1995a). It elicits both IgM and IgG1, but no IgG2, during infection. IgM has only been detected in immune complexes with congopain, while free IgG1 has consistently been detected as a major component of the immune response (Authié *et al.*, 1993a). Whilst the C-terminal domain is the most antigenic region, the catalytic domain is also antigenic in trypanotolerant cattle, which is surprising considering its high degree of conservation among cysteine proteases of the papain-family (Boulangé *et al.*, 2001).

Observations with congopain, and reports on cysteine proteases of other parasites, are beginning to confirm a pathogenic role for congopain, and other trypanosome cysteine proteases, as well as the hypothesis that anti-congopain antibodies may contribute to mechanisms of trypanotolerance (Lalmanach *et al.*, 2002). A further study supporting the pathogenic role of congopain found that plasma from *T. congolense* infected cattle contained parasite-specific cysteine protease activity and a precursor form of congopain. This suggests that procongopain may be extracellularly processed into its mature form in the presence of blood vessel glycosaminoglycans (Serveau *et al.*, 2003).

C2 is a 27 kDa recombinantly expressed truncated form of congopain containing only the catalytic domain. The proregion was included in the construct as it is likely to play an essential role in the correct folding and secretion of the enzyme (Vernet *et al.*, 1990). The proenzyme was expressed in a baculovirus system, and the proregion was removed by processing *in vitro* at acidic pH into the catalytically active enzyme. Kinetic studies have confirmed the relatedness between C2 and congopain (Boulangé *et al.*, 2001). In the present study, the interaction of congopain and C2 with α_2M was investigated. α_2M was also used as an adjuvant in the immunisation of rabbits with C2, to investigate the possibility of its use in an anti-trypanosomosis vaccine. Additionally, C2 was used to conjugate oligopeptidase B to α_2M, as discussed in Section 7.3. The properties of α_2M are discussed in Section 1.3.

1.2.2 Serine proteases

Serine proteases are those enzymes that depend on a serine residue in the active site for catalytic activity. The catalytic triad of serine proteases is usually completed with histidine and aspartate amino acid residues. These enzymes normally require no cofactors for activity, and the chemistry of the hydroxyl group of serine usually leads to these enzymes being most active at neutral or slightly alkaline pH (Barrett, 2002).

It has become clear that serine-dependent proteases have many separate evolutionary origins, resulting in some fundamental differences among them. Five or six clans,

grouping proteases by their common ancestry, have been proposed (Barrett and Rawlings, 1995).

The prolyl oligopeptidases make up family S9 of clan C (Rawlings and Barrett, 1994). This family contains peptidases with a very varied range of restricted specificities, all having activity restricted to the hydrolysis of peptides, with members known from prokaryotic and eukaryotic organisms. The family contains soluble and membrane-bound peptidases. Included with the cytosolic peptidases are two oligopeptidases with different P1 specificities: prolyl oligopeptidase (EC 3.4.21.26), which cleaves prolyl bonds, and oligopeptidase B (EC 3.4.21.83), which cleaves arginyl and lysyl bonds. The active site serine, aspartate and histidine residues have been identified in family S9, with conservation of the amino acids around them.

1.2.2.1 Oligopeptidase B

The oligopeptidase B subfamily of enzymes show greater amino acid sequence homology to each other than to the prolyl oligopeptidases, and the kinetoplastid oligopeptidase B enzymes show the greatest similarity to each other (Morty *et al.*, 1999b).

Oligopeptidase B has to date only been found in gram negative bacteria (Pacaud and Richaud, 1975; Morty *et al.*, 2002) and ancient eukaryotic unicellular organisms (Kornblatt *et al.*, 1992; Burleigh *et al.*, 1997; Morty *et al.*, 1999b). Recently it was also identified in higher plants, namely wheat germ of *Triticum aestivum* (Tsuji *et al.*, 2004), where it is believed to play a role in embryo development.

The oligopeptidase B enzymes possess a GXSXGGZZ consensus sequence, where X is any residue, Z is a hydrophobic residue and S is the catalytic serine residue (Ser^{563} in *T. b. brucei*; Morty *et al.*, 1999b). Additionally, the enzymes exhibit considerable sequence conservation within the catalytic domain. The activity of oligopeptidase B is enhanced by reducing agents and by spermine and spermidine, and inhibited by thiol-reactive agents such as iodoacetate. Inhibition by thiol-reactive agents is explained by a crucial

cysteine residue in close proximity to the catalytic site in the folded enzyme, identified as Cys^{256} in *T. b. brucei* by Morty *et al.* (2005b).

The crystal structure of oligopeptidase B has not yet been determined. However, the crystal structure of the closely related prolyl oligopeptidase has been determined (Fülop *et al.*, 1998), and a 3D model for oligopeptidase B has been constructed by homology modelling (Gérczei *et al.*, 2000; Section 3.3). Two domains have been identified in the protein. The first is an N-terminal β-propeller domain which excludes proteins from the enzyme's active site. The second is a C-terminal α/β hydrolase domain containing substrate-binding and catalytic residues.

Oligopeptidase B has been isolated from the stercorarian trypanosome *T. cruzi* (Burleigh *et al.*, 1997), the etiological agents of Chaga's disease, where it is expressed in all life cycle stages. The enzyme has been attributed a role in the pathogenesis of the disease by generating a calcium signalling factor mobilising Ca^{2+} from intracellular calcium pools. This Ca^{2+} signalling is a prerequisite for trypanosome invasion.

Oligopeptidase B has also been isolated from the salivarian trypanosomes *T. b. brucei* (Troeberg *et al.*, 1996), *T. congolense* (Morty *et al.*, 1999a) and *T. evansi* (Morty *et al.*, 2005a). Oligopeptidase B has been implicated as a therapeutic target since drugs used to treat African trypanosomosis inhibit the enzyme (Morty *et al.*, 1998), and specific oligopeptidase B inhibitors exhibit anti-trypanosomal activity and improve the outcome in a mouse model of *T. b. brucei* infection (Morty *et al.*, 2000). Oligopeptidase B has also been validated as a virulence factor in African trypanosomes, since it is released into the host circulation by dead or dying parasites, where it retains catalytic activity (Morty *et al.*, 2001) due to the enzyme's insensitivity to serum protease inhibitors (Troeberg *et al.*, 1996). The enzyme probably degrades host regulatory peptides, such as vasopressin, neurotensin and atrial natriuretic factor (Troeberg *et al.*, 1996), resulting in serious consequences to the metabolic homeostasis of the host. Atrial natriuretic factor levels in infected hosts have indeed been found to be depressed by oligopeptidase B, abrogating the prohypotensive properties of atrial natriuretic factor and leading to increased blood

volume (Morty *et al.*, 2005a). Elevated blood volume, a characteristic of infection with bloodstream trypanosomes, could lead to circulatory system lesions and further circulatory complications associated with trypanosome infections. In the present study oligopeptidase B from *T. congolense* and *T. vivax* was recombinantly expressed, purified and characterised. The enzymes were used for immunological studies in chickens and rabbits, also involving α_2M as an adjuvant (see Section 1.4).

1.3 α_2-Macroglobulin

α_2-Macroglobulin (α_2M) is a high molecular weight plasma glycoprotein and the major representative of the group of plasma proteins collectively termed the α-macroglobulins (αMs) (Sottrup-Jensen, 1989). This group has also been termed the thioester plasma protein family because the proteins have a unique cyclic thioester bond. The αM group includes complement components C3 and C4, pregnancy zone protein (Borth, 1992), and a cell surface antigen CD109, which is suggested to play a role in T cell-B cell interactions (Lin *et al.*, 2002). Most αMs are tetramers assembled from pair-wise disulfide-bridged 180 kDa subunits, but dimeric and monomeric αMs are also known to exist (Sottrup-Jensen, 1989). α_2M is a tetramer made up of four identical subunits of 180 kDa that are linked in pairs by disulfide bonds. The pairs are associated non-covalently (Fig. 1.5) (Barrett *et al.*, 1979). The molecular weight of the α_2M tetramer is approximately 720 kDa (Swenson and Howard, 1979).

The αMs have been isolated from invertebrates, reptiles, birds, and many mammalian species (Sottrup-Jensen *et al.*, 1989). Specifically, α_2M has been isolated and characterised from vertebrate species such as rabbit, dog, rat, mouse, horse, pig, cow and hedgehog (Starkey and Barrett, 1982), and ostrich (Van Jaarsveld *et al.*, 1994). The α_2Ms themselves show homology between species, with the bait region sequence being the only major segment of dissimilar sequence (Sottrup-Jensen *et al.*, 1989).

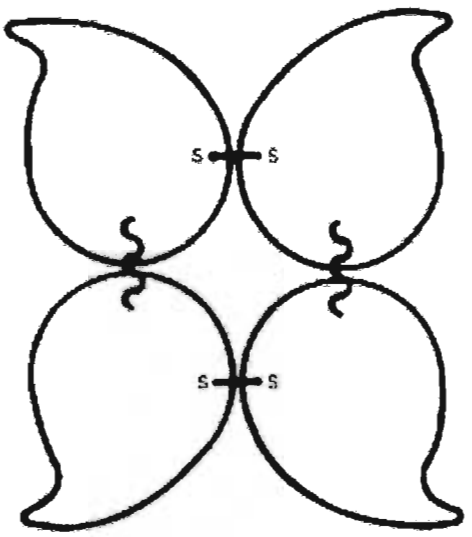

Figure 1.5. Diagrammatic representation of the α_2M tetramer. Subunits are linked in pairs by disulfide bonds (represented by S-S), which are non-covalently associated (represented by curved lines). The shapes indicated are not intended to resemble their actual shape (Barrett *et al.*, 1979).

α_2M was originally characterised as a broad spectrum protease inhibitor, as it is capable of inhibiting proteases from all mechanistic classes (Barrett and Starkey, 1973). Although there are many proposed functions of α_2M, there still remains considerable uncertainty regarding its primary function. Besides the ability of α_2M to "trap" proteases upon cleavage of the bait region, it is also capable of forming covalent complexes with proteases or non-proteolytic proteins and peptides via its thioester upon activation with a protease (Van Leuven *et al.*, 1982). This intrinsic ability of α_2M to irreversibly bind antigen, as well as the more recently identified role of α_2M in antigen delivery to antigen presenting cells (Chu and Pizzo, 1993), has sparked an interest in the use of α_2M in vaccines (Chu *et al.*, 1994; Cianciolo *et al.*, 2002; Liao *et al.*, 2002).

1.3.1 The α_2M subunit

Each of the four identical 180 kDa subunits of α_2M contains three major sites essential for its physiological function: the bait region, the internal thioester, and the receptor recognition site (Van Leuven *et al.*, 1986) (Fig. 1.6).

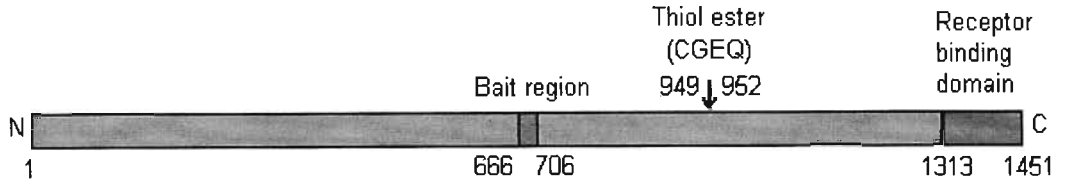

Figure 1.6. A schematic representation of the α_2M subunit, showing the locations of the features of the molecule that are vital to its function (Webb *et al.*, 1998).

The subunit polypeptide chains are cross-linked by the two disulfide bonds in an anti-parallel manner, presumably near the centre of the dimer's structure. The bait domains and thioester moieties are located near the structure's central cavity and the receptor binding domain in the C-terminal region of the subunits. The numerous intramolecular disulfide bonds associated with the subunit form loops in the polypeptide chains which probably contribute to its domain organisation and the stability of the subunit's architecture (Fig. 1.7) (Kolodziej *et al.*, 2002).

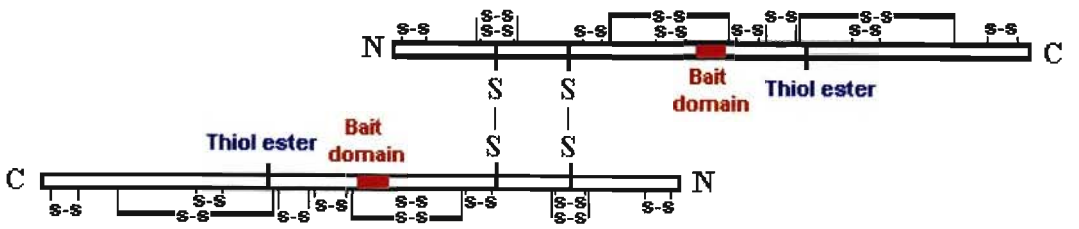

Figure 1.7. A diagram of the α_2M dimer showing the orientation of the subunits and positions of the inter- and intradisulfide bridges (Kolodziej *et al.*, 2002).

1.3.2 The bait region

The bait region is an exposed 25 amino acid stretch within the middle of each subunit that can be cleaved by virtually any active protease. Proteases can cleave the bait region whether they originate in the host or are released by microorganisms (Harpel, 1976; Barrett, 1981; Nyberg *et al.*, 2004). In human α_2M, the bait region is situated between residues 666 and 706. The bait regions of various species of α_2M, though functionally analogous to that of human α_2M, are dissimilar in sequence and are of different lengths. There is evidence that the bait region is flexible. Although restrictions of motility do

exist, residues 683-700 appear to constitute a highly flexible surface loop (Sottrup-Jensen *et al.*, 1989).

Complex formation with a variety of different proteases is initiated by specific cleavage in the bait region, and the sites of cleavage have been determined for many proteases (Sottrup-Jensen *et al.*, 1989; Tortorella *et al.*, 2004). Cleavage of the bait region results in an immediate and distinct conformational change of the α_2M molecule that is closely linked with the cleavage of the internal thioester. The instantaneous conformational change mediates non-covalent "trapping" of the protease and physical exclusion of large (but not small) substrates from the active centre of the enzyme. This "trapping" of the protease by α_2M does not block the protease's active site and thus contrasts with any other known mechanism of protease inhibitor-protease complex formation that involves irreversible active site inhibition (Borth, 1992).

The conformational change of α_2M can also be induced *in vitro* by treatment with methylamine (Barrett *et al.*, 1979). However, this leads to inactivation of α_2M with respect to protease binding (Steinbuch *et al.*, 1968). Although reaction of α_2M with methylamine or protease seems to be kinetically different, the final conformation assumed by α_2M after the reactions is indistinguishable in several aspects: electrophoretic mobility, pI, complexity of pattern in isoelectric focusing, and receptor-mediated endocytosis by cells in culture (Van Leuven *et al.*, 1981).

1.3.3 The internal β-cysteinyl-γ-glutamyl thioester

The thioester structure of α_2M forms a 15-membered thiol lactone ring involving the conserved segment Cys^{949}-Gln^{952} (Sottrup-Jensen, 1989). Activation of α_2M by proteases produces an intermediate ("nascent") α_2M with reactive glutamyl and cysteinyl residues in each of the four subunits. In the proteolytically activated "nascent" state, the thioester is highly reactive and can be rapidly cleaved by a wide range of predominantly nitrogen nucleophiles to form substituted Gln residues. Adjacent lysine residues belonging to

either the activating protease or adjacent proteins or peptides become covalently bound through ε-Lys-γ-Gln bonds (Fig. 1.8) (Sottrup-Jensen, 1989).

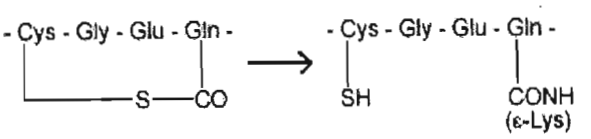

Figure 1.8. Structure and reaction of the internal thioester of α_2M. The reaction of the β-cysteinyl-γ-glutamyl thioester with a lysine residue of an adjacent protease or peptide upon proteolytic activation is shown (Sottrup-Jensen, 1989).

1.3.4 The receptor binding domain

The receptor binding site of α_2M is located in the 138-residue C-terminal receptor binding domain (RBD) expressed in each of the four subunits after cleavage of the thioester (Sottrup-Jensen, 1989). The conformational change that results from cleavage of the α_2M bait region by proteases, as well as activating the internal thioester in each subunit, exposes previously concealed recognition sites for the α_2M receptor (α_2MR) that is present on a variety of cells including macrophages, fibroblasts and hepatocytes (Debanne *et al.*, 1975; Van Leuven *et al.*, 1979). The activated form of α_2M is therefore referred to as receptor-recognised α_2M.

A study on the receptor recognition of human α_2M after dissociation into half molecules has led to the conclusion that contact zones between the α_2M dimers are essential for keeping α_2M in a native conformation. The contact zones are also necessary for the transfer of activation signals, by the cleavage of bait regions and/or thioesters, to the C-terminal part of the molecule with concomitant exposure of the receptor-binding domains (Jensen *et al.*, 2001).

1.3.5 The conformational changes of the α_2M molecule

The conformational change from the native form of α_2M to the receptor-recognised form also leads to an electrophoretic shift from a slow to faster form in native polyacrylamide gel electrophoresis (PAGE). This is as a result of the molecule becoming more compact, and the term "slow" α_2M has been assigned for the native form and "fast" α_2M for its

receptor-recognised activated form (Barrett *et al.*, 1979). Fig. 1.9 shows one of the models by which the conformational change and "trapping" mechanism is thought to occur (Feldman *et al.*, 1985).

The remarkable structural arrangement of α_2M has been the subject of numerous electron microscopy studies. Electron micrographs of the "fast" form of human α_2M and other tetramic αM's reveal well defined monogram-like objects resembling the Cyrillic letter Ж, measuring about 15.5 by 25 nm. Three-dimensional reconstructions of native and fully transformed α_2M reveal molecules of very different shapes, which has been confirmed by low-resolution X-ray mapping as well as other physicochemical studies (Qazi *et al.*, 1998). The native structure is more globular, with dense end regions that are connected by twisted strands (Boisset *et al.*, 1996). The transformed structure has a more compact central region of protein with four arms extending from its sides, similar to the letter "H" (Andersen *et al.*, 1995).

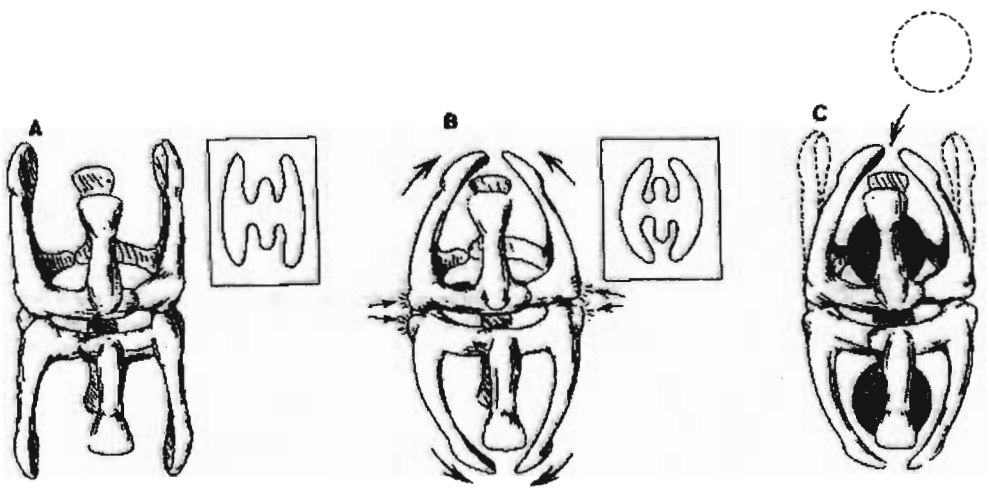

Figure 1.9. A model of protease-induced α_2M transformation (Feldman *et al.*, 1985). (A) The native conformation of α_2M, accompanied by a schematic presentation of the molecule as seen by electron microscopy (*Inset*). (B) The conformational change of α_2M accompanied by a schematic presentation of an electron micrograph of "fast" α_2M (*Inset*). Small arrows denote the positions postulated for the receptor-recognition sites. (C) Protease trapping by α_2M.

There is uncertainty surrounding the exact locations of the different regions of each α_2M subunit in the native and receptor-recognised structures, and the mechanism of protease

trapping. In a model proposed by Kolodziej *et al.* (2002), the RBDs are located within the join between the C-terminal ends of the subunits that form the chisel-like component of native α_2M. Upon cleavage of the thioesters, the ends separate to expose the RBDs at the ends of the transformed structure's arms (Fig. 1.10).

This model also suggests that the bait domains are externally exposed on the native structure, and are readily accessible to proteases irrespective of their size. Upon bait domain and thioester cleavages, the bait domains and their bound proteases are internalised through the large openings afforded by the half-transformed structure. Once inside, the geometric constraints of the cavity prevent the bait domain from binding to the protease's active site (Kolodziej *et al.*, 2002). Other studies have led to proposals that the bait region resides inside the native and transformed structure (Bowen and Gettins, 1998).

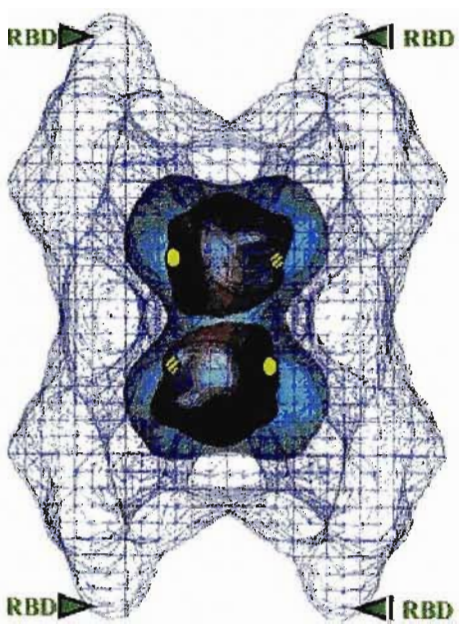

Figure 1.10. A wire frame display of the α_2M-chymotrypsin structure indicating the location of the sites of biological significance (Kolodziej *et al.*, 2002). The structure shows two chymotrypsin molecules (dark red) inside the cavity (blue) of transformed α_2M. The approximate locations of the receptor binding domains (RBD) are indicated by the arrows. The location of the Cys^{949} moieties on the wall of the cavity are indicated by the solid and hatched yellow ovals in front of and behind the chymotrypsin molecules, respectively.

1.3.6 Receptor-mediated endocytosis

Clearance of proteases after their inhibition by α_2M is mediated by a specific receptor that is expressed by fibroblasts, hepatocytes, adipocytes, astrocytes, dermal dendritic cells, syncytiotrophoblasts, monocytes and macrophages (Borth, 1992). These receptors recognise the changed structure of α_2M upon complex formation with a protease (Van Leuven *et al.*, 1986). The methylamine activated form of α_2M is recognised and internalised by the same mechanism as protease activated α_2M (Van Leuven *et al.*, 1981).

The α_2M receptor (α_2MR), also known as the low density lipoprotein receptor-related protein (LRP) or CD91 (Hart *et al.*, 2004), is composed of two subunits of approximately 515 and 85 kDa (Herz *et al.*, 1991). The 515 kDa N-terminal subunit contains the binding site for α_2M. It is composed of clusters of ligand binding cysteine-rich repeats and epidermal growth factor receptor like cysteine-rich repeats, separated by a cysteine-poor region that contains the tetrapeptide sequence YWTD (Herz *et al.*, 1990). The 515 kDa subunit remains attached to the membrane through non-covalent interaction with the 85 kDa subunit, which contains the membrane spanning region and cytoplasmic tail (Herz *et al.*, 1991). The cytoplasmic tail contains two copies of the sequence NPXY (where the X represents any amino acid), which are believed to direct the receptor to coated pits on the cell surface (Chen *et al.*, 1990).

The ligand binding regions in α_2MR occur in four clusters (clusters I-IV) containing between 2 and 11 individual ligand binding repeats. Nearly all of the ligands that bind this receptor interact with ligand binding clusters II and IV. The recognition of α_2M by its receptor requires the cooperation of two ligand binding cluster regions, clusters I and II (Mikhailenko *et al.*, 2001). A proposed model for the α_2M-receptor interaction is that the repeats from cluster I are in close proximity with the amino-terminal portion of cluster II to form a high affinity binding site for α_2M. A second model is that one subunit of α_2M recognises repeats within cluster I, while another subunit recognises repeats within the N-terminal region of cluster II.

The association of α_2M with its receptor is highly pH dependent, and acidification to pH 6.8 removes ligand receptor interaction, suggesting that upon endocytosis α_2M dissociates under the acidic conditions in the early stages of intracellular ligand sorting. This differs from other ligands that undergo receptor-mediated endocytosis which are released from their receptors at a pH of 6 or less (Borth *et al.*, 1990). Intact α_2M may also be sequestered for 24 hours and significant amounts of α_2M may be regurgitated intact (Maxfield *et al.*, 1981). Monoclonal antibodies directed against either the 515 kDa or the 85 kDa subunit of α_2MR are rapidly endocytosed by either subunit. Only the antibody against the larger subunit becomes degraded, whereas the antibody against the smaller subunit is recycled intact to the surface (Herz *et al.*, 1990). This finding indicates that the two subunits of α_2MR have different functions in the sorting and dispatching process in endosomes (Borth *et al.*, 1990).

Antigen presenting cells (APCs) that possess α_2MR, e.g. dendritic cells and macrophages, are proposed to present the antigen carried by α_2M (after processing of antigen) on MHC class II molecules, which in turn stimulate helper T cells ($CD4^+$ T cells), which leads to antibody synthesis. The implications of this are discussed further in the section on the biological functions of α_2M (Chu *et al.*, 1994).

"Fast" forms of α_2M can bind with both high and low affinity to α_2MR; high affinity when binding is dimeric to adjacent receptors, and low affinity when binding is monomeric due to scattered receptors or high receptor occupancy (Moestrup and Gliemann, 1991). Receptor-recognised αMs from different animal species cross-react with similar affinities for the α_2MR (Kaplan and Nielsen, 1979; Enghild *et al.*, 1989), since there is a high degree of receptor sequence homology between species.

In 1994, evidence was found that led to the conclusion that there is actually a second α_2MR, which was termed the α_2M signalling receptor (Misra *et al.*, 1994). Two classes of binding sites were identified on macrophages, one of high affinity and low capacity, and the other α_2MR which demonstrated lower affinity and higher binding capacity

(Howard *et al.*, 1996; Misra *et al.*, 1999). Some studies suggest that the α_2MR in association with one or more adaptor molecules constitute the α_2M signalling receptor (Trommsdorff *et al.*, 1998; Backsai *et al.*, 2000; Gotthardt *et al.*, 2000).

1.3.7 Biological functions of α_2M

There is evidence to suggest a role for α_2M in a wide variety of biological processes. Those functions suggested in the literature are briefly summarised in Table 1.2. An important development in the area regarding α_2M function is the finding that complexing antigen to α_2M results in an enhanced immune response (Chu and Pizzo, 1993; Mitsuda *et al.*, 1993; Chu *et al.*, 1994; Cianciolo *et al.*, 2002), leading to the suggestion that a biological function of α_2M is targeting antigen to APCs and enhancing antigen processing and presentation. α_2M may target potential antigen to macrophages (or any antigen presenting cell expressing α_2MR) within areas of inflammation (Chu and Pizzo, 1993). Areas of inflammation contain high levels of "slow" α_2M, both from increased plasma extravasation and from increased local synthesis by cells such as fibroblasts and macrophages (Chu *et al.*, 1994).

Table 1.2. Summary of the possible biological functions of α_2M.

Possible functions of α_2M	References
Counteracting proteases associated with infection and parasite growth	(Borth, 1992)
Enzyme inhibition, affecting a broad spectrum of biological processes	(Ikari *et al.*, 2001)
Regulation of growth factors and cytokines	(Liu *et al.*, 2001)
Growth factor	(Misra and Pizzo, 2002)
Regulation of immune cell development and function	(Hoffman *et al.*, 1987)
Affecting homeostasis of plasma cholesterol	(Krimbou *et al.*, 2001)
Protection against demyelination	(Gunnarsson and Jensen, 1998)
Role in progression of Alzheimer's disease	(Hughes *et al.*, 1998)
	(Van Uden *et al.*, 2000)
	(Mettenburg *et al.*, 2002)
Targeting antigen to antigen presenting cells	(Chu *et al.*, 1994)

By forming covalent complexes with different proteins, α_2M may be acting as a carrier or adaptor molecule that mediates rapid internalisation of these proteins. It has also been suggested that the unusual protein trapping mechanism of α_2M is primarily an antigen sampling device (Binder *et al.*, 2001). Because complex formation depends upon proteolytic activity, which is usually tightly controlled *in vivo*, the proteins carried by α_2M into the macrophages would be limited to those that are present in areas of enhanced proteolytic activity, as might be expected in areas of inflammation. Since α_2M can be activated by completely unrelated proteases, both pathogen- and host-derived proteases could serve this purpose (Chu and Pizzo, 1993). There is also a potential role for α_2M in antigen processing. Upon endocytosis into macrophages or other APCs that express the receptor, the bound proteins would be processed and presented for surveillance by T cells (Chu *et al.*, 1994).

1.4 Objectives of the present study

A vaccine for trypanosomosis in cattle, although difficult to develop due to parasite antigen variation, is likely to have a great impact on the control of the disease. An anti-disease vaccine, whilst unlikely to affect the survival of the parasite, would aim to neutralise pathogenic factors of the disease, such as parasite proteases. Congopain and oligopeptidase B have been identified as contributing to pathogenesis, and are attractive anti-disease vaccine candidates (Authié *et al.*, 2001; Morty *et al.*, 2001). The aim of the present study was to raise antibodies in rabbits against these enzymes that would inhibit their activity against peptide substrates *in vitro*. C2 was used as an immunogen, rather than the whole protein congopain. Congopain has a highly immunogenic C-terminal extension that is unlikely to elicit antibodies that will inhibit the enzyme. Therefore, a truncated form of the enzyme, C2, was used to avoid misdirection of the immune system. The possibility of enhancing the immunogenicity of C2 and of oligopeptidase B by using α_2M as an adjuvant (by proteolytic complexation of C2 and oligopeptidase B to α_2M) was assessed. α_2M, specifically human and murine, has been used to varying degrees of success to target different antigens to antigen presenting cells and enhance immunogenicity (Chu *et al.*, 1994; Cianciolo *et al.*, 2002; Liao *et al.*, 2002). Since α_2M is a naturally occurring protease inhibitor in bovine plasma, it is likely that it would play

a role in inhibition of congopain *in vivo*, and may be responsible for limitation of pathology in trypanosomosis as has been suggested for cruzipain in Chaga's disease (Ramos *et al.*, 2002).

The computer program Predict7 was used to identify probable immunogenic regions of oligopeptidase B from *T. congolense* (Chapter 3). The seven best peptide regions were selected and used to produce anti-peptide antibodies in chickens, to assess their immunogenicity. These anti-peptide antibodies were used in assays to determine their inhibitory effect on recombinant oligopeptidase B, produced as described in Chapter 4. It was hoped that a peptide epitope of oligopeptidase B would be found that produced antibodies that were inhibitory towards the enzyme, as such a peptide could be used in an anti-disease vaccine. The anti-peptide antibodies were also used in western blotting throughout the study, to identify oligopeptidase B. A 3D model of *E. coli* oligopeptidase B was used to identify the locations of the peptides on the enzyme, and also the secondary structures that they may form (Chapter 3). This was done to aid in the explanation of the results of the anti-peptide antibody assays.

Large amounts of oligopeptidase B were required in the present study for the formation of oligopeptidase B-C2-α_2M complexes for immunisation. Also, the use of oligopeptidase B in an anti-disease vaccine would require the production of large amounts of the enzyme. To this end, the oligopeptidase B open reading frame (ORF) was cloned from the major causative agents of cattle trypanosomosis, *T. congolense* and *T. vivax*. The ORFs were expressed in *E. coli* and the recombinant proteins were purified (Chapter 4). Recombinant C2 was also expressed and purified in large quantities for use in assays with α_2M, and the preparation of complexes with α_2M (and oligopeptidase B) for immunisation. Small quantities of native oligopeptidase B were isolated from *T. b. brucei*, *T. congolense* and *T. vivax* lysates. These native enzymes were used in assays to determine their K_m for the substrate Z-Arg-Arg-AMC for comparison with the recombinant enzymes (Chapter 5). They were also used in assays to determine whether antibodies raised against recombinant oligopeptidase B would inhibit the native enzymes (Chapter 8).

The purified recombinant oligopeptidases from *T. congolense* (rOPC) and *T. vivax* (rOPV) were kinetically characterised as reported in Chapter 5. This was done to ensure that the recombinant enzymes showed similar enzymatic behaviour to the trypanosomal oligopeptidases that have previously been characterised. This was necessary to ensure that the recombinant enzymes were an accurate replacement for the native enzymes in a vaccine. Also, since native oligopeptidase B from *T. vivax* has not yet been isolated and characterised, characterisation of the recombinant enzyme would offer some insight into the enzymatic behaviour of the native protein. Native oligopeptidases that were isolated (Chapter 4) were assayed to acquire comparative K_m values for the substrate Z-Arg-Arg-AMC (Chapter 5).

α_2M was purified from both bovine and rabbit plasma (Chapter 6) for use in assays to assess their interaction with C2 and other cysteine proteases (Chapter 7) and to prepare complexes with C2 and oligopeptidase B for immunisation of rabbits (Chapter 8). The ultimate use of the anti-disease vaccine would be in cattle, therefore, α_2M was purified from bovine plasma for comparative evaluation. α_2M was purified from rabbit plasma to obtain a reflection of the use of 'self' α_2M in the context of an anti-disease vaccine, since rabbits were used in the present study to raise antibodies.

The interaction of bovine α_2M with the cysteine proteases papain, cathepsin L, congopain, and C2 (the catalytic domain of congopain) was studied using enzymatic activity assays with various substrates of differing molecular sizes, as well as PAGE and SDS-PAGE (Chapter 7). This was necessary in order to establish the interaction of C2 with α_2M before preparing α_2M-C2 complexes for use in immunisation (Chapter 8). Complexes of oligopeptidase B, C2 and bovine α_2M were also prepared (Chapter 7) to assess the efficiency of α_2M as an antigen delivery system for multiple antigens by raising antibodies against the complexes in rabbits (Chapter 8).

Antibodies were raised in chickens against rOPC and rOPV (Chapter 8), and were affinity purified for use in immunological assays and the immunoaffinity purification of

native oligopeptidase from parasite lysates (Chapter 4). The main objective of the present study was to produce antibodies against C2 and oligopeptidase B in a mammalian system that would recognise the native enzymes and inhibit their activity *in vitro*. Another objective was to establish the effectiveness of α_2M as an antigen delivery system for C2 and oligopeptidase B. The efficiency of several adjuvant systems was assessed and compared (Chapter 8); i.e. Freund's adjuvant, bovine α_2M, rabbit α_2M and alum. Antibodies levels were monitored by ELISA, and their ability to inhibit the activity of C2, congopain, rOPC, rOPV and native oligopeptidase B from *T. b. brucei*, *T. congolense* and *T. vivax* was determined in assays against fluorogenic peptide substrates.

CHAPTER 2
GENERAL MATERIALS AND METHODS

A variety of general biochemical and molecular biology techniques which were used throughout the present study are described in this chapter. More specific experimental procedures are described in their appropriate chapters.

2.1 Materials

Buffer salts and other common chemicals were from Saarchem (South Africa), BDH (England), ICN Biomedicals, (USA), and Roche Diagnostics (Germany), and were of the highest purity available. Unless otherwise specified, distilled water (dH_2O), produced by a Milli-Q Plus ultra pure water system from Millipore (USA), was used throughout this study.

Iminodiacetic acid, Sephacryl S-300 HR, Coomassie blue R-250, Serva blue G, cysteine.HCl, TPCK-treated trypsin, papain, hide powder azure, 4-chloro-1-naphthol, *N*-ethylmaleimide, pepstatin A, iodoacetamide, sodium iodoacetate, benzamidine.HCl, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), soy bean trypsin inhibitor (SBTI), lime bean trypsin inhibitor (LBTI), 1,10-phenanthroline, $NaCNBH_3$, $NaBH_4$, kanamycin, SDM-79, sodium bicarbonate, 4-methylumbelliferyl-*p*-guanidobenzoate (MUGB), 4-methylumbelliferone, L-ornithine, 1,4-diaminobutane, 2-(*N*-morpholino)ethanesulfonic acid (MES), sorbitol, yeast extract, yeast nitrogen base powder, tetracycline, chloramphenicol, agarose, lysozyme, glutathione agarose, reduced glutathione, antithrombin III, spermine, spermidine, adenosine triphosphate (ATP), guanidine triphosphate (GTP), Freund's complete and incomplete adjuvants, Bz-Pro-Phe-Arg-pNA, Z-Phe-Arg-AMC, Z-Arg-Arg-AMC, and Boc-Gly-Lys-Arg-AMC were from Sigma (USA). Other synthetic peptide substrates were from Peninsula Laboratories (USA) and Bachem (Switzerland). 2,2′-Azinobis[3-ethyl-2,3-dihydrobenzthiazole-6-sulfonate (ABTS), bovine serum albumin (BSA), dithiothreitol (DTT), phenyl-methylsulfonylfluoride (PMSF), aprotinin, 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF), D-biotin, FastStart Taq DNA polymerase, the Expand High Fidelity PCR

system, dNTP mix, 25 mM $MgCl_2$, shrimp alkaline phosphatase and the Rapid DNA ligation kit were from Roche (Germany). PEG 20 000, PEG 6 000 and zinc chloride were from Saarchem (South Africa), and Triton X-100 was from BDH (England). Tween 20, Brij 35, bacto-tryptone, 3,4-dichloroisocoumarin (DCI), glutaraldehyde, and heparin (sodium salt, from porcine intestinal mucosa) were from Merck (Germany). Chymostatin, leupeptin, antipain and H-Arg-AMC were from Cambridge Research Chemicals (England). Alhydrogel® was from Brenntag Biosector (Denmark) and EndoTrap® red columns were from Profos AG (Germany). Native PAGE and high range molecular weight markers, High-range Rainbow™ molecular weight markers, pGEX4T1, and SP Sephadex™ C-25 were from GE Healthcare (USA). LMP agarose, the Wizard® *Plus* SV Minipreps DNA purification system, the Wizard® DNA Clean-Up System, the Wizard® PCR Preps DNA Purification System, ethidium bromide solution, *Eco*R1, *Not*1, *Sal*1, and 1 kb DNA step ladder were from Promega Corporation (USA). Restriction grade thrombin was from Novagen (USA), pPIC9 was from Invitrogen (USA), MassRuler™ from Fermentas (Lithuania), and fetal bovine serum from Gibco (USA). SDS-PAGE molecular weight markers (high range), Precision Plus molecular weight markers and Poly-Prep® chromatography columns were from Bio-Rad Laboratories (USA). Dimethylsulfoxide (DMSO), ampicillin, and hemin were from Fluka Chemicals (Germany), and Aminolink® and Sulfolink® were from Pierce Chemical Company (USA). DNA primers were from Proligo (France).

K3E Vacutainer™ tubes were from BD Vacutainer Systems (UK), Whatman No. 1 filter paper was from Whatman International Ltd (UK), and Nunc-Immuno™ 96-well plates and FluorNunc® 96-well fluorometry plates were from Nunc Intermed (Denmark). Sterile disposable plastic ware for cell culture was from Greiner Bio-one (Germany), and Stericup™ 0.22 µm filtration systems and Amicon Centripreps (YM 30) were from Millipore (USA).

HRPO-conjugated rabbit anti-chicken IgY and HRPO-conjugated goat anti-rabbit IgG were from Jackson Immunochemicals (USA). Sheep liver cathepsin L, chicken anti-bovine α_2M antibodies, and affinity purified chicken anti-C2 peptide antibodies were in-

house preparations. Recombinant congopain was supplied by Dr Edith Authié, International Livestock Research Institute, Nairobi, Kenya. Genomic DNA from *T. congolense* IL3000 and *T. vivax* IL4186, trypanosomal lysates for SDS-PAGE, *T. congolense* IL3000 and *T. vivax* IL4186 parasites for protein isolation, and *T. b. brucei* strain EATRO 1125 HN T70 procyclics for culture were provided by Dr Alain Boulangé (parasite material was prepared at the International Livestock Research Institute, Nairobi, Kenya).

Gel and blot images were captured with a VersaDoc imaging system using Quantity One software from Bio-Rad (USA). Absorbance readings were taken with an Ultrospec 2100 pro spectrophotometer. PCR was carried out in a GeneAmp 9700 PCR system from Applied Biosystems (USA). Sonication was carried out with a VirSonic 60 from The Virtis Company (USA). ELISA plates were read with a Versamax microplate reader using SOFTmax® software from Molecular Devices (USA).

Ethical approval for the procedures carried out in the present study was obtained from the University of KwaZulu-Natal animal ethics committee (reference AE/Coetzer/01/02).

2.2 Protein assays

2.2.1 Bradford protein assay

The Bradford protein assay was routinely used for protein quantitation of samples. The Bradford protein determination method involves the binding of dye to protein, chiefly to arginine residues (Compton and Jones, 1985), causing a shift in the absorption maximum of the dye from 465 nm (red) to 595 nm (blue). The assay is highly reproducible, rapid, and stable for up to 1 h. It is also highly sensitive due to the high extinction coefficient of the dye-protein complex (Bradford, 1976). The original assay utilising the dye Coomassie brilliant blue G-250 was subject to variations in response to different proteins. For this reason the assay was modified to include Serva blue G dye which resulted in increased sensitivity so that a protein concentration of 2 μg/ml could be detected in 50 μl of sample volume (Read and Northcote, 1981).

2.2.1.1 Reagents

Dye reagent. Serva blue G dye (50 mg) was completely dissolved in 85% phosphoric acid (50 ml) and 95% ethanol (23.5 ml). The solution was made up to 500 ml with dH_2O and stirred for 30 min, filtered through Whatman No. 1 filter paper and stored in a brown bottle at room temperature. It is stable for several weeks but regular visual checks were made for precipitation before use. If precipitation was visible, the reagent was filtered and re-calibrated before use.

Standard protein stock solution. A 1 mg/ml ovalbumin solution was made up in dH_2O, and further diluted to 100 μg/ml for the assay.

2.2.1.2 Procedure

A standard curve was constructed in the range of 1-5 μg of protein (Fig. 2.1). Quintuplicate samples of 0-50 μl of the 100 μg/ml protein standard solution were diluted to 50 μl with dH_2O and 950 μl dye reagent was added. Solutions were mixed and colour was allowed to develop for 2 min. The A_{595} was read in 1 ml plastic micro-cuvettes.

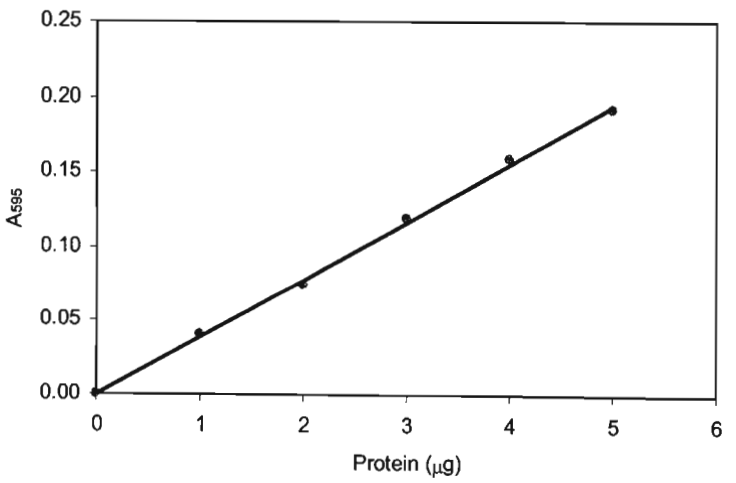

Figure 2.1. Standard curve for the Bradford assay, relating protein (1-5 μg) to absorbance at 595 nm. The equation of the trend line is given by $y = 0.0388x + 0.0002$, with a correlation coefficient of 0.9985.

Samples of unknown concentration were diluted to 50 µl with dH_2O and assayed with 950 µl dye reagent as above. The A_{595} was related to protein concentration by linear regression analysis of the standard curve.

2.2.2 Extinction coefficient

To determine the concentration of a purified protein, the equation $A = \varepsilon Lc$ can be used, where,

A is the absorbance at 280 nm,

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

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

c the concentration of the absorbing solution ($mg.ml^{-1}$).

The concentration of a number of proteins was determined by their absorbance at 280 nm, using their respective extinction coefficient (Table 2.1). A light path of 1 cm was used.

Table 2.1. The extinction coefficients used to determine the concentration of purified proteins.

Protein	ε	Reference
IgY	1.25	(Coetzer, 1993)
IgG	1.43	(Hudson and Hay, 1980)
Bovine α_2M	1.00	(Nagasawa *et al.*, 1970)

2.3 Concentration of proteins

It was often necessary to concentrate dilute protein samples before subjecting them to further purification, or for SDS-PAGE analyses. Dialysis against polyethylene glycol (PEG) 20 000 is a simple, low cost technique. PEG 20 000 is a dry, hydrophilic polymer that draws water from the sample through the dialysis membrane. An alternative technique is dialysis against sucrose, but the sucrose can diffuse through the dialysis membrane into the sample, and this is undesirable. SDS/KCl precipitation is a quick, small-scale method of concentration that was used where additional concentration was

required before reducing SDS-PAGE. The method is based on the precipitation of SDS-protein complexes by KCl, and results in 50-fold concentration.

2.3.1 Dialysis against PEG 20 000

The protein sample was placed in a dialysis bag (12 kDa cut-off) and completely covered by a thick layer of PEG 20 000 in a polypropylene tray at 4°C. After sufficient concentration of the sample, the dialysis bag was rinsed in dH_2O and the sample removed. Gloves were worn throughout the procedure to prevent contamination by skin keratins.

2.3.2 SDS/KCl precipitation

This procedure was used when the volume of a sample of protein required for reducing SDS-PAGE was too large for loading onto the gel.

2.3.2.1 Reagents

5% (w/v) SDS. SDS (0.5 g) was dissolved in dH_2O to a final volume of 10 ml.

3 M KCl. KCl (2.24 g) was dissolved in dH_2O to a final volume of 10 ml.

2.3.2.2 Procedure

5% SDS (10% of sample volume) was mixed with the sample in a 1.5 ml microfuge tube. 3 M KCl (10% of sample volume) was added and the mixture was centrifuged (12 000 *g*, 2 min, RT). The supernatant was discarded and the precipitate dissolved in stacking gel buffer (10 µl) (solution C, Section 2.4.1) and the appropriate treatment buffer (10 µl) (Section 2.4.1).

2.4 Laemmli SDS-PAGE

The Laemmli method was devised in order for PAGE and SDS-PAGE to be carried out with the same set of reagents, with the inclusion or omission of SDS (Laemmli, 1970). SDS-PAGE is dependent on the interaction of the protein molecule with SDS, an anionic detergent. The SDS interacts with the protein at a level that masks the charge of the

protein with the negative charge of SDS, thus giving all proteins the same charge to mass ratio and anionic migration. Thus, SDS-PAGE can be used for the separation of proteins based solely on size differences, and can be used for determination of molecular weight. More reliable molecular weight determination can be achieved by treatment of samples with 2-mercaptoethanol, a reducing agent, breaking the proteins into their constituent polypeptide chains by reducing interchain disulfide bonds (Weber and Osborne, 1969).

The SDS-PAGE system uses two types of gels containing Tris-HCl buffers: the stacking gel of large pore size, and the running gel of smaller pore size and higher pH. When an electrical field is applied to the system, an interface is formed between the Cl^- ion of high mobility, also known as the 'leading' ion, and the glycine ion, also known as the 'trailing' ion. As the interface migrates through the gel protein molecules, having a mobility intermediate to the 'leading' and 'trailing' ions, are concentrated into a thin band. The proteins in this band become stacked according to their mobilities. When the interface reaches the running gel the pH increases, resulting in increased mobility of the 'trailing' ion. Also, the proteins encounter the sieving effect of the smaller gel pores. As a result, the interface overtakes the stacked proteins, leaving them to separate according to their mobilities (Dennison, 1999).

2.4.1 Reagents

Solution A: Monomer solution [30% (w/v) acrylamide, 2.7% (w/v) bis-acrylamide]. Acrylamide (73 g) and bis-acrylamide (2 g) were dissolved and made up to 250 ml with dH_2O. The solution was filtered through Whatman No. 1 filter paper.

Solution B: 4 × Running gel buffer (1.5 M Tris-HCl buffer, pH 8.8). Tris (45.37 g) was dissolved in approximately 200 ml dH_2O, adjusted to pH 8.8 with HCl and made up to 250 ml with dH_2O. The solution was filtered through Whatman No. 1 filter paper.

Solution C: 4 × Stacking gel buffer (500 mM Tris-HCl buffer, pH 6.8). Tris (3 g) was dissolved in approximately 40 ml dH_2O, adjusted to pH 6.8 with HCl and made up to 50 ml with dH_2O. The solution was filtered through Whatman No. 1 filter paper.

Solution D: 10% (w/v) SDS. SDS (5 g) was dissolved in 50 ml dH_2O.

Solution E: Initiator [10% (w/v) ammonium persulfate]. Ammonium persulfate (0.1 g) was dissolved in 1 ml dH_2O.

Solution F: Tank buffer [250 mM Tris, 192 mM glycine, 0.1% (w/v) SDS]. Tris (3 g) and glycine (14.4 g) were dissolved and made up to 1 l with dH_2O. Prior to use, 5 ml of solution D (10% SDS) was added to 500 ml for use in the Bio-Rad Mini Protean® II.

Solution G: Reducing treatment buffer [125 mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol]. Solution C (2.5 ml), Solution D (4 ml), glycerol (2 ml) and 2-mercaptoethanol (1 ml) were made up to 10 ml with dH_2O.

Solution H: Non-reducing treatment buffer [125 mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol]. Solution C (2.5 ml), Solution D (4 ml) and glycerol (2 ml) were made up to 10 ml with dH_2O.

2.4.2 Procedure

SDS-PAGE was carried out according to Laemmli (1970) using a Bio-Rad Mini Protean® II vertical slab electrophoresis unit. Running and stacking gels were prepared as described in Table 2.2, depending on the concentration of acrylamide gel required. A stacking gel concentration of 3% was used only with a running gel of 5%.

For reducing SDS-PAGE, protein samples of at least 5 μg for Coomassie staining, or 500 ng for silver staining, were combined in a 1:1 ratio with solution G (reducing treatment buffer) and boiled for 2 min. For non-reducing SDS-PAGE, protein samples were combined in a 1:1 ratio with solution H (non-reducing treatment buffer). Standard curves were constructed for molecular weight markers, relating relative mobility to log of molecular weight for accurate estimation of molecular weight. This was carried out for each type of marker, at each percentage of acrylamide. A representative standard curve is shown in Fig. 2.2.

Table 2.2. Preparation of Laemmli SDS-PAGE running and stacking gels.

Reagent	Volume (ml)					
	Running gel (%)				Stacking gel (%)	
	12.5	10	7.5	5	4	3
A	6.25	5	3.75	2.5	0.94	0.71
B	3.75	5	3.75	3.75	0	0
C	0	0	0	0	1.75	1.75
D	0.15	0.15	0.15	0.15	0.07	0.07
E	0.075	0.075	0.075	0.075	0.035	0.035
dH_2O	4.75	6	7.25	8.5	4.3	4.53
TEMED	0.0075	0.0075	0.0075	0.0075	0.015	0.015

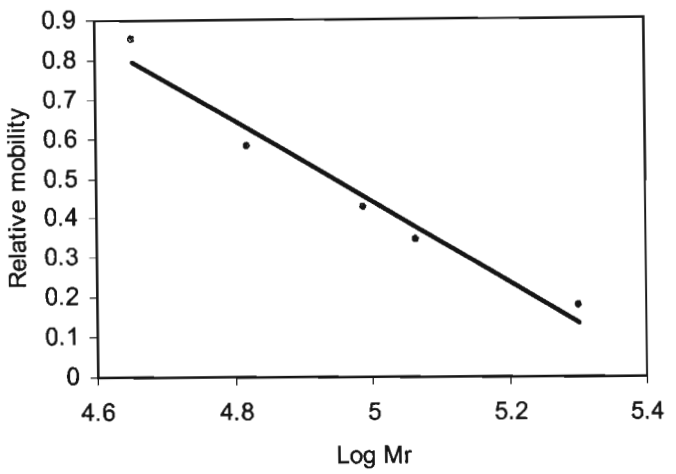

Figure 2.2. Standard curve relating relative mobility to log of molecular weight for high-range SDS-PAGE molecular weight markers (Bio-Rad) on a 7.5% SDS-PAGE gel. The markers consisted of myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (45 kDa). The equation of the trend line is given by $y = -1.0246x + 5.5638$, with a correlation coefficient of 0.968.

2.5 Laemmli PAGE

Laemmli PAGE was performed in exact accordance with Laemmli SDS-PAGE as described in Section 2.4, except that SDS (solution D, Section 2.4.1) was omitted from the system. That is, solution D was not added to the tank buffer and the volume of solution D prescribed for treatment buffer and running and stacking gels was replaced with dH_2O.

2.6 Protein staining procedures

2.6.1 Coomassie blue R-250 staining of proteins

Coomassie staining was used to detect protein bands after electrophoresis when the sample size was between 5-15 μg. This technique is simple but not very sensitive, with the capacity to detect down to 1 μg per protein band. The structure of the dye is shown in Fig. 2.3.

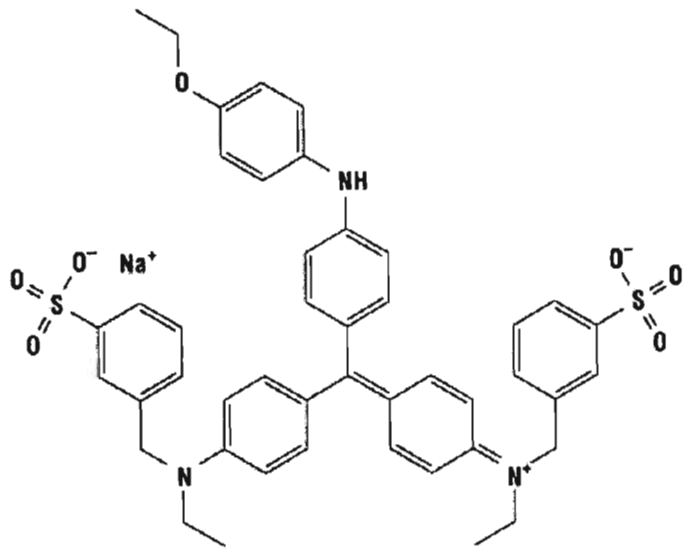

Figure 2.3. Structure of Coomassie blue R-250 dye (Syrovy and Hodny, 1991).

In the acidic staining solution the Coomassie blue R-250 dye becomes anionic, with two negatively charged sulfonic acid groups. The amino groups of the proteins in the gel become protonated, and thus positively charged. The dye binds to the protein by electrostatic interaction. The methanol in the staining solution allows the dye to penetrate the gel, whilst the acetic acid serves to fix the protein in the gel (Syrovy and Hodny, 1991).

2.6.1.1 Reagents

Stain stock solution [1% (w/v) Coomassie blue R-250]. Coomassie blue R-250 (1 g) was dissolved in 100 ml dH_2O by stirring for 1 h at room temperature. The solution was filtered through Whatman No. 1 filter paper.

Staining solution [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) acetic acid]. Stain stock (62.5 ml) was mixed with methanol (250 ml) and acetic acid (50 ml), and made up to 500 ml with dH_2O.

Destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid]. Methanol (500 ml) and acetic acid (100 ml) were made up to 1 l with dH_2O.

Destaining solution II [5% (v/v) methanol, 7% (v/v) acetic acid]. Methanol (50 ml) and acetic acid (70 ml) were made up to 1 l with dH_2O.

2.6.1.2 Procedure

Following electrophoresis, gels were immersed in staining solution (4 h). The gels were subsequently soaked in destaining solution I (overnight) followed by destaining solution II until background staining was completely removed.

2.6.2 Silver staining of proteins

The silver stain procedure was used for visualisation of small amounts of protein (around 500 ng) after electrophoretic separation. The silver staining technique has a considerably higher degree of sensitivity than staining with Coomassie blue R-250 (Blum *et al.*, 1987). This technique relies on the reduction of ionic silver in solution to its insoluble metallic form. This technique differs from other silver staining procedures by the inclusion of a pre-treatment step with sodium thiosulfate. This step considerably reduces the intensity of background staining by preventing the precipitation of insoluble silver complexes on the surface of the gel.

2.6.2.1 Reagents

Fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.0185% (v/v) formaldehyde]. Methanol (50 ml), glacial acetic acid (12 ml), and formaldehyde (50 µl of a 37% solution) were made up to 100 ml with dH_2O.

Washing solution 1 [50% (v/v) ethanol]. Absolute ethanol (50 ml) was made up to 100 ml with dH_2O.

Pretreatment solution [0.02% (w/v) $Na_2S_2O_3.5H_2O$]. $Na_2S_2O_3.5H_2O$ (20 mg) was dissolved in 100 ml dH_2O.

Impregnation solution [0.2% (w/v) $AgNO_3$, 0.02775% (v/v) formaldehyde]. $AgNO_3$ (200 mg) was dissolved in 100 ml dH_2O, and formaldehyde (75 µl of a 37% solution) was added.

Developing solution [6% (w/v) Na_2CO_3, 0.0004 % (w/v) $Na_2S_2O_3.5H_2O$, 0.0185% (v/v) formaldehyde]. Na_2CO_3 (6 g) was dissolved in 95 ml of dH_2O, $Na_2S_2O_3.5H_2O$ (2 ml of pretreatment solution) and formaldehyde (50 µl of a 37% solution) were added and the volume made up to 100 ml.

Stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid]. Methanol (50 ml) and glacial acetic acid (12 ml) were made up to 100 ml with dH_2O.

Washing solution 2 [50% (v/v) methanol]. Methanol (50 ml) was made up to 100 ml with dH_2O.

2.6.2.2 Procedure

All steps were carried out on an orbital shaker (50 rpm, RT). Following electrophoresis, the gel was soaked in fixing solution in a meticulously cleaned glass container to minimise background staining (1 h or overnight). The gel was washed in washing solution 1 (3 × 20 min), soaked in pretreatment solution (1 min), rinsed in dH_2O (3 × 20

s), and soaked in impregnation solution (20 min). The gel was rinsed in dH_2O (3 × 20 s) and immersed in developing solution until the protein bands became visible. The gel was washed in dH_2O (2 × 2 min), and development was stopped by soaking in stopping solution (10 min). Gels were washed in washing solution 2 (> 20 min).

2.7 Western blotting

Electrophoretic transfer of protein from polyacrylamide gels onto nitrocellulose was first described by Towbin *et al.* (1979). Protein bands separated on SDS-PAGE gels are transferred by electrophoresis to produce a replica on a nitrocellulose membrane. The gel and nitrocellulose are placed together in a "sandwich" which is placed in the blotting apparatus so that the gel is on the cathode side and the nitrocellulose on the anode side. Thus, negatively charged SDS-coated proteins migrate out of the gel towards the positive anode, subsequently binding to the nitrocellulose.

A blocking step is required to block all of the remaining sites on the nitrocellulose with a protein solution. This prevents the non-specific binding of antibodies to the nitrocellulose. In the present study a 5% (w/v) solution of non-fat milk powder was used for blocking. This method is cheap and gives a clean background, but can elute the protein from the membrane with excess incubation (Harlow and Lane, 1999). The immobilised proteins can subsequently be detected by their respective antibodies, followed by enzyme labelled anti-species antibodies. The appropriate substrate solution is used to visualise bound labelled antibodies. This method is sensitive enough to detect as little as 100 pg of electrophoretically separated antigen (Towbin *et al.*, 1979).

The most common enzymes used for labelling of antibodies are horseradish peroxidase (HRPO) and alkaline phosphatase. Visualisation of protein bands with alkaline phosphatase-labelled antibodies with bromochloroindoyl phosphate/nitro blue tetrazolium substrate is the more sensitive option. The substrate generates an intense black/purple precipitate at the site of enzyme binding that does not fade. HRPO-labelled antibody with 4-chloro-1-naphthol substrate is more economical, but less sensitive and is susceptible to fading.

2.7.1 Reagents

Blotting buffer [45 mM Tris, 173 mM glycine, 18% (v/v) methanol, 0.1% (w/v) SDS]. Tris (5.45 g) and glycine (12.96 g) were dissolved in 800 ml dH_2O and methanol (180 ml) was added. The volume was made up to 1 l, and 10% (w/v) SDS (1 ml solution D, Section 2.4.1) was added prior to use.

Ponceau S stain [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. Ponceau S (0.1 g) was dissolved in glacial acetic acid (1 ml) and made up to 100 ml with dH_2O.

Tris buffered saline (TBS) (20 mM Tris-HCl buffer, pH 7.4, 200 mM NaCl). Tris (2.42 g) and NaCl (11.69 g) were dissolved in 950 ml dH_2O, adjusted to pH 7.4 with HCl, and made up to 1 l.

Blocking solution [5% (w/v) non-fat milk]. Non-fat milk powder (5 g) was dissolved in 100 ml TBS.

Antibody diluent [0.5% (w/v) BSA]. BSA (0.5 g) was made up to 100 ml with TBS.

4-Chloro-1-naphthol substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) H_2O_2]. 4-chloro-1-naphthol (0.012 g) was dissolved in methanol (4 ml). This solution was diluted to 20 ml with TBS, with the addition of 30% hydrogen peroxide (8 µl).

2.7.2 Procedure

Following electrophoresis, gels were assembled in a blotting sandwich, whilst immersed in blotting buffer. Two pieces of blotting paper were placed on top of a piece of Scotchbrite™ foam. Gels were placed on top of the blotting paper and an equal sized piece of nitrocellulose was placed on top of the gel, taking care to remove any air bubbles. Two pieces of blotting paper, followed by another piece of Scotchbrite™ foam were placed on top of this. The sandwich was placed in the western blotting apparatus and immersed in blotting buffer. The apparatus was immersed in ice for cooling and blotted for 16 h and 200 mA.

After blotting was completed, the nitrocellulose was stained with Ponceau S and the positions of the molecular mass marker bands marked in pencil. Destaining was achieved by adding concentrated NaOH dropwise to the membrane immersed in distilled water. The membrane was rinsed with TBS, blocked for 1 h in blocking solution, washed (3 × 5 min) with TBS and incubated for 2 h with primary antibody made up in antibody diluent. It was further washed (3 × 5 min) with TBS, incubated for 1 h with an HRPO-linked secondary antibody made up in antibody diluent, washed (3 × 5 min) with TBS and incubated with 4-chloro-1-naphthol substrate solution until colour was sufficiently developed. It was finally rinsed with distilled water and dried between filter paper discs.

2.8 Enzyme-linked immunosorbent assays (ELISAs)

The ELISA technique, originally introduced by Engvall and Perlmann (1971) was used to evaluate the progress of polyclonal antibody production during the immunisation procedure and to verify the recognition of antigens by antibodies. Briefly, antigen is coated onto wells of an ELISA plate, followed by incubation with the sera or IgG preparations of interest. The degree of specific antibody bound to the antigen is detected by an enzyme-linked secondary anti-species antibody, which, when combined with substrate, will result in a coloured product that can be detected and quantified spectrophotometrically.

2.8.1 Reagents

Carbonate coating buffer (50 mM carbonate buffer, pH 9.6). A 50 mM solution of $NaHCO_3$ (0.21 g in 50 ml dH_2O) was titrated against a 50 mM solution of Na_2CO_3 (0.13 g in 50 ml dH_2O) to pH 9.6.

Phosphate buffered saline (PBS), pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in a total volume of 1 l of dH_2O.

0.5% (w/v) BSA-PBS. BSA (5 g) was dissolved in a total volume of 100 ml of PBS.

0.1% (v/v) PBS-Tween. Tween 20 (0.5 ml) was made up to 500 ml with PBS.

Citrate-phosphate buffer (150 mM citrate-phosphate, pH 5). A 150 mM solution of citric acid.H_2O (21 g/l) was titrated with a 150 mM solution of $NaH_2PO_4.2H_2O$ (35.6 g/l) to pH 5.

Substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2, in citrate-phosphate buffer]. ABTS (7.5 mg) was dissolved in citrate-phosphate buffer, pH 5 (15 ml) and H_2O_2 (7.5 µl) was added.

Stopping buffer [0.1% (w/v) NaN_3 in citrate-phosphate buffer]. NaN_3 (0.1 g) was dissolved in 100 ml citrate-phosphate buffer, pH 5.

2.8.2 Procedure

Test antigens were coated to the wells of 96 well Nunc-Immuno™ ELISA plates at a concentration of 1 µg/ml (150 µl per well) in either carbonate coating buffer, at pH 9.6 (cysteine proteases), or PBS (oligopeptidase B and peptides) for 16 h at 4°C. Non-specific binding of antibody was prevented by blocking with BSA-PBS (200 µl per well) for 1 h at 37°C. Wells were washed 3 times with PBS-Tween. Primary antibodies (chicken IgY or rabbit IgG) were diluted appropriately with 0.5% BSA-PBS and incubated in the wells for 2 h at 37°C (100 µl per well). Wells were washed 3 times with PBS-Tween. The HRPO-conjugated anti-species secondary antibody (goat anti-rabbit IgG or rabbit anti-chicken IgY), diluted in 0.5% BSA-PBS, was added (120 µl per well) and incubated for 1 h at 37°C. Wells were washed 3 times with PBS-Tween. Substrate solution was added (150 µl per well) and colour was allowed to develop in the dark. The enzyme reaction was stopped with the addition of stopping buffer (50 µl per well) and the A_{405} of each well was measured with an automated Versamax microplate reader using SOFTmax® software from Molecular Devices (USA).

2.9 Active site titration of proteases

It is important to know the active molarity of an enzyme preparation, since during purification, a proportion of the enzyme becomes denatured. A suitable titrant should be a stable, well-characterised molecule that combines rapidly with the active site of the enzyme with 1:1 stoichiometry, to yield an inactive or slowly turned-over complex (Knight, 1995).

2.9.1 Active site titration of cysteine proteases with E-64

The active concentration of cysteine proteases was determined by active site titration with E-64. E-64 is a powerful irreversible inhibitor of cysteine proteases that was discovered by Hanada *et al.*, (1978). It is known to be an excellent active site titrant, binding covalently to the active site cysteine residue of susceptible enzymes, and it does not react with non-active site cysteine residues, free reducing agents or other classes of proteases (Barrett *et al.*, 1982). When a linear plot is constructed of activity against E-64 molarity, the active molarity of the enzyme present can be determined where the plot reaches zero activity (Barrett and Kirschke, 1981).

2.9.1.1 Reagents

Cathepsin L assay buffer [340 mM Na-acetate, 60 mM acetic acid, 4 mM Na_2EDTA, pH 5.5, 0.02% (w/v) NaN_3, 8 mM DTT]. Na-acetate.$3H_2O$ (4.626 g) and glacial acetic acid (0.344 ml) and Na_2EDTA (0.15 g) were dissolved in dH_2O (90 ml), and titrated to pH 5.5 with NaOH. NaN_3 (0.02 g) was added and dissolved, and the volume made up to 100 ml with dH_2O. DTT (0.0062 g) was added to assay buffer (5 ml) just before use.

NaN_3 forms the toxic gas HN_3 in acid solution. It is therefore essential to add NaN_3 after titration of a buffer. This precaution is especially important when preparing acetate buffers from acetic acid.

Papain assay buffer [100 mM sodium phosphate buffer, pH 6.5, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. $NaH_2PO_4.H_2O$ (1.5601 g) and Na_2EDTA (0.15 g) were dissolved in dH_2O (90 ml) and titrated to pH 6.5 with NaOH. NaN_3 (0.02 g) was added and dissolved, and the volume made up to 100 ml with dH_2O. DTT (0.0062 g) was added to assay buffer (5 ml) just before use.

Congopain assay buffer [100 mM Bis-Tris buffer, pH 6, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. Bis-Tris (2.092 g) and Na_2EDTA (0.15 g) were dissolved in dH_2O (90 ml), and titrated to pH 6 with HCl. NaN_3 (0.02 g) was added and dissolved, and the volume made up to 100 ml with dH_2O. DTT (0.0062 g) was added to assay buffer (5 ml) just before use.

0.1% (w/v) Brij 35. Brij 35 (0.1 g) was dissolved in dH_2O (100 ml).

1 mM Z-Phe-Arg-AMC stock solution. Z-Phe-Arg-AMC (1 mg) was dissolved in DMSO (1.5 ml).

20 μM Z-Phe-Arg-AMC. Substrate stock solution (0.04 ml) was diluted to 2 ml with dH_2O.

10 mM E-64. E-64 (1.5 mg) was dissolved in DMSO (40 μl), and diluted up to 400 μl with dH_2O. This was diluted appropriately to 0-1 μM (in 0.1 μM increments) with dH_2O when required.

Stopping reagent (100 mM monochloroacetate, 30 mM Na-acetate, 70 mM acetic acid, pH 4.3). Monochloroacetate (0.945 g), Na-acetate (0.408 g) and glacial acetic acid (0.4 ml) were dissolved in 90 ml dH_2O, titrated to pH 4.3 with NaOH, and made up to 100 ml with dH_2O.

2.9.1.2 Procedure

Enzyme (1 μM made up to a volume of 25 μl with 0.1% Brij) was combined with E-64 (made up to 25 μl with 0.1% Brij) and assay buffer (50 μl), and incubated at 37°C for 30 min. The solution was diluted with 0.1% Brij (400 μl) and 10 μl aliquots were taken and combined with 25 μl substrate. After incubation at 37°C for 10 min, stopping solution was added (100 μl) and fluorescence was read (excitation at 360 nm and emission at 460 nm) with a 7620 microplate fluorometer from Cambridge Technology (UK). The active molarity of the enzyme was determined as described from a plot of activity against E-64 molarity.

2.9.2 Active site titration of serine peptidases with MUGB

Chase and Shaw (1970) described the use of *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) for the active site titration of serine proteinases such as trypsin, plasmin and thrombin. In the reaction, the enzyme becomes acylated and *p*-nitrophenol is released and quantified spectrophotometrically. This technique is limited, however, by its lack of sensitivity. 4-Methylumbelliferyl *p*-guanidobenzoate (MUGB) was first described as a burst titrant of serine peptidases by Jameson *et al.* (1973). Since it is a fluorogenic titrant, it has far greater sensitivity, allowing measurement of enzyme concentrations of 0.02 to 0.2 μM. It was for this reason that MUGB was used as a titrant in the present study.

2.9.2.1 Reagents

Titration buffer (0.05 M sodium phosphate buffer, pH 7.7): $NaH_2PO_4.2H_2O$ (0.39 g) was dissolved in dH_2O (40 ml) and titrated to pH 7.7 with NaOH. The final volume was made up to 50 ml with dH_2O.

1 mM HCl: A 32% (v/v) solution of HCl (2 μl) was added to dH_2O (20 ml).

Stock MUGB (10 mM 4-methylumbelliferyl *p*-guanidobenzoate in DMF): MUGB (3.7 mg) was dissolved in DMF (1 ml). The storage bottle was wrapped in aluminium foil to protect the contents against exposure to light.

MUGB working solution (0.1 mM in 1 mM HCl): Before use, 10 mM stock MUGB (10 µl) was diluted to 0.1 mM with 1 mM HCl (990 µl).

MU standard [10 mM 4-methylumbelliferone in dry DMF]. 4-Methylumbelliferone (17.6 mg) was dissolved in dry DMF (10 ml). The bottle was wrapped in aluminium foil to protect the contents against exposure to light.

2.9.2.2 Procedure

Oligopeptidase B (4.2 µM; 10 µg) was made up to 30 µl with titration buffer and mixed completely with 3 ml titration buffer in a fluorometer cuvette. MUGB working solution (30 µl) was added and the cuvette mixed by inversion. The progression of the fluorescence (excitation at 365 nm, emission at 445 nm) was monitored over a 10 min period using an SFM 25 fluorometer from Bio-Tek Kontron Instruments (Switzerland), and compared with that of a control containing no enzyme. Fluorescence was measured intermittently to avoid photobleaching (Knight, 1995).

For the enzymes in this study, a burst of fluorescence was observed after 1 min, followed by a constant turnover of fluorescence over time. The reading at 1 min was therefore used to represent the amount of active enzyme. Fluorescence was quantified using a standard curve relating fluorescence to 25-125 pmol of 4-methylumbelliferone (Fig. 2.4). MU standard was diluted appropriately with dilution buffer to the final reaction volume of the titration assay (3.06 ml).

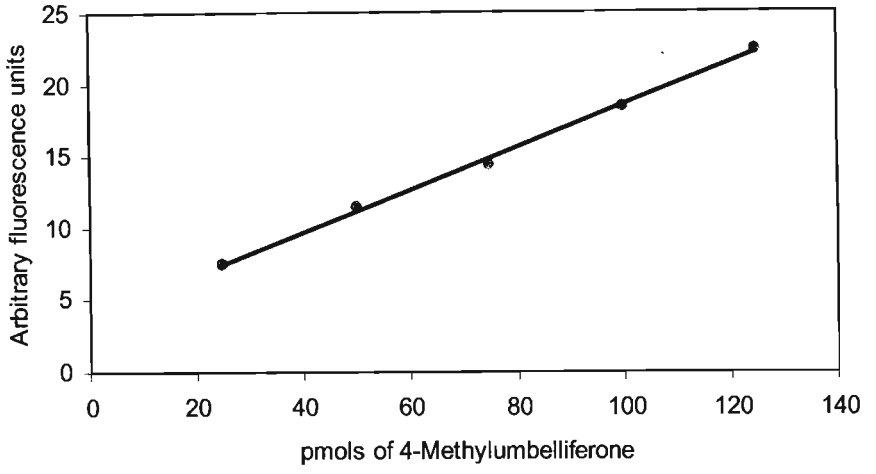

Figure 2.4. Standard curve relating the amount of 4-methylumbelliferone to fluorescence (excitation at 365 nm and emission at 445 nm). The equation of the trend line is given by y = 0.148x + 3.7, with a correlation coefficient of 0.9978.

2.10 Isolation of IgY from egg yolk

Chicken antibodies, IgY, were isolated from egg yolk by precipitation with PEG, as described by Polson *et al.* (1985). The procedure consists of three precipitation steps, the first removing vitellin and fats, and the second and third precipitating the antibodies. PEG is a hydrophilic linear polymer available as preparations of various average molecular weights. PEG 6 000 has been found to be the most suitable precipitant as it causes virtually no denaturation at room temperature (Polson *et al.*, 1964). PEG is thought to precipitate proteins by excluding them from water, and thus bringing them to their solubility limits. Since large proteins tend to be precipitated at lower concentrations of PEG, precipitation by this method is especially suited to the isolation of large proteins, such as antibodies (Dennison, 1999).

2.10.1 Reagents

100 mM Sodium phosphate buffer, pH 7.6, 0.02% (w/v) NaN_3. $NaH_2PO_4.2H_2O$ (15.601 g) was dissolved in dH_2O (950 ml) and titrated to pH 7.6 with NaOH. NaN_3 (0.2 g) was added and dissolved, and the final volume made up to 1 l with dH_2O.

100 mM Sodium phosphate buffer, pH 7.6, 0.1% (w/v) NaN_3. $NaH_2PO_4.2H_2O$ (15.601 g) was dissolved in dH_2O (950 ml) and titrated to pH 7.6 with NaOH. NaN_3 (1 g) was added and dissolved, and the final volume made up to 1 l with dH_2O.

2.10.2 Procedure

Egg yolks were separated from the egg white and rinsed under running tap water. The yolk sac was punctured, the yolk collected and the volume determined. Yolk was mixed with two volumes of 100 mM sodium phosphate buffer, pH 7.6, 0.02% NaN_3. PEG 6 000 was added to 3.5% (w/v) and dissolved with stirring. The mixture was centrifuged (4 420 *g*, 30 min, RT), and the supernatant filtered through absorbent cotton wool. The PEG concentration was increased to 12% (w/v), i.e. 8.5% (w/v) was added and dissolved. The solution was centrifuged (12 000 *g*, 10 min, RT) and the pellet dissolved in a volume of 100 mM sodium phosphate buffer, pH 7.6, 0.02 % NaN_3 equal to the yolk volume. PEG 6 000 was added to 12% (w/v) and dissolved. The solution was centrifuged (12 000 *g*, 10 min, RT) and the pellet dissolved in a volume of 100 mM sodium phosphate buffer, pH 7.6, 0.1% NaN_3 equal to a sixth of the yolk volume and stored at 4°C.

2.11 Isolation of IgG from rabbit serum

Rabbit IgG was isolated from serum by the method developed by Polson *et al.* (1964) in their studies on PEG precipitation of serum components.

2.11.1 Separation of serum

Following collection into glass tubes, blood was incubated for 30 min at 37°C. The clot was loosened from the sides of the test tube with a sealed Pasteur pipette. After storage (4°C, overnight), clots were removed and serum was clarified by centrifugation (10 000 *g*, 10 min, 4°C).

2.11.2 Reagents

Borate buffered saline, pH 8.6. Boric acid (2.16 g), NaCl (2.19 g), NaOH (0.7 g) and 37% HCl (0.62 ml) were dissolved in dH_2O (950 ml). The pH was adjusted if necessary to 8.6 using either NaOH or HCl, and the solution was made up to 1 l with dH_2O.

100 mM Sodium phosphate buffer, pH 7.6, 0.02% (w/v) NaN_3. As per Section 2.10.1.

2.11.3 Procedure

Serum was combined with double its volume of borate buffered saline, pH 8.6, and PEG 6 000 was added to 14% (w/v). After the PEG was dissolved, the solution was centrifuged (12 000 *g*, 10 min, 4°C), and the resulting pellet redissolved in the original serum volume using 100 mM sodium phosphate buffer, pH 7.6. PEG 6 000 was added to 14% (w/v) and dissolved. The solution was centrifuged (12 000 *g*, 10 min, 4°C), and the pellet was dissolved in half the serum volume using 100 mM sodium phosphate buffer, pH 7.6, containing 60% (v/v) glycerol, and stored at -20°C.

2.12 Preparation of affinity matrices

In the present study, antigens or antibodies were routinely coupled to affinity matrices for the purpose of either the affinity purification of antibodies or the immunoaffinity purification of antigens.

2.12.1 Coupling to Aminolink®

The Aminolink® support matrix is composed of 4% cross-linked beaded agarose containing aldehyde functional groups. Primary amine groups of peptides or proteins react with the aldehyde groups on the matrix to form a labile Schiff base. The reducing agent $NaCNBH_3$ is used to reduce the Schiff base intermediate to form stable secondary amine bonds between the matrix and the peptide or protein. Unreacted aldehyde groups are quenched with Tris-HCl, and the resulting Schiff bases are reduced by further addition of $NaCNBH_3$. $NaCNBH_3$ is the preferred reducing agent because it is considered gentle and has specificity towards the Schiff base (Hermanson, 1996).

2.12.1.1 Reagents

Aminolink® washing buffer [100 mM sodium phosphate buffer, pH 7, 0.02% (w/v) NaN_3]. $NaH_2PO_4.2H_2O$ (31.202 g) was dissolved in dH_2O (1.99 l) and titrated to pH 7 with NaOH. NaN_3 (0.4 g) was added and dissolved and the final volume made up to 2 l with dH_2O.

1 M Sodium cyanoborohydride. $NaCNBH_3$ (0.0628 g) was dissolved in 1 ml dH_2O.

1 M Tris-HCl buffer, pH 7.4. Tris (6.055 g) was dissolved in dH_2O (40 ml), titrated to pH 7.4 with HCl, and made up to 50 ml with dH_2O.

1 M NaCl. NaCl (2.922 g) was made up to 50 ml with dH_2O.

0.05% (w/v) NaN_3. NaN_3 (0.025 g) was dissolved in dH_2O (50 ml).

2.12.1.2 Procedure

Aminolink® (2 ml of a 50% slurry) was drained and washed with Aminolink® washing buffer (3 ml). Protein (5 mg, diluted to 1 ml with Aminolink® washing buffer) was combined with the resin and 1 M $NaCNBH_3$ was added (0.1 ml). Peptide was dissolved in DMSO (100 µl), and made up to 1 ml with Aminolink® washing buffer, before combining with Aminolink® resin and $NaCNBH_3$. The slurry was mixed for 2 h by end-over-end rotation, and left to stand for 4 h (RT). The gel was drained and washed with 1 M Tris-HCl buffer, pH 7.4 (2 ml). To block unreacted sites, the gel was combined with 1 M Tris-HCl buffer, pH 7.4 (1 ml) and 1 M $NaCNBH_3$ (0.1 ml), and mixed for 30 min (RT). The gel was drained and washed with 1 M NaCl (10 ml), followed by washing with 0.05% NaN_3 (10 ml), and stored in Aminolink® washing buffer at 4°C.

2.12.2 Coupling to Sulfolink®

The Sulfolink® support matrix, containing iodoacetyl functional groups, is used for the immobilisation of ligands containing sulfhydryl groups. Peptides with terminal cysteine residues are reduced with DTT prior to coupling and the sulfhydryl groups react with the iodoacetyl agarose to form stable thioester bonds (Hermanson, 1996). 5,5′-Dithiobis(2-nitrobenzoic acid), commonly known as Ellman's reagent, provides a sensitive assay for thiol groups. It is used in this protocol to detect the presence of reduced peptide after chromatography to separate peptide from DTT. The reagent is an aromatic disulfide which reacts with aliphatic thiols to form a mixed disulfide of the protein/peptide and 2-nitro-5-thiobenzoate, which has an intense yellow colour (Ellman, 1959).

2.12.2.1 Reagents

50 mM Tris-HCl buffer, pH 8.5, 5 mM Na_2EDTA. Tris (0.605 g) and Na_2EDTA (0.186 g) were dissolved in dH_2O (90 ml), titrated to pH 8.5 with HCl, and made up to 100 ml with dH_2O.

MEC buffer [100 mM sodium phosphate buffer, pH 7, 0.02% (m/v) NaN_3]. $NaH_2PO_4.2H_2O$ (31.202 g) was dissolved in dH_2O (1.99 l) and titrated to pH 7 with NaOH. NaN_3 (0.4 g) was added and dissolved and the final volume made up to 2 l with dH_2O.

Reducing buffer [100 mM Tris-HCl buffer, pH 8, 1 mM Na_2EDTA, 0.02% (m/v) NaN_3]. Tris (1.211 g) and Na_2EDTA (0.037 g) were dissolved in dH_2O (90 ml) and the pH adjusted to 8 with HCl. NaN_3 (0.02 g) was added and dissolved, and the final volume made up to 100 ml with dH_2O.

10 mM DTT. DTT (7.7 mg) was dissolved in 5 ml reducing buffer.

Ellman's reagent buffer [100 mM Tris-HCl buffer, pH 8, 10 mM Na_2EDTA, 1% (m/v) SDS]. Tris (1.211 g) and Na_2EDTA (0.37 g) were dissolved in dH_2O (950 ml) and the pH adjusted to 8 with HCl. SDS (0.1 g) was added and dissolved, and the final volume made up to 100 ml with dH_2O.

10 mM Ellman's reagent. Ellman's reagent (10 mg) was dissolved in 2.5 ml Ellman's reagent buffer.

50 mM Cysteine.HCl. Cysteine.HCl (0.044 g) was dissolved in 50 mM Tris-HCl buffer, 5 mM Na_2EDTA, pH 8.5 (5 ml).

1 M NaCl. As per Section 2.12.1.1.

0.05% (w/v) NaN_3. As per Section 2.12.1.1.

Sulfolink® washing buffer [100 mM sodium phosphate buffer, pH 6.5, 0.02% (w/v) NaN_3]. $NaH_2PO_4.2H_2O$ (15.601 g) was dissolved in dH_2O (950 ml) and titrated to pH 6.5 with NaOH. NaN_3 was added (0.2 g) and the volume made up to 1 l with dH_2O.

2.12.2.2 Procedure

Sulfolink® resin (2 ml of 50% slurry per 5 mg of peptide) was brought to RT and washed with 6 column volumes of 50 mM Tris-HCl buffer, 5 mM Na_2EDTA, pH 8.5. Peptide (5 mg or 7.5 mg) was dissolved in DMSO (100 µl) and reducing buffer was added (400 µl). 10 mM DTT in reducing buffer was added (500 µl) and the solution incubated at 37°C for 1.5 h. Reduced peptide was separated from DTT on a Sephadex G-10 column (1.5 × 11 cm; 10 ml/h), equilibrated with MEC buffer and 0.5 ml fractions collected. A 10 µl sample of each fraction was tested with 10 mM Ellman's reagent (10 µl). The first fractions to give a yellow reaction, i.e. reduced peptide, were pooled.

The reduced peptide was added to the resin and mixed by end-over-end rotation for 15 min, and left to stand for 30 min (RT). The resin was drained and washed with 3 column volumes of 50 mM Tris-HCl buffer, 5 mM Na_2EDTA, pH 8.5. The resin was combined with an equal volume of 50 mM cysteine to block unreacted sites and mixed by end-over-end rotation for 15 min, and left to stand for 30 min (RT). The resin was drained and washed with 16 column volumes of 1 M NaCl and 16 columns volumes of 0.05% (m/v) NaN_3, and stored in Sulfolink® washing buffer at 4°C.

2.13 Affinity purification of antibodies

When purified antigens are covalently bound to a suitable chromatography matrix, affinity purification can be used to isolate antibodies that are specific for the antigen. IgY isolated from egg yolk is a mixture of polyclonal antibodies with multiple specificities. Recognition of multiple antigens can interfere with many immunochemical techniques, therefore some antibody pools were affinity purified using matrices of immobilised peptide or protein.

2.13.1 Reagents

Sulfolink® washing buffer [100 mM sodium phosphate buffer, pH 6.5, 0.02% (w/v) NaN_3]. As per Section 2.12.2.1.

Aminolink® washing buffer [100 mM sodium phosphate buffer, pH 7, 0.02% (w/v) NaN_3]. As per Section 2.12.1.1.

Elution buffer (100 mM glycine–HCl buffer, pH 2.8). Glycine (7.507 g) was dissolved in dH_2O (950 ml), titrated to pH 2.8 with HCl, and made up to 1 l with dH_2O.

Neutralisation buffer (1 M sodium phosphate buffer, pH 8.5). $NaH_2PO_4.2H_2O$ (31.202 g) was dissolved in dH_2O (180 ml), titrated to pH 8.5 with NaOH, and made up to 200 ml with dH_2O.

10% (w/v) NaN_3. NaN_3 (5 g) was dissolved in dH_2O (50 ml).

2.13.2 Procedure

The affinity matrix was washed for several hours with the appropriate washing buffer, at a flow rate of 10 $ml.h^{-1}$. IgY (60-70 ml) was cycled through the affinity matrix overnight in the reverse flow direction to avoid blocking of the column. The column was washed with washing buffer (10 column volumes), and eluted with elution buffer (0.9 ml fractions) into microfuge tubes containing neutralisation buffer (0.1 ml). The elution was monitored by measuring the A_{280} of each fraction. Fractions with the highest A_{280} values were pooled and stored at 4°C with the addition of 10% (w/v) NaN_3 to a final concentration of 0.1%.

2.14 Preparation of media

2.14.1 Preparation of antibiotics

50 mg/ml Ampicillin stock. Ampicillin (500 mg) was dissolved in dH_2O (10 ml) and sterilised by passing through a 0.22 μm filter. The stock was stored at -20°C.

10 mg/ml Tetracycline stock. Tetracycline (100 mg) was dissolved in absolute ethanol (10 ml). The stock was stored at -20°C.

35 mg/ml Chloramphenicol stock. Chloramphenicol (350 mg) was dissolved in methanol (10 ml). The stock was stored at -20°C.

2.14.2 Preparation of bacterial media

Luria-Bertini (LB) medium. Bacto-tryptone (10 g), yeast extract (5 g), NaCl (5 g) and D-glucose (2 g) were dissolved in dH_2O (1 l) and autoclaved.

2 × Yeast extract, tryptone (2YT) medium. Bacto-tryptone (16 g), yeast extract (10 g), NaCl (5 g) and D-glucose (20 g) were dissolved in dH_2O (1 l) and autoclaved.

Super optimal broth (SOB). Bacto-tryptone (2 g), yeast extract (0.5 g), NaCl (0.058 g), KCl (0.019 g) and D-glucose (0.36 g) were dissolved in dH_2O (100 ml) and autoclaved.

2.14.3 Preparation of yeast media

10 × Yeast extract, peptone (YP). Yeast extract (100 g) and bacto-peptone (200 g) were dissolved in dH_2O (1 l) and autoclaved.

2 M sterile glucose. D-glucose (36 g) was dissolved in dH_2O (100 ml) and sterilised by passing through a 0.22 μm filter.

1 M KH_2PO_4. KH_2PO_4 (136.09 g) was dissolved in dH_2O (1 l).

1 M K_2HPO_4. K_2HPO_4 (174.18 g) was dissolved in dH_2O (1 l).

1 M Potassium phosphate buffer, pH 6.5. A 1 M solution of KH_2PO_4 (1 l) was titrated with 1 M K_2HPO_4 to pH 6.5 and autoclaved.

Yeast nitrogen base (YNB) stock. Yeast nitrogen base powder (67 g) was dissolved in dH_2O (500 ml) and sterilised by passing through a 0.22 μm filter.

50% (v/v) Sterile glycerol. Glycerol (250 ml) was mixed with dH_2O (250 ml) and autoclaved.

1000 × Biotin. Biotin (20 mg) was dissolved in dH_2O (50 ml), assisted by heating the solution to a maximum of 50°C for a few minutes. The solution was sterilised by passing through a 0.22 μm filter.

Yeast extract, peptone, dextrose (YPD) medium. 10 × YP (100 ml), 2 M glucose (50 ml) and ampicillin (1 ml) were made up to 1 l with sterile dH_2O, in a sterile measuring cylinder using aseptic technique.

Buffered glycerol complex (BMGY) medium. 1 M Potassium phosphate (100 ml), YNB stock (20 ml), 10 × YP (100 ml), 50% (v/v) glycerol (20 ml), 1000 × biotin (1 ml) and tetracycline (1 ml) were made up to 1 l with sterile dH_2O, in a sterile measuring cylinder using aseptic technique.

Buffered minimal medium (BMM). 1 M Potassium phosphate (100 ml), YNB stock (100 ml), 1000 × biotin (1 ml), and ampicillin or chloramphenicol (1 ml) were made up to 1 l with sterile dH_2O, in a sterile measuring cylinder using aseptic technique. Methanol (5 ml) was added at the end to prevent precipitation of dissolved compounds.

2.14.4 Preparation of media-agar plates

2.14.4.1 Reagents

LB agar. As per Section 2.14.2 with the addition of 15 g/l bacto-agar.

YPD agar. As per Section 2.14.3 with the addition of 15 g/l bacto-agar.

10 × Dextrose. D-glucose (20 g) was dissolved in dH_2O (100 ml). The solution was sterilised by passing through a 0.22 μm filter.

Minimal dextrose (MD) agar. Bacto-agar (3 g) was autoclaved in dH_2O (160 ml) and cooled to 55°C. YNB stock (20 ml; Section 2.14.3), 1000 × biotin (0.2 ml; Section 2.14.3) and 10 × dextrose (20 ml) were added and mixed well.

2.14.4.2 Procedure

After autoclaving, media were allowed to cool to approximately 55°C. If required, antibiotic was added aseptically to the cooled medium diluting the antibiotic stock 1000 times to the appropriate final concentration. Media was poured into sterile 90 mm plates until the base was covered. The plates were allowed to cool and set, and stored inverted at 4°C.

2.15 Agarose gel electrophoresis

Agarose gels have a lower resolving power than polyacrylamide gels, but have a greater range of separation. DNA from 200 bp to 50 kb can be separated on agarose gels of various concentrations (Sambrook *et al.*, 1989). Agarose is a linear polymer of D-galactose and 3,6-anhydro L-galactose, extracted from seaweed. An agarose gel is prepared by melting the agarose in buffer until the solution becomes transparent. When the agarose solution is cooled (in a mold) it hardens into a matrix, of which the density is determined by the concentration of agarose. When an electric field is applied across the gel, DNA, which is negatively charged, migrates towards the anode. The rate of migration is determined by a number of parameters including the molecular size of the DNA, the agarose concentration, the conformation of the DNA, the voltage applied, and the composition of the electrophoresis buffer (Sambrook *et al.*, 1989).

The most convenient way to visualise DNA in agarose gels is by staining with ethidium bromide. It is a fluorescent dye that intercalates between the bases of DNA, increasing the fluorescence of the dye in comparison to its free form (Sambrook *et al.*, 1989). The

dye is incorporated into the gel and the electrophoresis buffer at a concentration of 0.5 μg/ml.

2.15.1 Reagents

50 × TAE buffer (2 M Tris-acetate buffer, pH 8, 50 mM Na_2EDTA). Tris (242 g) was dissolved in dH_2O (800 ml). Glacial acetic acid (57.1 ml) was added and made up to 900 ml with dH_2O. Na_2EDTA (18.612 g) was dissolved separately in dH_2O (80 ml), the pH adjusted to 8 with glacial acetic acid, and the volume made up to 100 ml with dH_2O. The Na_2EDTA solution (100 ml) was combined with the Tris-acetate solution (900 ml).

1 × TAE buffer (40 mM Tris-acetate buffer, pH 8, 1 mM Na_2EDTA). 50 × TAE buffer (40 ml) was diluted to 2 l with dH_2O. Ethidium bromide solution (150 μl) was added.

1% (m/v) Agarose. Agarose (0.3 g) was placed with 1 × TAE buffer (30 ml) in an Erlenmeyer flask. The flask was heated in a microwave until all of the agarose was melted. The total volume was brought back to 30 ml by the addition of dH_2O. The flask was swirled gently to ensure complete mixing.

2.15.2 Procedure

The molten agarose solution was cooled to approximately 55°C and poured into a clean Perspex casting tray with a 10 well comb. When the agarose had solidified at RT the casting tray was disassembled and the comb removed. The gel was positioned in an electrophoresis tank, and 1 × TAE was poured into the tank to submerge the gel. 6 × Blue/Orange loading dye (Promega) was added to DNA samples (1 μl per 5 μl of sample) prior to loading into the wells. Loading dye contained glycerol to cause samples to sink into the wells, and tracking dyes to visualise the progress of electrophoresis. Electrophoresis was carried out at 100 V until the dye had migrated sufficiently through the gel. The gel was examined on an ultraviolet transilluminator, and the image captured with a Versadoc system. Standard curves were constructed relating relative mobility of the DNA markers to log of base pair number, for accurate estimation of DNA size. A representative standard curve is shown for the 1 kb DNA step ladder (1-10 kb; Fig. 2.5).

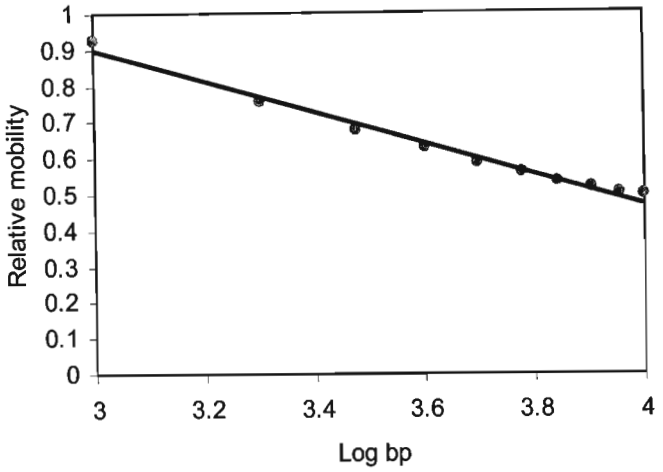

Figure 2.5. Standard curve relating relative mobility to log of base pair number for the 1 kb DNA step ladder (Promega) on a 1% agarose gel. The equation of the trend line is given by y = -0.4268x + 2.177, with a correlation coefficient of 0.9851.

2.16 Preparation of glycerol stocks

Bacteria and yeast can be stored indefinitely in culture containing glycerol at -70°C (Sambrook *et al.*, 1989).

2.16.1 Reagents

50% (v/v) Sterile glycerol. As per Section 2.14.3

2.16.2 Procedure

Cultures of bacteria were grown overnight at 37°C (500 µl) and combined with 50% sterile glycerol (500 µl) in an aseptic manner. Likewise, cultures of yeast grown to saturation (48 - 72 h at 30°C) in YPD were combined with 50% sterile glycerol in a 1:1 ratio. Glycerol stocks were stored at -70°C.

2.17 Isolation of plasmid DNA (Wizard® *Plus* SV Minipreps DNA purification system)

The small-scale purification of plasmid DNA is commonly known as a miniprep. There are a variety of miniprep protocols available (Ausubel *et al.*, 1994), but there are few that have proven to be reliable and reproducible. The Wizard® *Plus* SV Minipreps DNA

purification system from Promega is a kit that enables reliable plasmid DNA purification in 15 minutes or less, which is ideal for performing large numbers of minipreps in parallel. The procedure involves the lysis of bacterial cells in an alkaline solution containing SDS, and binding of plasmid DNA to resin in a column, where it is washed with an ethanol solution, and finally eluted (Wizard® *Plus* SV Minipreps DNA purification system technical bulletin, Promega).

2.17.1 Reagents

All reagents were provided in the Wizard® *Plus* SV Minipreps DNA purification system kit from Promega.

Cell Resuspension Solution (50 mM Tris-HCl buffer, pH 7.5, 10 mM EDTA, 100 µg/ml RNase A).

Cell Lysis Solution [0.2M NaOH, 1% (m/v) SDS].

Akaline Protease Solution.

Neutralisation Solution (4.09 M guanidine hydrochloride, 0.759 M potassium acetate, 2.12 M glacial acetic acid, pH 4.2)

Column Wash Solution [60 mM potassium acetate, 8.3 mM Tris-HCl, 0.04 mM EDTA, 60% (v/v) ethanol].

2.17.2 Procedure

An overnight culture of *E. coli* carrying a plasmid (10 ml) was centrifuged (1 000 *g*, 5 min, RT). The supernatant was discarded and the cell pellet was resuspended in Cell Resuspension Solution (250 µl) and transferred to a 1.5 ml microfuge tube. Cell Lysis Solution was added (250 µl) and mixed by inverting the tube 4 times. Alkaline Protease Solution was added (10 µl), mixed by inverting the tube 4 times, and incubated for 5 min at RT. Neutralisation Solution was added (350 µl) and mixed by inverting the tube 4

times. The solution was centrifuged (12 000 *g*, 10 min, RT). Clarified lysate was transferred to the Spin Column and centrifuged (12 000 *g*, 1 min, RT). Column Wash Solution (750 µl) was added to the Spin Column and centrifuged (12 000 *g*, 1 min, RT). This was repeated with 250 µl of Column Wash Solution. The Spin Column was dried by centrifugation (12 000 *g*, 2 min, RT) and plasmid DNA was eluted into a sterile microfuge tube by placing 100 ml Nuclease-Free Water in the Spin Column and centrifuging (12 000 *g*, 1 min, RT). DNA was stored at -20°C.

2.18 Restriction endonuclease digestion of DNA

Type II restriction endonucleases are bacterial enzymes that cut DNA molecules internally at specific base pair sequences. One of the first of these enzymes to be characterised was *Eco*RI, from *E. coli*. Type II restriction endonucleases are vital for gene cloning. When two different DNA samples are digested with the same restriction endonuclease that produces an overhang, or sticky end, and then mixed together, new DNA combinations can be formed as a result of base pairing between the overhang regions (Glick and Pasternak, 1998). In the present study the restriction endonucleases *Eco*RI (G↓AATTC), *Not*I (GC↓GGCCGC) and *Sal*I (G↓TCGAC) were utilised. Of these enzymes, *Eco*RI and *Sal*I are known to exhibit star activity. That is, the ability of restriction enzymes to cleave DNA sequences that are similar, but not identical, to their recognition sites under conditions of high glycerol concentration, excess of enzyme, presence of organic solvents, and/or non-optimal buffering (Roche Applied Science Lab FAQS). The manufacturer's recommendations for restriction digestion were followed carefully to avoid star activity.

2.18.1 Reagents

10 × Buffer D (6 mM Tris-HCl buffer, pH 7.9, 6 mM $MgCl_2$, 150 mM NaCl, 1 mM DTT), Promega.

10 × Buffer H (90 mM Tris-HCl buffer, pH 7.5, 10 mM $MgCl_2$, 50 mM NaCl), Promega.

2.18.2 Procedure

Restriction endonuclease digestion of DNA was carried out according to the manufacturer's instructions for each enzyme. The volumes of reagents used depended on the amount of DNA in the specific reaction. The reactions consisted of DNA, 10 × restriction buffer (10% of the final reaction volume), enzyme, and sterile dH_2O to adjust the final volume. Buffer D was used for restriction with *Not*I and *Sal*I, and Buffer H was used for restriction with *Eco*RI. For overnight incubations, 100 × BSA was included to increase stability of the DNA. Enzyme was added last so that it was correctly buffered. The volume of enzyme used could not exceed 10% of the reaction volume, due to the presence of glycerol in the enzyme storage buffer which can decrease endonuclease activity. Reactions were mixed by finger tapping and pulse spinning and incubated at 37°C for as long as required. Completion of the digestions were routinely analysed by running a minimum amount of the reaction sample on an agarose gel (Section 2.15).

2.19 Purification of plasmid DNA

2.19.1 Wizard® DNA Clean-Up System

The Wizard® DNA Clean-Up System was routinely used to purify linear and circular DNA from solution containing restriction enzymes, phosphatase, and salts. The kit employs a resin to which the DNA is bound in a column. The DNA on the column is washed with 80% (v/v) isopropanol and eluted with sterile dH_2O, heated to 65°C. The binding capacity of 1 ml of resin is approximately 20 µg of DNA. The sample volume must be between 50 and 500 µl. If the sample volume was less than 50µl, the volume was brought up to 50 µl with sterile water.

2.19.1.1 Reagents

80% (v/v) Isopropanol. Isopropanol (16 ml) was made up to 20 ml with dH_2O.

2.19.1.2 Procedure

Wizard® DNA Clean-Up Resin (1 ml) was added to a microfuge tube. The DNA sample was added to the resin and mixed by gently inverting several times. The resin containing the bound DNA was pipetted into a syringe barrel. The syringe plunger was used to

gently push the slurry into the Minicolumn. The syringe was further used to push 2 ml of 80% (v/v) isopropanol through the Minicolumn. The Minicolumn was dried by centrifugation (10 000 *g*, 2 min, RT). DNA was eluted by applying sterile dH_2O, prewarmed to 65°C, incubating for 1 min, and centrifuging (10 000 *g*, 20 s, RT).

2.19.2 Purification of plasmid DNA: Wizard® PCR Preps DNA Purification System

The Wizard® PCR Preps DNA Purification System was routinely used to purify DNA from low melting point agarose after electrophoretic separation. It is based on the same principles as the Wizard® DNA Clean-Up System (Section 2.19.1).

2.19.2.1 Procedure

DNA was separated by agarose gel electrophoresis (Section 2.15), using low melting point agarose. The desired band was excised using a clean, sterile scalpel in a maximum of 300 µl of agarose. The agarose was incubated at 70°C in a microfuge tube until completely melted. Wizard® PCR Preps DNA Purification Resin (1 ml) was added to the molten agarose and the procedure was carried out according to Section 2.19.1.

2.20 Preparation of competent *E. coli* cells

E. coli cells were made competent for transformation with DNA. In the present study two methods were used for competent cell preparation, as two methods of transformation were used. Pretreatment with TSS is not very efficient, but is simpler than other methods of competent cell preparation. The TSS method was only used for transformation of supercoiled DNA, for which high efficiency was not required and transformation was by heat shock (Section 2.22.1). The TSS solution creates holes in the bacterial cell wall, through which DNA can pass. The glycerol method, followed by electroporation (Section 2.22.2), is a more efficient method of transformation, and was used to transform ligation reactions for which high efficiency is required. The glycerol solution serves to wash the bacterial cells and remove traces of salt before electroporation. JM109 cells were used due to availability and their high transformation efficiency.

2.20.1 TSS method (for transformation with supercoiled DNA)

2.20.1.1 Reagents

TSS [85% (v/v) LB, 10% (m/v) PEG 6 000, 5% (v/v) DMSO, 50 mM $MgCl_2$]. Bacto-tryptone (0.85 g), yeast extract (0.425 g), NaCl, (0.425 g), D-glucose (0.17 g), PEG 6 000 (10 g), and $MgCl_2.6H_2O$ (1.0165 g) were dissolved in 85 ml of dH_2O. DMSO (5 ml) was added and the final volume brought to 100 ml with dH_2O. The solution was autoclaved.

2.20.1.2 Procedure

A 10 ml 2YT culture of *E. coli* JM109 was grown overnight at 37°C with shaking. Overnight culture (500 µl) was used to inoculate fresh 2YT (50 ml), which was incubated at 37°C until the A_{600} reached 0.5. The culture was chilled on ice for 30 min, and centrifuged (1 500 *g*, 10 min, 0°C). Cells were resuspended in ice cold TSS and transferred to sterile microfuge tubes for transformation in 150 µl aliquots.

2.20.2 Glycerol method (for transformation with a ligation reaction)

2.20.2.1 Reagents

10% (v/v) Glycerol. Glycerol (10 ml) was mixed thoroughly with dH_2O (90 ml) and autoclaved.

2.20.2.2 Procedure

A 10 ml 2YT culture of *E. coli* JM109 was grown overnight at 37°C with shaking. Overnight culture (500 µl) was used to inoculate fresh 2YT (100 ml), which was incubated at 37°C until the A_{600} reached 0.5. The culture was chilled on ice for 30 min, and centrifuged (3 600 *g*, 10 min, 0°C). Cells were resuspended in ice cold sterile dH_2O (100 ml). Cells were centrifuged (3 600 *g*, 10 min, 0°C) and resuspended in ice cold sterile dH_2O (50 ml). Cells were centrifuged (3 600 *g*, 10 min, 0°C) and resuspended in ice cold sterile 10% glycerol (1 ml). Cells were aliquotted (50 µl) into sterile microfuge tubes for transformation.

2.21 Preparation of competent *Pichia pastoris* GS115

The method used for the preparation of competent *P. pastoris* cells was adapted from (Wu and Letchworth, 2004). Transformation efficiency for *P. pastoris* is typically very low. The pre-treatment of yeast cells with a lithium acetate and dithiothreitol buffer was shown to enhance transformation efficiency dramatically. The method was altered in the present study, using lithium chloride instead of lithium acetate due to availability.

2.21.1 Reagents

10 mM Tris-HCl, 100 mM $LiCl_2$, 600 mM sorbitol, pH 7.5, 10 mM DTT. Tris (0.061 g), $LiCl_2$ (0.212 g), and sorbitol (5.465 g) were dissolved in dH_2O (40 ml), and the pH adjusted to 7.5 with HCl. The volume was brought to 49 ml with dH_2O, and the solution was autoclaved. Before use, DTT (0.077 g) was dissolved in dH_2O (1 ml), filter sterilised (0.22 μm) and added to the solution.

1 M Sorbitol. Sorbitol (18.217 g) was dissolved in dH_2O (100 ml) and autoclaved.

2.21.2 Procedure

YPD (10 ml) was inoculated with a single colony of *P. pastoris* GS115 and incubated overnight at 30°C with shaking. YPD (100 ml) was inoculated with overnight culture (200 μl) and incubated at 30°C with shaking until the A_{600} reached 1. Cells were collected by centrifugation (1500 *g*, 5 min, 4°C), resuspended in 100 mM $LiCl_2$, 10 mM DTT, 600 mM sorbitol, 10 mM Tris-HCl, pH 7.5 (50 ml), and incubated at RT for 30 min. Cells were collected by centrifugation (1500 *g*, 5 min, 4°C) and resuspended in ice cold 1 M sorbitol (1.5 ml). This was repeated 3 times and the cells resuspended in a final volume of 500 μl ice cold 1 M sorbitol.

2.22 Transformation of *E. coli* with DNA

2.22.1 Transformation by heat shock

The mechanism of action of the heat shock transformation procedure in *E. coli* is not known. It is assumed that the bacterial cell wall is broken down in localised regions, allowing the plasmid DNA in solution to be taken inside the bacterial cell (Glick and

Pasternak, 1998). Pretreatment of the bacteria to make them competent is necessary for this to take place.

2.22.1.1 Procedure

Heat shock was routinely used to transform supercoiled DNA into TSS-competent *E. coli* cells (Section 2.20.1). DNA (2 µl) was combined with cells (150 µl) and incubated on ice for 30 min. The mixture was heat-shocked by placing in a water bath at exactly 42°C for 2 min, and incubating on ice for 2 min. SOB medium (800 µl; Section 2.14.2) was added and cells were allowed to recover at 37°C with shaking for 1 h. Amounts of 200 µl were spread onto LB plates with 50 µg/ml ampicillin and incubated overnight at 37°C.

2.22.2 Electroporation

Electroporation is the process by which bacteria or yeast are able to take up free DNA upon the application of a high-strength electric field. Little is known about the mechanism of DNA uptake during electroporation. It is thought that transient pores are formed in the cell wall as a result of the electroshock, and DNA that has come into contact with the lipid bilayer of the cell membrane is taken into the cell (Glick and Pasternak, 1998). The experimental protocols for electroporation are different for various bacterial species.

2.22.2.1 Procedure

DNA (2 µl) was added to freshly prepared competent *E. coli* cells (50 µl), mixed gently by pipetting, and incubated on ice for 30 min. Cells and DNA were transferred to a pre-chilled electroporation cuvette (0.2 cm gap). A single pulse of 25 microfarads, 2.5 kilovolts, and 200 ohms was administered for about 4.8 milliseconds, using a Bio-Rad Gene Pulser. SOB medium (700 µl; Section 2.14.2), prewarmed to 37°C, was added and the cells were incubated for 1 h at 37°C. Amounts of 200 µl were spread onto LB plates with 50 µg/ml ampicillin. Plates were incubated at 37°C overnight.

2.23 Transfection of *P. pastoris* GS115 with DNA

The two most commonly used techniques for *P. pastoris* transformation are spheroplasting and electroporation (Cregg *et al.*, 1985). Spheroplasting is a method by which the host cells have their cell walls removed by enzymatic digestion. Electroporation is a process by which the host cell is subjected to short pulses of electric current, resulting in the formation of transient pores in the outer membrane through which DNA can enter. Electroporation was employed in the present study owing to the speed, simplicity and reproducibility of the technique. Slightly different conditions are required for the electroporation of yeast cells as compared to *E. coli* cells.

2.23.1 Procedure

DNA (10 µl) was added to freshly prepared competent *P. pastoris* GS115 cells (100 µl), mixed gently by pipetting, and incubated on ice for 5 min. Cells and DNA were transferred to a pre-chilled electroporation cuvette (0.2 cm gap). A single pulse of 125 microfarads, 2.5 kilovolts, and 400 ohms was administered for about 8 milliseconds, using a Bio-Rad Gene Pulser. Ice cold 1 M sorbitol (1 ml; Section 2.21.1) was added, and amounts of 200 µl were spread onto MD plates. Plates were incubated at 30°C for 3 days.

2.24 Colony PCR

Colony PCR is a rapid and simple method that was used in the present study to screen bacterial colonies directly for recombinant clones.

2.24.1 Reagents

FastStart Taq DNA polymerase (Roche).

10 × PCR reaction buffer with $MgCl_2$ (100 mM Tris-HCl buffer, pH 8.3, 15 mM $MgCl_2$, 500 mM KCl) (Roche).

2 mM dNTP mix (Roche). dNTP mix (with 10 mM each of dATP, dGTP, dCTP and dTTP) (20 µl) was diluted to 2 mM by the addition of sterile dH_2O (80 µl).

10 μM Primer solutions. As per Table 2.3. Primers were provided at a concentration of 100 μM. Stocks of primers at 10 μM were made up by diluting the original primer solution (10 μl) with sterile dH_2O (90 μl).

2.24.2 Procedure

The primers that were used, shown in Table 2.3, amplified a 1.1 kb PCR product when the coding sequence for oligopeptidase B was present. The following PCR conditions were used: 95°C for 5 min, 25 cycles of the following: 95°C for 1 min (denaturation); 55°C for 30 s (annealing); 72°C for 2 min (extension), and 70°C for 5 min, ending at 4°C.

Table 2.3. The base pair sequences of the primers used for screening by colony PCR.

Name	Direction	Sequence (5'-3')
T. congolense (1.1)[a]	Forward	ACATGCTGGTGGCGTACTATGTTG
T. congolense (1.1)	Reverse	ACCAGAGCAAATAAGATGCTCGGC
T. vivax (1.1)	Forward	CAAGTGGAGCCAGCCCCGCCC
T. vivax (1.1)	Reverse	CTTCACAGGCCAGCTGGTTGGGCG

[a]Primers designed to amplify a 1.1 kb product from the oligopeptidase B coding sequence

Template DNA was provided by touching a colony with the tip of a sterile toothpick and touching the PCR reaction mix, made up according to Table 2.4, with the same toothpick. Genomic and plasmid DNA were released from the bacteria in the initial denaturation step. PCR products were analysed by agarose gel electrophoresis.

Table 2.4. Components of PCR reaction used to screen bacterial colonies for the oligopeptidase B DNA sequence.

Reaction component	Volume (μl)	Final concentration
10 × PCR reaction buffer	2.5	1×
dNTP mixture	2.5	200 μM
Forward primer	1.0	400 nM
Reverse primer	1.0	400 nM
FastStart Taq DNA polymerase	0.1	
Sterile dH_2O	17.9	
Total reaction volume	25.0	

CHAPTER 3

Epitope mapping and bioinformatic studies of oligopeptidase B from *Trypanosoma congolense*

3.1 Introduction

Bioinformatics involves the use of techniques from applied mathematics, informatics, statistics, and computer science to solve biological problems. Major research efforts in the field include sequence alignment, gene finding, genome assembly, protein structure alignment, protein structure prediction, prediction of gene expression and protein-protein interactions, and the modelling of evolution (Claverie and Notredame, 2003).

This chapter describes a number of bioinformatic techniques that were used in the present study. The first technique used was epitope mapping of oligopeptidase B from *T. congolense* with Predict7 software (Carmenes *et al.*, 1989), to identify several immunogenic regions of the enzyme. Anti-peptide antibodies were produced in chickens against these regions of oligopeptidase B to assess their immunogenicity. The second bioinformatic technique used was amino acid sequence alignment using CLUSTAL W software (Thompson *et al.*, 1994), to compare the sequences of oligopeptidase B from different trypanosomal species, as well as closely related sequences for which the 3D structures are known. The third technique was visualisation and manipulation of 3D images of protein structures with PyMOL software (DeLano, 2004). This was used firstly, to display the 3D structure of oligopeptidase B (from *E. coli*), and secondly, to map the peptide sequences (that were identified by epitope mapping) on the 3D structure of the protein.

3.2. Epitope mapping of oligopeptidase B from *T. congolense*

3.2.1 Introduction

The immunochemical properties of synthetic peptides can be utilized in various ways. Peptides can be used in a diagnostic capacity, as antigenic probes to detect antibodies directed to the complete protein. Alternatively, they can be used as immunogens to elicit antibodies. Anti-peptide antibodies may react with either the native protein or with the

denatured protein. Both types of interaction are useful (Shinnick *et al.*, 1983). If the anti-peptide antibodies are capable of interacting with the protein in its native form, immunisation with the peptide may serve as a vaccine. Peptides that recognise the denatured protein can serve a role in protein detection by western blotting.

To be effective, a synthetic peptide vaccine must possess a high level of immunogenicity and induce antibodies that cross-react extensively with the pathogen (Van Regenmortel, 1989). The guidelines put forward for the selection of peptides to be used as immunogens are as follows: the region selected should be accessible to antibodies when presented in the native, full-length protein; the peptide should contain a sufficient number of polar amino acids to render it soluble in aqueous solutions, and; the peptide should be longer than 10 residues in order to have a high probability of inducing antibodies that would bind to the native protein (Shinnick *et al.*, 1983).

Epitopes can be distinguished as either continuous or discontinuous. A continuous epitope is considered to be any short linear peptide fragment of the antigen that is able to bind to antibodies raised against the intact protein (Pellequer *et al.*, 1991). Discontinuous epitopes consist of a group of residues that are not adjacent in the sequence but are brought together by the folding of the polypeptide chain or by combination of two separate peptide chains. Most antibodies to discontinuous epitopes bind to the native protein only when it is intact and its conformation is preserved.

Although neutralisation epitopes in a pathogen (those inducing neutralising antibodies) are probably only preserved in antigens with an intact tertiary or quaternary structure, it remains unclear to what extent it is necessary to mimic the native conformation of neutralising epitopes in order to achieve protective immunity by means of synthetic peptides. Since in some cases a peptide can adopt the suitable conformation in solution, it may not be necessary to exactly reproduce the native conformation in order to elicit protein-reactive antibodies (Shinnick *et al.*, 1983).

In order to accurately select peptide candidates for vaccines, methods have been developed to predict the properties of the amino acid sequences of a protein. Parameters such as hydrophilicity, accessibility and mobility of short segments of polypeptide chains have been correlated with the location of continuous epitopes in a few well-characterised proteins (Pellequer *et al.*, 1991). This has led to the development of some empirical rules that allow the position of continuous epitopes to be predicted from certain features of the protein sequence. All prediction calculations are based on propensity scales for each of the 20 amino acids. These scales describe the tendency of each residue to be associated with properties such as surface accessibility, hydrophilicity, or segmental atomic mobility. Each scale consists of 20 values assigned to each of the 20 amino acid residues based on their relative propensity to possess the property described by the scale.

In the present study, the prediction software Predict7 was used to locate continuous epitopes of oligopeptidase B from *T. congolense*. Predict7 analyses protein structural features using seven different algorithms: secondary structure analysis, hydrophilicity, hydropathy, side-chain flexibility, surface probability, antigenicity and location of putative N-glycosylation sites (Carmenes *et al.*, 1989).

The hydrophilicity scale of Hopp and Woods (1981) was the first scale to be based on the observed link between the location of antigenicity at the surface of proteins and the hydrophilic character of these regions. It was suggested that charged amino acids were likely to be antigenic because such residues were mainly located at the surface of proteins. The authors constructed a scale for the hydrophilicity of the 20 amino acids. Although the peaks in the profiles constructed with this scale correspond to hydrophilic regions, the authors remarked that not all the peaks correspond to epitopes, and that all known epitopes of a protein were not located in the most hydrophilic regions.

Karplus and Schulz (1985) developed the flexibility scale; a method for predicting mobility in which the 20 amino acid residues could be split into 10 flexible (Lys, Ser, Thr, Arg, Glu, Pro, Gly, Asp, Asn, Gln) and 10 rigid residues (Ala, Leu, His, Val, Tyr,

Ile, Phe, Cys, Trp, Met). The prediction takes into account the flexible nature of a stretch of residues and not only the propensity of single residues.

Welling *et al.* (1985) constructed an antigenicity scale on the basis of structural analysis of the 606 amino acids found in 69 continuous epitopes of 20 well studied proteins. Each amino acid was characterised by its frequency of appearance in antigenic regions and the scale values were obtained by dividing this frequency by the frequency of each amino acid in all the proteins. Prediction of surface probability is based on a formula by Emini *et al.* (1985) which incorporates surface probability values proposed by Janin *et al.* (1978) for the different amino acid residues.

In the present study, several immunogenic regions of oligopeptidase B from *T. congolense* were identified by epitope mapping. These epitopes were synthesised as peptides and used to raise anti-peptide antibodies in chickens (Section 3.3).

3.2.2. Selection of peptide sequences

The values for hydrophilicity, surface probability, flexibility and antigenicity, that were obtained for the 715 amino acids in the *T. congolense* oligopeptidase B sequence were plotted graphically (Appendix 1, Figs. A1-A5). The seven best peptide sequences were chosen based on their predicted hydrophilicity, surface probability, flexibility and antigenicity (Figs. 3.1-3.7). The sequences of the chosen peptides, shown in Table 3.1, were assessed for similarity to other proteins, specifically trypanosomal or chicken proteins, using a BLAST (basic local alignment search tool) search. BLAST is a program that compares nucleotide or protein sequences to sequence databases and calculates the statistical significance of matches. The seven peptides were found not to have a similar sequence to any relevant proteins (results not shown). Peptides OpBTc2-7 were synthesised with an additional cysteine residue (Table 3.1) for coupling purposes, using MBS.

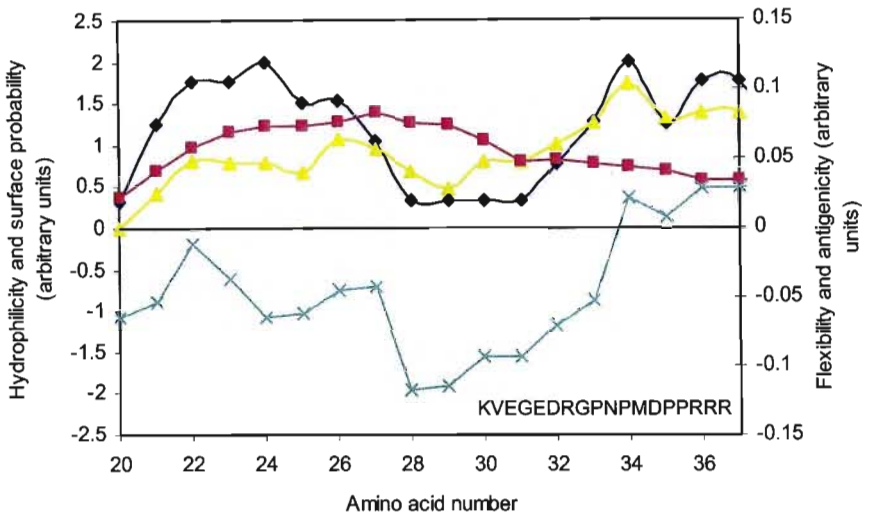

Figure 3.1. Peptide OpBTc1, corresponding to amino acids 20-37 of oligopeptidase B from *T. congolense*. Hydrophilicity (◆), surface probability (▲), flexibility (■) and antigenicity (×), as predicted with Predict7, are depicted. The sequence of the peptide is displayed.

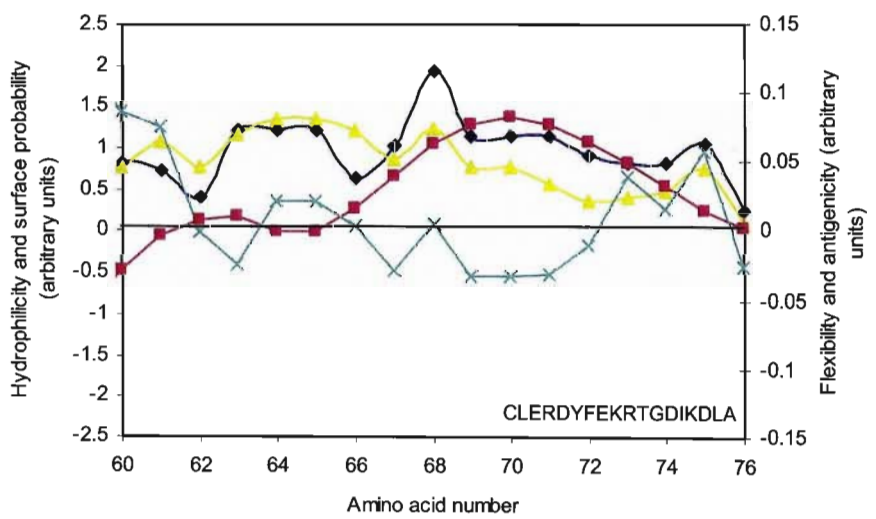

Figure 3.2. Peptide OpBTc2, corresponding to amino acids 60-76 of oligopeptidase B from *T. congolense*. Hydrophilicity (◆), surface probability (▲), flexibility (■) and antigenicity (×), as predicted with Predict7, are depicted. The sequence of the peptide is displayed.

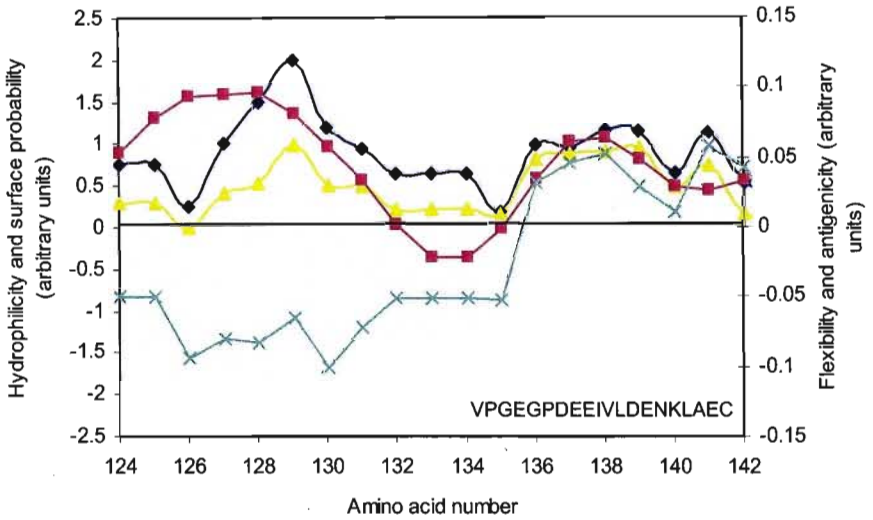

Figure 3.3. Peptide OpBTc3, corresponding to amino acids 124-142 of oligopeptidase B from *T. congolense*. Hydrophilicity (◆), surface probability (▲), flexibility (■) and antigenicity (×), as predicted with Predict7, are depicted. The sequence of the peptide is displayed.

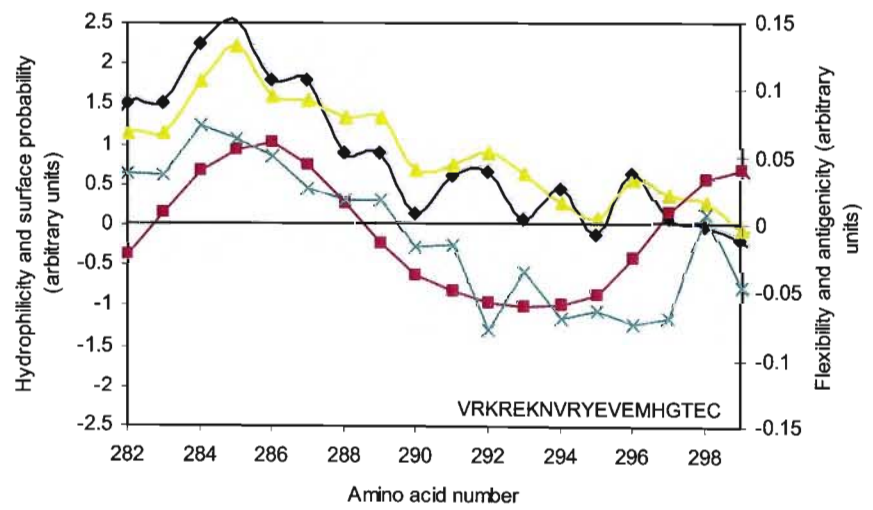

Figure 3.4. Peptide OpBTc4, corresponding to amino acids 282-299 of oligopeptidase B from *T. congolense*. Hydrophilicity (◆), surface probability (▲), flexibility (■) and antigenicity (×), as predicted with Predict7, are depicted. The sequence of the peptide is displayed.

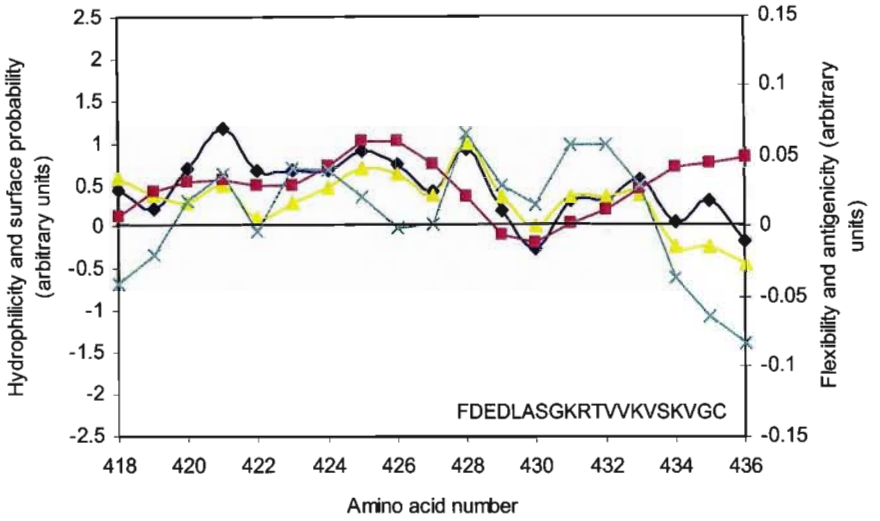

Figure 3.5. Peptide OpBTc5, corresponding to amino acids 418-436 of oligopeptidase B from *T. congolense*. Hydrophilicity (◆), surface probability (▲), flexibility (■) and antigenicity (×), as predicted with Predict7, are depicted. The sequence of the peptide is displayed.

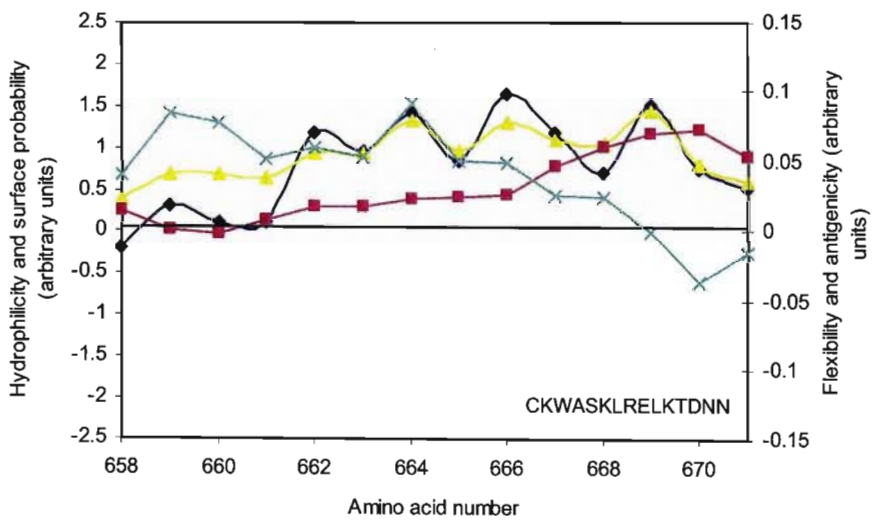

Figure 3.6. Peptide OpBTc6, corresponding to amino acids 658-671 of oligopeptidase B from *T. congolense*. Hydrophilicity (◆), surface probability (▲), flexibility (■) and antigenicity (×), as predicted with Predict7, are depicted. The sequence of the peptide is displayed.

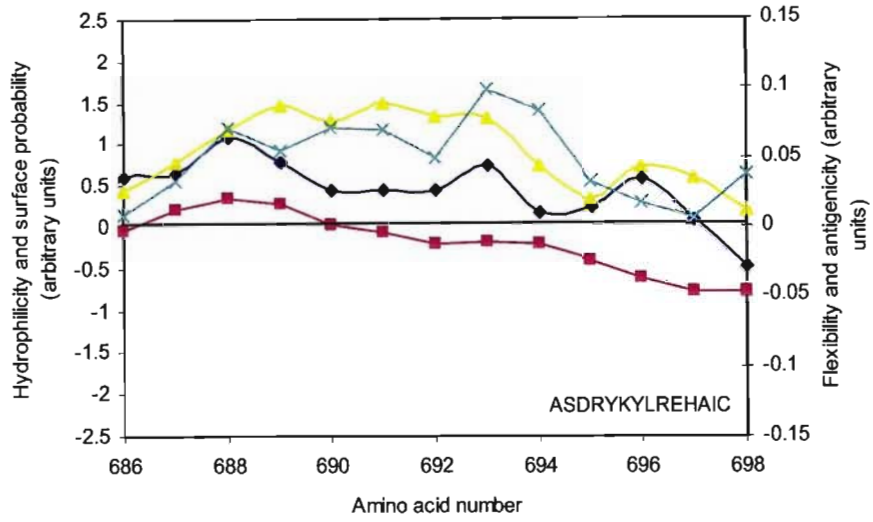

Figure 3.7. Peptide OpBTc7, corresponding to amino acids 686-698 of oligopeptidase B from *T. congolense*. Hydrophilicity (◆), surface probability (▲), flexibility (■) and antigenicity (×), as predicted with Predict7, are depicted. The sequence of the peptide is displayed.

Table 3.1. The amino acid sequences of the oligopeptidase B peptides that were synthesised.

Peptide name	Amino acid sequence[a]	Amino acid number
OpBTc1	KVEGEDRGPNPMDPPRRR	20-37
OpBTc2	CLERDYFEKRTGDIKDLA	60-76
OpBTc3	VPGEGPDEEIVLDENKLAEC	124-142
OpBTc4	VRKREKNVRYEVEMHGTEC	282-299
OpBTc5	FDEDLASGKRTVVKVSKVGC	418-436
OpBTc6	CKWASKLRELKTDNN	658-671
OpBTc7	ASDRYKYLREHAIC	686-698

[a] Cysteine residues that were introduced for the purpose of coupling peptides using MBS are underlined

3.3 Production of anti-peptide antibodies in chickens

Chickens were used to produce antibodies against peptides based on the *T. congolense* oligopeptidase B sequence that were selected in Section 3.2. The usual method of raising

antibodies against peptides is to immunise animals with peptide-carrier conjugates. It has, however, been reported that it is possible to immunise animals with peptides that are 6-36 residues in length without conjugating them to macromolecular carriers (Briand *et al.*, 1985). Despite this, in the present study peptides were coupled to rabbit albumin using either glutaraldehyde or MBS. Rabbit albumin was used because of its large size (68.2 kDa) and its lack of similarity to chicken proteins. Table 3.2 describes the amounts of peptide and carrier used in the coupling reactions. One peptide, OpBTc1, was coupled to rabbit albumin by its N-terminus using glutaraldehyde (Section 3.3.1). The other six peptides were coupled to rabbit albumin by a terminal cysteine residue using MBS (Section 3.3.2). In each case, the ratio of carrier to peptide was 1:40.

Table 3.2. The amounts of peptide, carrier, and coupling agent used in the coupling reactions.

Peptide name	Molecular weight of peptide	Mass of peptide (mg)	Mass of carrier (mg)	Mass of MBS (mg)
OpBTc1	2105	4	3.20	na
OpBTc2	2171	4	3.14	0.58
OpBTc3	2155	4	3.16	0.58
OpBTc4	2362	4	2.89	0.50
OpBTc5	2137	4	3.20	0.58
OpBTc6	1805	4	3.78	0.70
OpBTc7	1724	4	4.00	0.70

3.3.1 Coupling of peptides to rabbit albumin with glutaraldehyde

The use of glutaraldehyde as a coupling agent is considered simple and effective, with the antigenicity of the protein or peptide being preserved (Reichlin, 1980). The coupling reaction involves amino groups, and thus, glutaraldehyde is used for peptides with a single free amino group at the N-terminus. Glutaraldehyde cannot be used when the

peptide has any internal lysine residues as this will lead to the formation of large multimeric complexes (Thorpe, 1994).

3.3.1.1 Reagents

PBS, pH 7.2. As per Section 2.8.1.

2% (v/v) Glutaraldehyde. 25% Glutaraldehyde (80 µl) was made up to 1 ml with dH_2O.

10 mg/ml $NaBH_4$. $NaBH_4$ (2.5 mg) was dissolved in dH_2O (250 µl).

3.3.1.2 Procedure

OpBTc1 (4 mg) was dissolved in DMSO (100 µl) and PBS (400 µl) was added. Rabbit albumin (3.2 mg) was dissolved in PBS (500 µl) and combined with the peptide. 2% (v/v) Glutaraldehyde (1 ml) was added dropwise with stirring, and stirring was continued for 2 h at 4°C. The reaction was stopped with the addition of 10 mg/ml $NaBH_4$ (200 µl) and stirred for 1 h at 4°C. The solution was dialysed overnight against PBS with the addition of 0.02% (m/v) NaN_3 (3 changes). The dialysed solution was divided into 4 equal aliquots and stored at -20°C.

3.3.2 Coupling of peptides to rabbit albumin with M-maleimidobenzoyl acid N-hydroxy succinimide ester (MBS)

The coupling agent MBS, developed by Kitagawa and Aikawa (1976), is used as a coupling reagent for peptides that have a single, terminal cysteine residue. MBS reacts with the primary amine group on the carrier protein to form a stable amide bond. The MBS-activated carrier protein reacts with sulfhydryl containing peptides to form the conjugate (Liu *et al.*, 1979). It is necessary to reduce the peptide for the coupling reaction to take place. This is carried out in the same manner as in Section 2.12.2, where peptide is reduced with DTT and purified by MEC on Sephadex G-10.

3.3.2.1 Reagents

As per Section 2.12.2.1.

3.3.2.2 Procedure

OpBTc2-7 (4 mg) was dissolved in DMSO (100 µl) and reducing buffer (900 µl) was added. 10 mM DTT (1 ml) was added and incubated for 1.5 h at 37°C. Reduced peptide was separated from DTT on a Sephadex G-10 column as per Section 2.12.2.2.

MBS (40:1 rabbit albumin, molar ratio) was dissolved in DMF (200 µl) and made up to a final volume (500 µl per mg of MBS) with PBS. Rabbit albumin was dissolved in an amount of PBS to bring the total volume to 2 ml when combined with the MBS. The combined solutions were stirred for 30 min (RT). Activated rabbit albumin was separated from MBS on a Sephadex G-25 column (1.5 × 13 cm; 10 ml/h), with the collection of 1 ml fractions. Elution was monitored by reading A_{280} of the fractions. The first peak at 280 nm was pooled.

Reduced peptide and activated rabbit albumin were combined and stirred for 3 h (RT). The solution was divided into 4 equal aliquots and stored at -20°C.

3.3.3 Preparation of immunogen and immunisation of chickens

3.3.3.1 Reagents

Sterile PBS, pH 7.2. As per Section 2.8.1. PBS was sterilised by filter-sterilisation through a 0.22 µm filter.

3.3.3.2 Procedure

Immunogen was prepared just before use by bringing the total volume to 1 ml with sterile PBS, combining with 1 ml Freund's complete adjuvant, and triturating to give a stable water-in-oil emulsion. Two chickens were immunised, receiving 1 ml each. OpBTc peptides coupled to rabbit albumin (equivalent to 500 µg of peptide per immunisation) were injected intramuscularly at two sites on either side of the breast bone. Booster injections were given at weeks 2, 4, and 6, using Freund's incomplete adjuvant. Eggs were collected prior to immunisation, and throughout the immunisation schedule.

3.3.4 Affinity purification of antibodies

Peptide OpBTc1 (5 mg) was coupled to Aminolink® resin as in Section 2.12.1.2. Peptides OpBTc2-7 (7.5 mg for OpBTc2, 3, 6 and 7; 5 mg for OpBTc 4 and 5) were coupled to Sulfolink® resin as in Section 2.12.2.2. Affinity purification was carried out according to Section 2.13.

3.3.5 ELISA evaluation of antibody production

3.3.5.1 Reagents

As per Section 2.8.1.

3.3.5.2 Procedure

As per Section 2.8.2.

3.3.6 Western blotting evaluation of antibody production

3.3.6.1 Reagents

As per Section 2.7.1.

3.3.6.2 Procedure

Protein (rOPC or rOPV; 2 μg) separated by Laemmli SDS-PAGE (Section 2.4) was electroblotted onto nitrocellulose (200 mM, 16 h), and treated as per Section 2.7.2. Affinity purified anti-peptide antibodies were chosen for incubation with the membrane on the basis of good recognition of the respective peptide in ELISA, and a high concentration of the antibody preparation. The following affinity purified IgY preparations were used at 10 μg/ml: OpBTc1-2, weeks 8-12; OpBTc2-1, weeks 4-7; OpBTc3-2, weeks 4-7; OpBTc4-1, weeks 4-7; OpBTc5-1, weeks 4-7; OpBTc6-1, weeks 4-7; OpBTc7-1, weeks 8-12.

3.3.7 Inhibition of oligopeptidase B activity by anti-peptide antibodies

Antibodies directed against enzymes can inhibit, activate or have no effect on an enzyme's activity. This depends on factors such as the epitopes targeted by the antibodies, concentration of the antibody, and the substrate used in the assay (Richmond,

1977). Antibody-mediated inhibition of enzymes is thought to be a result of occlusion of the active site, or by unfavourable conformational changes (Oppenheim and Nachbar, 1977). Anti-peptide antibodies were used in assays to assess their effect, if any, on the activity of oligopeptidase B (rOPC, produced as described in Chapter 4) against the substrate Z-Arg-Arg-AMC.

3.3.7.1 Reagents

Assay buffer [200 mM sodium phosphate buffer, pH 7.2, 4 mM Na_2EDTA, 0.1% (v/v) Tween, 8 mM DTT]. $NaH_2PO_4.2H_2O$ (3.12 g) and Na_2EDTA (0.15 g) were dissolved in dH_2O (90 ml) and titrated to pH 7.2 with NaOH. Tween 20 (0.1 ml) was added and dissolved, and the final volume made up to 100 ml with dH_2O. DTT (0.0062 g) was added to assay buffer (5 ml) just before use.

1 mM Z-Arg-Arg-AMC stock solution. Z-Arg-Arg-AMC (0.9 mg) was dissolved in DMSO (1.5 ml).

20 µM Z-Arg-Arg-AMC. 1 mM Z-Arg-Arg-AMC stock solution (0.04 ml) was diluted to 2 ml with dH_2O.

All other reagents as per Section 2.9.1.1.

3.3.7.2 Procedure

The inhibition of enzyme activity by specific antibodies was assayed in a stopped-time format with serial two-fold dilutions of chicken IgY from 1 mg/ml. All assays were performed in duplicate. Recombinant OPC (20 ng), diluted to 75 µl in 0.1% Brij, was combined with 75 µl of IgG or IgY, diluted with assay buffer without DTT, to yield final antibody concentrations of 1 mg/ml, 500 µg/ml, 250 µg/ml and 125 µg/ml. The samples were incubated for 15 min at 37°C. Aliquots (50 µl) were removed from the incubation mixture and combined with 25 µl assay buffer containing 8 mM DTT in a microplate, and activated for 1 min at 37°C. Substrate was added (25 µl) and the microplate was incubated for 5 min at 37°C. Fluorescence (emission at 460 nm with excitation at 360

nm) was read with a 7620 microplate fluorometer from Cambridge Technology (UK). Inhibition was expressed as a percentage of the activity in the presence of non-immune antibody at the same concentration.

3.4 Alignment of amino acid sequences using CLUSTAL W

The simultaneous alignment of multiple amino acid or nucleotide sequences is an essential tool in molecular biology. The many useful applications include the design of oligonucleotide primers for PCR, prediction of secondary and tertiary structures of new sequences, detection of homology between new sequences and the discovery of diagnostic patterns to characterise protein families (Thompson *et al.*, 1994). CLUSTAL W is a highly sensitive sequence alignment program that is commonly used for alignment of both amino acid and base pair sequences (http://www.ebi.ac.uk/clustalw; 1/6/2006). In the present study, CLUSTAL W was used for the comparison between oligopeptidase B amino acid sequences. An alignment was used to compare the sequences of the peptides (Section 3.1.2) between the different trypanosomal species, and to identify differences or similarities at certain positions in the sequences (Fig. 3.8). Oligopeptidase B sequences for *T. congolense* and *E. coli* were compared with a prolyl oligopeptidase sequence (Fig. 3.9) to determine the degree of similarity between them, and to locate the positions of the peptides for 3D visualisation (Section 3.5). Symbols are displayed in the alignment to denote the degree of conservation observed in each column. A star (*) means that the residues in that column are identical in all sequences in the alignment. A colon (:) means that conserved substitutions have been observed, and a point (.) that semi-conserved substitutions have been observed. A conserved substitution is replacement with an amino acid of similar properties, i.e. small and hydrophobic (AVFPMILW); acidic (DE); basic (RHK); basic with a hydroxyl or amine groups (STYHCNGQ).

```
T. brucei       MQTERGPIAAHRPHEVVFGKVEGEDRGANPMDPPRRRVDPLFWLRDDNRADPEVLAHLHL 60
T. congo        MTSDRGPIAAHKPYEVVFGKVEGEDRGPNPMDPPRRRVDPLFWLRDDSRTNPDVLAHLHL 60
T. vivax        MPAPCGPVAEQQDHEVIFGKVEGEDRGPNPMDPPLRRNDPLFWLRDDNRTNPKVLAHLHL 60
                * :  **:* :: :**:**********.****** ** *********.*::*.*******

T. brucei       EKDYYEKRAVDIKDLAETIYQEHISHIEETDMSAPYVYDRFLYYTRDVKGLSYKLHCRVP 120
T. congo        ERDYFEKRTGDIKDLAETIYQEHISHIEETDMSPPYTYNRFVYYTRRVKGLSYKIHCRVP 120
T. vivax        EKAYFEECTVDLKDLSETVYQEHLSHIQETDMSAPYVHDNYMYYKREVKGLSYKIHCRVP 120
                *: *:*: : *:***:**:****:***:*****.**.::.::**.* *******:*****

T. brucei       AGKTPGEGEDEEIVLDENKLAEGKSFCVVGCVAPAPPEHALVAYSVDYCGDEVYSIRFVR 180
T. congo        LGKVPGEGPDEEIVLDENKLAEGKAFCDVRSVAPAPPEHMLVAYSVDHLGDELYSIQFVG 180
T. vivax        LGKTPG-SSDEQVVLDENKLAEGKAFCHVRQVEPAPPKHSLVAYSVDYTGNEVYTIRFVG 179
                 **.**.. **::**********:** *  * ****:* *******: *:*:*:*:**

T. brucei       DVVADKVEGTNGSVVWGPNAECFFYITKDASKRDNKVWRHIIGQPQSEDVCLYTDDDPLF 240
T. congo        DASPDKLEGTTGSIIWGTNAECFFYVTPDSTKRSNKVWRHIIGQSQSEDVCLYTDDDPLF 240
T. vivax        DDVPDVVEGTNGHIVWGPDGGCFFYTTKDAAQRDYKVWRHVIGQQQSEDVCLYTEVDAVF 239
                *  .* :***.* ::**.:. **** * *:::*. *****:*** *********: *.:*

T. brucei       SVGVGRSGDGKTLIICSMSSETSESHLLDLRKGVKHNTLEMVRPREKGVRYTVEMHGTDT 300
T. congo        SVAASKSGDGHTLLISSSSSETTELHLLDLRKGLNNTTLEVVRKREKNVRYEVEMHGTET 300
T. vivax        SACMSKSGDGNTLLITSSSSETTEVHLLDLRGGVAHNELETVRPREKGVRYDVALHGTDT 299
                *.  .:****:**:* * ****:* ****** *: :. ** ** ***.*** * :***:*

T. brucei       LIVLTNKDKCVNGKVVLTKRSAPTDWGTVLIPHDDKVTIDDVAVFAKFAVLSGRRDGLTR 360
T. congo        LVILTNKDKCINGKVVLAKRASPSEWTNVLVPHDDKVFIDDIAVFAKFAVLSGRRDGLTR 360
T. vivax        LLVLTNKDKCINGQVLIVQRSAPSDWTRVLVPHSEEVFIEEIAVFSTFAVLAGRRAGLSR 359
                *::*******:**:*::.:*::*::*  **:**.::* *:::***:.****:*** **:*

T. brucei       VWTVRLGPDNLFSSATLKELHFDEPVFTAHVVCSQMKTYDASLLRLRYSSMTTPTVWYDE 420
T. congo        VWTVQVGPDNCFSAGTLRELQFDEPVFTAHVITSQMKTYDTSSLRLEYSSMTTPTTWFDE 420
T. vivax        IWTMQVGPDNTFSNSLVNELQFDEPVFTTHVVLSQMHMYNTATLRLTYSSMTTPTTWYDV 419
                :**:::**** ** . :.**:*******:**: ***: *::: *** ********.*:*

T. brucei       DVLSGERKVVKARKVGGGFESKNYVCRRELATAPDGTKVPISLVYDTSIDLKKPNPTMLY 480
T. congo        DLASGKRTVVKVSKVGGGFDSKNYVCQRRLATAPDGTTIPLSILYDVSLDMKKPHPTMLY 480
T. vivax        HVAEGGRTPVKIREVGGGFNPAHYVSKRLLATAPDGTKIPISLVYDAALDLTKPHPTMLY 479
                .: .* *. **  :*****:. :**.:* ********.:*:*::**.::*:.**:*****

T. brucei       GYGSYGICIEPEFNSRFLPYVDRGMIYAIAHVRGGGEMGRTWYEVGGKYLTKRNTFMDFI 540
T. congo        GYGSYGICVEPQFDIRCLPYVDRGVIYAIAHVRGGGEMGRAWYEIGGKYLTKRNTFMDFI 540
T. vivax        GYGSYGICVEPEFNIQYLPYVDRGVIFAIAHVRGGGEMGRAWYELGAKYLTKRNTFMDFI 539
                ********:**:*: : *******:*:**************:***:*.*************

T. brucei       ACAEHLISSGLTTPAQLSCEGRSAGGLLVGAVLNMRPDLFHVALAGVPFVDVMTTMCDPS 600
T. congo        SCAEHLISSGVTTPPQLACEGRSAGGLLVGAVLNMRPDLFRVAVAGVPFVDVMTTMCDPS 600
T. vivax        ACAEHLISSGLTTPNQLACEGRSAGGLLIGAVLNMRPDLFHVALAGVPFVDVMTTMCDPT 599
                :*********:*** **:**********:***********:**:***************:

T. brucei       IPLTTGEWEEWGNPNEYKFFDYMNSYSPIDNVRAQDYPHLMIQAGLHDPRVAYWEPAKWA 660
T. congo        IPLTTGEWEEWGNPNEYKFFDYMNSYSPIDNVRPQDYPNLIIQAGLHDPRVAYWEPAKWA 660
T. vivax        IPLTTFEWEEWGNPNEYKYFDYMNSYSPIDNVRAQAYPHLMIQAGLHDPRVAYWEPVKWA 659
                ***** ************:**************.* **:*:***************.***

T. brucei       SKLRELKTDSNEVLLKMDLESGHFSASDRYKYLRENAIQQAFVLKHLNVRQLLRK 715
T. congo        SKLRELKTDNNEVLLKMDLDSGHFSASDRYKYLREHAIQQAFVLKHLGVRRLLRH 715
T. vivax        SRLRQLKTDGNEVLVKMDLDSGHFSSADRYKYWREMAIQQAFVLKHLNVRCLLRR 714
                *:**:****.****:****:*****::***** ** ***********.** ***:
```

Figure 3.8. Alignment of the oligopeptidase B sequences from the African trypanosomes *T. b. brucei*, *T. congolense* and *T. vivax*. The active-site residues are shown in red (Ser[563], Asp[648] and His[683] for *T. b. brucei* and *T. congolense*; Ser[562] Asp[647] and His[682] for *T. vivax*), and the peptide sequences (OpBTc1-7) are shown in order, in blue. The alignment was prepared using CLUSTAL W software.

Figure 3.9. Sequence alignment of *T. congolense* oligopeptidase B with related enzymes for which 3D models have been constructed. Oligopeptidase B from *T. congolense* (T. congo_OPB) was aligned with oligopeptidase B from *E. coli* (E.coli_OPB) and prolyl oligopeptidase from porcine brain (Porcine_POP) using CLUSTAL W software. The three active-site residues are shown in red, and the peptide sequences (OpBTc1-7) are shown in order, in blue. The alignment was prepared using CLUSTAL W software.

Fig. 3.8 is an alignment of the sequences of oligopeptidase B from *T. b. brucei, T. congolense* and *T. vivax*. There is a high degree of similarity between the enzymes from the three species. The sequence of *T. b. brucei* oligopeptidase B (715 amino acids) has 81% similarity with the *T. congolense* oligopeptidase B sequence (715 amino acids), and 74% similarity with the 714 amino acid *T. vivax* sequence. The *T. congolense* and *T. vivax* sequences share 73% sequence similarity. Most of the amino acid substitutions between the sequences are to conserved residues.

Fig. 3.9 is an alignment of the sequences of oligopeptidase B from *T. congolense* with those of *E. coli* oligopeptidase B and prolyl oligopeptidase from porcine brain, for which the 3D structures have been determined. The most similarity occurs between the two oligopeptidase B sequences (34%). Porcine brain prolyl oligopeptidase shows 19% similarity with *T. congolense* oligopeptidase B, and 23% similarity with *E. coli* oligopeptidase B.

3.5 Three-dimensional visualisation of proteins

Three-dimensional structures were viewed and manipulated using PyMOL software from DeLano Scientific (http://pymol.sourceforge.net; accessed 1/02/2006). The crystal structure is available for prolyl oligopeptidase from porcine brain (Fülop *et al.*, 1998), which belongs to the same family of enzymes as oligopeptidase B. The crystal structure of oligopeptidase B has not yet been determined, but with the use of homology modelling, Gérczei *et al.* (2000) have constructed a 3D model for oligopeptidase B from *E. coli* (Fig. 3.10). Since oligopeptidase B from *T. congolense* has 34% sequence similarity to the *E. coli* oligopeptidase B and 19% similarity to porcine brain prolyl oligopeptidase, the model of *E. coli* oligopeptidase B was the most suitable representation of the 3D structure of *T. congolense* oligopeptidase B.

The two structural domains of oligopeptidase B, mentioned previously in Section 1.2.2.1, are shown in Fig 3.10. The N-terminal β-propeller domain is represented in blue, green and yellow. The C-terminal α/β hydrolase domain, containing substrate-binding and catalytic residues, is represented in dark blue (the N-terminus), orange and red (the C-

terminus). The 3D model shows clearly how the N- and C-terminal residues form the second domain, whilst the 'central' residues make up the first domain.

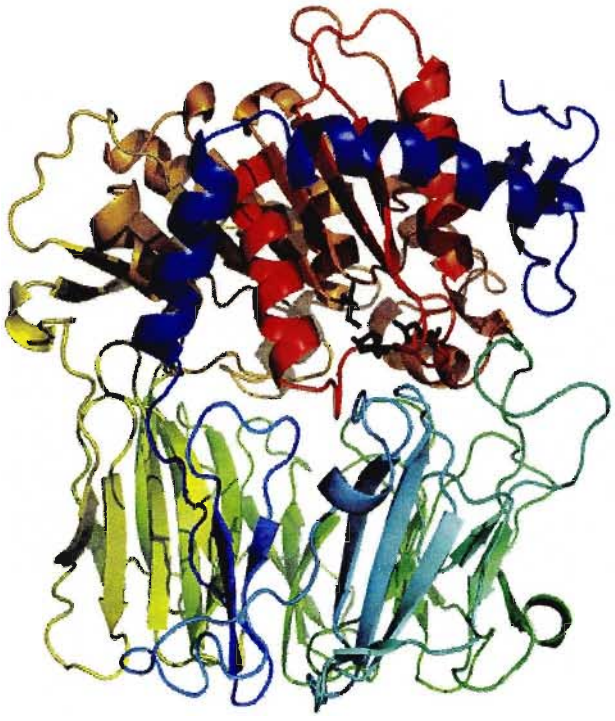

Figure 3.10. Stereo representation of the structure of oligopeptidase B from *E. coli*, as modelled by Gérczei *et al.* (2000). The ribbon structure is shown in a spectrum of colour, ranging from blue at the N-terminus, to red at the C-terminus. The three active site residues (Ser^{554}, Asp^{641}, and His^{680}) are represented in black.

Using an alignment between the *T. congolense* and *E. coli* oligopeptidase B sequences (Fig. 3.9), the position of each of the *T. congolense* peptide sequences, identified in Section 3.1, was determined on the *E. coli* sequence and highlighted on the 3D structure, shown individually (Fig. 3.11). The first peptide, OpBTc1, only had 4 out of the total 18 amino acids represented in the *E. coli* sequence since that region of the *E. coli* sequence appeared to be absent (Fig. 3.9, *E. coli* sequence between residues 16 and 19). All of the other peptides were adequately represented in the *E. coli* sequence.

Peptide OpBTc1 appears to be part of a β sheet and loop in the catalytic domain (Fig. 3.11A). Peptide OpBTc2 is mostly represented as an α helix, but it also contains a loop region and a small part of an adjoining α helix in the catalytic domain (panel B). OpBTc3 is mostly a loop region with a small α helix, situated in the β-propeller domain (panel C). OpBTc4 is a β strand within a β sheet with loop regions at each end, in the β-propeller domain (panel D). OpBTc5 is an entire β sheet, 2 connecting loop regions and part of second β strand within a β sheet in the β-propeller domain (panel E). OpBTc6 is an α helix and loop region in the catalytic domain (panel F). OpBTc7 is also an α helix and loop region in the catalytic domain (panel G).

The PyMOL software was used to predict the surface of the oligopeptidase B molecule showing the peptide sequences (Fig. 3.12). Peptides OpBTc1, 2, 3, 5 and 6 appeared to have a good proportion of their sequences exposed on the surface of the molecule (panels A, B, C, E, and F). Peptides OpBTc4 and 7, whilst partially visible on the surface, were not well exposed on the surface of the molecule (panels D and G).

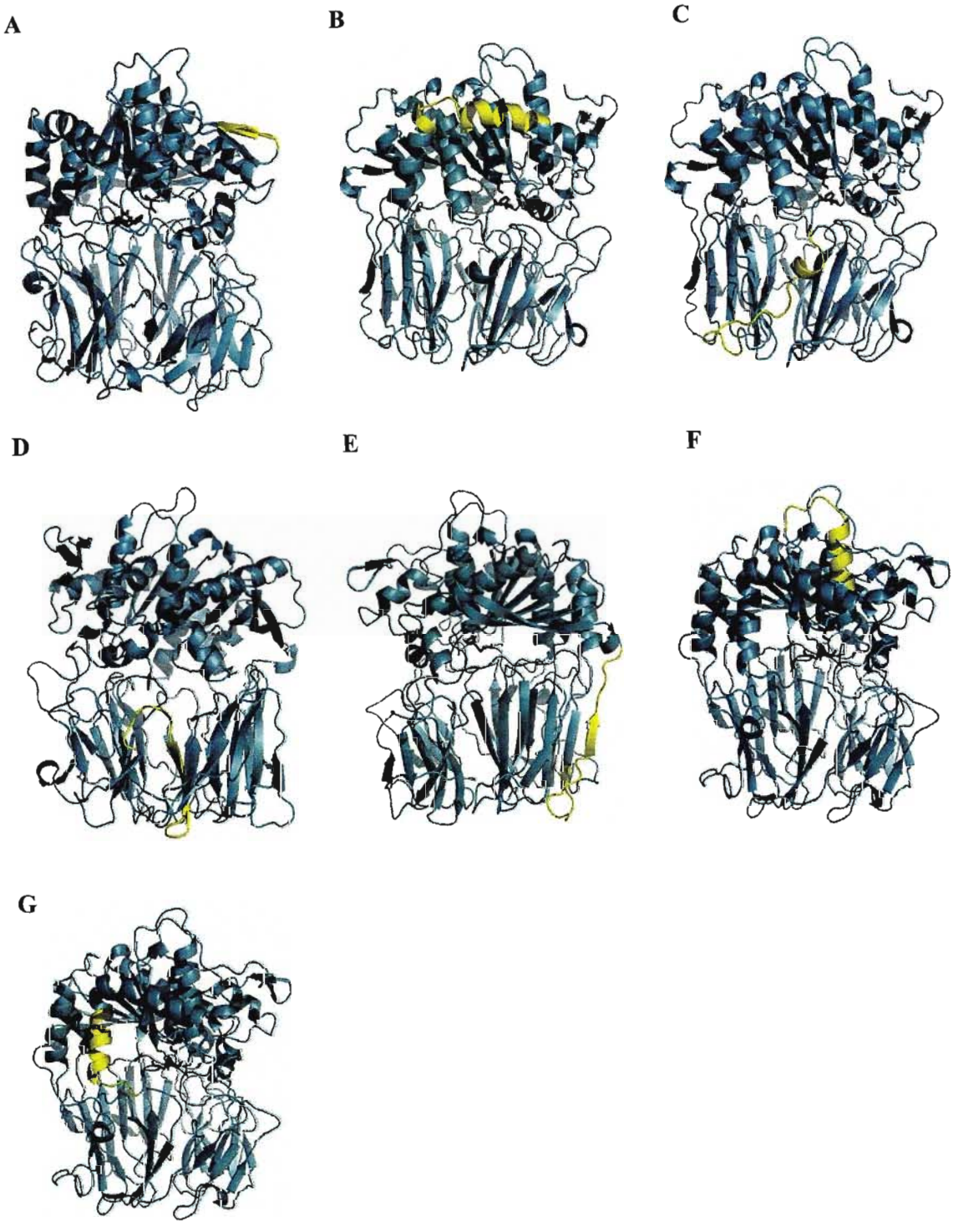

Figure 3.11. Stereo representation of the structure of oligopeptidase B from *E. coli* showing the location of the seven peptide sequences. The peptide sequences corresponding to the *T. congolense* oligopeptidase B peptides are represented on the ribbon structure of *E. coli* oligopeptidase B in yellow, and the three active site residues are represented in black. The structure was positioned to best show the location of the peptide on the structure (Diagrams A-G correspond to the peptides OpBTc1-7 respectively).

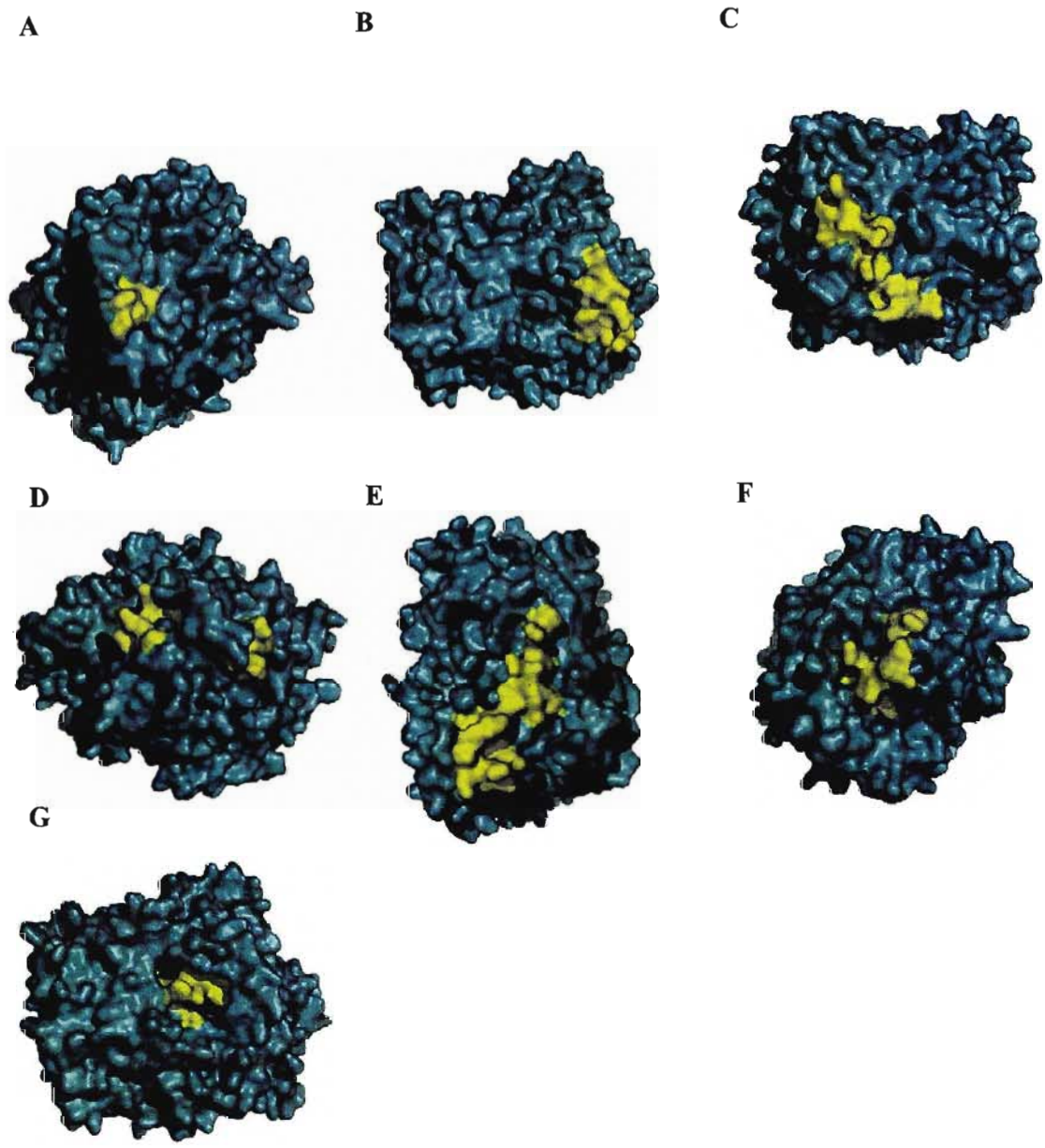

Figure 3.12. Surface representation of the structure of oligopeptidase B from *E. coli* showing the location of the seven peptide sequences. The peptide sequences corresponding to the *T. congolense* oligopeptidase B peptides are represented on the structure of *E. coli* oligopeptidase B in yellow. The structure was positioned to best show the location of the peptide on the structure (Diagrams A-G correspond to the peptides OpBTc1-7 respectively).

3.6 Results

3.6.1 ELISA evaluation of chicken anti-peptide antibodies

Anti-peptide antibody production in chickens over the 12 week immunisation schedule was monitored by ELISA. Two ELISAs were conducted to illustrate the progress of antibody production. The first ELISA used an IgY concentration of 100 µg/ml (Fig. 3.13). The second ELISA used an IgY concentration of 500 µg/ml for samples with lower antibody levels (Fig. 3.14).

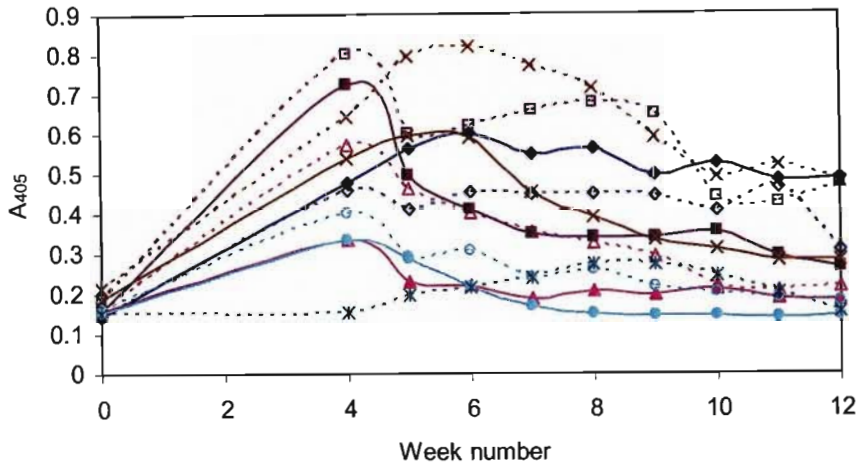

Figure 3.13. ELISA of anti-peptide antibodies in IgY isolated from immunised chickens, at 100 µg/ml. Peptide was coated onto ELISA plates (Section 2.8.2) and primary antibody was IgY from weeks 4-12 at 100 µg/ml (in BSA-PBS, 100 µl per well, 2 h, 37°C). Rabbit anti-chicken-HRPO secondary antibodies (in BSA-PBS, 120 µl per well, 1 h, 37°C) and ABTS/H_2O_2 (150 µl per well) were used as the detection system. Absorbance readings at 405 nm of IgY against OpBTc1 (◆;◇), OpBTc3 (▲;△),OpBTc4 (■;□), OpBTc5 (●;○), OpBTc6 (×) and OpBTc7 (✳) represent the average of duplicate experiments. IgY from chicken 1 is represented by a solid line, while chicken 2 is represented as a broken line.

Antibody production against peptide OpBTc1 was high in both chickens, reaching a peak at around week 6 (Fig. 3.13). Antibody production against peptide OpBTc2 was poor in the first chicken, peaking at week 8, and undetectable in the second (Fig 3.14). Anti-OpBTc3 antibody levels were moderate to high, peaking at week 4 (Fig. 3.13). Anti-OpBTc4 antibody levels were very high, peaking at week 4. Levels of anti-OpBTc5 antibodies were consistently low over the 12 week period, peaking at week 4. Antibody production against peptide OpBTc6 peaked at week 6, with consistently high antibody levels observed over the 12 week period. Low levels of anti-OpBTc7 antibody were

observed in one chicken, peaking at week 9 (Fig. 3.13), while remaining undetectable in the other chicken (Fig. 3.14).

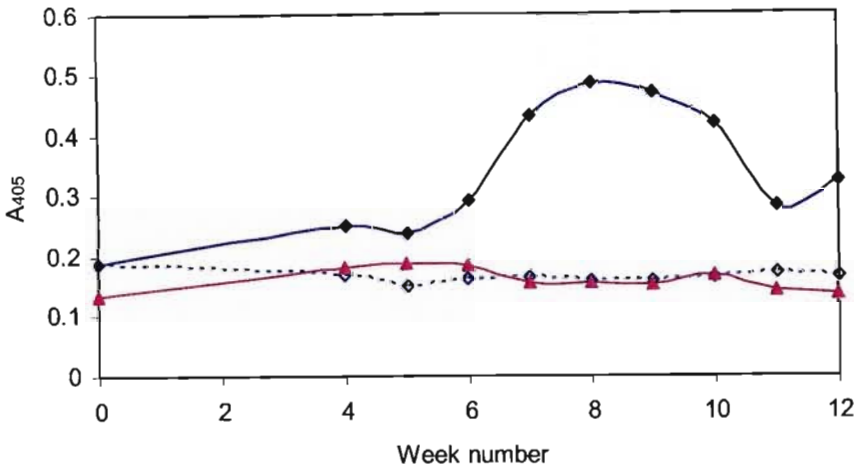

Figure 3.14. ELISA of anti-peptide antibodies in IgY isolated from immunised chickens, at 500 µg/ml. Peptide was coated onto ELISA plates (Section 2.8.2) and primary antibody was IgY from weeks 4-12 at 500 µg/ml (in BSA-PBS, 100 µl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 3.13. Absorbance readings at 405 nm of IgY against OpBTc2 (◆;◇) and OpBTc7 (▲) represent the average of duplicate experiments. IgY from chicken 1 is represented by a solid line, while chicken 2 is represented as a broken line.

Affinity purification of the anti-peptide antibodies, over a matrix of their respective immobilised peptides, greatly enhanced their recognition of the respective peptides in ELISA (Fig. 3.15). Anti-peptide antibodies gave very high signals, even at very low concentrations. Also, antibodies that previously showed no recognition of their respective peptides (i.e. OpBTc2, both chickens, and OpBTc7, one chicken) showed detectable levels of specific antibodies after affinity purification (Fig. 3.15, B and G). The affinity purified anti-peptide antibodies were used in ELISAs to detect the recombinant oligopeptidases, rOPC and rOPV, produced and purified as described in Chapter 4. Recombinant OPC was well recognised by antibodies against OpBTc1, 2 and 3 (Fig. 3.16, A-C), and recognised to a small degree by antibodies against OpBTc4 and OpBTc7 (Fig. 3.16, D and G). Antibodies against OpBTc5 and 6 did not recognise rOPC (Fig. 3.16, E and F). All of the anti-peptide antibodies showed recognition of rOPV (Fig. 3.17), especially antibodies against OpBTc1 and OpBTc3.

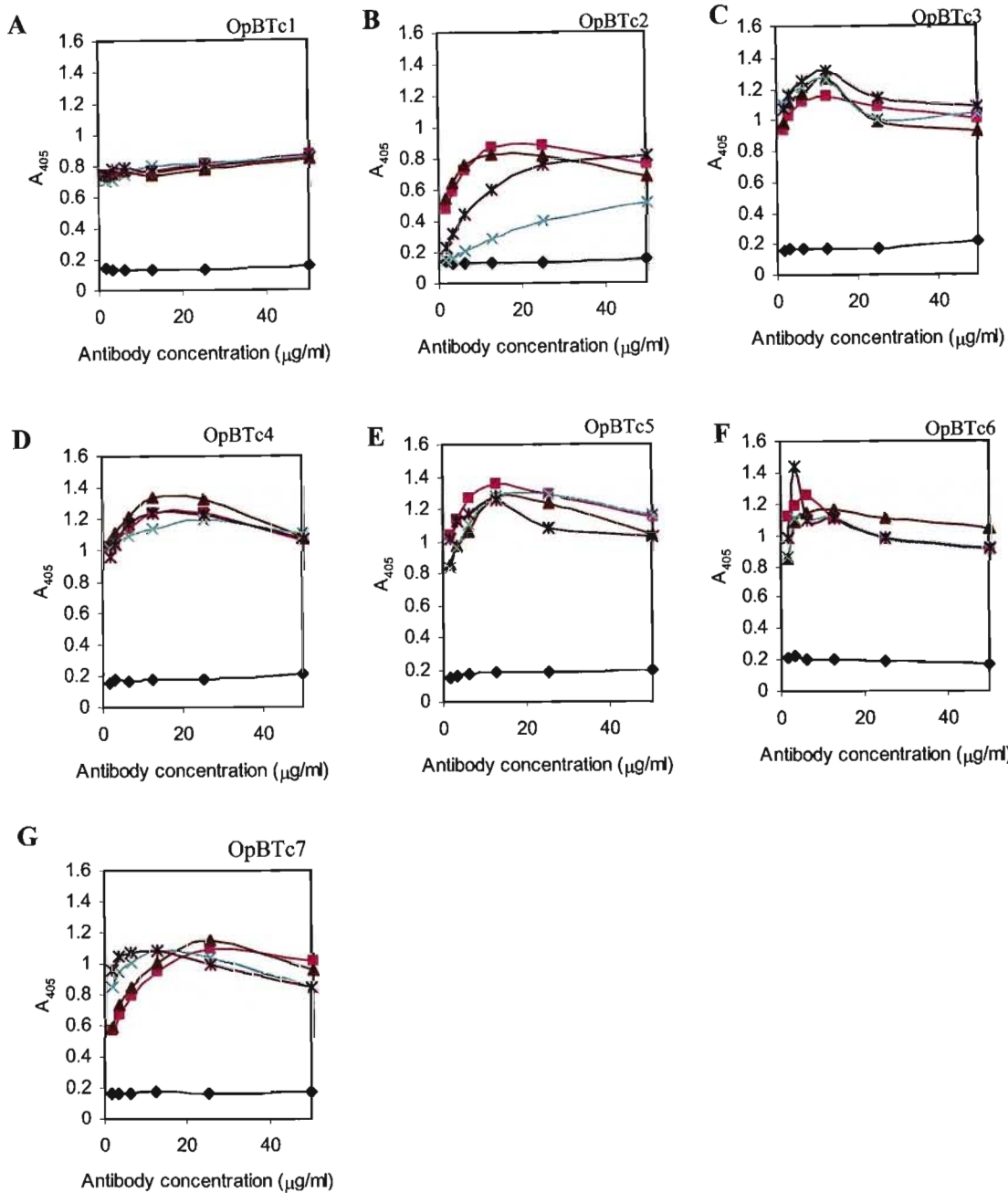

Figure 3.15. ELISA of affinity purified anti-peptide antibodies. Peptide was coated onto an ELISA plate (Section 2.8.2) and affinity purified IgY diluted serially from 50 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 3.13. Absorbance readings at 405 nm of affinity purified IgY from chicken 1, weeks 4-7 (■) and weeks 8-12 (▲); and chicken 2, weeks 4-7 (×), and weeks 8-12 (✳); and non-immune IgY (◆) represent the average of duplicate experiments. Panels A-G show antibodies against peptides OpBTc1-7 respectively, as indicated.

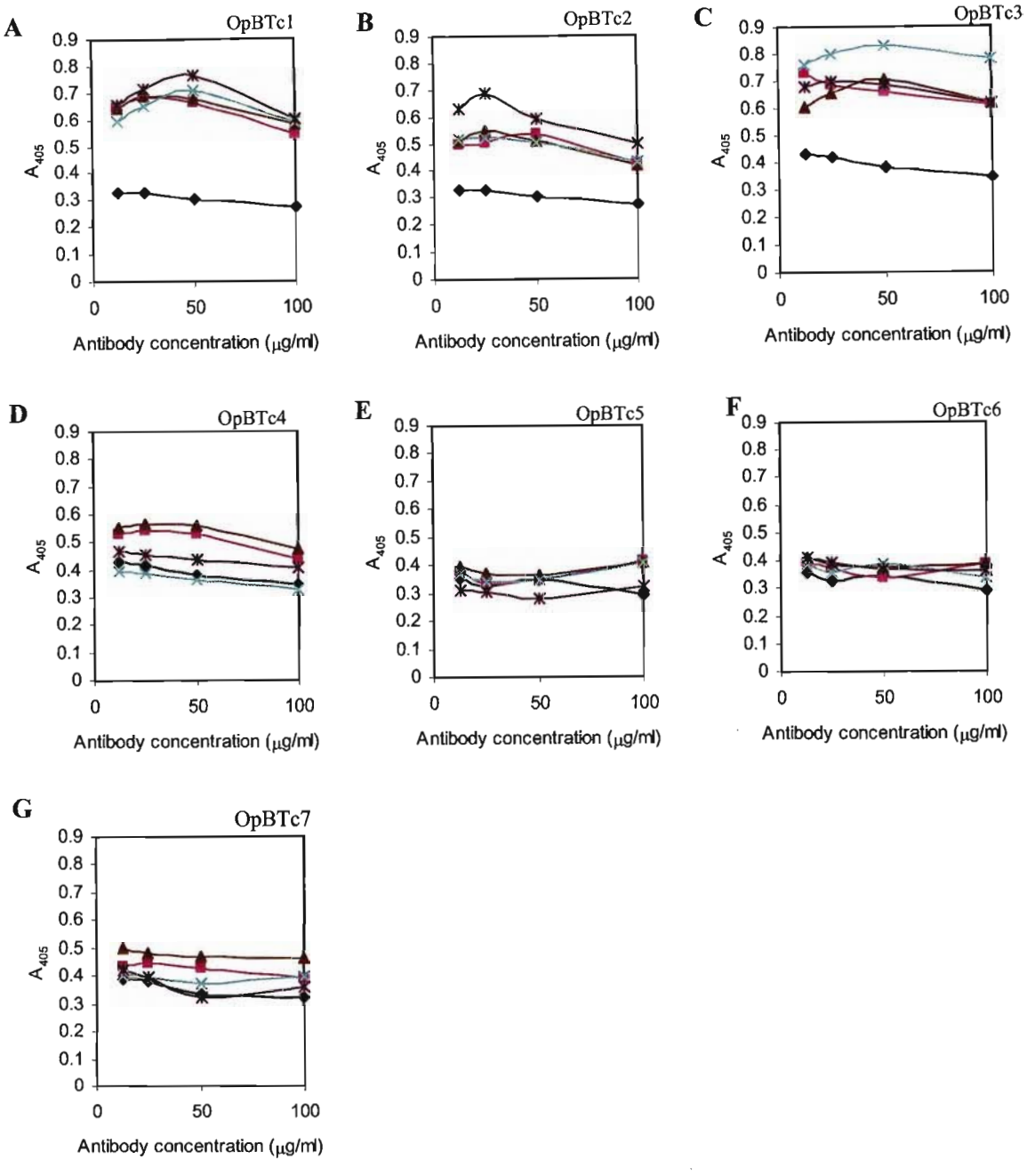

Figure 3.16. Recognition of rOPC by affinity purified anti-peptide antibodies. rOPC was coated onto an ELISA plate (Section 2.8.2) and affinity purified anti-peptide IgY diluted serially from 100 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 3.13. Absorbance readings at 405 nm of affinity purified IgY from chicken 1, weeks 4-7 (■) and weeks 8-12 (▲); and chicken 2, weeks 4-7 (×), and weeks 8-12 (∗); and non-immune IgY (◆) represent the average of duplicate experiments. Panels A-G show antibodies against peptides OpBTc1-7 respectively, as indicated.

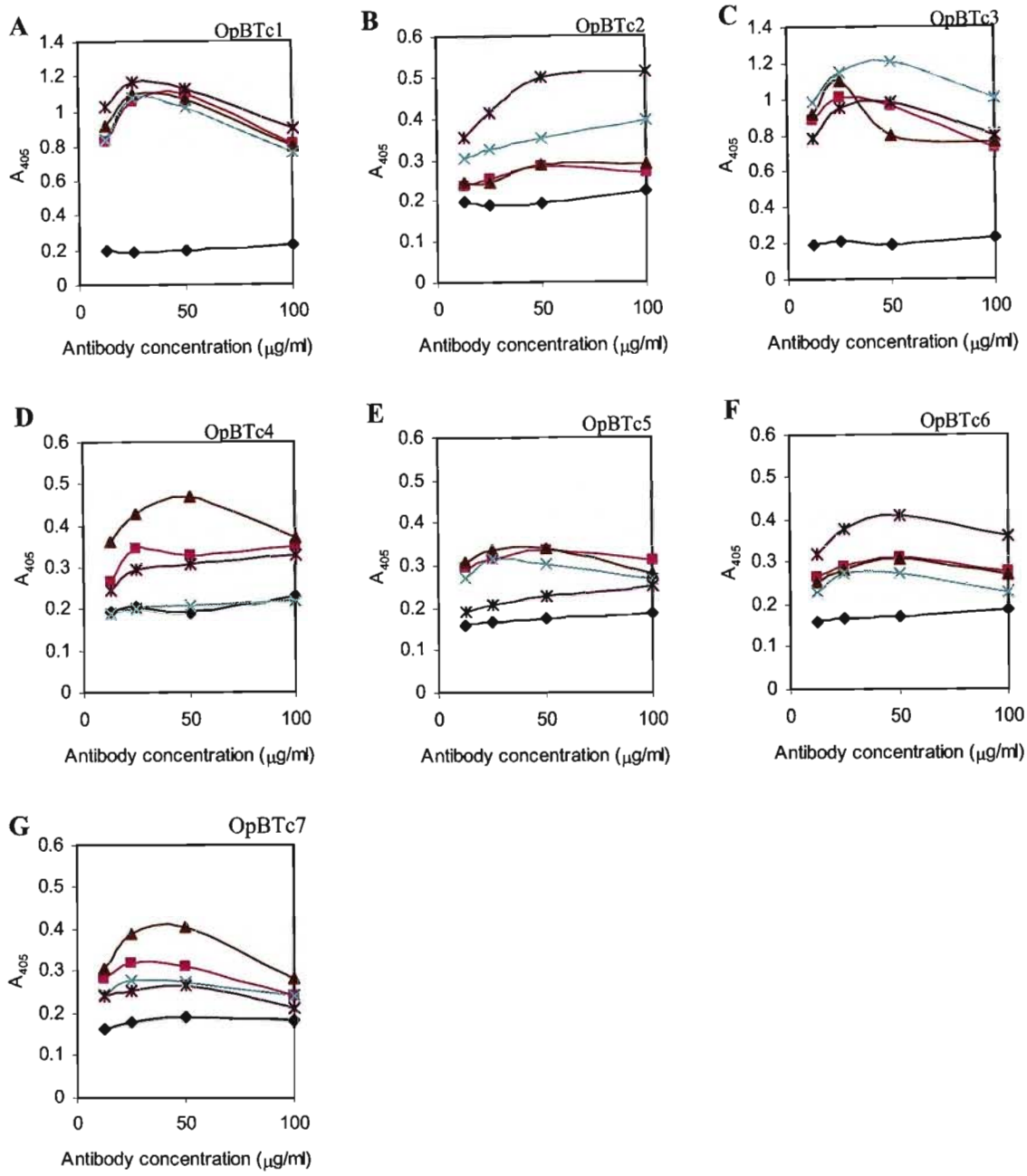

Figure 3.17. Recognition of rOPV by affinity purified anti-peptide antibodies. rOPV was coated onto an ELISA plate (Section 2.8.2) and affinity purified anti-peptide IgY diluted serially from 100 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 3.13. Absorbance readings at 405 nm of affinity purified IgY from chicken 1, weeks 4-7 (■) and weeks 8-12 (▲); and chicken 2, weeks 4-7 (×), and weeks 8-12 (✳); and non-immune IgY (◆) represent the average of duplicate experiments. Panels A-G show antibodies against peptides OpBTc1-7 respectively, as indicated.

3.6.2 Western blotting evaluation of anti-peptide antibodies

Identical amounts of affinity purified anti-peptide antibodies were used in western blots to probe whole recombinant rOPC or rOPV to assess their ability to recognise the denatured protein. A band at 80 kDa, corresponding to rOPC was observed in blots for all anti-peptide antibodies (Fig. 3.18). Antibodies against peptides OpBTc5, 6 and 7 gave very light signals, however (lanes 5-7). Recombinant OPV was recognised strongly by antibodies against peptides OpBTc1 and 3 (Fig. 3.19, lanes 1 and 3), while faint signals were observed for antibodies against OpBTc4 and 6 (lanes 4 and 6), and no signals for antibodies against OpBTc5 and OpBTc7. The results of the western blots parallel the observations made by ELISA (Fig. 3.16 and Fig. 3.17)

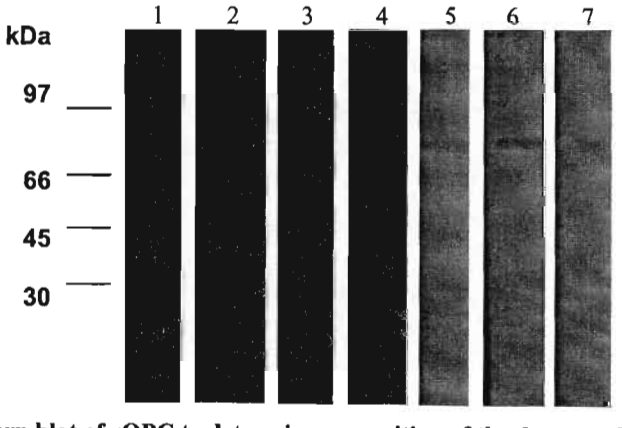

Figure 3.18. Western blot of rOPC to determine recognition of the denatured protein by anti-peptide antibodies. Protein, rOPC (2 μg), was electrophoresed on 10% acrylamide and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with affinity purified IgY (10 μg/ml) corresponding to OpBTc1-7 in lanes 1-7 respectively, followed by HRPO-conjugated rabbit anti-chicken IgY. Bands were developed with 4-chloro-1-naphthol/H_2O_2.

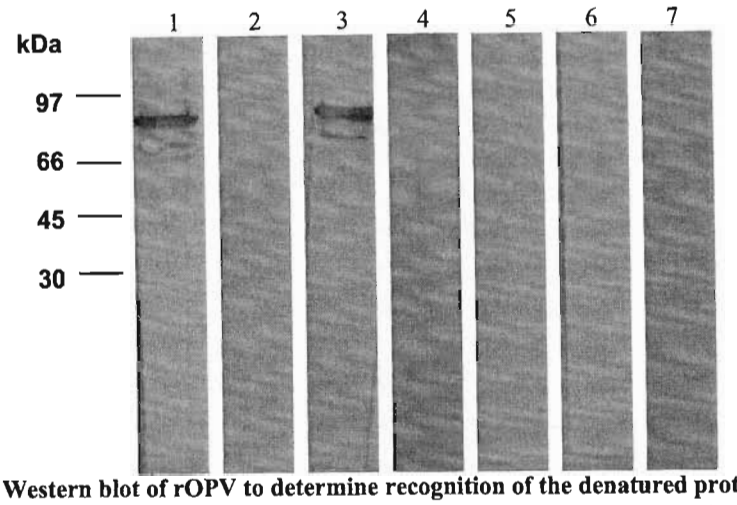

Figure 3.19. Western blot of rOPV to determine recognition of the denatured protein by anti-peptide antibodies. Protein, rOPV (2 μg), was electrophoresed on 10% acrylamide and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with affinity purified IgY (10 μg/ml) corresponding to OpBTc1-7 in lanes 1-7 respectively. Bands were detected as per Fig. 3.18.

3.6.3. Inhibition of oligopeptidase B by anti-peptide antibodies

All of the anti-peptide antibodies were assayed for their ability to inhibit the peptidolytic activity of rOPC. None of the antibodies were found to have any significant inhibitory or activating effect on the enzyme (result not shown).

3.7 Discussion

The alignment of the oligopeptidase B amino acid sequences from the three African trypanosomal species *T. b. brucei*, *T. congolense* and *T. vivax* revealed a high degree of similarity. From this information it would be expected that all three enzymes would show very similar characteristics, especially since the areas around the catalytic residues are conserved. Indeed, it has been found that oligopeptidase B from *T. b. brucei* and *T. congolense* are very similar with respect to substrate specificity and inhibitor profiles, although the *T. congolense* enzyme appears to be less efficient at cleaving peptide substrates (Morty *et al.*, 1999a).

The amino acid sequence of oligopeptidase B from *T. congolense* was aligned with that of *E. coli* oligopeptidase B and prolyl oligopeptidase; enzymes of predicted and known 3D structure respectively. From the alignment, the degree of similarity observed allowed for selection of the most appropriate protein for comparison. Oligopeptidase B from *E.*

coli was the most similar to the *T. congolense* protein, which was expected since they are part of the same subfamily, oligopeptidase B, of the S9 serine peptidase family. The alignment of the two proteins was used as a tool to identify the sequences on the *E. coli* protein that correspond to the sequences of the peptides, to be highlighted on the 3D structure. The secondary structures that the peptide sequences may form, according to Fig. 3.11, provide some insight into the results obtained in the production of anti-peptide antibodies. Similarly, the locations of the peptides with reference to the surface of the intact protein also assist in the interpretation of the immunochemical results.

In the present study, several immunogenic epitopes of oligopeptidase B from *T. congolense* were identified. These epitopes, 14 to 20 residues in length, were selected on the basis of their hydrophilicity, surface probability, flexibility and antigenicity according to Predict7. Their suitability for use as immunogens in the present study was confirmed by their lack of similarity to any chicken proteins or other trypanosomal proteins. Chicken proteins were significant because chickens were the animal species chosen for raising the anti-peptide antibodies. If the peptides had shown similarity to any chicken proteins, they would probably not be recognised as foreign when used for immunisations. It was also not desirable for the peptides to have similarity to other trypanosomal proteins since this could result in cross-reactivity.

The 3D representations of oligopeptidase B, showing the peptide locations relative to the surface of the molecule (Fig. 3.12), lend credit to the prediction of surface probability made with Predict7 because all the peptide are represented on the surface of the molecule to some degree. All of the peptides have positive values for surface probability, but some have lower surface probability than others (Figs. 3.1-3.7). The lowest surface probability was predicted for peptides OpBTc3 and OpBTc5, and the N-terminal half of OpBTc4 showed the highest surface probability overall (Figs. 3.3-3.5). The visualisation of peptide OpBTc1 on the surface of the molecule offers a poor demonstration of which residues may be on the surface since so few of the amino acid residues are represented in the *E. coli* sequence (Fig. 3.12). Peptides OpBTc3 and OpBTc5 appear to be well represented on the surface of the molecule, whereas OpBTc4 does not (Fig. 3.12). This is

in direct opposition to the surface probability results for those peptides. The other peptides had comparably mediocre predicted values for surface probability, and, besides OpBTc7, appear to have fairly good representation on the surface of the molecule. Therefore, it would appear that the strength of the surface probability values does not have a direct correlation to the degree of surface location of the peptide as modelled according to *E. coli* oligopeptidase B.

Not all of the peptides that were used for immunisation were found to be immunogenic in chickens, even with the carrier protein. Strong antibody responses were observed for peptides OpBTc1, 3, 4 and 6, whereas peptides OpBTc2, 5 and 7 resulted in moderate to poor antibody levels. Only a few of the anti-peptide antibodies, namely against OpBTc1, OpBTc2 and OpBTc3, were able to recognise the full protein, rOPC, in ELISA format. This would suggest that certain epitopes, corresponding to OpBTc5 and 6, are not accessible to the antibodies in ELISA, where the protein is not fully denatured. From the ELISA results it could be argued that peptides OpBTc2, 5 and 7 are not actually located on the surface of oligopeptidase B, although they showed high surface probability with Predict7 software. According to the location of the peptides on the 3D structure of the protein (Fig. 3.12), peptides OpBTc4 and 7 are partially buried in the structure and not easily accessible, whereas OpBTc2 and 5 appear to be adequately represented on the surface of the molecule. An alternative explanation for the poor recognition of the full protein in the native conformation is that the epitopes corresponding to peptides OpBTc2 and 7 are α helices (Fig. 3.11). It might be difficult for the antibodies to recognise their corresponding epitopes in a helical, non-linear conformation. The poor recognition of the protein by anti-OpBTc5 antibodies cannot be explained by its secondary structure since it consists of a β strand and loop regions.

Oddly, all of the anti-peptide antibodies were able to recognise rOPV when adsorbed to the well of a multititre plate in ELISA, albeit to varying degrees. This could suggest that rOPC and rOPV adopt different conformations in this format, whereby these epitopes are more accessible in rOPV. Alternatively, the anti-peptide antibodies may be cross reacting with non-linear, conformational epitopes in rOPV at very low levels. The results

of western blotting confirmed the ability of all of the anti-peptide antibodies to recognise the full protein, rOPC, in a denatured and linearised conformation. The lack of the ability of some of the anti-peptide antibodies to recognise denatured rOPV lends support to the argument that the anti-peptide antibodies were not recognising the specific linear epitopes in ELISA.

The *T. congolense* oligopeptidase B peptides all had a high degree of similarity with the corresponding sequences from *T. vivax* (Fig. 3.11). It would therefore be expected that the anti-peptide antibodies would be able to cross-react with rOPV. This was true for the anti-peptide antibodies against OpBTc1 and OpBTc3 which showed good recognition of both rOPC and rOPV in ELISA (Figs. 3.16 and 3.17). However, in western blotting, denatured rOPV was only recognised by anti-peptide antibodies against peptides OpBTc1, 3, 4 and 6 (Fig. 3.19). The strongest cross-reactivity was observed by anti-OpBTc1 and anti-OpBTc3 antibodies. OpBTc1 has only one amino acid that is different between the *T. vivax* and *T. congolense* sequences, so this was expected. OpBTc3 has a number of differences in the N-terminal half of the peptide, but is identical at the C-terminal half, which is a stretch of 9 residues. The peptides that did not show immunological cross-reactivity are quite similar in sequence but the amino acid substitutions occur intermittently in the sequence and there is probably not a long enough stretch of amino acids in common for cross-reactivity to occur.

None of the anti-peptide antibodies that were raised in the present study were able to inhibit the activity of rOPC against Z-Arg-Arg-AMC. This is probably because the epitopes to which these antibodies were directed are not in a suitable location to block entry of the substrate to the active site of the enzyme. There is a narrow hole at the bottom of the central tunnel of the β-propeller domain, which restricts access to the active site (Fülop *et al.*, 1998). None of the peptides are located adjacent to the hole (Fig. 3.20), although peptides OpBTc3 (amino acids 124-142), OpBTc4 (amino acids 282-299) and OpBTc5 (amino acids 418-436) are close, with OpBTc4 being the closest. It is likely that antibodies that inhibit the peptidolytic activity of oligopeptidase B block the entry of substrate through this hole. It may be possible to direct antibodies against a specific

peptide, corresponding to an epitope in this region, that would prevent entry of substrate through the hole. Selection of such a peptide is further discussed in Chapter 9. Identification of an inhibitory peptide sequence may be difficult since the hole is flanked by amino acid residues that are not sequential, and would likely form conformational rather than linear epitopes. Some short stretches of amino acids were, however, identified by examination of the 3D model, and these corresponded to amino acids 154-160, 248-254 and 396-400 on the *T. congolense* sequence. Upon reference to the Predict7 results (Appendix 1), it was observed that residues 154-160 and 396-400 correspond to immunogenic regions of the protein, but 248-254 does not.

In this chapter a considerable amount of speculation was possible based on comparisons with the 3D model of *E. coli* oligopeptidase B. Elucidation of the 3D structure of oligopeptidase B from trypanosomal sources would aid in the accurate identification of the epitopes that elicit antibodies capable of inhibiting the peptidolytic activity of the enzyme.

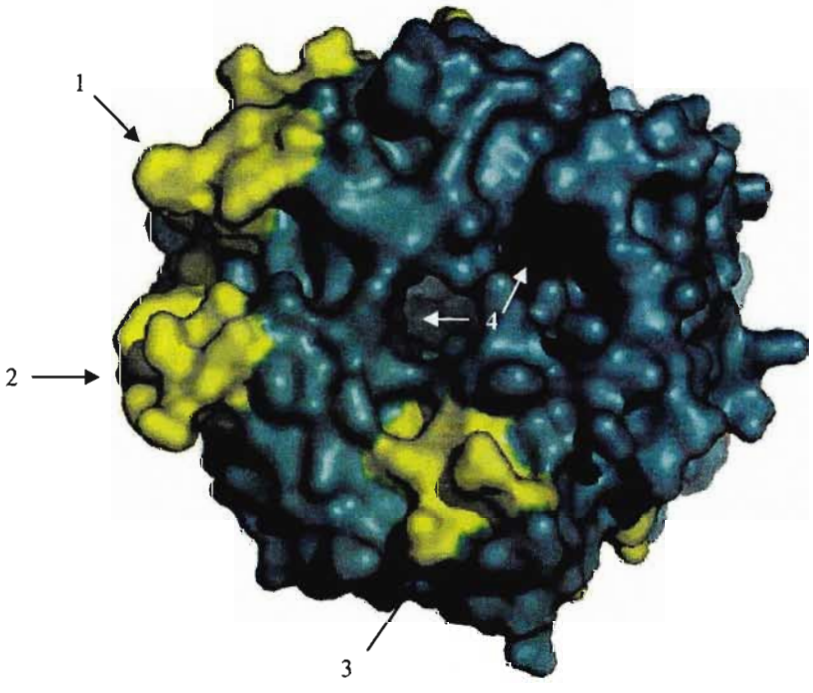

Figure 3.20. Surface representation of the 3D structure of oligopeptidase B showing the hole that restricts access to the active site within the enzyme's structure. Peptides are highlighted in yellow and important features are designated by arrows. (1) Peptide OpBTc3, (2) peptide OpBTc5, (3) peptide OpBTc4, (4) the hole leading to the active site.

CHAPTER 4

Recombinant expression and purification of oligopeptidase B and the catalytic domain of congopain

4.1 Introduction

This chapter describes the recombinant expression and purification of oligopeptidase B from both *T. congolense* and *T. vivax*, and the catalytic domain of congopain, C2. C2 has previously been expressed in *P. pastoris* (Alain Boulangé, manuscript in preparation), and was produced and purified in the present study to obtain sufficient amounts for use in experimentation and immunisation, to investigate its potential role in an anti-disease vaccine. As discussed in Section 1.2.1.1, C2 is a truncated form of congopain, containing only the catalytic domain and the proregion, to ensure correct folding. The C-terminal domain of the protease was excluded because, while it is highly immunogenic, antibodies directed against it are unlikely to inhibit the enzyme's activity.

Oligopeptidase B, a validated virulence factor of African trypanosomes, was also of significant interest in the present study for application to an anti-disease vaccine for trypanosomosis. Prior to the present study, oligopeptidase B from *T. vivax* had not been isolated from the parasite, and oligopeptidase B from neither *T. congolense* nor *T. vivax* had been recombinantly expressed. Large amounts of both proteins were required for kinetic characterisation as well as immunisation of experimental animals and subsequent immunochemical analysis. Recombinant expression and purification of oligopeptidase B was a much more viable way to obtain the amount of protein required for the present study, since native protein isolation is laborious and results in low yields (Morty, 1998). The ORFs of oligopeptidase B from *T. congolense* and *T. vivax* were amplified by PCR, and cloned into a PCR cloning vector (pCAP^{S}), and subsequently, the expression vectors pPIC9 and pGEX4T1. Both bacterial and yeast expression systems were employed in the present study for optimal expression of the oligopeptidase B ORFs. Recombinant enzymes, rOPC and rOPV, were purified following expression in *E. coli*.

This chapter also describes the isolation of native oligopeptidase B from trypanosomal lysates (*T. b. brucei, T. congolense* and *T. vivax*). Although recombinant enzyme was used throughout the majority of the study, native enzyme was isolated for comparative purposes, and used to assay inhibition by specific antibodies (Chapter 8) and to establish K_m values against the substrate Z-Arg-Arg-AMC (Chapter 5).

4.1.1 Bacterial expression systems

A major objective of gene cloning is the expression of the cloned gene in a selected host organism. Currently, although a wide range of both prokaryotic and eukaryotic organisms can express foreign genes, most commercially important proteins produced by recombinant technology are synthesised in *E. coli* where possible. Reasons for this include that many proteins can be produced by *E. coli* rapidly and inexpensively, and it is one of the best studied organisms in the world (Glick and Pasternak, 1998).

During expression the foreign protein may undergo degradation by host proteases. One way to avoid this problem is to fuse the cloned ORF to a sequence coding for an *E. coli* protein, called a carrier protein. The resulting fusion protein is stable and recognised as self by the bacterium, protecting the foreign protein from attack by host cell proteases (Murby *et al.*, 1996). The carrier protein can also act as a tag for the gene product and assist during the purification of the gene product. In the case of pGEX vectors, the gene product is expressed as a fusion protein with glutathione S-transferase (GST, 25 kDa). The GST-fusion protein can be purified by affinity chromatography on a matrix with a glutathione ligand (Smith and Johnson, 1988).

Expression of the fusion protein in the pGEX system is under the control of the *tac* promoter. The *tac* promoter is a widely used, strong, inducible promoter consisting of the -10 region (10 nucleotide pairs upstream from the site of initiation of transcription) of the *lac* promoter and the -35 region of the *trp* promoter. The hybrid *tac* promoter can be repressed by the *lac* repressor and can be derepressed by IPTG, and shows increased efficiency compared to both parental promoters (De Boer *et al.*, 1983).

The strategy employed to remove the carrier protein from the expressed protein is the inclusion of a cleavage site for a specific protease in between the two proteins at the DNA level. In the case of pGEX4T1, the proteolytic cleavage site is specific for thrombin (Smith and Johnson, 1988), which hydrolyses the peptide bond between the Arg and Gly residues of the conserved Leu-Val-Pro-Arg-Gly-Ser sequence. Other proteases commonly used for this purpose are factor X_a, which cleaves after the Arg residue of the Ile-Glu/Asp-Gly-Arg sequence (Eaton *et al.*, 1986), and enterokinase, which cleaves after the Lys residue of the Asp-Asp-Asp-Asp-Lys sequence (Hopp *et al.*, 1988).

4.1.2 Eukaryotic expression systems

Proteins synthesised in bacteria are often unstable or lack biological activity, due to misfolding and/or the absence of post-translational modifications, such as glycosylation. Also, bacterial compounds, particularly lipopolysaccharide, commonly contaminate the final product. In view of this problem, several eukaryotic systems have been developed for protein expression, including yeast, baculovirus and mammalian cells. The advantage of a eukaryotic expression system is that any post-translational modifications required for stability or activity are possible. These include correct disulfide bridge formation, cleavage of precursor forms, glycosylation and phosphorylation (Glick and Pasternak, 1998).

The brewer's yeast, *Saccharomyces cerevisiae*, has been used extensively as a host cell for the expression of cloned eukaryotic genes. Although it is a successful system for many proteins, it generally gives a low yield of expressed protein and plasmid loss can easily occur. Expressed proteins often appear hyperglycosylated with mannose residues, resulting in altered activity and immunogenicity. Also, proteins that are meant to be secreted often remain in the periplasmic space, resulting in purification difficulties (Glick and Pasternak, 1998). The problems associated with the *S. cerevisiae* expression system have led to the investigation of alternative yeast species for protein expression. One such alternative is the methylotrophic yeast *P. pastoris* (Cregg *et al.*, 1985).

There are several features of *P. pastoris* that make it an appealing host for expressing recombinant proteins. Heterologous gene expression in *P. pastoris* is driven by the alcohol oxidase 1 (*AOX1*) promoter, which is a very strong, inducible promoter. Transcription from this promoter is induced by the addition of methanol to the culture, which is relatively inexpensive. The yeast is grown for expression in a minimal, protein-free medium leading to fewer protein contaminants. Aberrant glycosylation is also less likely to occur (Cregg *et al.*, 1985).

Commonly used expression vectors for *P. pastoris*, such as pPIC9 (Fig. 4.1), include a) the *AOX1* promoter to drive transcription of the recombinant gene insert (indicated by "5′ AOX1" in Fig. 4.1); b) the *AOX1* transcriptional terminator sequence (indicated by "TT" in Fig. 4.1); c) a functional histidinol dehydrogenase gene (*HIS4*) which encodes an enzyme required for the synthesis of histidine; and d) a signal sequence derived from the pre-pro α mating factor of *S. cerevisiae* to facilitate secretion of the desired protein into the medium (indicated by "S" in Fig. 4.1) (Rosenfeld, 1999). Some plasmids, such as pHIL-D2, do not include a signal sequence for secretion and are typically used for the expression of cytosolic, non-glycosylated proteins. The *P. pastoris* host strain, e.g. GS115, has a mutation in the his4 gene, preventing it from synthesising histidine. Positive transformants will therefore be able to grow in a medium lacking in histidine, which is a trait used for selection (Cregg *et al.*, 1985). Another mechanism of selection is resistance to the antibiotic zeocin, where a plasmid carrying zeocin resistance, such as pPICZ, has been employed (Drocourt *et al.*, 1990).

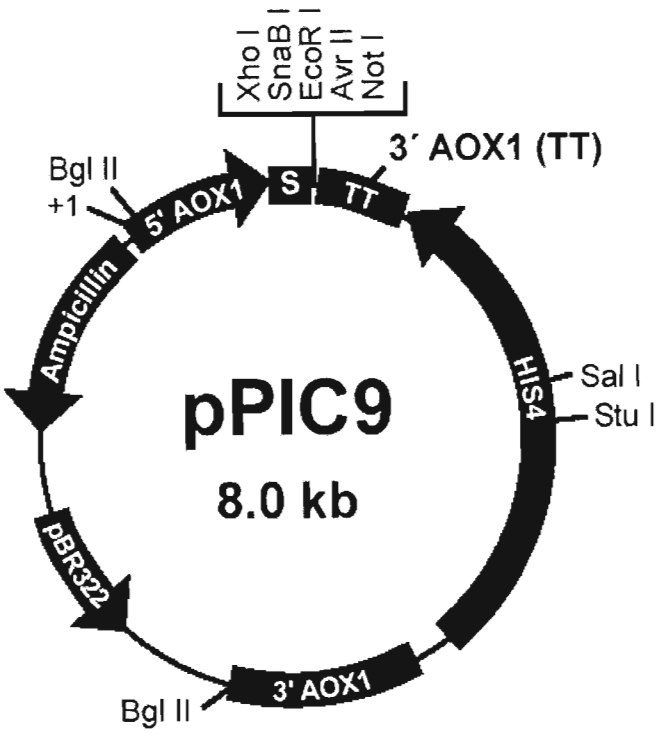

Figure 4.1. Map of the pPIC9 expression vector (Invitrogen), detailing the multiple cloning site.

Transformation of *P. pastoris* using cloning vectors occurs via integration of the transforming DNA into the host genome by homologous recombination. The site of integration into the host genome can be directed by restriction digestion of the vector DNA so that its ends are homologous to the corresponding chromosomal site of integration. Useful sites of chromosome integration include the *HIS4* gene and the *AOX1* gene. Directed single crossover recombination events with the *AOX1* or *HIS4* genes may be achieved by restriction digesting the plasmid DNA such that a single cut is introduced within these genes (Cregg *et al.*, 1985). In pPIC9, this is achieved by digesting the recombinant plasmid with the restriction enzymes *Sac*I or *Sal*I for integration into the *AOX1* and *HIS4* genes respectively. The resulting transformants have a His^+Mut^+ phenotype in the GS115 host strain. That is, the ability to synthesise histidine (His^+) and thus grow in media not supplemented with histidine, and the ability to grow on methanol as a sole carbon source (Mut^+: methanol utilisation plus) (Rosenfeld, 1999). The fact that transformation of yeast occurs by integrative transfection with the gene of interest, as described, was the limiting factor of the technique until improvements were made by

treatment of yeast cells with lithium and DTT (Wu and Letchworth, 2004) which enhance yeast cell permeability through an unknown mechanism.

4.2 Production and purification of recombinant C2

The catalytic domain of congopain, C2, has previously been expressed in a baculovirus system (Boulangé *et al.*, 2001). In the present study, C2 was expressed in *P. pastoris* GS115 that was previously transfected with recombinant pPIC9 with the coding sequence for C2, obtained from Dr Alain Boulangé (manuscript in preparation). The change to the yeast system was made due to the cost and complexity of the baculovirus system, in addition to a very low yield. C2 was expressed as an inactive proenzyme, as was previously done using the baculovirus system (Boulangé *et al.*, 2001). Maturation of the inactive proform of C2 into active C2 was affected by treatment at acidic pH to remove the pro-peptide.

4.2.1 Expression of C2 in *P. pastoris*

4.2.1.1 Reagents

As per Section 2.14.

4.2.1.2 Procedure

P. pastoris GS115 cells containing the coding sequence of C2 were streaked from a glycerol stock onto YPD-agar plates and incubated at 30°C. A single colony was used to inoculate YPD medium (50 ml) and incubated for 3 days at 30°C with shaking. This pre-culture was used to inoculate BMGY medium (500 ml), and incubated for a further 3 days. The culture was centrifuged in sterile containers (700 *g*, 10 min, RT). To induce expression, the cell pellet was resuspended in BMM medium (500 ml) and incubated for 4 days at 30°C. Methanol (0.5% final concentration) was added to the BMM culture every 24 h after inoculation to compensate for consumption and evaporation. The final culture was centrifuged in sterile containers (700 *g*, 10 min, 4°C). The resulting supernatant was clarified by centrifugation (4500 *g*, 10 min, 4°C) and stored at -20°C. The cell pellet could be used for a second round of expression by resuspending it in BMM as above.

4.2.2 Purification of recombinant C2 from culture supernatant

After expression of C2, the protein was purified from the culture supernatant by ion exchange chromatography on SP Sephadex™ C-25 resin. SP Sephadex™ C-25 is a strong cation exchanger based on a charged sulfopropyl group. The pI of C2 is predicted to be 4.89, and therefore should bind to the negatively charged resin at low pH. The low pH also serves to release the pro-segment of the proenzyme by auto-activation (Boulangé *et al.*, 2001), as reported for maturation of recombinant pro-papain (Vernet *et al.*, 1990). The bound protein is eluted from the resin by using a buffer with an increased pH as well as a higher concentration of salt.

4.2.2.1 Reagents

500 mM Potassium phosphate buffer, pH 4.2. A 1 M solution of KH_2PO_4 (approximately 500 ml) was titrated with 1 M K_2HPO_4 (Section 2.14.3) to pH 4.2, and diluted with an equal volume of dH_2O.

50 mM Potassium phosphate buffer, pH 4.2. 500 mM Potassium phosphate, pH 4.2 (500 ml) was diluted to 5 l with dH_2O.

SP Sephadex™ C-25. SP Sephadex™ C-25 (100 g) was added to dH_2O (4 l), and stirred gently overnight at 4°C. Beads were allowed to sediment and the water was poured off. 50 mM Potassium phosphate buffer, pH 4.2 (1 l) was added and beads were stirred gently for 5 min. The beads were allowed to sediment and the buffer was poured off. Washing with 50 mM potassium phosphate buffer, pH 4.2 (1 l) was repeated 3 times, and the beads were autoclaved.

50 mM Potassium phosphate buffer, pH 7, 500 mM KCl. KH_2PO_4 (6.805 g) and KCl (37.275 g) were dissolved in dH_2O (950 ml), and titrated to pH 7 with KOH. The final volume was made up to 1 l with dH_2O.

50 mM MES buffer, pH 6.5, 150 mM NaCl, 1 mM $HgCl_2$. MES (9.76 g), NaCl (8.766 g) and $HgCl_2$ (0.272 g) were dissolved in dH_2O (950 ml), and titrated to pH 6.5 with NaOH. The final volume was made up to 1 l with dH_2O.

4.2.2.2 Procedure

Thawed culture supernatant (500 ml) was diluted 4 times with dH_2O and the pH adjusted to 4 with phosphoric acid. SP Sephadex^{TM} C-25 (50 ml), prepared as described above, was added to the supernatant and stirred gently overnight at 4°C. The beads were allowed to sediment and the supernatant was poured off. The beads were packed into a glass column and washed with 50 mM potassium phosphate buffer, pH 4.2 until the A_{280} was less than 0.01. Bound protein was eluted with 50 mM potassium phosphate buffer, 500 mM KCl, pH 7. The collective eluate was dialysed overnight against 50 mM MES buffer, 150 mM NaCl, 1 mM $HgCl_2$, pH 6.5. C2 was concentrated to approximately 2 mg/ml using an Amicon Centriprep YM-30. Sucrose was added to 20% (m/v) and C2 was stored at -70°C.

4.3 Cloning of the open reading frame (ORF) of oligopeptidase B from *T. congolense* and *T. vivax*

The cloning strategy, and subsequent steps, used in the present study for the oligopeptidase B ORFs are outlined in Fig. 4.2. The *T. b. brucei* oligopeptidase B gene (accession number AF078916) was submitted to a BLAST search against the *T. congolense* (http://www.sanger.ac.uk/Projects/T_ congolense; April 2004) and *T. vivax* (http://www.sanger.ac.uk/Projects/T_vivax; April 2004) databases of the Sanger trypanosome genome sequencing project. Relevant reads and contigs were selected, and the consensus sequence for each gene was constructed using Sequencher 4.5. By comparison with the known ORF of *T. b. brucei* oligopeptidase B, the ORF of each gene was established using CLUSTAL W (as in Section 3.3; see Appendix 2). Amino acid sequences (see Fig. 3.8, Chapter 3) were derived from translation of the nucleotide sequences with BioEdit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html; April 2004).

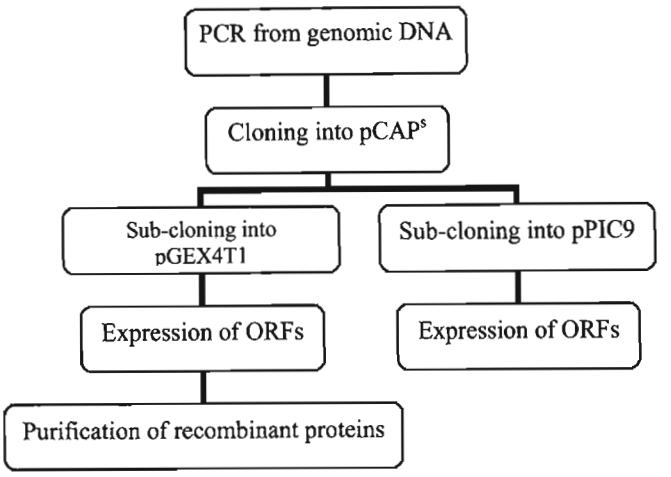

Figure 4.2. The strategy employed for the cloning and expression of the oligopeptidase B ORF from *T. congolense* and *T. vivax*.

4.3.1 PCR

The Expand High Fidelity PCR System from Roche, used in the present study, is composed of a unique enzyme mix containing Taq DNA polymerase and Tgo DNA polymerase. Tgo is a thermostable DNA polymerase with proofreading activity. The polymerase mixture is designed to generate PCR products of high yield, high fidelity and high specificity, up to 5 kb. In the present study, the forward primer was designed to contain an *Eco*RI site (Table 4.1) followed by the methionine initiation codon and 18 base pairs homologous to the gene sequence (corresponding to the N-terminus of oligopeptidase B). The reverse primer was designed to contain a *Not*I site followed by a stop codon and 18 base pairs homologous to the gene sequence (corresponding to the C-terminus of the peptidase).

Table 4.1. The base pair sequences of the primers used in the PCR amplification of the oligopeptidase B ORF.

Name	Direction	Sequence (5'-3')
T. congolense	Forward[a]	ACCGAATTCATGACATCTGATCGCGGCCCC
T. congolense	Reverse[b]	CGCGGCCGCTTAATGCCGCAGCAACCGGCG
T. vivax	Forward[a]	CTGAATTCATGCCAGCTCCCTGCGGTCCGG
T. vivax	Reverse[b]	CGCGGCCGCCTAGCGCCGCAAAAGACAGCG

[a] The *Eco*RI site is shown in red, and start codon is underlined.
[b] The *Not*I site is shown in blue, and the stop codon is underlined.

4.3.1.1 Reagents

Expand High Fidelity Enzyme mix (Roche).

Expand High Fidelity Buffer (10×) without $MgCl_2$ (Roche).

2 mM dNTP mix. As per Section 2.24.1.

10 µM Primer solutions. See Table 4.2. Diluted as per Section 2.24.1.

25 mM $MgCl_2$ (Roche).

4.3.1.2 Procedure

Genomic DNA from *T. congolense* IL3000 (Fish *et al.*, 1989) or *T. vivax* IL4186 (see Appendix 3) was used as template DNA (50 ng) in a total reaction volume of 100 µl. Additional components of the PCR reaction were added as described in Table 4.2. The following PCR conditions were used: 95°C for 5 min, 25 cycles of the following: 95°C for 1 min (denaturation); 55°C for 1 min (annealing); 72°C for 2 min (extension), and 70°C for 5 min, ending at 4°C.

Table 4.2. Components of the PCR reaction used to amplify the oligopeptidase B ORF from trypanosomal genomic DNA.

Reaction component	Volume (µl)	Final concentration
Template DNA	Variable	50 ng
10 × PCR reaction buffer	10	1×
dNTP mixture	10	200 µM
Forward primer	4	400 nM
Reverse primer	4	400 nM
Expand High Fidelity Enzyme mix	1.5	5 U
25 mM $MgCl_2$	10	2.5 mM
Sterile dH_2O	To make up to 100	
Total reaction volume	100	

4.3.2 Cloning into pCAP^s (Roche)

This blunt end PCR cloning system is based on a specially designed suicide vector (pCAP^s) containing the lethal mutant gene of the catabolite activator protein, CAP (Fig. 4.3). Ligation of a blunt ended DNA fragment into the *Mlu*N1 restriction site disrupts the expression of the CAP gene. Therefore, only positive recombinants can grow after transformation (Roche PCR cloning kit (blunt end) instruction manual). The PCR cloning kit (blunt-end) is suitable for cloning PCR products generated with the Expand High Fidelity PCR system, which results in blunt-ended PCR products. The *Mlu*N1 restriction site of the cloning kit provides blunt ends for ligation with the PCR product.

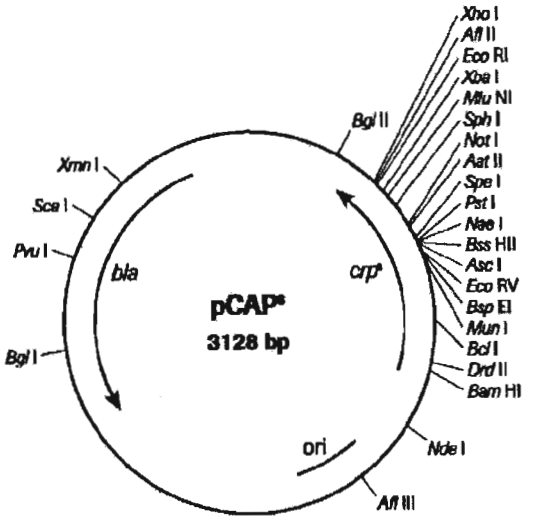

Figure 4.3. Map of the blunt-end PCR cloning vector pCAP^s (Roche), detailing the multiple cloning site.

4.3.2.1 Procedure

Following PCR, the 2.15 kb amplified product was purified from low melting point agarose (Section 2.19.2.1) to remove all of the components of the PCR reaction. The pCAP^s vector was linearised with *Mlu*N1 restriction enzyme (Section 2.18), and ligated with the PCR product as per manufacturer's instructions (PCR cloning kit (blunt end), Roche). The product of the ligation reaction was transformed into competent *E. coli* JM109 cells (20 µl; Promega) by heat shock as per Section 2.22.1.1, except that incubation at 42°C was reduced to 45 s.

Initial screening to assess the success of cloning was conducted by colony PCR (Section 2.24). Positive clones were assessed for the presence of the insert following a miniprep and subsequent restriction digestion with *Eco*RI and *Not*I (Section 2.18). Final confirmation of the insert was obtained by sequencing of pCAP-OPC and pCAP-OPV with the aid of primers provided in the PCR cloning kit (blunt end) from Roche.

4.3.3 Sub-cloning of oligopeptidase B coding sequences, OPC and OPV, into the pPIC9 expression vector

The properties of the expression vector pPIC9 were discussed in Section 4.1.2. Most significantly, the 8 kb vector has a *HIS4* gene for positive selection of the His^+ phenotype in *P. pastoris* and has unique cleavage sites that enable integration into the host genome in both the *AOX1* and *HIS4* position (Fig. 4.1). The presence of the pre-pro α mating factor of *S. cerevisiae* triggers secretion of the expressed protein (Rosenfeld, 1999).

4.3.3.1 Reagents

Shrimp alkaline phosphatase (Roche).

Shrimp alkaline phosphatase 10 × buffer (Roche).

Rapid DNA Ligation Kit (Roche).

4.3.3.2 Procedure

2YT media (50 ml) containing 50 μg/ml ampicillin was inoculated with pPIC9 transformed *E. coli*, and incubated at 37°C overnight. An overnight culture (10 ml) was centrifuged (1 000 *g*, 5 min, RT) to pellet cells. A plasmid miniprep was performed as per Section 2.17. The pPIC9 plasmid was completely linearised with *Not*I (as per Section 2.18) and purified with the Wizard® DNA clean-up system (Section 2.19.1). The purified plasmid was further digested with *Eco*RI (as per Section 2.18). Plasmid DNA was dephosphorylated with shrimp alkaline phosphatase (as per manufacturer's instructions) and purified with a Wizard® DNA clean-up system (Section 2.19.1). Plasmid, insert DNA, 1 × DNA dilution buffer, 2 × ligation buffer, and DNA ligase were combined as per manufacturer's instructions (Rapid DNA ligation kit, Roche) and incubated overnight at 16°C. The product of the ligation reaction was transformed into *E. coli* JM109 cells by electroporation (Section 2.22.2).

Initial screening to assess the success of cloning was conducted by colony PCR (Section 2.24). Positive clones were assessed for the presence of the insert by miniprep and

subsequent restriction digestion with *Eco*RI and *Sal*I (Section 2.18). *Sal*I was used in this case as a cheaper alternative to *Not*I.

4.3.4 Transfection of pPIC9-OPC and pPIC9-OPV into *P. pastoris* GS115

Recombinant plasmid DNA was linearised with *Sal*I (Section 2.18) and purified with the Wizard® DNA clean-up system (Section 2.19.1). Transformation of *P. pastoris* GS115 by electroporation was carried out as per Section 2.23.1.

4.3.5 Sub-cloning of oligopeptidase B coding sequences into pGEX4T1

The properties of the pGEX expression vector (Fig. 4.4) were discussed in Section 4.1. Using pGEX4T1, the protein of interest is expressed as a fusion protein with GST, which can be cleaved using thrombin. Expression is regulated with the *tac* promoter, which is derepressed by the use of IPTG (De Boer *et al.*, 1983).

4.3.5.1 Reagents

As per Section 4.3.3.1.

4.3.5.2 Procedure

Plasmid DNA of pGEX4T1 was prepared, and ligated with insert DNA in the same manner as for pPIC9 (Section 4.3.3.2). Initial screening to assess the success of cloning was conducted by colony PCR (Section 2.24). Positive clones were assessed for the presence of the insert by miniprep and subsequent restriction digestion with *Eco*RI and *Not*I (Section 2.18)

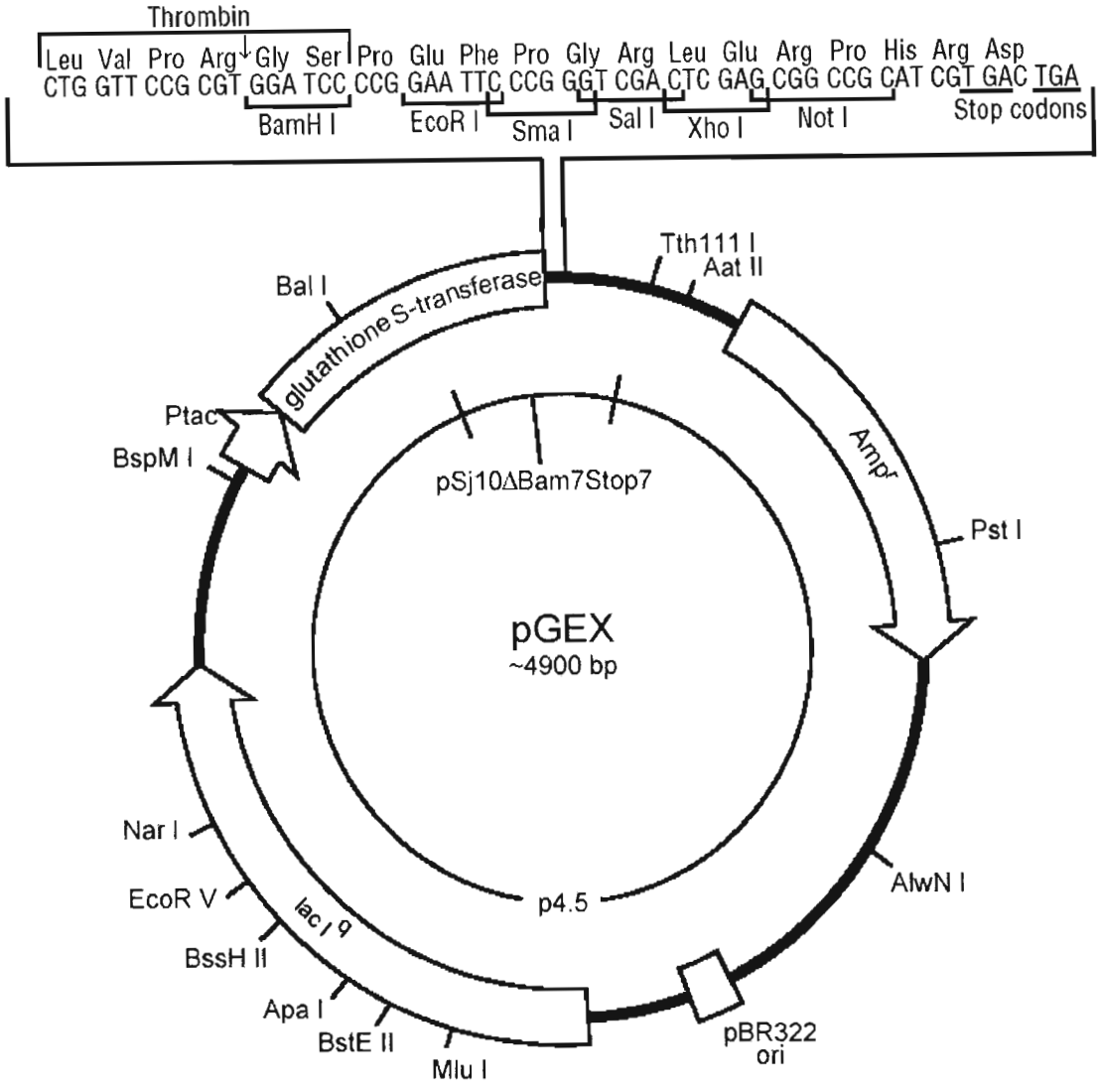

Figure 4.4. Map of the pGEX4T1 expression vector (Promega), detailing the multiple cloning site.

4.4 Expression and purification of recombinant oligopeptidase B from *T. congolense* and *T. vivax*

4.4.1 Expression of recombinant oligopeptidase B by *P. pastoris*

4.4.1.1 Reagents

See Section 2.14.

4.4.1.2 Procedure

The procedure used for expression of recombinant oligopeptidase B by *P. pastoris* was the same as that used for C2 (Section 4.2.1.2.).

4.4.2 Expression of recombinant oligopeptidase B in *E. coli* using pGEX4T1

4.4.2.1 Reagents

See Section 2.14.

0.1 M IPTG. IPTG (0.476 g) was dissolved in dH_2O (20 ml) and sterilised by passing through a 0.22 μm filter.

4.4.2.2 Procedure

2YT medium (100 ml) with 50 μg/ml ampicillin was inoculated with a single colony of recombinant *E. coli* JM109 pGEX-OPC or pGEX-OPV, and incubated at 37°C overnight. The 100 ml culture was used to inoculate 900 ml of 2YT medium containing 50 μg/ml ampicillin. The culture was incubated at 37°C until an OD_{600} of 1 was reached. 0.1 M IPTG (3 ml) was added to induce the expression and the culture was incubated for a further 4 h at 37°C. Ampicillin (50 μg/ml) was added to the culture with the IPTG, as well as 2 h afterwards.

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

Purification of GST-tagged proteins is carried out using an affinity matrix of glutathione immobilised on agarose. The oligopeptidase B-GST fusion protein was adsorbed onto glutathione agarose resin and cleaved with thrombin while still immobilised on the resin, to facilitate separation of the recombinant protein from the GST carrier. The most common procedure is purification of the fusion protein by affinity chromatography, followed by cleavage of the fusion protein in solution and subsequent purification steps to separate the recombinant protein from GST. However, by this method, the purification of oligopeptidase B after cleavage from the fusion protein was found to be difficult. The 'on-column' method of cleavage of the fusion protein eliminated these difficulties and significantly shortened the length of the purification procedure.

4.4.3.1 Reagents

PBS, pH 7.2, 0.02% (w/v) NaN_3. As per Section 2.8.1, but with NaN_3 (0.2 g) dissolved in 1 l of buffer.

PBS-T [PBS with 1% (v/v) Triton X-100]. Triton X-100 (1 ml) was completely homogenised in PBS (100 ml).

Glutathione agarose column. Lyophilised glutathione agarose (Sigma; 74 mg/ml of required resin) was swollen in dH_2O (14 ml per 74 mg) overnight at 4°C. Swollen resin (0.8 ml) was placed in a Poly-Prep® Chromatography column (Bio-Rad) and equilibrated with 20 column volumes of PBS (16 ml).

Thrombin cleavage buffer (20 mM Tris-HCl buffer, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). Tris (0.6055 g), NaCl (2.1915 g) and $CaCl_2$ (0.0694 g) were dissolved in dH_2O (230 ml). The pH was titrated to 8.4 with HCl and the final volume made up to 250 ml with dH_2O.

Thrombin dilution buffer [50 mM citrate buffer, pH 6.5, 200 mM NaCl, 1 mg/ml PEG, 50% (v/v) glycerol] (Novagen).

Thrombin (1 U/µl) (Novagen). Thrombin (34.7 µl) at 1.44 U/µl was diluted to 1 U/µl with thrombin dilution buffer (15.3 µl).

Antithrombin III (0.01 U/µl) (Sigma). Antithrombin III (10 U) was reconstituted in dH_2O (1 ml).

50 mM Tris-HCl buffer, pH 8, 0.02% (w/v) NaN_3. Tris (0.6055 g) was dissolved in dH_2O (90 ml) and titrated to pH 8 with HCl. NaN_3 (0.02 g) was added and dissolved, and the final volume made up to 100 ml with dH_2O.

10 mM Reduced glutathione. Reduced glutathione (0.154 g) was dissolved in 50 mM Tris-HCl buffer, pH 8 (50 ml).

100 mM Sodium borate buffer, pH 8, 500 mM NaCl. Boric acid (0.6183 g) and NaCl (2.922 g) were dissolved in dH_2O (90 ml). The pH was adjusted to 8 with NaOH and the final volume brought to 100 ml with dH_2O.

100 mM Sodium acetate buffer, pH 4, 500 mM NaCl. Sodium acetate (1.361 g) and NaCl (2.922 g) were dissolved in dH_2O (90 ml). The pH was adjusted to 4 with glacial acetic acid and the final volume brought to 100 ml with dH_2O.

4.4.3.2 Procedure

After expression of the fusion protein, cultures were centrifuged (2 000 *g*, 10 min, 4°C) to pellet the cells. The cells were resuspended in PBS-T (40 ml per litre of culture) and lysozyme was added to a final concentration of 1 mg/ml. The cells were incubated at RT for 10 min and stored at -20°C. The lysate was thawed, sonicated (4 × 30 s) using a VirSonic 60 sonicator, and clarified by centrifugation (10 000 *g*, 10 min, 4°C). Additional clarification of the lysate was carried out by filtering the supernatant through Whatman No. 1 filter paper. The lysate (20 ml) was cycled over the glutathione agarose column (0.8 ml) overnight at 4°C. The resin was washed with PBS-T (15 ml) until the A_{280} reached 0.01, and equilibrated with thrombin cleavage buffer (20 ml). Thrombin cleavage buffer (0.8 ml) was used to gently resuspend the resin into a 50% slurry to which thrombin was added (4 U). The column was incubated overnight at RT with gentle rocking. Cleaved recombinant oligopeptidase B present in the buffer was directly collected from the column. A wash fraction of 3 column volumes was also collected.

Bound GST and any uncleaved fusion protein was eluted from the resin with 10 mM reduced glutathione. The column was regenerated by washing with 5 column volumes of 100 mM Sodium borate buffer, 500 mM NaCl, pH 8, followed by 5 column volumes of dH_2O, 5 column volumes of 100 mM Sodium acetate buffer, 500 mM NaCl , pH 4, and 5 column volumes of dH_2O. The resin was stored in PBS with 0.02% (w/v) NaN_3 at 4°C.

The thrombin that was present in the recombinant oligopeptidase B sample was inactivated by the addition of an equimolar amount of antithrombin III.

4.4.4 N-terminal sequencing of recombinant oligopeptidase B

The use of a semi-dry blotter in the present study to transfer proteins onto PVDF membrane for N-terminal sequencing negated any need for special preparation of the gels and the use of an alternative blotting buffer without glycine.

4.4.4.1 Reagents

Blotting buffer [45 mM Tris, 173 mM glycine, 18% (v/v) methanol, 0.1% (w/v) SDS]. As per Section 2.7.1.

PVDF stain [0.1% (m/v) Coomassie Brilliant Blue R-250, 50% (v/v) methanol]. Coomassie Brilliant Blue R-250 (0.1 g) was dissolved in a solution containing methanol (50 ml) and dH_2O (50 ml).

PVDF destain [50% (v/v) methanol, 10% (v/v) glacial acetic acid]. Methanol (50 ml) and glacial acetic acid (10 ml) were made up to 100 ml with dH_2O.

4.4.4.2 Procedure

Recombinant oligopeptidase B (2 μg) was electrophoresed on a 10% SDS-PAGE gel, under reducing conditions, as per Section 2.4. PVDF membrane was activated by submersion in methanol for a few seconds before being soaked in blotting buffer for 10 min, along with blotting paper and the gel. The gel and PVDF were arranged in a sandwich as per Section 2.7.2, but were blotted using a Sigma semi-dry blotter at 5 mA for 16 h.

The PVDF membrane was washed extensively with dH_2O (6 × 5 min) to remove traces of glycine and stained for 5 min in Coomassie. The membrane was destained in PVDF destain for 10 min, rinsed with dH_2O and air dried. The membrane was sent for N-terminal sequencing to Michèle Brillard of the Université de Tours, France.

4.5 Isolation of native oligopeptidase B

Frozen aliquots of bloodstream forms of *T. congolense* strain IL3000 (5×10^9) and *T. vivax* strain IL4186 (2×10^9) were obtained from Dr Alain Boulangé, and used to isolate oligopeptidase B. Procyclic forms of *T. b. brucei* strain EATRO 1125 HN T70 were obtained by culturing the parasites as described below.

4.5.1 Parasite culture

In vitro culture of the tsetse midgut, or procyclic forms of *T. b. brucei* was employed in the present study to acquire enough parasites for the isolation of native oligopeptidase B. This method was suitable because oligopeptidase B is reported to be expressed in all life cycle stages of the parasite (Burleigh *et al.*, 1997), and large numbers of parasites could be obtained without the need for propagation in rats. The culture of procyclic salivarian trypanosomes was considered difficult (Cross and Manning, 1973) until some key changes in culture technique were brought about, including the development of semi-defined media such as SDM-79 (Brun and Schonenberger, 1979).

4.5.1.1 Reagents

50 mM NaOH. NaOH (0.1 g) was dissolved in dH_2O (50 ml).

2.5 mg/ml Hemin. Hemin (0.025 g) was dissolved in 50 mM NaOH (10 ml).

Culture medium [SDM-79 with 2.2 g/l $NaHCO_3$, 0.062 g/l kanamycin, 10% (v/v) FCS, 0.005 g/l hemin]. SDM-79 (12.7 g), $NaHCO_3$ (1.1 g), and kanamycin (0.031 g) were dissolved in dH_2O (430 ml) and titrated to pH 7.2 with NaOH. The solution was made up to 450 ml with dH_2O, and filter-sterilised through a 0.22 μm filter. Before use, FCS (10 ml) and 2.5 mg/ml hemin (200 μl) were added aseptically to 90 ml of sterile media.

4.5.1.2 Procedure

Culture medium was aliquotted into disposable polystyrene tissue culture flasks with vented caps containing 0.22 μm filters. Culture medium was inoculated with procyclic parasites thawed from cryo-preserved stabilates (provided by Dr Alain Boulangé).

Cultures were maintained at 27°C in 5% (v/v) CO_2. Cultures were not allowed to drop below around 104 cells per ml (which would result in parasite death).

4.5.2 Immunoaffinity purification of oligopeptidase B from trypanosomal lysate

Immunoaffinity purification has been used successfully for the isolation of trypanosomal enzymes such as oligopeptidase B (Morty *et al.*, 1999a) and congopain (Authié *et al.*, 1992). The procedure used in the present study is based on that used for congopain, except that basic elution conditions were used, since oligopeptidase B is unstable at acidic pH (Morty *et al.*, 1999b). Affinity purified antibodies from chickens against rOPV (Chapter 8) were used for the immunoaffinity purification of oligopeptidase B from all three species of African trypanosomes because they showed very strong cross-reactivity with these enzymes (results not shown) and were available in large quantities after affinity purification.

4.5.2.1 Reagents

0.1% (m/v) Brij 35. As per Section 2.9.1.1.

10 × PBS, pH 7.2. NaCl (8 g), KCl (0.2 g), $Na_2HPO_4.2H_2O$ (1.15 g) and KH_2PO_4 (0.2 g) were dissolved in dH_2O (90 ml) and made up to a final volume of 100 ml.

PBS, pH 7.2. As per Section 2.8.1.

Elution buffer (50 mM sodium carbonate buffer, pH 10, 1 M NaCl). $NaHCO_3$ (0.21 g) and NaCl (2.922 g) were dissolved in dH_2O (50 ml). Separately, Na_2CO_3 (0.13 g) and NaCl (2.922 g) were dissolved in dH_2O (50 ml). The two solutions were titrated to pH 10.

Neutralisation buffer (1 M sodium phosphate buffer, pH 6.8). $NaH_2PO_4.2H_2O$ (15.601 g) was dissolved in dH_2O (90 ml), titrated to pH 6.8 with NaOH, and made up to 100 ml with dH_2O.

50 mM Tris-HCl buffer, pH 8, 0.02% (w/v) NaN_3. As per Section 4.4.3.1.

4.5.2.2 Procedure

Parasites (5×10^9 of either procyclic *T. b. brucei* or bloodstream form *T. congolense*; 2×10^9 *T. vivax*) were suspended in ice cold 0.1% (m/v) Brij 35 (9 ml) and incubated for 10 min on ice to facilitate lysis. 10 × PBS (1 ml) was added and the suspension was clarified by centrifugation (10 000 *g*, 5 min, 4°C). A Poly-Prep® chromatography column (Bio-Rad Laboratories) of Aminolink®-immobilised affinity purified anti-rOPV antibodies (Chapter 8) was prepared as per Section 2.12.1.2, and equilibrated in PBS. The lysate was circulated over the column for 16 h at 4°C. Unbound antigen was eluted with cold PBS. Bound antigen was eluted with 50 mM sodium carbonate buffer, 1 M NaCl, pH 10 (900 µl fractions collected into 100 µl 1 M sodium phosphate buffer, pH 6.8). Eluted fractions with activity against Z-Arg-Arg-AMC were pooled and concentrated with an Amicon Centriprep YM-30. Salt was removed by dialysis against 50 mM Tris-HCl buffer, pH 8, 0.02% NaN_3 (overnight, 4°C).

4.6 Results

4.6.1 Production and purification of recombinant C2

The catalytic domain of congopain, C2, was expressed in the *P. pastoris* system. The recombinant protein was purified by ion exchange chromatography on SP Sephadex™ C-25 and analysed by SDS-PAGE to assess both purity and molecular weight (Fig. 4.5). C2 electrophoresed under non-reducing conditions appeared as a band at approximately 22 kDa. Under reducing conditions, the protein appeared as a band at approximately 27 kDa. The enzyme showed significant activity against the substrate Z-Phe-Arg-AMC (result not shown), as expected.

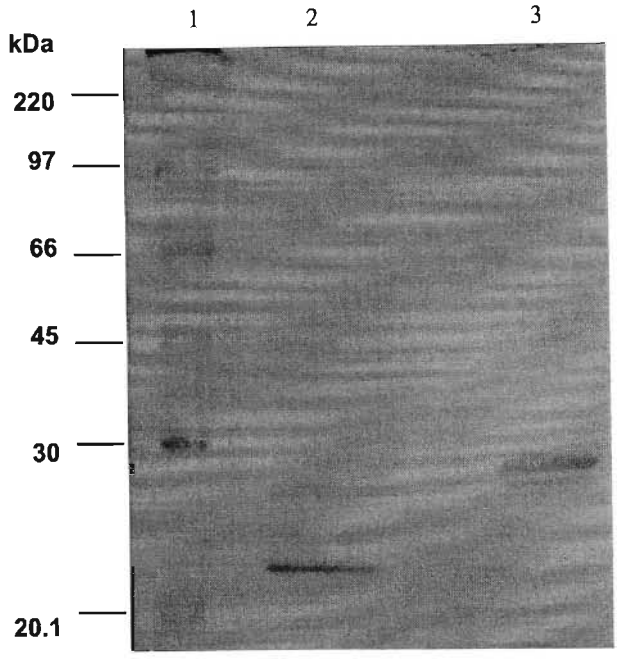

Figure 4.5. Analysis of recombinantly expressed C2 by SDS-PAGE on a 12.5% gel. Lane 1, high-range Rainbow™ markers corresponding to myosin (220 kDa), phosphorylase B (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), and trypsin inhibitor (20.1 kDa); lane 2, non-reduced C2 (2.5 μg); lane 3, reduced C2 (2.5 μg). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

4.6.2 Cloning of the open reading frame (ORF) of oligopeptidase B from *T. congolense* and *T. vivax*

A blunt-ended PCR product of 2.15 kb in size was amplified from genomic DNA from both *T. congolense* and *T. vivax* using the Roche High Fidelity PCR system (Fig. 4.6). Due to the difficulty of digesting a PCR product with restriction enzymes, which would be necessary for cloning into an expression vector, the PCR product was first cloned into a PCR cloning vector. Restriction digestion of pCAP-OPV with *Eco*RI alone, when analysed by agarose gel electrophoresis, resulted in two DNA bands: one at approximately 3 kb and one at approximately 2.15 kb (Fig. 4.7). These bands correspond to the sizes of the pCAP^{s} vector and the OPV insert respectively. Restriction digestion of pCAP-OPC with *Eco*RI alone, when analysed by agarose gel electrophoreses, resulted in a single band of approximately 5.2 kb (Fig. 4.8), corresponding to the size of linearised recombinant pCAP^{s}. This shows that the two inserts had opposite orientations in the multiple cloning site. Restriction digestion of pCAP-OPC with *Eco*RI and *Not*I resulted

in two bands (Fig. 4.9), corresponding to the sizes of the pCAP^s vector and the OPC insert as seen for *Eco*RI cleavage of pCAP-OPV, as well as a band at about 5.2 kb.

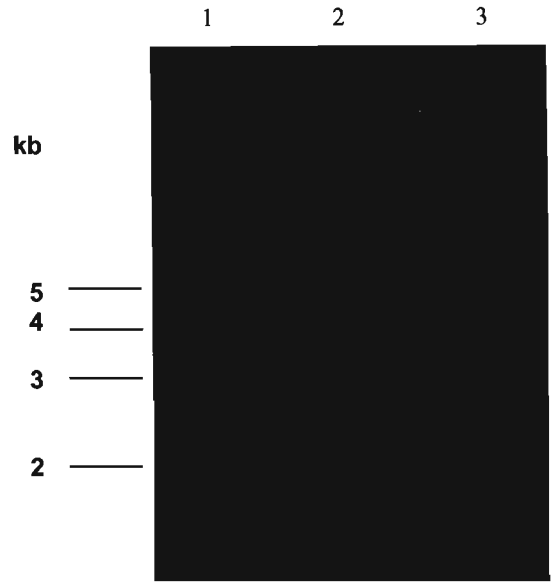

Figure 4.6. Analysis of PCR amplification of the oligopeptidase B ORF from trypanosomal genomic DNA. Samples from PCR to amplify the oligopeptidase B ORF from genomic DNA were electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lane 2, PCR product of *T. congolense* DNA; lane 3, PCR product of *T. vivax* DNA. DNA was stained with ethidium bromide (Section 2.15).

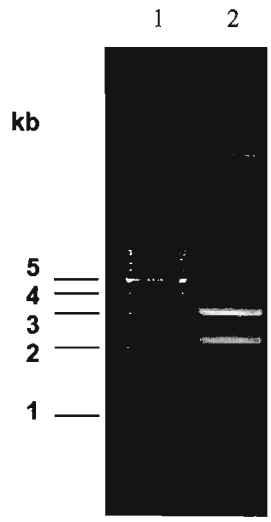

Figure 4.7. Analysis of *Eco*RI-restricted pCAP-OPV by agarose gel electrophoresis. Recombinant pCAP^s plasmid was restriction digested with *Eco*RI and electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lane 2, pCAP-OPV digested with *Eco*RI. DNA was stained with ethidium bromide (Section 2.15).

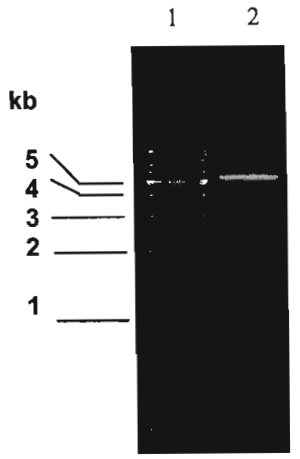

Figure 4.8. Analysis of *Eco*RI-restricted pCAP-OPC by agarose gel electrophoresis. Recombinant pCAP^s plasmid was restriction digested with *Eco*RI and electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lane 2, pCAP-OPC digested with *Eco*RI. DNA was stained with ethidium bromide (Section 2.15).

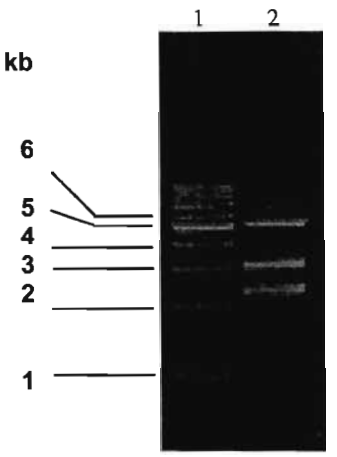

Figure 4.9. Agarose gel analysis of pCAP-OPC digested with *Eco*RI and *Not*I. Recombinant pCAP^s plasmid was restriction digested with *Eco*RI and *Not*I, and electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lane 2, pCAP-OPC digested with *Eco*RI and *Not*I. DNA was stained with ethidium bromide (Section 2.15).

4.6.3 Sub-cloning of the oligopeptidase B ORF into pPIC9

Before ligation into the pPIC9 vector, the linearised plasmid (cut with both *Eco*RI and *Not*I) and OPC and OPV inserts (also cut with *Eco*RI and *Not*I) were analysed by agarose gel electrophoresis (Fig. 4.10). From the relative quantities of DNA that were estimated (Fig. 4.10), it was decided to use 0.5 µl of the vector and either 8 µl of the OPC insert or 4 µl of the OPV insert in the ligation reaction. These amounts were used in order to have a vector to insert molecular ratio of approximately 1:3.

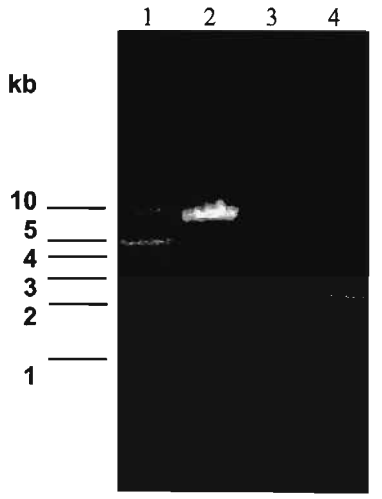

Figure 4.10. Analysis of linearised pPIC9 and OPC and OPV inserts for the estimation of relative amounts of DNA before ligation. Vector and inserts were digested with both *Eco*RI and *Not*I and electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lane 2, pPIC9 (2 µl); lane 3, OPC (2 µl); lane 4, OPV (2 µl). DNA was stained with ethidium bromide (Section 2.15).

Ampicillin resistant colonies were screened by PCR, and 2 out of 13 showed an amplification product of 1.1 kb upon analysis by agarose gel electrophoresis (Fig. 4.11, lanes 7 and 12). Similarly, four recombinant pPIC9 clones with the OPV insert were found out of the 16 colonies tested (Fig. 4.12, lanes 3, 5, 10 and 17), though some gave a faint signal and were not visible when documented.

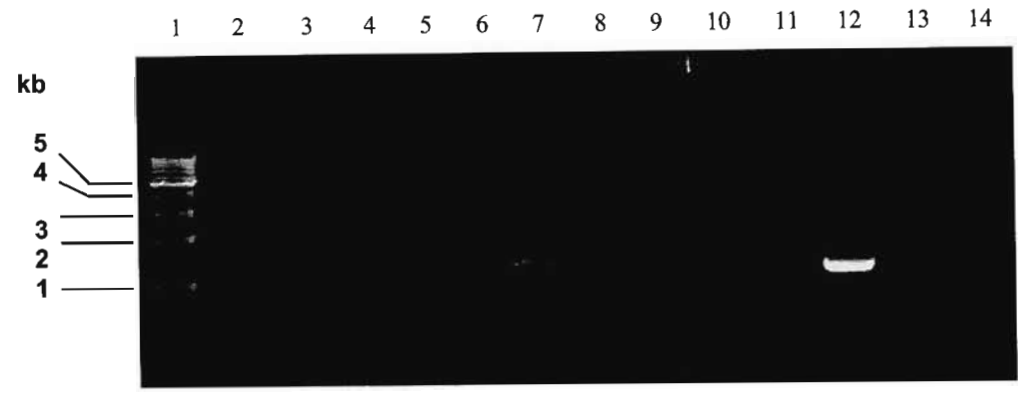

Figure 4.11. Screening for recombinant OPC clones of pPIC9 by colony PCR. Following PCR of *E. coli* colonies, samples were electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lanes 2-14, PCR of 13 individual colonies. DNA was stained with ethidium bromide (Section 2.15).

Figure 4.12. Screening for recombinant OPV clones of pPIC9 by colony PCR. Following PCR of *E. coli* colonies, samples were electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lanes 2-17, PCR of 16 individual colonies. DNA was stained with ethidium bromide (Section 2.15).

The colonies that tested positive by PCR were used to start cultures from which the plasmid DNA was purified by miniprep. The purified DNA was digested with *Eco*RI and *Sal*I to confirm the presence of the insert (Fig. 4.13). When analysed by agarose gel electrophoresis, positive clones appeared as 3 DNA bands at approximately 10 kb, 6.5 kb and 4.5 kb. The band at 10 kb corresponds to linearised recombinant plasmid, whereas the 6.5 kb and 4.5 kb bands correspond to the expected sizes of the recombinant plasmid cleaved at the *Eco*RI and *Sal*I restriction sites.

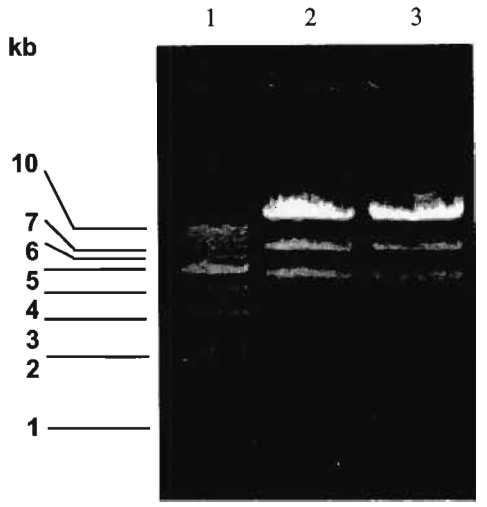

Figure 4.13. Analysis of *Eco*RI and *Sal*I-restricted recombinant clones to confirm the presence of the insert after ligation into pPIC9 and transformation into *E. coli* JM109. Recombinant pPIC9 was restriction digested with *Eco*RI and *Sal*I, and electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lane 2, pPIC9-OPC; lane 3, pPIC9-OPV. DNA was stained with ethidium bromide (Section 2.15).

The DNA of the recombinant pPIC9 clones, linearised with *Sal*I, was transfected into *P. pastoris* and the His^+ phenotype selected for on minimal media (which does not contain histidine). Only yeast cells that were successfully transfected with the plasmid were able to form colonies on the MD plates.

4.6.4 Expression of oligopeptidase B in the *P. pastoris* system

Expression of the oligopeptidase B ORF was induced in *P. pastoris* with methanol, and resulting 4-day old culture supernatants were analysed by SDS-PAGE and western blotting. SDS-PAGE of culture supernatants expressing rOPC and rOPV revealed a faint band of protein at the expected size of oligopeptidase B (80 kDa) and a very high molecular weight protein at the top of the gel (Fig. 4.14, i). Western blotting of the supernatants with an affinity purified antibody against peptide OpBTc1 (Section 3.2) revealed a band at approximately 80 kDa in the supernatant from the expression of both rOPC and rOPV (Fig. 4.14, ii).

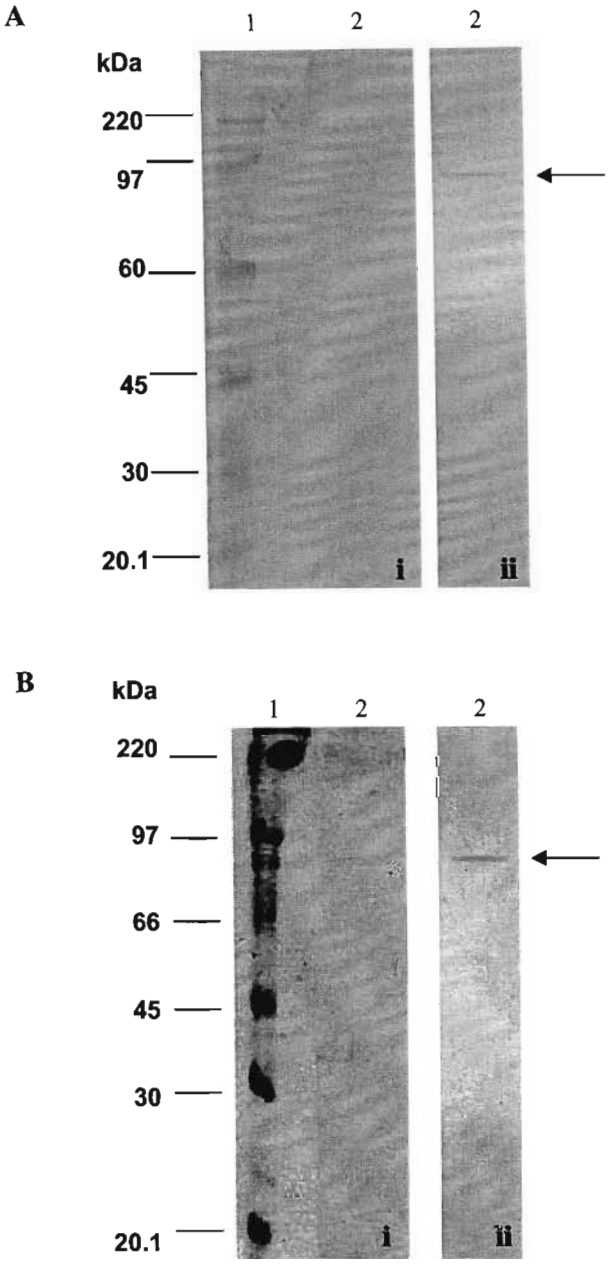

Figure 4.14. Reducing SDS-PAGE and western blotting to detect the presence of rOPC (A) and rOPV (B) in the supernatant from the expression by *P. pastoris*. Culture supernatant from pPIC9-OPC (A) or pPIC9-OPV expression was electrophoresed on a 10% acrylamide gel after reduction (i), and electroblotted onto a nitrocellulose membrane (ii). Lane 1, high-range Rainbow™ markers (as per Fig. 4.4); lane 2, culture supernatant (30 µl). (i) Proteins were stained with Coomassie blue R-250 (Section 2.6.1). (ii) The membrane was incubated with affinity purified chicken anti-OpBTc1 peptide antibody (Section 8.2), followed by HRPO-conjugated rabbit anti-chicken IgY. Bands were developed with 4-chloro-1-naphthol/H_2O_2. The arrows depict a band at approximately 80 kDa.

4.6.5 Sub-cloning of the oligopeptidase B ORF into pGEX4T1

Before ligation into the pGEX4T1 vector, the linearised plasmid (cut with both *Eco*RI and *NotI*) and OPC and OPV inserts (also cut with *EcoRI* and *Not*I) were analysed by agarose gel electrophoresis (Fig. 4.15). From the relative quantities of DNA that were apparent on the gel it was decided to use 4.5 µl of the vector and 4.5 µl of either the OPC or OPV inserts in the ligation reaction. These amounts were used in order to have a vector to insert molecular ratio of approximately 1:3.

Analysis by PCR of ampicillin resistant colonies after transformation of the ligation reaction revealed four recombinant pGEX4T1 clones containing the OPC insert out of the seven colonies tested. This was shown by the presence of a 1.1 kb PCR product upon analysis by agarose gel electrophoresis (Fig. 4.16, lanes 1, 4, 6 and 7). Similarly, three recombinant pGEX4T1 clones with the OPV insert were found out of the 12 colonies tested (Fig. 4.17, lanes 5, 11 and 12).

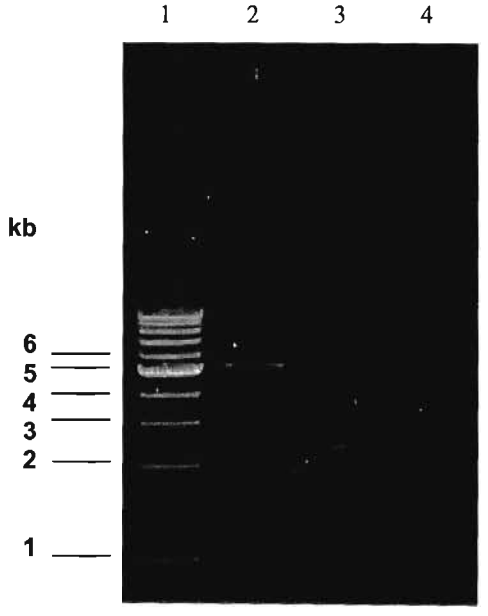

Figure 4.15. Estimation of the relative quantities of pGEX4T1, OPC and OPV, digested with *NotI* and *Eco*RI by agarose gel electrophoresis. Vector and inserts were restriction digested with *Eco*RI and *Not*I, and electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lane 2, pGEX4T1 (5 µl); lane 3, pGEX-OPC (5 µl); lane 4, pGEX-OPV (5 µl). DNA was stained with ethidium bromide (Section 2.15).

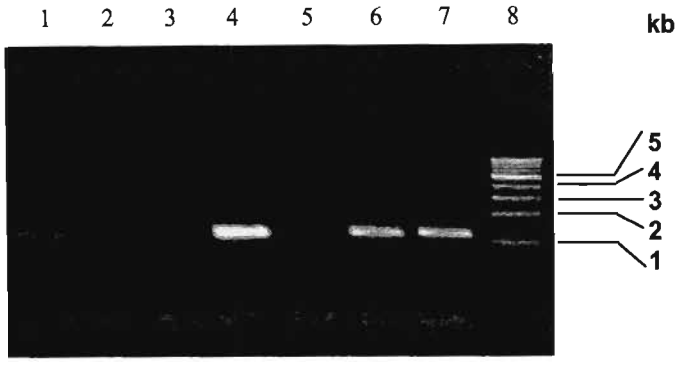

Figure 4.16. Screening for recombinant OPC clones of pGEX4T1 by colony PCR. Following PCR, samples were electrophoresed on a 1% agarose gel. Lanes 1-7, PCR of seven individual colonies; lane 8, 1 kb DNA step ladder. DNA was stained with ethidium bromide (Section 2.15).

The colonies that tested positive by PCR for the presence of the insert were used to inoculate cultures from which the plasmid DNA was purified by miniprep. The purified DNA was digested with *Eco*RI and *Not*I to confirm the presence of the insert. When analysed by agarose gel electrophoresis (Fig. 4.18), positive clones appeared as 3 bands of DNA at approximately 7 kb, 5 kb and 2.15 kb. The band at 7 kb corresponds to linearised recombinant plasmid, due to incomplete digestion, whereas the 5 kb and 2.15 kb bands correspond to the expected sizes of the pGEX4T1 plasmid and OPC or OPV inserts respectively.

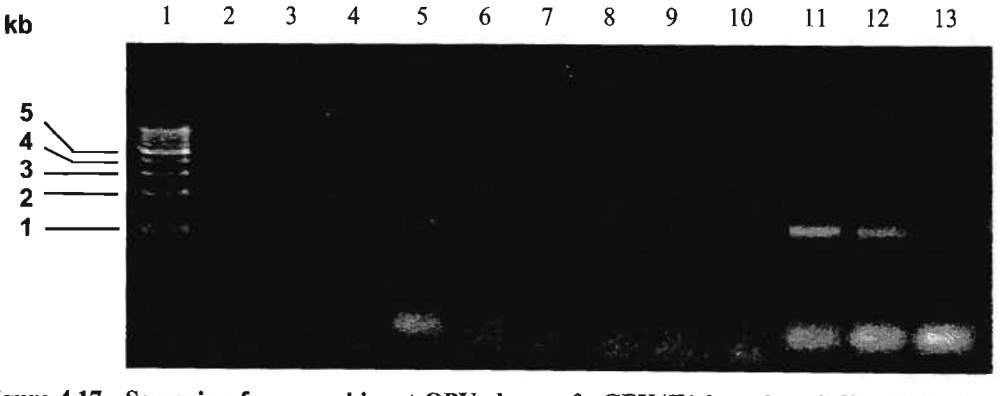

Figure 4.17. Screening for recombinant OPV clones of pGEX4T1 by colony PCR. Following PCR, samples were electrophoresed on a 1% agarose gel. Lane 1, 1 kb DNA step ladder; lanes 2-13, PCR of 12 individual colonies. DNA was stained with ethidium bromide (Section 2.15).

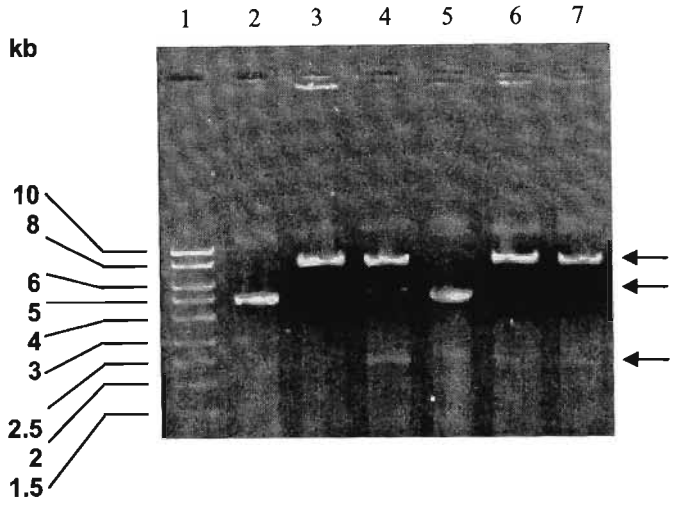

Figure 4.18. Analysis of purified recombinant pGEX-OPC and pGEX-OPV plasmids by agarose gel electrophoresis. Purified recombinant plasmid DNA was electrophoresed alongside restriction digested recombinant plasmid DNA on a 1% agarose gel, to confirm the presence of the 2.15 kb insert. Lane 1, MassRuler™; lane 2, uncut pGEX-OPC; lane 3, pGEX-OPC restricted with *Eco*RI; lane 4, pGEX-OPC restricted with *Eco*RI and *Not*I; lane 5, uncut pGEX-OPV; lane 6, pGEX-OPV restricted with *Eco*RI; lane 7, pGEX-OPV restricted with *Eco*RI and *Not*I. DNA was stained with ethidium bromide (Section 2.15). The arrows indicate the position of significant bands.

4.6.6 Expression of oligopeptidase B in *E. coli*

Expression of rOPC and rOPV in the pGEX system was induced in cultures by the addition of IPTG. After expression, cells were collected and lysed, and run on an SDS-PAGE gel for analysis (Fig. 4.19). Cells transformed with non-recombinant pGEX4T1 were used as a control to show that induction was successful and resulted in the expression of a protein at approximately 26 kDa, corresponding to GST (lane 3). Induction of pGEX-OPC and pGEX-OPV resulted in the appearance of a protein with an apparent molecular weight of 106 kDa (lanes 5 and 7), which corresponds to the expected size of the fusion protein (80 kDa + 26 kDa). The fusion protein for rOPV was produced in a larger quantity than that for rOPC, though neither fusion protein was produced in such a large quantity as GST alone.

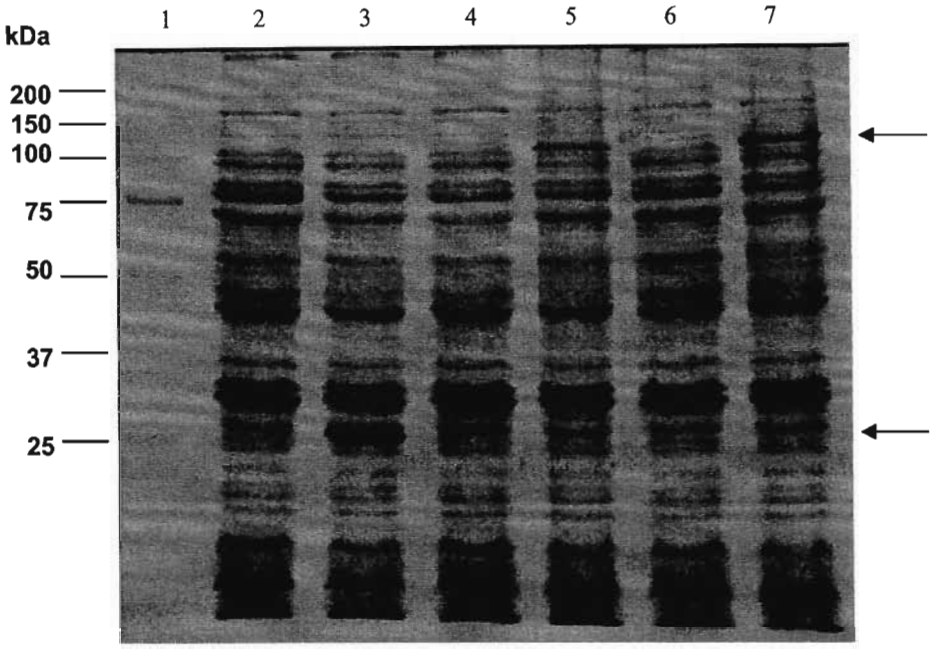

Figure 4.19. Analysis of expression of recombinant OPC and OPV fusion proteins by reducing SDS-PAGE. Expression was carried out as in Section 4.4.2.2, except that a duplicate culture in each case was not induced. Following centrifugation, cells were resuspended in buffer C (Section 2.4.1), reduced, and electrophoresed. Lane 1, Bio-Rad precision plus markers; lane 2, pGEX4T1 uninduced; lane 3, pGEX4T1 induced with IPTG; lane 4, pGEX-OPC uninduced; lane 5, pGEX-OPC induced with IPTG; lane 6, pGEX-OPV uninduced; lane 7, pGEX-OPV induced with IPTG. Proteins were stained with Coomassie blue R-250 (Section 2.6.1). Arrows indicate bands at approximately 26 kDa and 106 kDa.

4.6.7 Purification of recombinant oligopeptidase B

Following on-column cleavage of the GST fusion proteins, the recombinant oligopeptidase B proteins were analysed by SDS-PAGE to assess their molecular weight and purity (Fig. 4.20). Both proteins had an apparent molecular weight of 80 kDa, the expected size of oligopeptidase B, and were judged to be >95% pure.

4.6.8 N-terminal sequencing of rOPC and rOPV

The first 10 N-terminal amino acid residues of rOPC and rOPV were found to be GSPEFMTSDR and GSPEFMPAPC respectively. The first five amino acid residues of each recombinant protein (GSPEF) were those amino acids linking the carrier protein to oligopeptidase B that were left after thrombin cleavage, coded for by a number of bases in the multiple cloning site (Fig. 4.4).

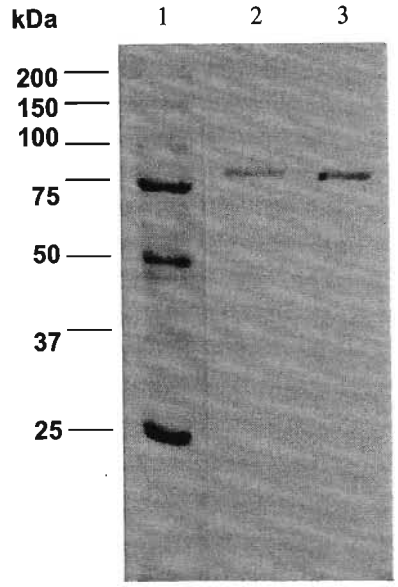

Figure 4.20. Assessment of purity of rOPC and rOPV expressed with the pGEX system by reducing SDS-PAGE. Recombinant oligopeptidase B enzymes, rOPC and rOPV, were electrophoresed on 10% SDS-PAGE after reduction. Lane 1, Bio-Rad Precision Plus markers; lane 2, rOPC (5 μg); lane 3, rOPV (5 μg). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

4.6.9 Isolation of native oligopeptidase B

Lysates of procyclic *T. b. brucei* parasites were analysed by SDS-PAGE and western blotting to confirm the presence of oligopeptidase B in the samples before attempting to isolate the enzyme. Western blotting of the lysate with an anti-peptide (OpBTc1) antibody (antibodies directed against residues 20-37 in the oligopeptidase B sequence) that was prepared in Chapter 3, confirmed the presence of oligopeptidase B at the expected size of 80 kDa (Fig. 4.21).

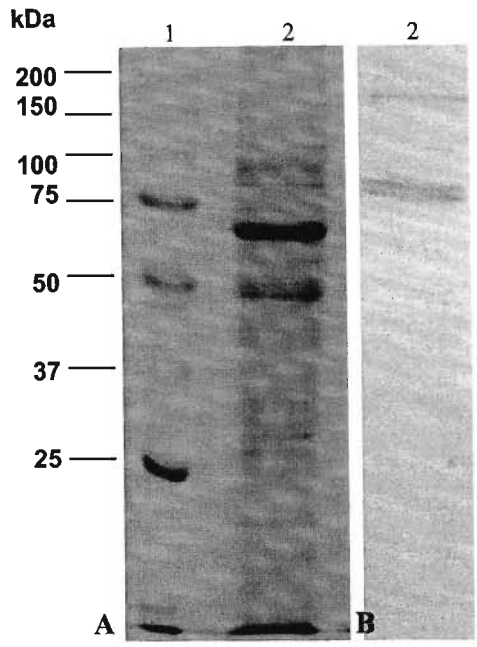

Figure 4.21. SDS-PAGE and western blotting of procyclic *T. b. brucei* lysate. Reduced protein was electrophoresed on a 10% acrylamide gel (A), and electroblotted onto nitrocellulose membrane (B). Lane 1, Bio-Rad precision plus markers; lane 2, lysate (5 µl). (A) Proteins were stained with Coomassie blue R-250 (Section 2.6.1). (B) The membrane was incubated with affinity purified chicken anti-OpBTc1 peptide antibody (Section 8.2). Bands were detected as for Fig. 4.14.

Procyclic *T. b. brucei* lysate was passed over a column of immobilised affinity purified anti-rOPV antibodies. Bound protein was eluted and analysed by SDS-PAGE and western blotting (Fig. 4.22). SDS-PAGE revealed the presence of several protein bands between about 120 kDa and 50 kDa in size. The major band occurred at about 70 kDa, and a band was visible at about 80 kDa (panel A). Western blotting, using anti-OpBTc1 IgY (panel B) and anti-rOPV IgY (panel C), confirmed the presence of oligopeptidase B at 80 kDa. No further purification was carried out as the sample size was very small and the activity of the sample was sufficient for the analysis that was required in the present study.

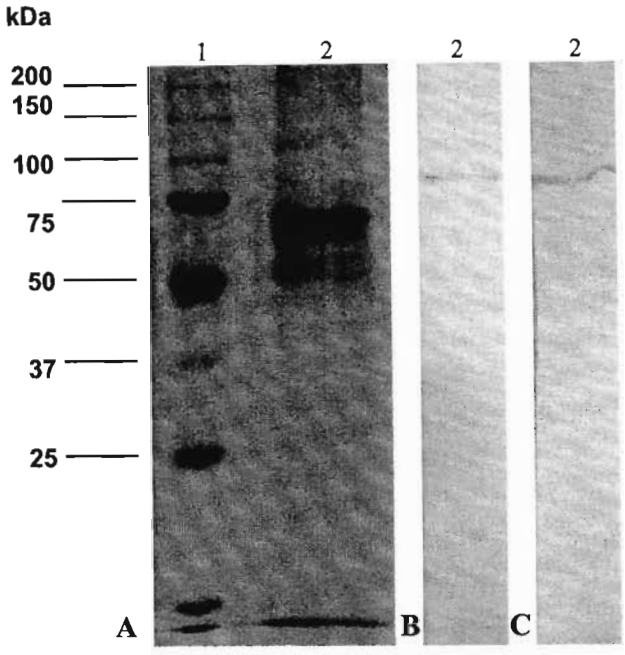

Figure 4.22. SDS-PAGE and western blotting of protein isolated by immunoaffinity chromatography of procyclic *T. b. brucei* lysate. Reduced protein was electrophoresed on a 10% acrylamide gel (A), and electroblotted onto nitrocellulose membrane (B and C). Lane 1, Bio-Rad precision plus markers; lane 2, eluted protein (5 μg). (A) Proteins were stained with Coomassie blue R-250 (Section 2.6.1). (B) The membrane was incubated with affinity purified chicken anti-OpBTc1 peptide antibody (Section 8.2). (C) The membrane was incubated with affinity purified chicken anti-rOPV antibody (Section 8.2) and developed as in Fig. 4.14.

Oligopeptidase B was isolated from bloodstream from *T. congolense* and *T. vivax* parasites in the same manner as for *T. b. brucei* procyclics. A single-step immunoaffinity isolation resulted in the elution of a partially purified protein of about 80 kDa, as detected by SDS-PAGE and western blotting (Fig. 4.23). There was a second band at approximately 65 kDa in the samples of both *T. congolense* and *T. vivax*.

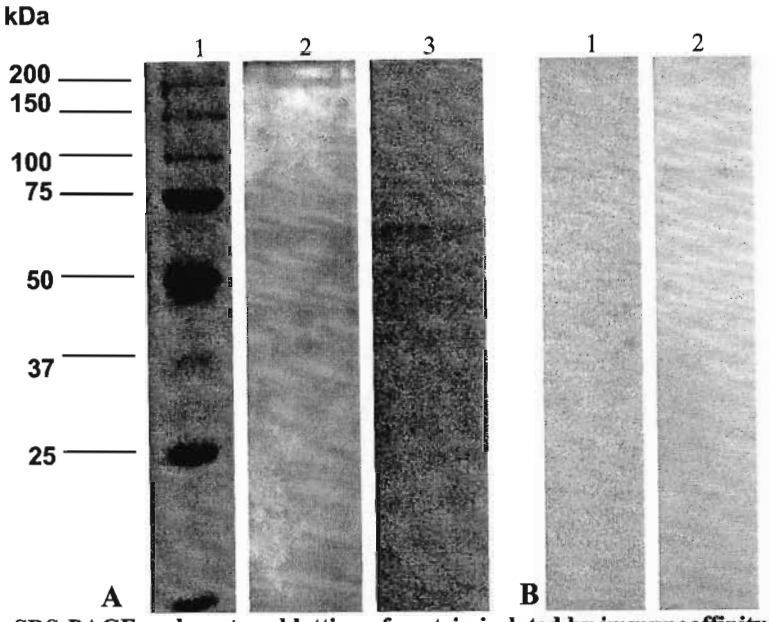

Figure 4.23. SDS-PAGE and western blotting of protein isolated by immunoaffinity chromatography of *T. congolense* and *T. vivax* lysate. Protein was electrophoresed on a 10% acrylamide gel after reduction (A), and electroblotted onto a nitrocellulose membrane (B). (A) Lane 1, Bio-Rad precision plus markers; lane 2, eluted protein from *T. congolense* (1 μg); lane 3, eluted protein from *T. vivax* (5 μg). Proteins were silver-stained (lane 2; Section 2.6.2) or stained with Coomassie blue R-250 (lane 3; Section 2.6.1). (B) Lane 1, eluted protein from *T. congolense* (1 μg); lane 2, eluted protein from *T. vivax* (5 μg). The membrane was incubated with affinity purified chicken anti-OpBTc1 peptide antibody (Section 8.2). Bands were detected as for Fig. 4.14.

4.7 Discussion

The catalytic domain of congopain, C2, was expressed in the *P. pastoris* system as a proenzyme of approximately 35 kDa. This was necessary for the correct folding of the enzyme, and hence activity, as is the case for other cysteine proteases (Vernet *et al.*, 1990). The pro-peptide was cleaved from the enzyme by decreasing the pH to 4.2 before purification of the mature enzyme by ion-exchange chromatography, as described for cathepsin L (Mason *et al.*, 1987).

Analysis by SDS-PAGE showed that C2 migrates as a 22 kDa protein under non-reducing conditions, while it appears as a 27 kDa protein after reduction (Fig. 4.5). The C2 molecule has 6 cysteine residues, and consequently, 3 intramolecular disulfide bridges. The disulfide bridges pull the molecule into a compact conformation (Dr Alain Boulangé, University of KwaZulu-Natal, South Africa, personal communication), and hence it is able to migrate further on a gel under non-reducing conditions. Reduced C2 is

fully denatured and linearised, reflecting a more accurate molecular weight. The size of reduced C2 observed by SDS-PAGE (27 kDa) was consistent with that reported for C2 expressed in the baculovirus system (Boulangé *et al.*, 2001).

The use of the Roche Expand High Fidelity PCR system for amplification of the oligopeptidase B ORF resulted in a blunt-ended amplicon of 2.15 kb. Restriction enzymes cut poorly at the ends of a DNA fragment, which necessitated the use of an intermediate vector such as pCAP^{s}. Once cloned into the intermediate vector the insert could easily be excised and cloned into an expression vector. Confirmation of the insert in the pCAP^{s} vector was obtained by sequencing of clones that were shown to have the insert by restriction digestion with *Eco*RI and *Not*I. Further sequencing after sub-cloning was not considered necessary since the insert DNA in pCAP-OPC and pCAP-OPV was found not to have any mutations.

The oligopeptidase B ORFs from *T. congolense* and *T. vivax* were successfully cloned into both the pPIC9 and pGEX4T1 expression vectors, as confirmed by restriction digestion. Integration of the recombinant pPIC9 plasmids (pPIC9-OPC and pPIC9-OPV) was likely to have occurred in the *HIS4* gene, since the plasmids were linearised with *Sal*I. Relatively low levels of the recombinant enzymes were expressed in the yeast system of *P. pastoris*. However, activity of the culture supernatants against the substrate Z-Arg-Arg-AMC and recognition of the protein by anti-peptide antibodies were firm indications of successful expression. Due to the relatively low levels of expression of the enzymes, purification was not feasible. The low yields of protein that would result did not justify the expense of time and reagents. Therefore, purification of the enzymes from the *P. pastoris* expression system was not pursued.

Expression of the recombinant enzymes as fusion proteins in *E. coli* with pGEX4T1 was successful, as shown by SDS-PAGE (Fig. 4.19). The use of the non-recombinant pGEX4T1 control during expression confirmed that induction took place, since a protein of 26 kDa, likely to be GST, was visible on the gel. The fusion proteins that were produced upon induction of expression appeared at the expected size of approximately 106 kDa. Levels of expression of the rOPV-fusion protein appeared to be approximately

twice as high as those observed for the rOPC-fusion protein. Purification of rOPC and rOPV by cleavage of the fusion proteins while adsorbed to the glutathione agarose affinity matrix was found to be a very rapid and efficient technique. The protein was judged to require no further purification, even though there were small amounts of thrombin present after cleavage of the fusion protein. The specific inhibitor antithrombin III was used to inhibit the thrombin to ensure that it would not affect the kinetic characterisation of the two enzymes.

N-terminal sequencing of the purified recombinant proteins confirmed their identity as oligopeptidase B from *T. congolense* and *T. vivax*. Each protein had five extra amino acids (GSPEF) on their N-termini as a result of translation of the region upstream from the *Eco*RI restriction site of the multiple cloning region, as can be seen in Fig. 4.4. The upstream sequence coded for the thrombin hydrolysis site, and the Gly and Ser residues were left after cleavage of the recognition site by thrombin. The Pro residue was coded for by the three base pairs in between the thrombin site and the *Eco*RI site, and the Glu and Phe residues were translated from the *Eco*RI site itself. The five additional residues were not considered likely to affect the enzymatic activity of the recombinant proteins. This assumption was based on studies where recombinant oligopeptidase B from *T. b. brucei, T. evansi*, and *T. cruzi* were expressed with a His-tag (10 His residues) on their N-termini, and the enzymes were found to have unaltered activity (Morty *et al.*, 1999b; Burleigh *et al.*, 1997; Morty *et al.*, 2005a).

The final yields of the purified recombinant enzymes, rOPC and rOPV, were 2.6 mg and 3.1 mg per litre of culture respectively. These yields are lower than the 12 mg/l and between 30-40 mg/l yields reported for recombinant oligopeptidase B from *T. b. brucei* and *T. evansi* respectively (Morty *et al.*, 1999b; Morty *et al.*, 2005a). However, a true comparison of yields cannot be made since the latter recombinant oligopeptidase B enzymes have been expressed with a different vector, pET19b.

Western blotting of procyclic forms of *T. b. brucei* with anti-peptide antibodies against oligopeptidase B confirmed the presence of oligopeptidase B in this lifecycle stage of the parasite. The enzyme has previously been found to be expressed in all life cycle stages of

T. cruzi (Burleigh *et al.*, 1997), but similar studies had not been undertaken for salivarian trypanosomes. Oligopeptidase B from *T. b. brucei*, *T. congolense* and *T. vivax* was isolated to a partially purified state from parasite lysates. The isolations were carried out in a single step, utilising affinity purified antibodies that were produced against recombinant oligopeptidase B from *T. vivax* in the present study. Elution was effected with carbonate buffer at pH 10, since oligopeptidase B is unstable at acidic pH. The chaotropic agent sodium thiocyanate has previously been used as an elution agent (Morty *et al.*, 1999a), but this was considered undesirable in the present study since thiocyanate interferes with absorbance readings as well as activity assays.

Since the same antibodies were found not to recognise the contaminating proteins in the preparation it was concluded that these proteins were bound to the affinity matrix by non-specific interaction. In an attempt to minimise the amount of non-specific interaction with the matrix, the lysate was passed over a non-specific IgY column before specific immunoaffinity purification (results not shown). However, this did not improve the purity of the sample.

Purity could probably have been improved by subjecting the lysate to clean-up steps prior to immunoaffinity purification. Three-phase partitioning has been used successfully as a fractionation step in the purification of oligopeptidase B from bloodstream forms of *T. b. brucei* (Morty *et al.*, 1999b), but would require re-optimisation for use with procyclic parasites (as used in the present study for the purification from *T. b. brucei*). When bloodstream form parasites differentiate into procyclic forms, they lose their VSG coat and develop a new coat of procyclin molecules (Acosta-Serrano *et al.*, 2001). Therefore the protein repertoire of procyclic parasites would differ from that of bloodstream form parasites, for which three-phase partitioning has been optimised. Owing to the small supply of parasites and time constraints, however, further purification of the native enzyme was not deemed necessary for the assays for which it was required.

The possibility that the major contaminant in the *T. b. brucei* preparation (of approximately 70 kDa) was the heavy chain of IgY was negated by western blotting,

since IgY would have been detected by the enzyme-labelled secondary antibody. The contaminating protein appears to correspond to the major protein present in procyclic parasites, as observed by SDS-PAGE, and may be a surface protein. Where bloodstream form parasites were used, the oligopeptidase B preparations also contain a protein at approximately 65 kDa which is likely to be VSG.

Native oligopeptidase B was isolated from *T. b. brucei*, *T. congolense* and *T. vivax* parasites, and was used for K_m determination (Chapter 5) and to assay for inhibition by specific antibodies (Chapter 8). Purified rOPC and rOPV were used in the present study for enzymatic characterisation (Chapter 5) as well and immunochemical studies (Chapter 8).

CHAPTER 5

Enzymatic characterisation of recombinant oligopeptidase B from *T. congolense* and *T. vivax*

5.1 Introduction

Thorough enzymatic characterisation has been undertaken for native, and recombinant oligopeptidase B from the African trypanosomes *T. b. brucei* (Morty *et al.*, 1999b) and the causative agent of surra, *T. evansi* (Morty *et al.*, 2005b), as well as native enzyme from *T. congolense* (Morty *et al.*, 1999a). Recombinant, and native oligopeptidase B from *T. cruzi* (Burleigh *et al.*, 1997) and *E. coli* (Pacaud and Richaud, 1975; Kanatani *et al.*, 1991), as well as recombinant oligopeptidase B from *Salmonella enterica* (Morty *et al.*, 2002) and *Moraxella lacunata* (Yoshimoto *et al.*, 1995), and native oligopeptidase B from *Triticum aestivum* (Tsuji *et al.*, 2004), have been characterised to some degree.

It was important for the present study to ensure that the recombinant oligopeptidases from *T. congolense* and *T. vivax*, expressed and purified in Chapter 4, showed similar enzymatic characteristics to the native and recombinant salivarian trypanosomal enzymes that have previously been studied. This was to ensure that the recombinant enzymes in the present study displayed characteristic oligopeptidase B behaviour, and thus, were an accurate representation of the native enzymes. Native oligopeptidase B from *T. vivax* has not yet been isolated, so no comparison can be made with the recombinant enzyme, but the characteristics of the recombinant enzyme would give insight into the properties of the native enzyme. In the following chapter, rOPC and rOPV were enzymatically characterised by assaying their fluorogenic peptide specificities and inhibitor profiles, and the effect of ionic strength, pH, reducing agents, effector molecules and metal ions on their activity.

5.2 Fluorogenic peptide specificity

The parameters K_m (the Michaelis constant) and V_{max} are of value in characterising the specificity of an enzyme for a particular substrate and can indicate a role for an enzyme in metabolism (Price and Stevens, 1989). V_{max} varies with the total concentration of

enzyme present, whereas K_m is independent of enzyme concentration. When an active-site titrant is available to enable accurate evaluation of the active enzyme concentration, the value of V_{max} can be converted to a value for k_{cat} (the turnover number of the enzyme) from the following relationship

$$k_{cat} = \frac{V_{max}}{[E]_0} \text{ (Salvesen and Nagase, 1989)}$$

where $[E]_0$ represents the active concentration of enzyme. The apparent second order rate constant (k_{cat}/K_m) describes the rate in terms of the concentrations of the free enzyme and free substrate, and is used as an accurate measure of specificity of an enzyme for a substrate.

Determination of the kinetic constants of an enzyme is accomplished for single-substrate reactions by the determination of initial steady-state velocity (v_0) over a range of substrate concentrations. The Briggs-Haldane revision of the Michaelis-Menten equation

$$v_0 = \frac{V_{max}[S]}{[S] + K_m} \text{ (Briggs and Haldane, 1925)}$$

relates the variables to K_m and V_{max}, allowing their estimation from a direct plot of v_0 versus substrate concentration, $[S]$.

The determination of K_m and V_{max} from a direct plot is often not considered to be entirely satisfactory, and a number of alternative plots have been developed to fit the data to a straight line: the Lineweaver-Burk plot (Lineweaver and Burk, 1934); the Eadie-Hofstee plot (Eadie, 1942; Hofstee, 1952); the Hanes plot (Hanes, 1932); and the Eisenthal-Cornish-Bowden plot (Eisenthal and Cornish-Bowden, 1974). In recent years, software has been designed that fits data directly to the Briggs-Haldane equation above, allowing accurate determination of K_m and V_{max} by hyperbolic regression. This was the method used in the present study for determination of K_m and V_{max} for various synthetic peptide

substrates, using Hyper32 software (obtained from Dr J. Easterby, University of Liverpool, UK).

5.2.1 Reagents

0.1 % (m/v) Brij 35. As per Section 2.9.1.1.

4 × Assay buffer [200 mM Tris-HCl buffer, pH 8, 10 mM DTT, 0.02% (m/v) NaN_3]. Tris (2.422 g) and DTT (0.155 g) were dissolved in dH_2O (90 ml) and titrated to pH 8 with HCl. NaN_3 (0.02 g) was added and the solution was made up to 100 ml with dH_2O. Assay buffer was made freshly on the day of use due to the instability of DTT in solution.

1 mM Substrate stock solutions. Z-Arg-Arg-AMC (0.9 mg), Z-Gly-Gly-Arg-AMC (0.9 mg), Z-Phe-Arg-AMC (1 mg), Boc-Leu-Arg-Arg-AMC (1.2 mg), Boc-Leu-Gly-Arg-AMC (0.9 mg), Boc-Gly-Arg-Arg-AMC (1 mg), Boc-Val-Gly-Arg-AMC (0.9 mg), H-Ala-Phe-Lys-AMC (0.8 mg), Boc-Gly-Lys-Arg-AMC (1 mg), Boc-Val-Leu-Lys-AMC (0.9 mg), Z-Arg-AMC (0.7 mg), Boc-Val-Pro-Arg-AMC (0.9 mg), and Z-Ala-Ala-Arg-AMC (1.2 mg) were dissolved separately in DMSO (1.5 ml). H-Arg-AMC (0.5 mg) was dissolved in dH_2O (1.5 ml).

Working substrate solutions. Substrates were diluted from their 1 mM stock solutions with dH_2O to 4 times the appropriate final concentration, allowing for dilution in the assay.

1 mM AMC. AMC (1.8 mg) was dissolved in DMSO (10 ml) and stored at 4°C.

5.2.2 Procedure

All reagents were preincubated at 37°C and assays were carried out in FluorNunc® 96-well fluorometry plates. Enzyme (2.5 ng active rOPC or rOPV) made up to 50 µl with 0.1% brij, was activated in assay buffer with DTT (25 µl) for 10 min at 37°C. Substrate solution (25 µl) was added and the fluorescence (excitation at 360 nm, emission at 460 nm) was monitored continuously in a Cambridge Technology 7620 Microplate

Fluorometer for 6 min. The initial steady state velocity (v_0) was determined over a range of substrate concentrations (100 nM to 150 μM). K_m and V_{max} were determined by hyperbolic regression of the kinetic data using the software package Hyper32. The k_{cat} was determined from $k_{cat} = V_{max}/[E]_0$, where $[E]_0$ represents the active enzyme concentration.

To facilitate quantitation of the product (AMC), a calibration graph was constructed for the range of 5-250 pmol AMC, diluted in assay buffer. AMC concentration was determined by linear regression analysis of the standard data (Fig. 5.1).

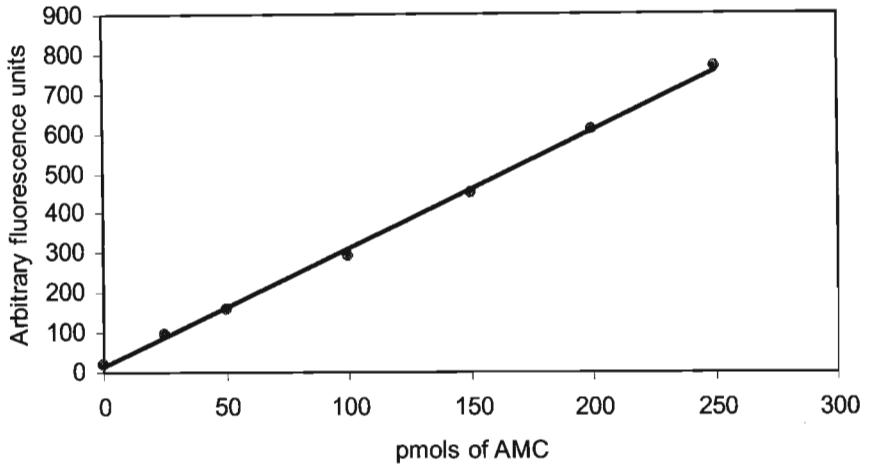

Figure 5.1. Standard curve relating the amount of AMC to fluorescence (excitation at 360 nm and emission at 460 nm). The equation of the trend line is given by y = 2.964x + 13.8, with a correlation coefficient of 0.9985.

5.3 Inhibitor profile

Any compound that decreases the measured rate of enzyme-catalysed hydrolysis of a particular substrate is an enzyme inhibitor. There are many different forms of enzyme inhibitors, and they are often grouped on the basis of their reaction mechanism, origin or structural similarity (Salvesen and Nagase, 2001). The kinetics of the interaction of oligopeptidase B from *T. b. brucei* and *T. congolense* have previously been determined (Morty *et al.*, 1999a; Morty *et al.*, 1999b). In the present study it was important to conduct inhibitor profiling of the previously uncharacterised oligopeptidase B from *T.*

vivax, and also to compare the inhibitor kinetics of rOPC to those previously determined for native oligopeptidase B from *T. congolense*.

5.3.1 Reagents

Inhibitor stock solutions. AEBSF (24 mg, 100 mM), benzamidine.HCl (42 mg, 100 mM), EDTA (37.2 mg, 100 mM), leupeptin (8.6 mg, 18 mM), soybean trypsin inhibitor (1 mg, 1 $mg.ml^{-1}$), lima bean trypsin inhibitor (1 mg, 1 $mg.ml^{-1}$), and aprotinin (1 mg, 1 $mg.ml^{-1}$) were each dissolved separately in dH_2O (1 ml). Antipain (12.1 mg, 18 mM), chymostatin (6 mg, 10 mM), 3,4-DCI (2.2 mg, 10 mM), *N*-ethylmaleimide (12.5 mg, 100 mM), E-64 (3.5 mg, 10 mM), and pepstatin A (6.8 mg, 10 mM) were each dissolved separately in DMSO (1 ml). PMSF (17.4 mg, 100 mM) was dissolved in ethanol (1 ml). 1,10-Phenanthroline (39.6 mg, 200 mM) and TPCK (3.5 mg, 10 mM) were each dissolved in methanol (1 ml). Inhibitor stocks were stored at -70°C. Due to instability, sodium iodoacetate (20.7 mg, 100 mM) and iodoacetamide (18.5 mg, 100 mM) were prepared on the day of use in dH_2O (1 ml), and TLCK.HCl (3.7 mg, 10 mM) in 1 mM HCl, pH 3 (1 ml).

5.3.2 Procedure

The effect of irreversible inhibitors (TLCK, DCI, AEBSF, PMSF, iodoacetamide, sodium iodoacetate, N-ethylmaleimide, and TPCK) was investigated under pseudo first-order conditions ($[I]>50[E]_0$), as described by Salvesen and Nagase (2001), by adding an aliquot of inhibitor (10 µl) to a buffered enzyme solution (140 µl, containing 50 ng rOPC or rOPV in 50 mM Tris-HCl, pH 8) to initiate the inactivation. Aliquots were removed at timed intervals and residual activity determined against 5 µM Z-Arg-Arg-AMC (v_t) as in Section 5.2.2. The reaction velocity of the uninhibited enzyme was also determined (v_0). The pseudo first-order inhibition rate constant (k_{obs}) was determined from the linear section of plots of ln v_t/v_0 versus time, which has a slope of $-k_{obs}$. The rate for complex association (k_{ass}) was obtained from the relationship:

$$k_{ass} = \frac{k_{obs}}{[I]}$$

where [I] represents the inhibitor concentration. The time required for the free enzyme concentration to decrease by 50%, i.e. half-life ($t_{½}$), was given by:

$$t_{½} = \frac{0.693}{k_{ass}[I]}$$

For competitive reversible inhibitors (leupeptin, antipain, aprotinin, benzamidine, E-64, amastatin, bestatin, chymostatin, EDTA, LBTI, SBTI, 1,10-phenanthroline and pepstatin A), the inhibition constant (K_i) was determined by the method of Salvesen and Nagase (2001). The enzyme-catalysed hydrolysis of Z-Arg-Arg-AMC was monitored continuously as described in Section 5.2.2 to establish an uninhibited reaction velocity (v_0). Inhibitor was added to the reaction (twenty-fold molar excess over enzyme in less than 5% of the total assay volume) and the new steady-state velocity in the presence of the inhibitor (v_i) determined. The apparent inhibition constant in the presence of substrate ($K_{i(app)}$) was calculated using the equation:

$$\frac{v_0}{v_i} = 1 + \frac{[I]}{K_{i(app)}}$$

The true K_i was calculated, catering for the presence of the substrate, from the relationship:

$$K_i = \frac{K_{i(app)}}{1 + \frac{[S]}{K_m}}$$

The rate constant for complex formation (k_{ass}) for competitive reversible inhibitors was assayed in the same manner as irreversible inhibitors, but was obtained from the relationship:

$$k_{ass} = \frac{k_{obs}}{[I]}\left(1 + \frac{[S]}{K_m}\right)$$

The rate constant for complex dissociation, k_{diss}, was determined from the relationship:

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

5.4 Effect of pH on oligopeptidase B activity and stability

Ionic strength is an important consideration when assessing the effect of pH on enzyme activity. Changes in ionic strength can affect the ionisation of protein side chains, and thus can affect the activity of an enzyme independent of pH changes (Dehrmann *et al.*, 1995). For this reason, AMT buffers of constant ionic strength were utilised across a broad pH spectrum to assay the effect of pH on both the activity and stability of oligopeptidase B. In this manner, one is assured of observing the true effect of pH, without the influence of ionic strength fluctuations (Ellis and Morrison, 1982).

5.4.1 Reagents

AMT buffers (100 mM acetate, 100 mM MES, 200 mM Tris, 10 mM DTT, 4 mM Na_2EDTA; I = 0.1). Glacial acetic acid (1.72 ml), MES (5.86 g), Tris (7.27 g) and Na_2EDTA (0.45 g) were dissolved in dH_2O (240 ml). This solution was divided into 12 aliquots of 20 ml each, which were titrated to pH values in the range of 4.0 to 12.0 using HCl or NaOH, and made up to 25 ml with dH_2O. DTT (7.7 mg) was added to a 5 ml aliquot of each buffer, if required, just before use and the pH adjusted if necessary.

20 µM Z-Arg-Arg-AMC. As per Section 3.3.7.1.

5.4.2 Procedure

The effect of pH on the activity of rOPC and rOPV against the substrate Z-Arg-Arg-AMC was conducted in the same manner as in Section 5.2.2, except that AMT buffers, at various pH values, replaced the 4 × assay buffer. The assays were conducted both in the presence and absence of DTT in the assay buffer. Activity was reflected as a percentage of maximum activity observed in the assay.

The effect of pH on the stability of rOPC and rOPV was assayed by incubating enzyme (5 ng active enzyme made up to 50 µl with 0.1% brij) in AMT buffers of various pH

values (25 µl) for 1 h at 37°C. AMT buffer at pH 8.5 (75 µl) was added to bring all of the samples to pH 8.5, and residual activity against Z-Arg-Arg-AMC was assayed as before.

5.5 Effect of ionic strength on oligopeptidase B activity

The effect of ionic strength on the activity of rOPC and rOPV was assayed with AMT buffers of varying ionic strengths (0.5, 0.2, 0.1, 0.05 and 0.025 in the final assay mixture), in the presence and absence of DTT. Ionic strength is known to affect the activity of proteases such as cathepsin B (Dehrmann *et al.*, 1995) and cathepsin L (Dennison *et al.*, 1992), and has been shown to influence the activity of oligopeptidase B from *T. b. brucei* (Morty, 1998). It was of interest to observe the relative activity of oligopeptidase B at physiological ionic strength, especially in comparison to that of oligopeptidase B from *T. b. brucei.*

5.5.1 Reagents

AMT buffer: I = 1 (500 mM acetate, 500 mM MES, 1 M Tris, 10 mM DTT, pH 8). Glacial acetic acid (2.866 ml), MES (9.767 g) and Tris (12.11 g) were dissolved in dH_2O (80 ml). The solution was titrated to pH 8 with NaOH, and made up to 100 ml with dH_2O. DTT (15.4 mg) was added to a 10 ml aliquot before use, where necessary.

AMT buffers: I = 0.4, 0.2, 0.1, 0.05. AMT buffer: I = 1 was diluted 2.5, 5, 10, and 20 times in dH_2O to give I = 0.4, 0.2, 0.1, 0.05 respectively. That is, 4 ml, 2ml, 1 ml, 0.5 ml of AMT buffer: I = 1 were made up to 10 ml with dH_2O. DTT (15.4 mg) was added to a 10 ml aliquot before use, where appropriate, and the pH adjusted if necessary.

5.5.2 Procedure

Enzyme (2.5 ng active rOPC or rOPV made up to 25 µl in brij) was preincubated in AMT buffers (50 µl) of varying ionic strengths for 10 min at 37°C. 20 µM Z-Arg-Arg-AMC (25 µl; Section 5.4.1) was added and the fluorescence monitored as described for Section 5.2.2.

5.6 Reductive activation

Reducing agents are known to enhance the activity of trypanosomal oligopeptidase B by up to 4-fold (Morty and Burleigh, 2004). In the present study the effect of three different reducing agents, DTT, cysteine.HCl and reduced glutathione, at final concentrations ranging from 0-25 mM, on the activity of rOPC and rOPV was assayed.

5.6.1 Reagents

4 × Assay buffer [200 mM Tris-HCl buffer, pH 8, 0.02% (m/v) NaN_3]. As per Section 5.2.1, but without DTT.

Stock 100 mM reduced glutathione. Reduced glutathione (0.144 g) was dissolved in assay buffer (5 ml) and the pH adjusted if necessary.

Stock 100 mM DTT. DTT (0.076 g) was dissolved in assay buffer (5 ml) and the pH adjusted if necessary.

Stock 100 mM cysteine.HCl. Cysteine.HCl (0.06 g) was dissolved in assay buffer (5 ml) and the pH adjusted if necessary.

5.6.2 Procedure

Activity of rOPC and rOPV against 5 μM Z-Arg-Arg-AMC was determined as described in Section 5.2.2, except that reduced glutathione, DTT or cysteine.HCl were included in the assay buffer at final concentrations of 0 to 25 mM. Stock reducing agents were diluted as necessary with assay buffer. Enzyme was preincubated with reducing agent for 10 min at 37°C to allow activation to occur before adding the substrate. Activity was reflected as a percentage of the activity observed without reducing agent.

5.7 Rate of activation with DTT

The rate of activation of rOPC and rOPV with 2.5 mM DTT was assayed in the present study to determine the optimal time of incubation. This was carried out because it was essential to assay the kinetic constants of the enzymes when they were fully activated.

5.7.1 Procedure

Enzyme was preincubated in assay buffer containing 10 mM DTT (2.5 mM final concentration) for various lengths of time (0-15 min), and activity against 5 μM Z-Arg-Arg-AMC was determined as described in Section 5.2.2. Activity was reflected as a percentage of the activity observed at time zero.

5.8 Potential activators of oligopeptidase B

It has previously been shown that polyamines, at micromolar concentrations, are able to weakly enhance the activity of trypanosomal oligopeptidase B (Morty *et al.*, 1999a; Morty *et al.*, 1999b). These compounds have also been found to inhibit the activity of an alkaline peptidase from *T. cruzi* at millimolar concentrations (Ashall, 1990). Heparin has also been found to enhance the activity of trypanosomal oligopeptidase B by up to 66% at 30 $\mu g.ml^{-1}$ (Morty *et al.*, 1999a; Morty *et al.*, 1999b). It was therefore considered of interest to determine if polyamines, as well as other effector molecules, such as ATP, GTP and heparin, had any effect on the activity of rOPC and rOPV.

5.8.1 Reagents

10 mM Stock solutions. Putrescine (also known as 1,4-diaminobutane; 1.016 ml), spermine.4HCl (35 mg), spermidine.3HCl (25.4 mg), ornithine.HCl (17 mg), ATP (55 mg), and GTP (52 mg) were each dissolved separately in dH_2O (10 ml). As heparin does not have a well-defined molecular weight, it was made up as a solution of 1 $mg.ml^{-1}$.

5.8.2 Procedure

The influence of potential activators on the hydrolysis of 5 μM Z-Arg-Arg-AMC by rOPC or rOPV (5 ng) was assayed as in Section 5.2.2, except that the activator was included in the assay buffer in the absence of DTT. The stock solutions of activators were diluted appropriately in assay buffer to 4 × the final concentration to allow for dilution following addition of substrate. Final concentrations were 50 μM putrescine, spermine, spermidine and ornithine, 1 mM ATP and GTP, and 30 $\mu g.ml^{-1}$ heparin. Activity was expressed as a percentage as compared with the activity of a control assay with no activator.

5.9 Influence of metal ions

The influence of metal ions on the activity of an enzyme can provide insight into the catalytic mechanism of the enzyme. The activity of oligopeptidase B from *T. b. brucei* and *T. congolense* was found to be inhibited by a variety of metal ions in previous studies (Morty *et al.*, 1999a; Morty *et al.*, 1999b), but no activation by metal ions was observed.

5.9.1 Reagents

10 mM Metal ion stock solutions. $MgCl_2.6H_2O$ (20 mg), $CaCl_2.2H_2O$ (15 mg), $ZnCl_2$ anhydrous (14 mg), $MnCl_2.4H_2O$ (20 mg), $HgCl_2$ anhydrous (27 mg), $CuCl_2.2H_2O$ (17 mg), $FeCl_2.4H_2O$ (12 mg), $NiCl_2.6H_2O$ (23 mg), $CdCl_2.2½H_2O$ (23 mg), and $BaCl_2.2H_2O$ (24 mg) were each dissolved separately in assay buffer without DTT (10 ml).

5.9.2 Procedure

The assay was carried out as in Section 5.2.2, except that the metal ions were included in assay buffer. DTT was omitted from the assay buffer because of its potential to form complexes with heavy-metal ions. As in Section 5.8.2, stock solutions of metal ions were diluted appropriately in assay buffer to 4 × the final concentration to allow for dilution following addition of substrate. The final concentration of the metal ions in the assay was 1 mM. Activity was expressed as a percentage as compared with the activity of a control with no metal ions.

5.10 Results

5.10.1 Fluorogenic peptide specificity

Since the substrate specificity of oligopeptidase B has previously been characterised as trypsin-like (Morty *et al.*, 1999b), only substrates with basic residues at the P1 position were assayed in the present study. From the kinetic data obtained for the various peptide substrates it was clear that Arg was strongly preferred over Lys at the P1 position (Table 5.1). The two enzymes rOPC and rOPV differed slightly in their affinities for the different substrates. Recombinant OPC showed the greatest affinity for the substrate Z-Arg-Arg-AMC, whilst, even though rOPV had a similar k_{cat}/K_m for this substrate, it

showed a higher k_{cat}/K_m for the substrate Z-Gly-Gly-Arg-AMC. However, the general trends for substrate preferences were similar for both enzymes.

A variety of residues were acceptable at the P2 position, including Arg, Ala, Gly, Lys, Phe, Leu and Pro. Both of the recombinant enzymes showed a preference for Arg over Phe, shown by comparing the k_{cat}/K_m for Z-Arg-Arg-AMC and Z-Phe-Arg-AMC. An Arg residue was also preferred over Lys, shown by comparing Boc-Gly-Arg-Arg-AMC and Boc-Gly-Lys-Arg-AMC. Comparison of the results obtained for Boc-Leu-Arg-Arg-AMC and Boc-Leu-Gly-Arg-AMC shows a definite preference for Arg over Gly at the P2 position, whereas comparing Boc-Val-Pro-Arg-AMC with Boc-Val-Gly-Arg-AMC reveals a preference for Pro over Gly at P2. There appeared to be little specificity for the P3 residue since substitutions at this position had variable effects on the k_{cat}/K_m values.

Both rOPC and rOPV showed very poor activity against H-Arg-AMC, but very strong activity against Z-Arg-AMC, where the N-terminus is blocked with a benzyloxycarbonyl group.

Table 5.1. Fluorogenic peptide specificity of recombinant oligopeptidases

Substrate	rOPC			rOPV		
	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.\mu M^{-1}$)	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}.\mu M^{-1}$)
Z-Arg-Arg-AMC	2.00	22.1	11.0	1.88	19.4	10.3
Boc-Leu-Arg-Arg-AMC	1.58	15.8	10.0	1.73	14.5	8.4
Z-Ala-Ala-Arg-AMC	2.29	19.6	8.6	3.01	18.1	6.0
Z-Gly-Gly-Arg-AMC	5.90	47.6	8.1	0.98	14.7	15.0
Boc-Gly-Arg-Arg-AMC	2.87	22.9	8.0	2.82	26.8	9.5
Boc-Gly-Lys-Arg-AMC	1.84	13.1	7.1	1.52	8.9	5.9
Z-Arg-AMC	9.31	58.1	6.2	3.07	24.5	8.0
Boc-Val-Gly-Arg-AMC	5.14	15.3	3.0	2.81	12.6	4.5
H-Ala-Phe-Lys-AMC	7.43	14.9	2.0	5.77	22.9	4.0
Boc-Val-Pro-Arg-AMC	9.44	14.3	1.5	5.08	13.0	2.6
Boc-Leu-Gly-Arg-AMC	6.58	9.1	1.4	1.19	2.2	1.8
Z-Phe-Arg-AMC	10.83	14.1	1.3	1.76	6.6	3.8
Boc-Val-Leu-Lys-AMC	11.38	6.8	0.6	9.55	10.6	1.1
H-Arg-AMC	93.58	2.6	0.03	70.91	2.1	0.03

Native oligopeptidase B, isolated from *T. b. brucei*, *T. congolense* and *T. vivax* in Chapter 4, was found to have K_m values of 1.93 μM, 1.95 μM and 1.99 μM respectively, against the substrate Z-Arg-Arg-AMC.

5.10.2 Inhibitor profile

The inhibition of recombinant oligopeptidase B from *T. congolense* (rOPC) and *T. vivax* (rOPV) by competitive, reversible inhibitors is shown in Table 5.2. The two enzymes were found to react similarly with the inhibitors. Recombinant oligopeptidase B was strongly inhibited by the peptide aldehydes leupeptin and antipain, but not by chymostatin. Antipain was found to have a lower K_i and higher k_{ass} than leupeptin for both enzymes. Aprotinin, a proteinaceous inhibitor with M_r 6500, was found to inhibit recombinant oligopeptidase B to a similar degree, having nanomolar K_i values (8.9 and 50.3 nM). Other high molecular weight, proteinaceous inhibitors, such as LBTI (M_r 9000) and SBTI (M_r 20 100) did not inhibit either of the two enzymes. Benzamidine and E-64 were found to inhibit both rOPC and rOPV, with micromolar K_i values. Amastatin, bestatin, EDTA, 1,10-phenanthroline and pepstatin A were found not to inhibit either of the recombinant enzymes.

Table 5.2. Inhibition of recombinant trypanosome oligopeptidase B by competitive, reversible inhibitors[a,b].

Inhibitor	rOPC			rOPV		
	K_i (μM)	k_{ass} ($M^{-1}.s^{-1}$)	k_{diss} (s^{-1})	K_i (μM)	k_{ass} ($M^{-1}.s^{-1}$)	k_{diss} (s^{-1})
Leupeptin	15.6×10^{-3}	5.6×10^{5}	8.74×10^{-3}	52.7×10^{-3}	5.63×10^{4}	2.97×10^{-3}
Antipain	5.3×10^{-3}	2.3×10^{6}	12.25×10^{-3}	8.6×10^{-3}	1.4×10^{6}	12.44×10^{-3}
Aprotinin	8.9×10^{-3}	n.d.	n.d.	50.3×10^{-3}	n.d.	n.d.
Benzamidine	290.9	n.d.	n.d.	345.2	n.d.	n.d.
E-64	51.5	n.d.	n.d.	64.0	n.d.	n.d.

[a]No inhibition was observed with amastatin (125 μM), bestatin (125 μM), chymostatin (100 μM), EDTA (1 mM), LBTI (100 μg/ml), 1,10-phenanthroline (1 mM), pepstatin A (1 μM) or SBTI (100 μg/ml).
[b]n.d., not determined. In these cases the k_{ass} was too fast to be determined experimentally.

The inhibition of rOPC and rOPV by irreversible inhibitors is shown in Table 5.3. The two recombinant enzymes showed very similar kinetic values for inhibition by TLCK, DCI, AEBSF, and PMSF. The chloromethylketone inhibitor TLCK was the most effective inhibitor of rOPC and rOPV, with k_{ass} values of 14 135 $M^{-1}.s^{-1}$ and 14 110 $M^{-1}.s^{-1}$, respectively. TPCK, however, was found not to inhibit either enzyme. The general serine peptidase inhibitor DCI was the next best inhibitor of rOPC and rOPV with k_{ass} values of 214.8 $M^{-1}.s^{-1}$ and 212.5 $M^{-1}.s^{-1}$ respectively. AEBSF was also an effective inhibitor of the two recombinant enzymes (k_{ass} = 21.6 $M^{-1}.s^{-1}$ for rOPC and 30.4 $M^{-1}.s^{-1}$ for rOPV), whereas PMSF was a comparatively weak inhibitor (k_{ass} = 1.4 $M^{-1}.s^{-1}$ for rOPC and 1.5 $M^{-1}.s^{-1}$ for rOPV). The thiol-reactive inhibitors iodoacetamide, sodium iodoacetate and *N*-ethylmaleimide were all found not to inhibit the activity of rOPC or rOPV.

Table 5.3. Inhibition of recombinant trypanosome oligopeptidase B by irreversible inhibitors[a].

Inhibitor	rOPC		rOPV	
	k_{ass}[b] ($M^{-1}.s^{-1}$)	$t_{½}$ (s)	k_{ass}[b] ($M^{-1}.s^{-1}$)	$t_{½}$ (s)
TLCK[c]	14 135 ± 742	4.9	14 110 ± 866	5
DCI[d]	214.8 ± 21	13	212.5 ± 41	13
AEBSF[d]	21.6 ± 3.9	128	30.4 ± 4.1	91
PMSF[d]	1.4 ± 0.28	2021	1.5 ± 0.46	2002

[a]No inhibition was observed with iodoacetamide, sodium iodoacetate, *N*-ethylmaleimide, or TPCK (250 μM), for which assays were conducted without DTT.
[b]Data reflect the mean k_{ass} ± S.D. (n=3).
[c]10 μM inhibitor concentration.
[d]250 μM inhibitor concentration.

5.10.3 Effect of pH on activity and stability

The activity of both rOPC and rOPV peaked between pH 8.5 and 9 (Fig. 5.2), and showed substantial activity at physiological pH (pH 7.4) with between 83% and 87% of maximum activity observed in the assay. The presence of DTT in the assay did not appear to make much difference in the response to pH overall. The most noteworthy difference was that the presence of DTT in the assay buffer resulted in a slightly narrower

optimal pH range for both enzymes. At pH 6, the enzymes showed similar activity of approximately 30%, decreasing to almost zero at pH 5. The major difference observed in the pH profiles of the two enzymes was that rOPV (panel B) was more sensitive to extremely high pH values, resulting in a narrower pH profile than for rOPC (panel A).

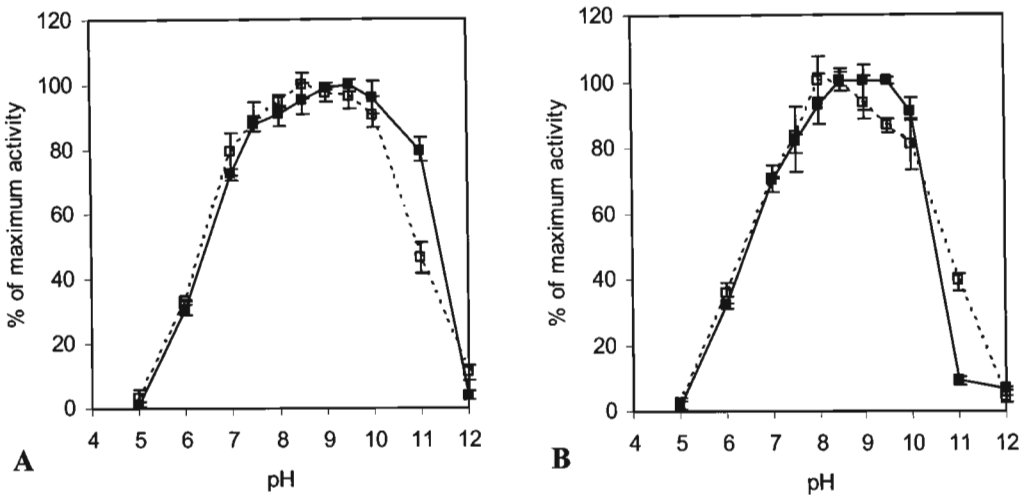

Figure 5.2. The effect of pH on the activity of recombinant trypanosomal oligopeptidase B. Enzyme (2.5 ng), rOPC (panel A) or rOPV (panel B), was assayed in AMT buffers (I=0.1) over the range of pH 5 to 12, in the presence (--□--) or absence (—■—) of 10 mM DTT. Activity against Z-Arg-Arg-AMC was expressed as a percentage of the maximum activity observed in the assay. Error bars represent the mean ± SEM (n=4).

The enzymes rOPC and rOPV showed stability over a narrow range of pH, i.e. pH 6-8 (Fig. 5.3). The presence of DTT during the assay served to shift the optimal range of stability to slightly more basic pH values, as well as reducing the stability of rOPV at pH values above 9 (panel B). Without DTT, pH stability peaked at about pH 6, whilst with DTT, pH stability peaked at about pH 8-8.5. At physiological pH (pH 7.4), rOPC showed approximately 97% maximum activity (94% with DTT), whereas rOPV showed approximately 85% maximum activity (95% with DTT).

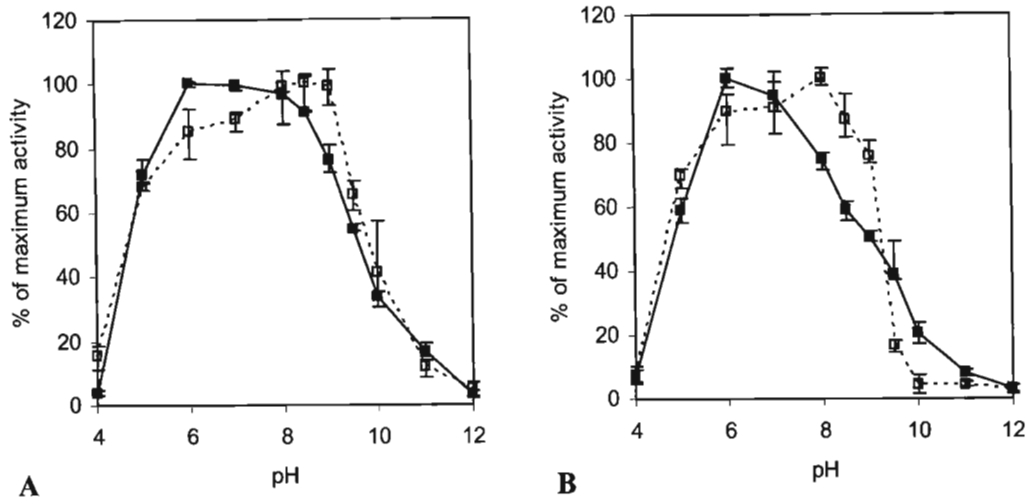

Figure 5.3. The effect of pH on the stability of recombinant trypanosome oligopeptidase B. Enzyme (2.5 ng), rOPC (panel A) or rOPV (panel B), was incubated in AMT buffers (I=0.1) over the range of pH 4 to 12, in the presence (--□--) or absence (—■—) of 10 mM DTT. Residual activity against Z-Arg-Arg-AMC was assayed in AMT buffer at pH 8.5, and expressed as a percentage of the maximum activity observed in the assay. Error bars represent the mean ± SEM (n=4).

5.10.4 Effect of ionic strength

The activity of rOPC and rOPV towards the substrate Z-Arg-Arg-AMC was sensitive to changes in ionic strength (Fig. 5.4). For both enzymes, an increase in ionic strength resulted in a decrease in activity, although to a lesser degree for rOPV. Oligopeptidase B activity was optimal at low ionic strength with little change in enzyme activity up to an ionic strength of 0.1 (physiological ionic strength; Cortese *et al.*, 1991). The presence or absence of DTT had no real influence on the effect of ionic strength on rOPC, but rOPV showed increased sensitivity to higher ionic strengths in the absence of DTT.

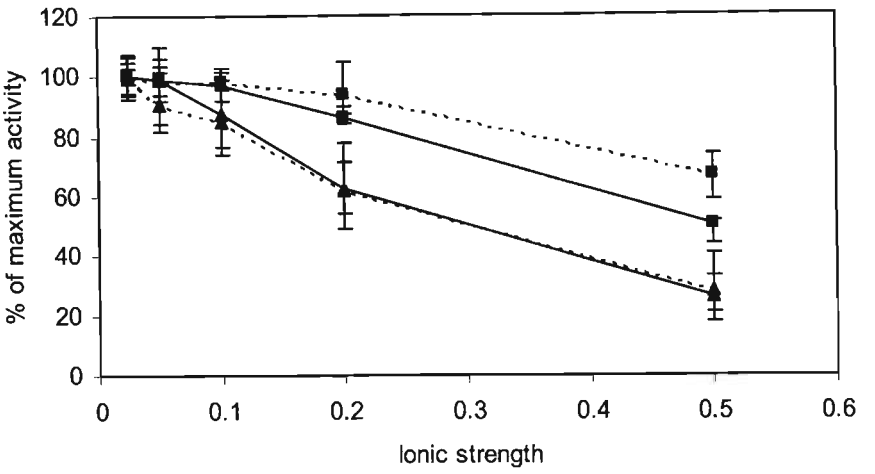

Figure 5.4. The effect of ionic strength on the activity of recombinant trypanosome oligopeptidase B. The activity of rOPC (▲) and rOPV (■) (4 ng) against Z-Arg-Arg-AMC was assayed, in the presence (---) or absence (—) of 10 mM DTT, in AMT buffers of varying ionic strengths at pH 8.5. Activity against Z-Arg-Arg-AMC was expressed as a percentage of the maximum activity observed in the assay. Error bars represent the mean ± SEM (n=4).

5.10.5 Reductive activation

The reducing agents reduced glutathione, DTT, and cysteine.HCl, all enhanced the activity of rOPC and rOPV at low concentrations (Figs. 5.5 and 5.6). DTT was the best reducing agent, with the maximal activation occurring between 2.5 and 5 mM. At higher concentrations, DTT had an inhibitory effect on the enzymes. Cysteine.HCl and reduced glutathione activated rOPC and rOPV with almost equal efficiency, with maximal activation occurring at 1 mM and 5 mM respectively. At higher concentrations, as with DTT, cysteine.HCl and reduced glutathione became inhibitory towards the enzymes. The activation by reducing agents was more enhanced for rOPV than for rOPC, with activity increasing by up to 2.5-fold, compared to a mere 1.5-fold increase in activity for rOPC.

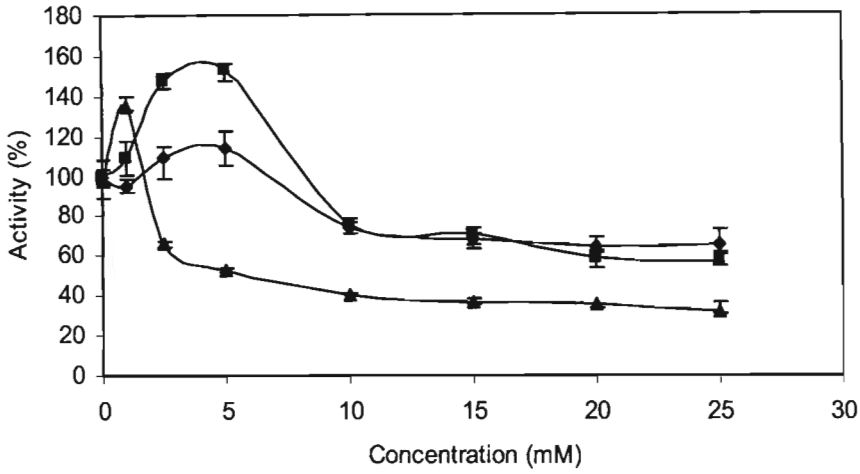

Figure 5.5. Effect of reducing agents on rOPC activity against Z-Arg-Arg-AMC. Enzyme (2.5 ng) was activated in 0-25 mM reduced glutathione (◆), DTT (■), or cysteine.HCl (▲) for 10 min at 37°C before activity against Z-Arg-Arg-AMC was assessed. Activity was expressed as a percentage of the activity observed for the control with no reducing agent. Error bars represent the mean ± SEM (n=3).

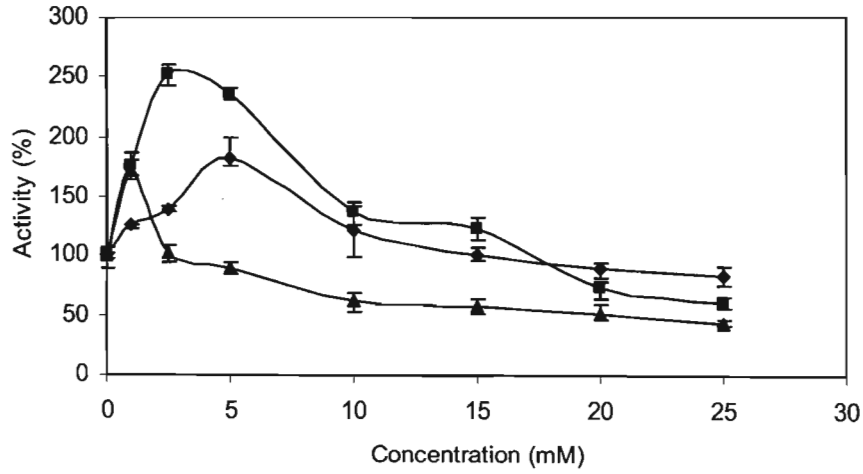

Figure 5.6. Effect of reducing agents on rOPV activity against Z-Arg-Arg-AMC. Enzyme (2.5 ng) was activated in 0-25 mM reduced glutathione (◆), DTT (■), or cysteine.HCl (▲) for 10 min at 37°C before activity against Z-Arg-Arg-AMC was assessed. Activity was expressed as a percentage of the activity observed for the control with no reducing agent. Error bars represent the mean ± SEM (n=3).

5.10.6 Rate of activation with DTT

The recombinant enzymes rOPC and rOPV were both rapidly activated in the presence of 2.5 mM DTT after 1 min (Fig. 5.7). There was a progressive increase in activity over time, which appeared to plateau after about 10 min of incubation at 37°C. The enzyme rOPV appeared to be activated to a greater extent than rOPC, reaching just over 220% activity as compared to the control without DTT, whereas rOPC reached just over 150% activity.

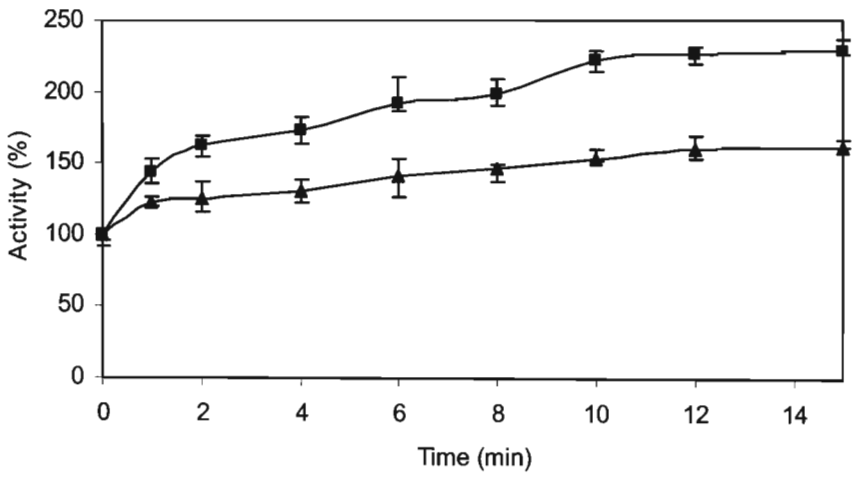

Figure 5.7. The effect of time of activation with DTT on the activity of rOPC and rOPV against Z-Arg-Arg-AMC. Enzyme (2.5 ng) was activated in 2.5 mM DTT for 0-15 min at 37°C before activity against Z-Arg-Arg-AMC was assessed. Activity of rOPC (▲) and rOPV (■) was expressed as a percentage of the activity observed for the control with no reducing agent. Error bars represent the mean ± SEM (n=4).

5.10.7 Potential activators

A number of polyamines, as well as heparin, ATP and GTP, were assessed for their ability to enhance the activity of rOPC and rOPV. None of the potential activators that were assayed had any significant activating or inhibitory effect on the enzymes.

5.10.8 Influence of metal ions

The influence of divalent metal ions on rOPC and rOPV activity is shown in Table 5.4. None of the metal ions that were tested had an activating effect on the recombinant

enzymes. Cadmium, mercury and zinc ions were strongly inhibitory, abolishing up to 100% of the peptidolytic activity against Z-Arg-Arg-AMC. Copper, iron and nickel ions were also inhibitory, but to a lesser extent. Manganese ions showed a mild inhibitory effect, whereas barium, calcium and magnesium ions had no effect on the activity of the enzymes.

Table 5.4. The effect of divalent metal ions on oligopeptidase B activity[a].

Ion	Parent compound	Activity (% relative to control)	
		rOPC	rOPV
Cd^{2+}	$CdCl_2.2½H_2O$	7.2	2.9
Cu^{2+}	$CuCl_2.2H_2O$	23.8	41.3
Fe^{2+}	$FeCl_2.4H_2O$	36.7	23
Hg^{2+}	$HgCl_2$ anhydrous	3.2	4.3
Mn^{2+}	$MnCl_2.2H_2O$	76.5	49.7
Ni^{2+}	$NiCl_2.6H_2O$	45.8	34.8
Zn^{2+}	$ZnCl_2$ anhydrous	2.5	0

[a]No effect on activity was observed in the presence of $BaCl_2.2H_2O$, $CaCl_2.2H_2O$ or $MgCl_2.2H_2O$.

5.11 Discussion

The two recombinant enzymes showed typical oligopeptidase B preference for the synthetic substrates that were assayed in the present study. That is to say, the recombinant enzymes showed cleavage of substrates on the carboxy side of basic residues, preferring Arg over Lys. A variety of residues were acceptable in the P2 position, with the basic residues Arg and Lys being favoured. The substrate specificities for the two enzymes are comparable with each other, except for the apparent preference of rOPV for the substrate Z-Gly-Gly-Arg-AMC over Z-Arg-Arg-AMC. The substrate preferences also compare well with those previously found for oligopeptidases from *T. congolense, T. b. brucei* and *T. evansi* (Morty *et al.*, 1999a; Morty *et al.*, 1999b; Morty *et al.*, 2005b).

A large difference, however, is apparent for the values for K_m and k_{cat}/K_m that were obtained in the present study and those in the literature. The kinetic constants that were obtained in the present study are more comparable to those obtained for oligopeptidase B from *S. enterica* (Morty *et al.*, 2002) than for trypanosomal enzymes. The K_m values of rOPC and rOPV for various substrates was higher than those obtained in other studies on the native enzyme from *T. congolense* (0.72 μM for Z-Arg-Arg-AMC, 1.19 μM for Z-Gly-Gly-Arg-AMC and 1.09 μM for Z-Phe-Arg-AMC; Morty *et al.*, 1999a), but comparable with those obtained for *T. cruzi* oligopeptidase B (5 μM for Z-Arg-Arg-AMC and 6 μM for Z-Gly-Gly-Arg-AMC and Z-Phe-Arg-AMC; Burleigh *et al.*, 1997). The k_{cat}/K_m for Z-Arg-Arg-AMC by native *T. congolense* enzyme is 133 $s^{-1}.\mu M^{-1}$ (Morty *et al.*, 1999a), which is much higher than the 11 $s^{-1}.\mu M^{-1}$ obtained in the present study.

Native oligopeptidase B from *T. b. brucei*, *T. congolense* and *T. vivax* that were isolated in the present study (Chapter 4) were used in assays to determine their K_m values against Z-Arg-Arg-AMC. The three enzymes had very similar values for K_m (between 1.93 and 1.99 μM), which were very close to K_m values obtained for the recombinant enzymes. The amount of native enzyme was insufficient for their active site titration, so values for k_{cat}, and hence k_{cat}/K_m, could not be determined.

Recombinant rOPC and rOPV were strongly inhibited by the peptide aldehydes leupeptin and antipain, where the aldehyde is contributed by an Arg residue in the P_1 position (Umezawa, 1976). As found previously for oligopeptidase B from *T. b. brucei* and *T. congolense*, antipain was the more effective of the two inhibitors, having lower K_i values and higher k_{ass} values (Morty *et al.*, 1999a; Morty *et al.*, 1999b). Chymostatin, which is also a peptide aldehyde, has an aldehyde contributed by a Phe residue in the P_1 position, and does not inhibit rOPC or rOPV, but does inhibit *T. cruzi* oligopeptidase B (Burleigh *et al.*, 1997).

Of the proteinaceous inhibitors, only aprotinin was able to inhibit oligopeptidase B, with an efficiency that is comparable with leupeptin. The other inhibitors that were tested have higher molecular weights than aprotinin, so are probably unable to gain access to the

active site of the enzymes. This is identical to the results obtained for oligopeptidase B from wheat (Tsuji *et al.*, 2004), and other salivarian trypanosomes (Morty *et al.*, 1999a; Morty *et al.*, 1999b). Oligopeptidase B from *T. cruzi*, however, is not inhibited by aprotinin (Burleigh *et al.*, 1997).

E-64 is typically known as an irreversible inhibitor of cysteine proteases, and was once thought not to inhibit any other type of protease (Barrett *et al.*, 1982). However, in the present study, E-64 was found to inhibit the activity of both rOPC and rOPV. This is in agreement with previous findings that E-64 is a competitive reversible inhibitor of native oligopeptidase B from salivarian trypanosomes (Morty *et al.*, 1999a; Morty *et al.*, 1999b), for which similar micromolar K_i values were observed to those determined for rOPC and rOPV. Wheat germ oligopeptidase B is also inhibited by E-64 (Tsuji *et al.*, 2004), but oligopeptidase B from *T. cruzi* and *E. coli* are not (Kanatani *et al.*, 1991; Burleigh *et al.*, 1997). Inhibition of cysteine proteases by E-64 results from occupancy of the enzyme subsites followed by alkylation of the catalytic cysteine by the *trans* epoxide group (Salvesen and Nagase, 2001). It is possible that alkylation of a key cysteine residue near the active site of oligopeptidase B by E-64 prevents access of substrate to the active site, since it is unlikely that the inhibitor is interacting directly with an active site residue. Oligopeptidases that are not inhibited by E-64 might not offer suitable binding sites for the inhibitor. Alternatively, binding of the inhibitor to the protein might not affect the access of the substrate to the active site.

Of all the reversible inhibitors (leupeptin, antipain, aprotinin, benzamidine and E-64) of rOPC and rOPV determined in the present study, benzamidine was found to be the weakest, since it showed the highest K_i value. Benzamidine has also been found to inhibit other trypanosomal oligopeptidases with similar efficiency (Morty *et al.*, 1999a; Morty *et al.*, 1999b; Morty *et al.*, 2005b) as well as oligopeptidase B from wheat germ (Tsuji *et al.*, 2004) and *S. enterica* (Morty *et al.*, 2002), but not from *M. lacunata* (Yoshimoto *et al.*, 1995) and *E. coli* (Kanatani *et al.*, 1991).

The effect of reversible, competitive inhibitors (leupeptin, antipain, aprotinin, benzamidine and E-64) on the activity of rOPC and rOPV was found to be comparable with previously characterised native oligopeptidase B from *T. b. brucei*, *T. congolense*, and *T. evansi*. Only small differences were observed for the kinetic values that were obtained. Generally, rOPC was more susceptible to the inhibitors since higher K_i values were obtained for rOPV with each reversible inhibitor.

The thiol-reactive, irreversible, inhibitors iodoacetamide, sodium iodoacetate and *N*-ethylmaleimide were expected to inhibit the activity of rOPC and rOPV, but were found not to. These inhibitors have been shown to inhibit the activity of native oligopeptidase B from *T. b. brucei* (Morty *et al.*, 1999b) and *T. congolense* (Morty *et al.*, 1999a), albeit with very low efficiency. It is interesting to note that the recombinant enzymes were not as efficiently activated (1.5-fold for rOPC and 2.5-fold for rOPV) by reducing agents as reported for native *T. b. brucei* (3-fold; Morty *et al.*, 1999b) and *T. congolense* (7-fold; Morty *et al.*, 1999a) enzymes. Oligopeptidase B from *S. enterica* is also not inhibited by thiol-reactive inhibitors, nor does it undergo reductive activation (Morty *et al.*, 2002). A recent study on oligopeptidase B from *T. b. brucei* showed that Cys^{256} in the *T. b. brucei* sequence mediates inhibition by thiol-reactive inhibitors such as *N*-ethylmaleimide and iodoacetate, and that Cys^{559} and Cys^{597} are responsible for thiol-enhancement of activity (Morty *et al.*, 2005b). According to the alignment of the African trypanosomal oligopeptidase B sequences (Fig. 3.8, Section 3.4), the *T. congolense* and *T. vivax* enzymes have Ser and Thr at this position respectively. In the same vein, *S. enterica* oligopeptidase B has a Gly at this position when aligned with the *T. b. brucei* sequence (Morty *et al.*, 2002). It is therefore not surprising that these enzymes are not inhibited by thiol-reactive inhibitors, and contradicts the finding that native *T. congolense* oligopeptidase B is sensitive to these inhibitors.

The association rate constants for irreversible inhibitors that were determined in the present study were highly comparable to those observed for native oligopeptidase B from *T. b. brucei* and *T. congolense* (Morty *et al.*, 1999a; Morty *et al.*, 1999b). Also, the k_{ass} for inhibition by DCI was very similar to values reported for bovine trypsin (198 $M^{-1}.s^{-1}$)

and rat mast cell protease I (260 $M^{-1}.s^{-1}$) (Harper *et al.*, 1985). Inhibition by TLCK, and not by TPCK, was expected because it has already been shown that the active site of oligopeptidase B prefers basic residues (such as lysine in TLCK). Also, TLCK is known to inhibit trypsin-like enzymes (such as oligopeptidase B), whereas TPCK is an inhibitor of chymotrypsin-like enzymes (Beynon and Salvesen, 1989). TLCK (10 μM) is reported to inhibit *T. congolense* oligopeptidase B with a k_{ass} of 4.49×10^5 $M^{-1}s^{-1}$ and $t_{½}$ of 7.97 s (Morty *et al.*, 1999a). This is approximately 30 times higher than the k_{ass} obtained in the present study, but the values for $t_{½}$ are comparable.

Like oligopeptidase B from most other sources, rOPC and rOPV show enhanced activity in the presence of reducing agents such as DTT (Morty and Burleigh, 2004). It is not currently understood how the enhanced effects of reducing agents are mediated by the cysteine residues of oligopeptidase B. It has however been proposed that the reduction of intramolecular disulfide bridges may promote conformational changes with activity-enhancing effects (Morty *et al.*, 2005b). In the present study, the optimum concentration of reducing agent was 5 mM for both DTT and reduced glutathione, and 1 mM for cysteine, above which the activity of the enzyme appeared to decline. Concentrations above 10 mM appeared to have an inhibitory effect on the enzymes, but whether this was due to enzyme denaturation or adoption of unfavourable conformations is unclear.

The pH profile for the activity of rOPC and rOPV against Z-Arg-Arg-AMC in the present study is highly similar to the profile determined for oligopeptidase B from *T. b. brucei* (Morty *et al.*, 1999b), with a basic pH optimum of around 8.5. The pH stability profile is very similar again, with optimal pH stability at pH 6, shifting to pH 8-8.5 in the presence of DTT. Therefore, oligopeptidase B is very active and stable at physiological pH 7.4. The enzymes also show high activity at physiological ionic strength (I = 0.1). This supports the findings that the enzyme could be active in the bloodstream of the infected host (Morty *et al.*, 2001).

The activity of the two recombinant enzymes in the present study was not affected by any of the potentially activating substances that were assayed. Previously it has been shown

that the activity of native oligopeptidase B from *T. b. brucei* and *T. congolense* was enhanced by 50 μM spermine and spermidine (between 60-80%) and in the presence of 30 $\mu g.ml^{-1}$ heparin by 58% and 66% respectively (Morty *et al.*, 1999a; Morty *et al.*, 1999b). The inhibition of rOPC and rOPV activity by certain metal ions was consistent with the literature, and this has been explained by the probable formation of mercaptides between heavy metal ions and a reactive thiol group (Barron, 1951).

Some slight differences in the enzymatic behaviour of rOPC and rOPV were observed throughout this chapter. Recombinant OPV was found to be more sensitive to extremely high pH values, showing less activity and stability then rOPC at higher pH. Recombinant OPV was also found to be less sensitive than rOPC to conditions of increasing ionic strength. Recombinant OPV showed the greatest affinity for the substrate Z-Gly-Gly-Arg-AMC, while still showing a similar affinity to rOPC for the substrate Z-Arg-Arg-AMC. Recombinant OPV showed greater activation by reducing agents than rOPC, and also appeared to be less sensitive to deactivation by reversible inhibitors (leupeptin, antipain, aprotinin, benzamidine and E-64). These differences could be accounted for by the differences in their amino acid sequences, since they only show 73% similarity (Chapter 3). Once the enzymes' 3D structures are elucidated, perhaps these subtle differences in behaviour can be explained.

In conclusion, despite some small discrepancies, rOPC and rOPV behave as expected for oligopeptidase B, with similar substrate preferences, as well as inhibitor and pH profiles. Therefore, these recombinant proteins can be produced and purified in relatively large amounts for further study of oligopeptidase B, as well as for use in an anti-disease vaccine. The present study represents the first enzymatic characterisation of oligopeptidase B from *T. vivax*.

CHAPTER 6

ISOLATION OF α_2-MACROGLOBULIN

6.1 Introduction

A major objective of the present study was to investigate the interaction of α_2M with several cysteine proteases, including congopain, and the serine protease oligopeptidase B. This was done ultimately to use α_2M as an adjuvant for anti-congopain and anti-oligopeptidase B antibody production. It was therefore necessary to purify α_2M from suitable sources. Bovine α_2M was used in the present study because its suitability for use in an anti-disease vaccine was to be assessed, since the vaccine is ultimately to be applied to cattle. Rabbit α_2M was also used in the present study, because rabbits were used as experimental animals for raising antibodies (Chapter 8), and the use of 'self' protein would serve as a comparison to the use of foreign, bovine protein. In this chapter the isolation of bovine and rabbit α_2M for the above studies is described.

The partial amino acid sequence of bovine α_2M, corresponding to residues 538-954 of human α_2M, has been predicted from its cDNA sequence. This sequence, containing the bait region, was found to be significantly similar to those of human, mouse and rat α_2Ms (59, 62 and 55% similarity, respectively). The bait regions, however, were of dissimilar sequence and of different length (Shibata *et al.*, 2003). The sequences of the RBD are known for many species, and show a high degree of sequence homology, which is why αMs from different animal species cross-react with similar affinities for the α_2M receptor (Kaplan and Nielsen, 1979; Enghild *et al.*, 1989). The three-dimensional structures for the RBD of human (Huang *et al.*, 2000) and bovine α_2M (Jenner *et al.*, 1998) have been determined and compared, and found to be very similar (Fig. 6.1). The one difference between the two structures is the orientation of helix H2 relative to the body of the protein in human RBD, probably due to the presence of an extra residue (His^{68}) in the loop that links the C-terminal end of the helix to the body of the protein in human RBD (Huang *et al.*, 2000).

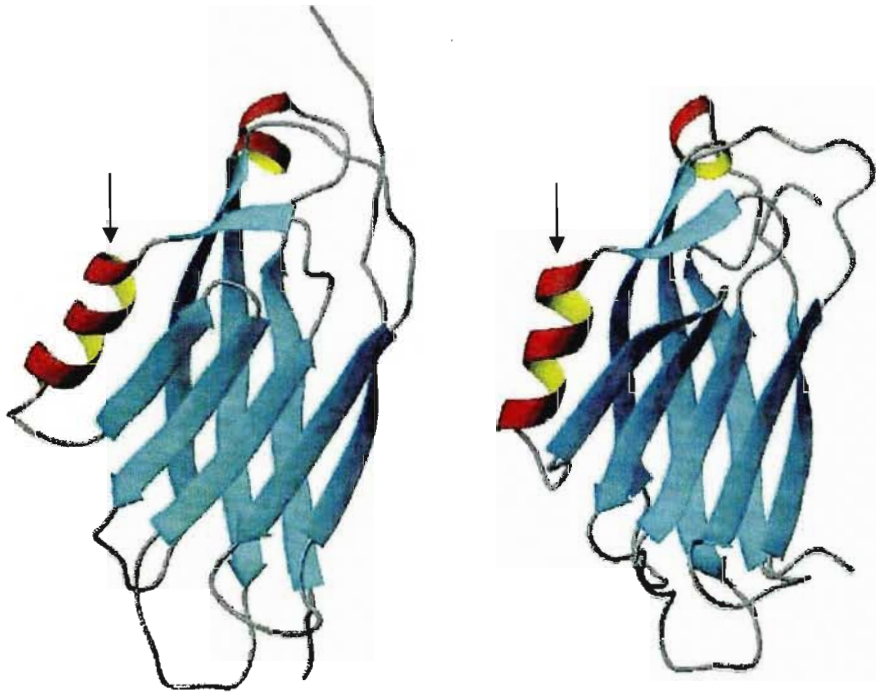

Figure 6.1. Ribbon diagram of the NMR structure of human RBD (left) and the crystal structure of bovine RBD (right) (Huang *et al.*, 2000). Helix H2 is shown in red on the extreme left of each molecule, indicated by the arrows.

Rabbit plasma contains two proteins, α_1M and α_2M, that are chemically and functionally homologous to human and bovine α_2M (Hinata *et al.*, 1987). However, studies suggest that rabbit α_1M and α_2M are immunologically distinct, since antibodies directed against rabbit α_1M are able to cross-react with human α_2M, but not with rabbit α_2M (Kaplan and Nielsen, 1979), and antibodies raised against rabbit α_2M do not react with rabbit α_1M (Kurdowska *et al.*, 2002). Despite immunological differences, rabbit α_1M and α_2M are taken up by the same receptor (Kaplan and Nielsen, 1979). The two proteins can be separated by ion exchange chromatography, as they elute at different salt concentrations, and can also be separated by PAGE on a low percentage gel. In SDS-PAGE, however, the two proteins occur as a single band (Kaplan and Nielsen, 1979). A recent study on the characteristics of the two rabbit α-macroglobulins has found that the properties of rabbit α_1M, upon activation, are different from those of rabbit and human α_2M (Banbula *et al.*, 2005). The study suggests that the primary function of rabbit α_1M may not be

protease inhibition. Due to the higher similarity of rabbit α_2M to human (and thus bovine) α_2M, the protein was purified in the present study for use as an adjuvant.

6.2 Isolation of bovine and rabbit α_2M

There are a variety of procedures for the isolation of α_2M. The procedure followed in this study was a combination of the human α_2M isolation procedures of Salvesen and Enghild (1993) and Barrett (1981). The starting material for the isolation was plasma. Serum was not suitable because it contains the "fast" or receptor recognised form of α_2M (Salvesen and Enghild, 1993). The first step in the isolation procedure used in this study was precipitation with PEG 6 000. The initial precipitate, containing β-lipoprotein, is removed by centrifugation, and the second precipitate (formed at 5.5-12.5% (w/v) PEG) is collected by centrifugation for further purification (Barrett, 1981). PEG 6 000 replaced ammonium sulfate in this fractionation step when it was discovered that ammonium sulfate reacts with the thioester (Harpel, 1976).

The next step in the isolation procedure was affinity purification by zinc chelate chromatography. Metal chelate chromatography was first described by Porath *et al.* (1975). A gel-matrix was developed whereby a ligand, such as iminodiacetic acid, is immobilised on epoxy-activated agarose, and can be converted to a metal chelate form by binding to metal ions. Binding to the matrix is based on interactions between electron donor groups on the surface amino acid side chains of the protein and the immobilised metal group as the electron acceptor. Elution is carried out with a decrease in pH, resulting in protonation of the electron donor atom on the protein, thereby disrupting the coordination process. This technique was applied to human α_2M isolation by Kurecki *et al.* (1979) on the basis of the zinc binding capacity of α_2M (Giroux, 1975). The final step in the α_2M purification procedure was gel filtration, which was added as a subsequent step to zinc chelate chromatography by Imber and Pizzo (1981), since the α_2M eluted from the zinc chelate column still contains some contaminating proteins.

6.2.1 PEG 6 000 precipitation

PEG is a hydrophilic polymer with different average molecular weights. It is thought that PEG precipitates proteins by excluding the proteins from water, which, with increasing PEG concentration, brings the protein to its solubility limit. PEG precipitation is especially suited to the isolation of large proteins (Polson, 1977), such as α_2M (720 kDa).

6.2.1.1 Reagents

Acid citrate dextrose (ACD). Citric acid.H_2O (8 g), tri-sodium citrate.H_2O (22 g) and glucose.H_2O (24.42 g) were dissolved in dH_2O (950 ml) and made up to 1 l.

Aqueous 25% (w/v) PEG 6 000. PEG 6 000 (62.5 g) was dissolved in dH_2O to a final volume of 250 ml.

Buffer A (20 mM sodium phosphate buffer, pH 6, 150 mM NaCl). $NaH_2PO_4.2H_2O$ (6.252 g) and NaCl (17.532 g) were dissolved in dH_2O (1.9 l), titrated to pH 6 with NaOH and made up to 2 l with dH_2O.

6.2.1.2 Procedure

Bovine blood was collected into a flask containing the anticoagulant ACD (7.5 ml for every 42.5 ml blood). Rabbit blood was collected in K3E Vacutainer™ tubes (containing EDTA). Blood was centrifuged to remove cells (bovine: 10 000 *g*, 20 min, 4°C; rabbit: 200 *g*, 30 min, 4°C). The resulting plasma was stored, if necessary, at –20°C until required.

Plasma was mixed with 0.28 volumes of aqueous 25% (w/v) PEG 6 000, stirred (10 min, RT), and left to stand (20 min, RT). The mixture was centrifuged (10 000 *g*, 20 min, 4°C). The supernatant was mixed with 0.72 volumes (based on the original plasma volume) 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 precipitate dissolved in a minimum volume of buffer A. Any remaining PEG was removed by dialysis against buffer A overnight at 4°C (2 changes). The final volume of sample was divided into aliquots not exceeding the equivalent of 50 ml plasma and stored at -20°C, if necessary, until required.

6.2.2 Zinc chelate chromatography

6.2.2.1 Reagents

Buffer A (20 mM sodium phosphate buffer, pH 6, 150 mM NaCl). As per Section 6.2.2.1.

Buffer B (50 mM Na_2EDTA buffer, pH 7, 500 mM NaCl). Na_2EDTA (9.306 g) and NaCl (14.612 g) were dissolved in dH_2O (450 ml), titrated to pH 7 with NaOH, and made up to 500 ml with dH_2O.

Buffer C (250 mM sodium acetate buffer, pH 5, 150 mM NaCl). Glacial acetic acid (7.2 ml) and NaCl (4.383 g) were dissolved in dH_2O (450 ml), titrated to pH 5 with NaOH, and the volume made up to 500 ml with dH_2O.

Buffer D (20 mM sodium cacodylate buffer, pH 5, 150 mM NaCl). Sodium cacodylate trihydrate (4.28 g) and NaCl (4.383 g) were dissolved in dH_2O (950 ml), titrated to pH 5 with HCl, and made up to 1 l with dH_2O.

3 mg/ml $ZnCl_2$. $ZnCl_2$ (0.3 g) was dissolved in dH_2O (100 ml).

2 M Na_2CO_3. Na_2CO_3 (2.08 g) was dissolved in dH_2O (10 ml).

1 M Sodium phosphate buffer, pH 7. $NaH_2PO_4.2H_2O$ (39 g) was dissolved in dH_2O (200 ml), titrated to pH 7 with NaOH, and made up to 250 ml with dH_2O.

Zinc chelate column. The supplied saline slurry of iminodiacetic acid immobilised on cross-linked 4% beaded agarose was packed into a glass column under gravity. The column bed was washed with several column volumes of buffer B, followed by several column volumes of distilled water. The matrix was converted to a zinc chelate form by passing several column volumes of 3 mg/ml $ZnCl_2$ through the column until the column effluent formed a precipitate with 2 M Na_2CO_3. The column bed was washed with several column volumes of buffer C and equilibrated with buffer A.

6.2.2.2 Procedure

The re-dissolved PEG 6 000 precipitate was loaded onto the zinc chelate column (15 × 95 mm, 50 $ml.h^{-1}$, RT), which had been prepared as described in Section 6.2.2.1. After elution of unbound protein with buffer A, elution of the bound protein was effected with buffer D. The eluted protein was titrated to pH 7 with 1 M sodium phosphate buffer, pH 7, and concentrated by dialysis against PEG 20 000 (Section 2.3.1).

6.2.3 Sephacryl S-300 HR chromatography

6.2.3.1 Reagents

MEC buffer [100 mM sodium phosphate buffer, pH 7, 0.02% (w/v) NaN_3]. As per Section 2.12.2.1.

Calibration solution. Blue dextran (6 mg), ferritin (15 mg), BSA (15 mg), ovalbumin (15 mg) and myoglobin (15 mg) were dissolved in dH_2O (3 ml).

6.2.3.2 Procedure

A Sephacryl S-300 HR column (25 × 840 mm, 25 $ml.h^{-1}$, 4°C) was calibrated with the calibration solution (3 ml) consisting of blue dextran (2 000 kDa), ferritin (440 kDa), BSA (66 kDa), ovalbumin (45 kDa), and myoglobin (18.8 kDa). The elution volume (V_e) of blue dextran was an indication of the void volume of the column (V_o). The availability constant (K_{av}) was calculated for each protein by the equation

$$K_{av} = \frac{V_e - V_o}{V_t - V_o}$$

where, K_{av} is the availability constant,
V_e the elution volume,
V_o the void volume, and
V_t the total column volume.

A Fischer plot of K_{av} versus log molecular weight was used as a standard curve (Fig. 6.2). Molecular weight could be estimated from the calculation of a protein's K_{av} from its elution volume (Dennison, 1999). The column was equilibrated with one column volume

of MEC buffer, where elution of the sample served as equilibration for the next sample. The concentrated sample from zinc chelate chromatography (between 2-5% of the column volume) was applied to the column and eluted with MEC buffer.

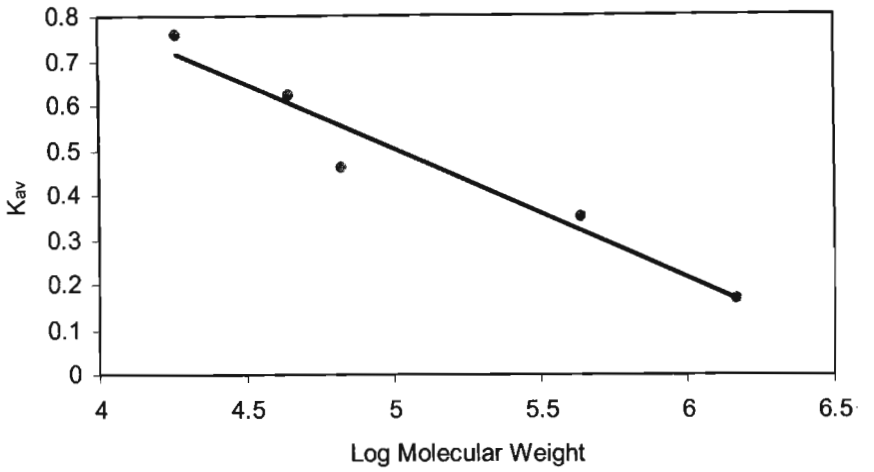

Figure 6.2. A Fischer plot relating log molecular weight to the availability constant, K_{av}. The equation of the trend line is given by y = -0.2892x + 1.9485, with a correlation coefficient of 0.9473.

6.2.4 Determination of inhibitory activity of α_2M by trypsin titration

Determination of the active concentration, i.e. the "slow" form of α_2M, was carried out by titration with trypsin (Salvesen and Enghild, 1993). The activity of α_2M was determined by its capacity to inhibit the hydrolysis of hide powder azure by trypsin. Hide powder azure is an insoluble particulate protein mixture that has been dyed blue. It is a highly sensitive general substrate for proteases that releases a dark blue colour into solution upon proteolytic degradation, which can be quantified by measurement of absorption at 595 nm. The trypsin used in this experiment was pre-treated with TPCK to inhibit any chymotrypsin activity in the sample.

6.2.4.1 Reagents

Assay buffer [100 mM Tris-HCl buffer, pH 8, 10 mM $CaCl_2$, 0.05% (v/v) Triton X-100].
Tris (6.055 g) and $CaCl_2$ (0.555 g) were dissolved in dH_2O (450 ml) and titrated to pH 8 with HCl. Triton X-100 (0.25 ml) was added and the volume was made up to 500 ml with dH_2O.

Substrate solution (12.5 mg/ml hide powder azure, 600 mM sucrose in assay buffer). Hide powder azure (0.1875 g) and sucrose (3 g) were dissolved in assay buffer (15 ml).

3 mg/ml Trypsin. TPCK-treated trypsin (3 mg) was dissolved in assay buffer (1 ml).

6.2.4.2 Procedure

Trypsin was diluted with assay buffer so that 100 µl contained 10-90 pmol. Ten aliquots of α_2M (50 pmol) were diluted to 500 µl with assay buffer in microfuge tubes. Trypsin (100 µl, i.e. 10-90 pmol) was added to the α_2M, with one aliquot receiving no trypsin, thus acting as a blank. After incubation (20 min, RT), substrate solution (400 µl) was added to each tube. Mixtures were incubated with agitation (1 h, RT) and unhydrolysed substrate collected by centrifugation (13 700 *g*, 5 min, RT). Supernatant absorbances at 595 nm were plotted against trypsin concentration. The concentration of trypsin required to saturate the inhibitory capacity of 50 pmol α_2M was multiplied by two to reflect a percentage inhibitory activity.

6.2.5 Assessment of cross-reactivity between α_2M from different species

An ELISA was conducted to establish whether antibodies raised in chickens against bovine α_2M would cross-react with rabbit α_2M.

6.2.5.1 Reagents

As per Section 2.8.1.

6.2.5.2 Procedure

As per Section 2.8.2. Serial two-fold dilutions of affinity purified chicken anti-bovine α_2M IgY, starting from 10 µg/ml, were prepared in the plate with 0.5% BSA-PBS. The HRPO-conjugated anti-chicken secondary antibody was diluted to 1:30 000 in 0.5% BSA-PBS.

6.2.6 N-terminal sequencing of rabbit α_2M

To identify which form of αM was purified from rabbit plasma in the present study, the N-terminus of the protein was sequenced, as in Section 4.4.4.

6.3 Results

6.3.1 Isolation of α_2M

Affinity chromatography of PEG precipitated plasma on a zinc chelate column resulted in a single, sharp protein peak after application of the elution buffer for bovine (Fig. 6.3) and rabbit (Fig. 6.4) α_2M isolation. Subsequent MEC on Sephacryl S-300 HR led to the separation of two major protein peaks in each case (bovine, Fig. 6.5; rabbit, Fig. 6.6). For both species, the high molecular weight peak corresponded to an elution volume of 220 ml, which gave an estimated molecular weight of approximately 720 kDa. The lower molecular weight peaks had molecular weights estimated between 45 and 66 kDa. The high molecular weight fractions for the bovine α_2M isolation were subjected to SDS-PAGE on 7.5% acrylamide gels under reducing conditions (Fig. 6.7). The major band on the gel had a molecular weight of approximately 170 kDa, which corresponds to the monomer of α_2M that is expected to result after reduction. At the peak of elution of α_2M, additional bands of approximately 120 and 60 kDa were observed, which correspond to the autolytic fragments of the monomer which can result from reducing conditions (Salvesen and Enghild, 1993).

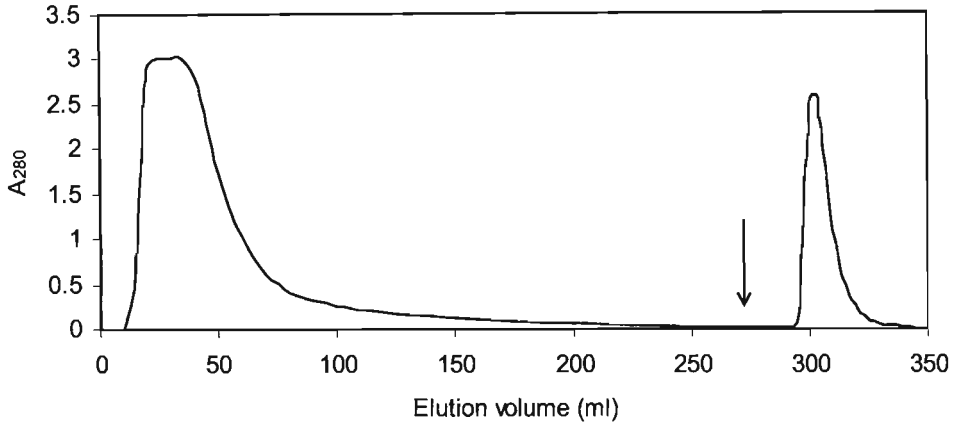

Figure 6.3. Affinity chromatography of bovine α_2M on a zinc chelate column. Zinc-chelated agarose-iminodiacetic acid (15×95 mm, 50 ml.h^{-1}) was equilibrated with buffer A (200 mM sodium phosphate buffer, 150 mM NaCl, pH 6). After elution of unbound protein with buffer A, bound protein was eluted by application of buffer D (20 mM sodium phosphate buffer, 150 mM NaCl, pH 5), applied at the point indicated by the arrow (↓).

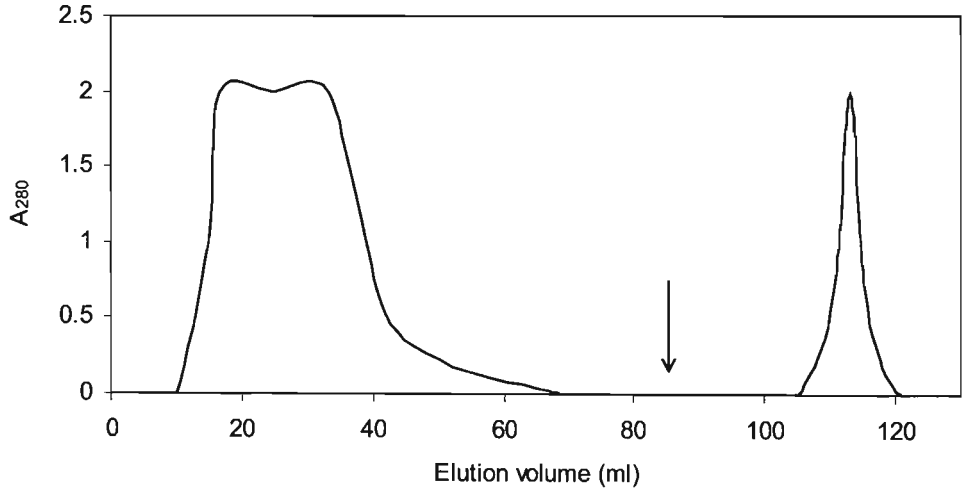

Figure 6.4. Affinity chromatography of rabbit α_2M on a zinc chelate column. Zinc-chelated agarose-iminodiacetic acid (15×95 mm, 50 ml.h^{-1}) was equilibrated with buffer A (200 mM sodium phosphate buffer, 150 mM NaCl, pH 6). After elution of unbound protein with buffer A, bound protein was eluted by application of buffer D (20 mM sodium phosphate buffer, 150 mM NaCl, pH 5), applied at the point indicated by the arrow (↓).

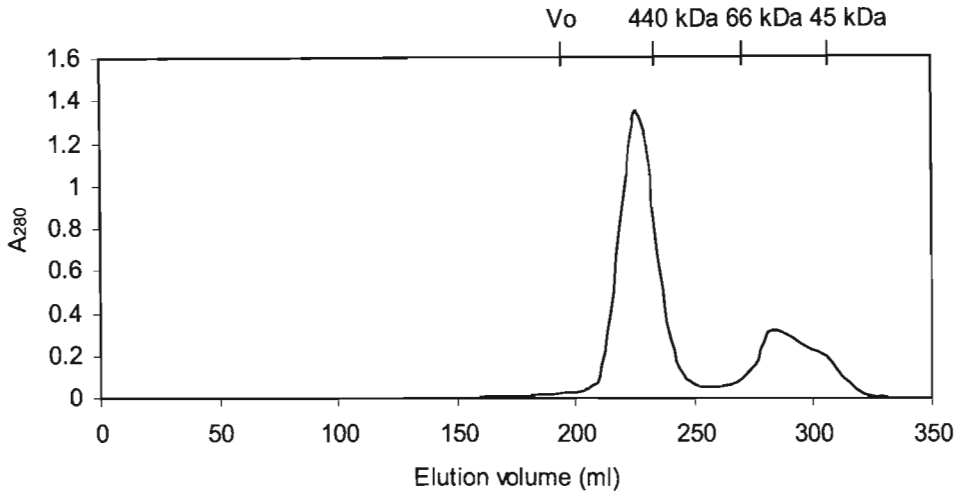

Figure 6.5. Molecular exclusion chromatography of bovine α_2M on Sephacryl S-300 HR. Sephacryl S-300 HR (25×840 mm, 25 ml.h^{-1}) was equilibrated with MEC buffer (100 mM sodium phosphate buffer, pH 7, 0.02% NaN_3). Sample (5 ml) was loaded, and protein eluted with MEC buffer. The molecular weights of the calibration proteins are indicated at the top.

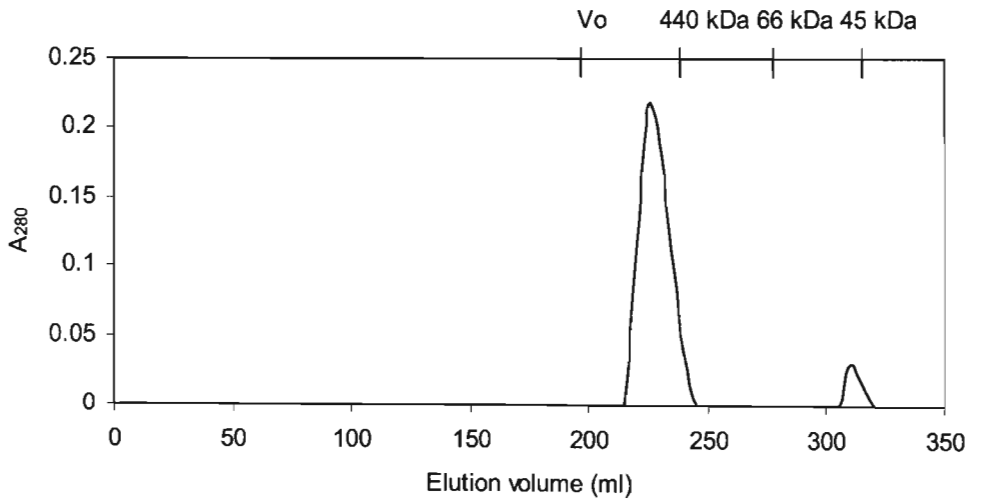

Figure 6.6. Molecular exclusion chromatography of rabbit α_2M on Sephacryl S-300 HR. Sephacryl S-300 HR (25×840 mm, 25 ml.h^{-1}) was equilibrated with MEC buffer (100 mM sodium phosphate buffer, pH 7, 0.02% NaN_3). Sample (5 ml) was loaded, and protein eluted with MEC buffer. The molecular weights of the calibration proteins are indicated at the top.

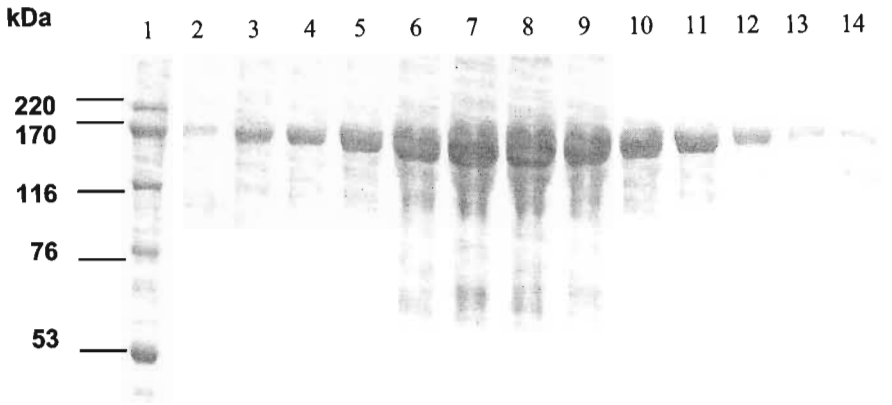

Figure 6.7. Reducing SDS-PAGE of bovine high molecular weight α_2M fractions from Sephacryl S-300 HR. Lane 1, high range molecular weight markers corresponding to myosin (220 kDa), α_2M (170 kDa), β-galactosidase (116 kDa), transferrin (76 kDa), and glutamic dehydrogenase (53 kDa); lanes 2-14, fractions eluting at 195, 200, 205, 210, 215, 220, 225, 230, 235, 240, 245, 250 and 255 ml, respectively, from the MEC column (10 µl). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

Reducing SDS-PAGE of high molecular weight fractions was not done for the rabbit α_2M isolation due to the small amount of protein present. The two peaks in the MEC elution profile for rabbit α_2M were considered to be sufficiently well separated to pool the high molecular weight peak without contamination by the low molecular weight peak. The maximum yield of bovine α_2M was 44 mg from 50 ml of plasma, whilst for rabbit α_2M the yield was 6 mg from 11 ml plasma. By trypsin titration bovine α_2M was found to be 100% active, whilst rabbit α_2M was found to be 40% active.

Samples were taken at each stage of the purification procedure for both rabbit and bovine α_2M. These samples were electrophoresed on 4-15% SDS-PAGE gradient gels under non-reducing conditions to evaluate the purification process (Figs. 6.8 and 6.9).

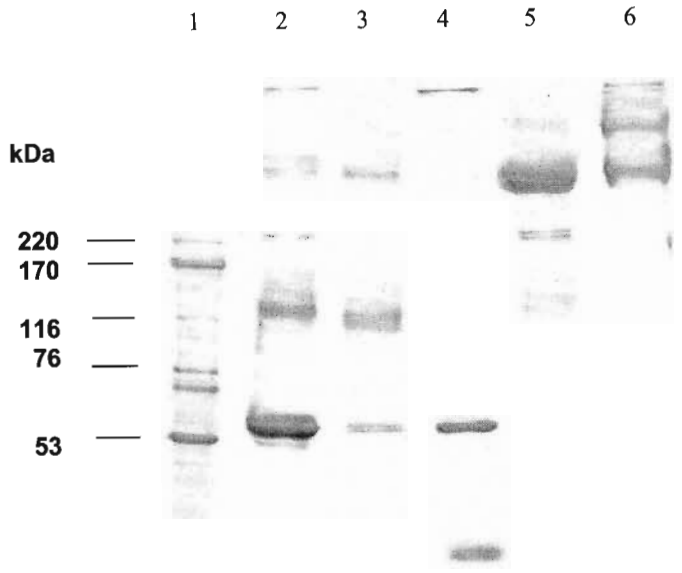

Figure 6.8. Non-reducing SDS-PAGE on a 4-15% acrylamide gradient gel of samples at each stage of the bovine α_2M purification procedure. Lane 1, high-range molecular weight markers (as per Fig. 6.7); lane 2, bovine plasma (15 μg); lane 3, resuspended PEG precipitate (10 μg); lane 4, unbound fraction from the zinc chelate column (10 μg); lane 5, adsorbed protein eluted from the zinc chelate column (10 μg); lane 6, pooled α_2M fractions after MEC (10 μg). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

The samples for both species displayed similar banding patterns (Figs. 6.8 and 6.9). The samples of plasma showed multiple bands, with prominent bands at approximately 60 and 120 kDa (lane 2). The main difference between the plasma and the redissolved final PEG precipitate (lane 3) appeared to be a reduction in the intensity of the band at 60 kDa, indicating that a large proportion of the protein of this size was removed by PEG precipitation. The sample of unbound protein washed from the zinc chelate column (lane 4) showed bands at approximately 120 and 60 kDa. In the case of bovine (Fig. 6.8) samples, the more prominent band was at approximately 60 kDa, whilst for the rabbit sample (Fig. 6.9), the more prominent band was at approximately 120 kDa. The bovine sample (Fig. 6.8) also showed a very low molecular weight band.

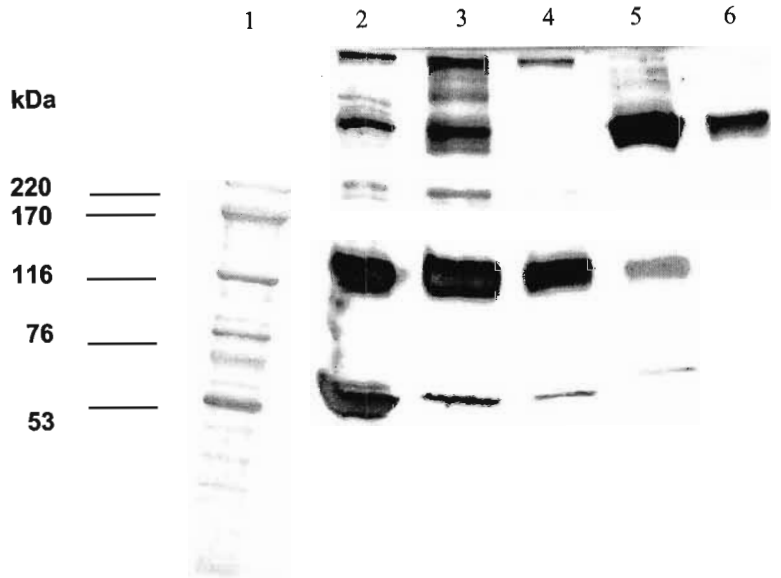

Figure 6.9. Non-reducing SDS-PAGE on a 4-15% acrylamide gradient gel of samples at each stage of the rabbit α_2M purification procedure. Lane 1, high-range molecular weight markers (as per Fig. 6.7); lane 2, rabbit plasma (1 μg); lane 3, resuspended PEG precipitate (750 ng); lane 4, unbound fraction from the zinc chelate column (750 ng); lane 5, adsorbed protein eluted from the zinc chelate column (750 ng); lane 6, pooled α_2M fractions after MEC (750 ng). Proteins were silver stained (Section 2.6.2).

The adsorbed sample eluted from the zinc chelate column (Figs. 6.8 and 6.9; Lane 5) showed a prominent high molecular weight band, possibly corresponding to the α_2M dimer of 360 kDa, which is expected to form under non-reducing conditions (Salvesen and Enghild, 1993). In the case of each species, though, there were a number of lower molecular weight contaminating proteins, illustrating the need for further purification of the sample after zinc chelate chromatography. The pooled high molecular weight peaks after MEC (Lane 6) showed the *ca.* 360 kDa dimer of α_2M for both species. Bovine (Fig. 6.8) α_2M also showed higher molecular weight bands, probably corresponding to the intact α_2M tetramer of 720 kDa, and possibly some dimers that interacted to form complexes of multiples of 360 kDa.

6.3.2 Evaluation of purity of α_2M samples

Purity of the resulting α_2M isolates from bovine and rabbit plasma was assessed by silver staining of the proteins after SDS-PAGE under reducing conditions (Fig. 6.10). The molecular weight of the α_2M monomer for each species appears to differ slightly as they did not have the same mobilities in the gel. Both samples showed a single band of

protein, with bovine α_2M at approximately 169.5 kDa (lane 2) and rabbit α_2M at approximately 172 kDa (lane 3), indicating that each isolation procedure resulted in an electrophoretically homogeneous protein. The difference in molecular weight should be more noticeable in the complex forms of the proteins, as the effect should be amplified. Unfortunately, due to the nature of the forces holding the α_2M tetramer together, i.e. non-covalent association, the molecular weight of the tetramer cannot be determined by reducing SDS-PAGE. Gradient PAGE could be used to this effect provided molecular weight markers of the appropriate size range were available for use in gradient gels.

The α_2M dimers from the two species were compared by SDS-PAGE under non-reducing conditions (Fig. 6.11). The resulting bands of the dimers were broad and not clearly defined, which made comparison of molecular weight difficult and inaccurate. However, it still seemed that the rabbit protein (lane 3) migrated a shorter distance in the gel, thus suggesting a higher molecular weight.

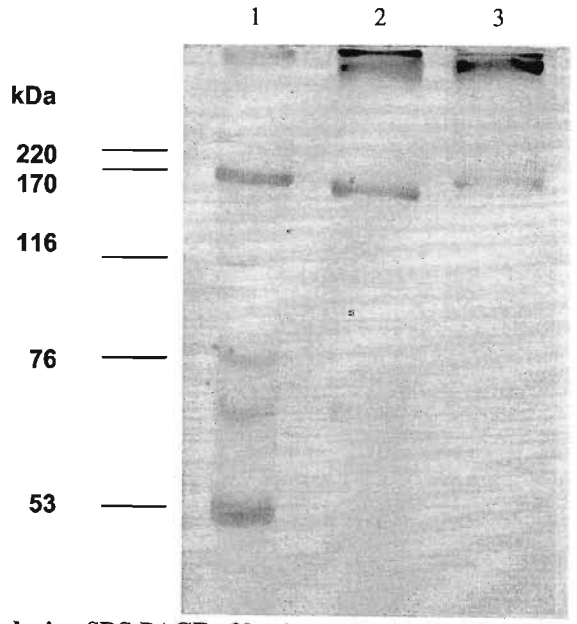

Figure 6.10. Reducing SDS-PAGE of bovine and rabbit α_2M to assess purity. Samples of bovine and rabbit α_2M were reduced and electrophoresed on a 7.5% acrylamide gel. Lane 1, high-range molecular weight markers (as per Fig. 6.7); lane 2, bovine α_2M (1 μg); lane 3, rabbit α_2M (1 μg). Proteins were silver stained (Section 2.6.2).

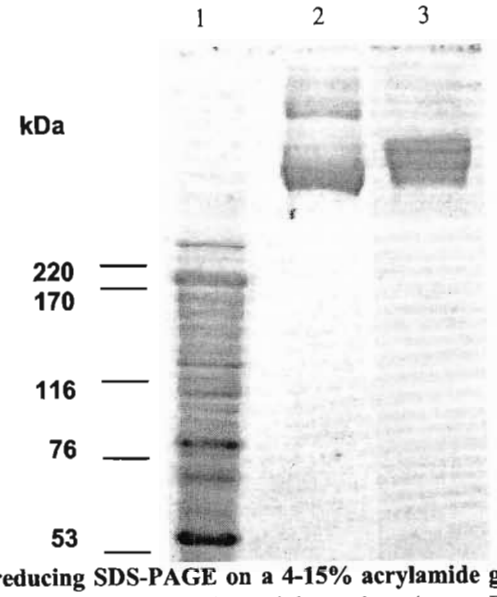

Figure 6.11. Non-reducing SDS-PAGE on a 4-15% acrylamide gradient gel comparing bovine and rabbit α_2M. Lane 1, high-range molecular weight markers (as per Fig. 6.7); lane 2, bovine α_2M (5 μg); lane 3, rabbit α_2M (5 μg). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

Cross-reactivity of affinity purified chicken anti-bovine α_2M antibodies with rabbit α_2M was examined by ELISA (Fig. 6.12). A strong signal was observed for bovine α_2M at very low levels of antibody (less than 10 μg/ml). No significant reactivity was observed for rabbit α_2M. Non-immune controls were included for each species of α_2M and found to be of negligible absorbance at 405 nm.

Bovine and rabbit α_2M were compared by non-denaturing PAGE on a 5% acrylamide gel (Fig. 6.13). Molecular weight markers were utilised in this system to illustrate the large size of the proteins, giving only a rough indication of size on the gel. They were also treated with a two times molar excess of papain for comparison of their "fast" and "slow" forms. Without treatment with papain, bovine α_2M (lane 2) appeared as one prominent band. With papain treatment (lane 3), the prominent band appeared lower down in the gel, indicating a more compact, "fast" form of α_2M. Without papain treatment, the rabbit protein (lane 4) appears as two distinct bands, the more prominent of which did not migrate as far in the gel. This could indicate the presence of both the "fast" and "slow" forms of the protein. With papain treatment, the rabbit protein (lane 5) shows a slight difference in migration distance for the upper, more prominent band, with the lower band still evident. The very high molecular weight bands were ignored for all lanes on the

basis that they were probably formed as a result of protein-protein interaction between α_2M molecules.

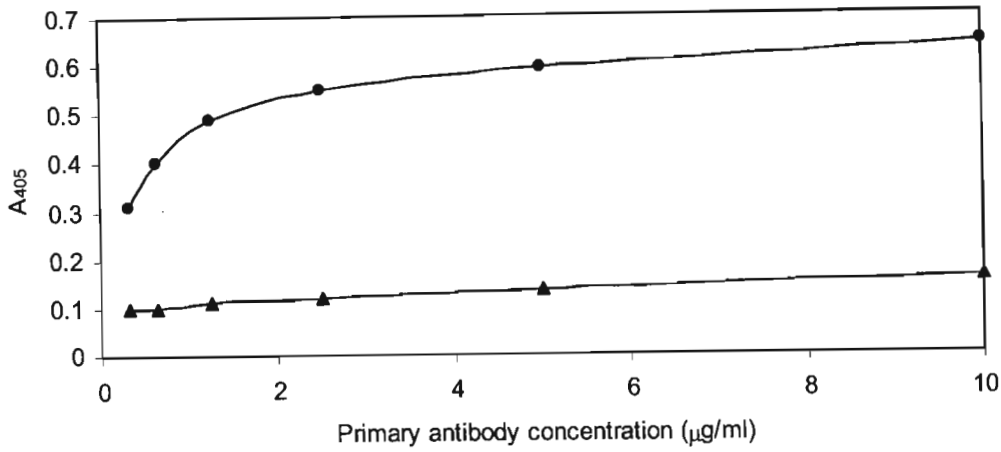

Figure 6.12. ELISA to determine cross-reactivity of anti-bovine α_2M antibodies with rabbit α_2M. Bovine (●) and rabbit α_2M (▲) were coated onto an ELISA plate (1 μg/ml in PBS, 150 μl per well, overnight, 4°C), and affinity purified chicken anti-bovine α_2M was titrated between 10 and 0.3125 μg/ml and incubated (in BSA-PBS, 100 μl per well, 2 h, 37°C). Rabbit anti-chicken-HRPO secondary antibodies (in BSA-PBS, 120 μl per well, 1 h, 37°C) and ABTS/H_2O_2 (150 μl per well) were used as the detection system. Absorbance readings at 405 nm represent the average of duplicate experiments.

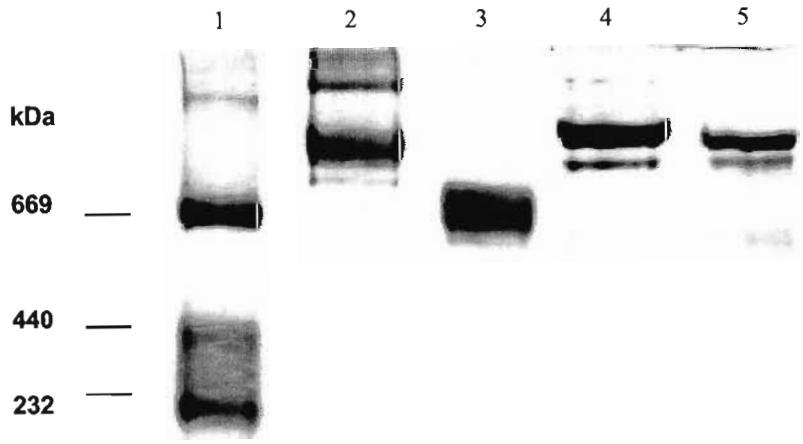

Figure 6.13. Non-denaturing PAGE on a 5% acrylamide gel comparing bovine, and rabbit α_2M. Samples of bovine and rabbit α_2M were electrophoresed alongside bovine and rabbit α_2M incubated at 37°C for 20 min with 2 times molar excess of activated papain. Lane 1, native PAGE markers consisting of thyroglobulin (669 kDa), ferritin (440 kDa), and catalase (232 kDa); lane 2, bovine α_2M (10 μg); lane 3, bovine α_2M treated with papain (10 μg); lane 4, rabbit α_2M (10 μg); lane 7, rabbit α_2M treated with papain (10 μg). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

6.3.3 N-terminal sequencing of rabbit α_2M

The first seven amino acids at the N-terminus of the protein isolated from rabbit plasma were found to be TNKPQYI. This is identical to the N-terminal sequence of rabbit α_2M, TNKPQYIVLVPSELY, reported by Banbula *et al.* (2005).

6.4 Discussion

The α_2M sample resulting from zinc chelate chromatography was found to be impure due to the presence of a low molecular weight peak in the MEC elution profile for each species, ranging between 45 and 66 kDa, and by non-reducing SDS-PAGE, necessitating further purification. MEC on Sephacryl S-300 HR was found to be an effective technique for the removal of the low molecular weight contaminating proteins. Proteins that could possibly be the cause of contamination of the sample after zinc chelate chromatography are albumin (66 kDa), and haemoglobin (64 kDa). The sizes of the two isolated α_2Ms under reducing conditions, i.e. monomeric forms, were approximately169.5 kDa (bovine) and 172 kDa (rabbit). This is very similar to the 170 kDa size reported for the bovine monomeric form (Shibata *et al.*, 2003), but less than the 180 kDa monomeric size reported for all species of α_2M (Salvesen and Enghild, 1993).

A higher yield of bovine α_2M compared to rabbit α_2M was obtained from the equivalent of 50 ml plasma, i.e. 44 mg and 27 mg respectively. Yields achieved by other authors for human α_2M range from 62.5 mg (Salvesen and Enghild, 1993) to 32 mg (Barrett, 1981) from 50 ml plasma. The bovine α_2M isolated in the present study was found to be 100% active, i.e. isolated in the "slow" form. Rabbit α_2M was found to be 40% active after isolation, which correlates with other rabbit α_2M isolations where activities of between 20 and 40% were reported (Kaplan and Nielsen, 1979). It is not likely for "fast" α_2M to be isolated from plasma since "fast" α_2M is rapidly removed from circulation by cells bearing the α_2MR (Barrett, 1981). Therefore, it appears that rabbit α_2M is more sensitive to conversion to the "fast" form during the isolation procedure than bovine and human α_2M. The combination of protein isolation techniques used in the present study has not been used for α_2M isolation from species other than human. Results presented here

suggest that the isolation technique used was effective for bovine and rabbit α_2M, yielding pure protein at moderate to high yields. This isolation technique would probably be suitable for isolation of α_2M from other animal species, especially those that are found to have a high degree of similarity to the bovine and human proteins.

At the outset of the present study it was not known whether rabbit α_1M or α_2M, or both, would be isolated by the techniques used. Rabbit α_1M and α_2M can be separated by native PAGE at low percentages of acrylamide (Kaplan and Nielsen, 1979). Native PAGE at 5% acrylamide showed the presence of two high molecular weight bands of protein in the rabbit α_2M sample. An important factor to note was that rabbit αMs are not stable under storage conditions after isolation (Chu *et al.*, 1994), so it is likely that the rabbit protein was partially converted to the "fast" form prior to 5% PAGE. The results presented here suggest that there was only one type of rabbit αM present. Owing to the lack of cross-reactivity of the rabbit protein with antibodies reacting with bovine α_2M, the isolated form was likely to be rabbit α_2M, since α_1M would be likely to show cross-reactivity with these antibodies (Kaplan and Nielsen, 1979). N-terminal sequencing of the protein confirmed that rabbit α_2M was purified in the present study, and not α_1M.

In conclusion, α_2M was purified to electrophoretic homogeneity from bovine and rabbit plasma. Bovine α_2M was 100% active, while rabbit α_2M was 40% active, which is comparable to reported values. Bovine α_2M was used in the present study in the investigation of its inhibitory effect on several cysteine proteases (Chapter 7). Bovine α_2M and rabbit α_2M were also used to investigate their efficiency as adjuvants for raising antibodies against C2 and oligopeptidase B in rabbits (Chapter 8).

CHAPTER 7
INTERACTION OF α_2M WITH CYSTEINE PROTEASES AND OLIGOPEPTIDASE B

7.1 Introduction

The interaction of α_2M, most commonly human α_2M, with a variety of proteases has been widely studied (Nagase *et al.*, 1994; Fryer *et al.*, 1996; Arbeláez *et al.*, 1997; Peloille *et al.*, 1997; Ramos *et al.*, 1997; Walter *et al.*, 1999; Rangarajan *et al.*, 2000; Ramos *et al.*, 2002; Shibata *et al.*, 2003). Members from all classes of proteases have been studied, but relatively few experiments have been performed with cysteine proteases. If α_2M is found to inhibit a protease with high efficiency it is commonly suggested that α_2M probably has a natural role in regulation of the enzyme, or of the process in which the enzyme is involved. Classic indicators of an interaction *in vitro* between a protease and α_2M are: conversion to the receptor recognised, "fast" form of α_2M; reduced enzyme activity towards high molecular weight substrates, and bait region cleavage, evidenced by 95 and 85 kDa fragments in reduced SDS-PAGE (Salvesen and Enghild, 1993).

Matrix metalloprotease (MMP)-1, involved in collagenolysis, as well as MMP-2 and MMP-9, involved in the degradation of the extracellular matrix, are inhibited by human α_2M (Nagase *et al.*, 1994; Arbeláez *et al.*, 1997). This raises the possibility that α_2M is a major regulator of tissue destruction by proteases. Other recent examples of enzymes inhibited by human α_2M are human chymase (Walter *et al.*, 1999), and two Arg-specific gingipains from *Porphyromonas gingivalis* W50, RgpAcat and HRgpA (Rangarajan *et al.*, 2000). Cruzipain, the major cysteine protease of *T. cruzi*, has been found to interact with human α_2M (Ramos *et al.*, 2002). Therefore, α_2M may have a role in regulating the activity of cruzipain and other parasitic proteases involved in inflammation, cell and host invasion and immune reaction events caused by *T. cruzi* in Chaga's' disease.

Trypanosomal cysteine proteases are structurally and functionally related to mammalian cathepsins B and L (Sajid and McKerrow, 2002). Congopain differs from cathepins B and L by its broad pH activity profile and high stability at neutral pH, together with the presence of a C-terminal extension whose function is unknown (Serveau *et al.*, 2003). In the present study, the interaction of congopain and C2, the recombinant congopain catalytic domain, with bovine α_2M was analysed. Since α_2M is a natural protease inhibitor it is likely that it would interact with congopain that has been released from lysed parasites during infection with *T. congolense*. Based on the similarity of congopain with cathepsin L and papain, the interaction of bovine α_2M with these cysteine proteases was also studied. In the scope of this study, it was critical to know whether bovine α_2M interacted with C2, and in what manner. This is because α_2M was to be used as an adjuvant or antigen delivery system to raise antibodies against C2 in rabbits. The use of α_2M as an antigen delivery system depends on the proteolytic cleavage of the bait region of α_2M followed by trapping of the enzyme. Knowledge of the molar ratios of the interaction between C2 and α_2M was also required to ensure optimum complex formation for use as an antigen delivery system. The investigation of the interaction of α_2M with papain and cathepsin L was performed to optimise the assays since limited amounts of congopain and C2 were available.

The activity of oligopeptidase B is not affected by α_2M (Troeberg *et al.*, 1996), and it has been shown that these proteins do not interact with each other. This is presumed to be because of the limited interaction of the active site of oligopeptidase B with higher molecular mass proteins. Indeed, with the elucidation of the 3D structure of oligopeptidase B (Chapter 3), it is obvious that α_2M would not have access to the active site of oligopeptidase, which is necessary for the bait region cleavage of the inhibitor, and hence, trapping and inhibition of the protease. In the present study an attempt was made to conjugate oligopeptidase B to α_2M in an alternative manner. When the bait region of α_2M is cleaved by a protease, neighbouring proteins or peptides containing lysine residues can become covalently associated with the glutamyl component of the thiol ester of α_2M (Section 1.3.3). This has previously been conducted by the use of porcine

pancreatic elastase for the incorporation of antigens such as insulin, cytochrome C, and hen egg lysozyme (Chu *et al.*, 1991; Chu and Pizzo, 1993; Mitsuda *et al.*, 1993; Chu *et al.*, 1994). These co-incubations have resulted in antigen becoming covalently linked to α_2M, in addition to the protease becoming trapped within the α_2M structure. This type of co-incubation was performed with C2 and oligopeptidase B, in order to assess the antigen delivery capacity of α_2M for oligopeptidase B. The efficiency with which α_2M delivered multiple antigens was also of interest in connection with a multiple component anti-disease vaccine.

7.2 Interaction of α_2M with papain, cathepsin L, congopain and C2

Papain is a 23.4 kDa single-chain, non-glycosylated cysteine protease from *Carica papaya*. The enzyme is stable and active over a wide pH range of pH 4 to 10, with an optimum range between pH 6 and 7 (Ménard and Storer, 1998). Little is known about the biology of papain. It is produced as an inactive precursor (Vernet *et al.*, 1990), and located in the plant within the latex of the laticifer system. It is speculated that papain plays a protective role, guarding the plant against attack by insects and fungi (Ménard and Storer, 1998).

Cathepsin L is a lysosomal cysteine protease that is reported to be catalytically active between pH 3 and pH 6.5, in the presence of thiol compounds (Kirschke *et al.*, 1995). In the physiological pH range the activity and stability of cathepsin L are markedly dependent on ionic strength (Dehrmann *et al.*, 1995). Cathepsin L is suggested to have major biological roles in normal lysosomal proteolysis, and in several diseases (Turk *et al.*, 2001). The protease appears to be ubiquitous in vertebrate cells, showing almost no differences in its properties between species (Kirschke *et al.*, 1998).

The source of cathepsin L used in the present study was sheep liver, and the enzyme was isolated in a single chain form of 26 kDa (Pike and Dennison, 1989). Sheep α_2M has been found to interact rapidly with sheep liver cathepsin L, with a k_{ass} value of $8.8\pm0.75\times10^6$ $M^{-1}.s^{-1}$. This value is reported to compare to the highest values for association between serine proteases and human α_2M (k_{ass} values of 4.2 and 3.7×10^7

$M^{-1}.s^{-1}$ for neutrophil elastase, (Salvesen *et al.*, 1983; Virca and Travis, 1984). This study led to the suggestion that α_2M could actively participate in the control of cathepsin L in the testis and in the first part of the epididymis of mammals (Peloille *et al.*, 1997).

The properties of congopain have already been discussed (Section 1.2.1.1). The enzyme was in short supply and of low activity at the time this part of the study was undertaken. Therefore, it was only assayed with Bz-Pro-Phe-Arg-pNA, the most sensitive substrate. As mentioned (Section 1.2.1.1), C2 is a recombinantly expressed truncated form of congopain, consisting of the region of the proteinase between the propeptide and C-terminal extension, i.e. the catalytic domain. C2 was the major focus of this study, as discussed in Chapter 8, and was in greater supply than congopain. Therefore, a more thorough investigation into its interaction with α_2M was conducted than for congopain.

7.2.1 Inhibition assays using hide powder azure as a substrate

As mentioned in Section 6.2.4, hide powder azure is a highly sensitive general substrate for proteases. Hide powder azure is typically excluded from the enzyme trapped within the α_2M complex (Enghild *et al.*, 1990; Bender and Bayne, 1996; Rangarajan *et al.*, 2000), and therefore provides an accurate indication of enzyme trapping by α_2M.

Determination of the effect of pH on the inhibition of papain by α_2M was done using acetate-MES-Tris (AMT) buffers. AMT buffers were designed by Ellis and Morrison (1982) to have constant ionic strength across a pH range since, as indicated above, ionic strength may have a profound effect on enzyme activity.

7.2.1.1 Reagents

Papain assay buffer [100 mM sodium phosphate buffer, pH 6.5, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3]. As per Section 2.9.1, without DTT.

Cathepsin L assay buffer [340 mM Na-acetate buffer, pH 5.5, 60 mM acetic acid, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3]. As per Section 2.9.1, without DTT.

Congopain assay buffer [100 mM Bis-Tris buffer, pH 6, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3]. As per Section 2.9.1, without DTT.

100 mM Cysteine. Cysteine.HCl (0.088 g) was dissolved in dH_2O (5 ml).

Substrate solution (12.5 mg/ml hide powder azure, 600 mM sucrose, in assay buffer). As per Section 6.2.4.1.

AMT buffers (100 mM acetate, 100 mM MES, 200 mM Tris-HCl, 4 mM Na_2EDTA). As per Section 5.4.1, but without DTT.

7.2.1.2 Procedure

The assay was performed in triplicate. Cysteine protease (Table 7.1), made up to 50 µl with assay buffer, was combined with assay buffer (400 µl) and 100 mM cysteine (100 µl), and activated for 5 min (RT). α_2M (Table 7.1), diluted in assay buffer to 50 µl, was added and incubated for 20 min at 37°C. Substrate solution (400 µl) was added and tubes were incubated for 1 h at 37°C, with agitation. Undigested substrate was collected by centrifugation (13 700 *g*, 5 min, RT) and the absorbance of the supernatant was read at 595 nm.

To investigate the effect of pH on the inhibitory activity of α_2M against papain, AMT buffers were used from pH 5-8, and equimolar amounts of papain and α_2M were used, i.e. 10 pmol.

Table 7.1. Quantities of cysteine proteases and α_2M used in the hide powder azure assays.

Cysteine protease	Active Enzyme (pmol)[a]	α_2M (pmol)
Papain	10	0-20
Cathepsin L	26	0-52
C2	100	0-200

[a] Amount of active enzyme determined by E-64 titration

7.2.2 Inhibition assays using Bz-Pro-Phe-Arg-pNA as substrate

Para-nitroanilide (pNA) substrates are N-acylated derivatives of 4-nitroanilide. They are chromogenic substrates and upon cleavage release a pNA group which has a high absorbance at 405 nm. Specificity of papain-like enzymes is primarily determined by P2/S2 interactions. The substrate Bz-Pro-Phe-Arg-pNA is suitable for papain-like enzymes due to their preference for bulky hydrophobic or aromatic residues, such as Phe, in the P2 position (Ménard and Storer, 1998). The enzymes will therefore cleave the substrate after the Arg residue, releasing the pNA group.

7.2.2.1 Reagents

Papain assay buffer [100 mM sodium phosphate buffer, pH 6.5, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. As per Section 2.9.1.

Cathepsin L assay buffer [340 mM Na-acetate buffer, pH 5.5, 60 mM acetic acid, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. As per Section 2.9.1.

Congopain assay buffer [100 mM Bis-Tris buffer, pH 6, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. As per Section 2.9.1.

Substrate stock (10 mM Bz-Pro-Phe-Arg-pNA). Bz-Pro-Phe-Arg-pNA (6.8 mg) was dissolved in DMSO (150 µl) and made up to 1 ml with dH_2O.

Substrate solution (3 mM Bz-Pro-Phe-Arg-pNA). Substrate stock (300 µl) was diluted to 1 ml with dH_2O.

Stopping solution [50% (v/v) acetic acid]. Glacial acetic acid (50 ml) was diluted to 100 ml with dH_2O.

7.2.2.2 Procedure

This assay was performed in triplicate. Cysteine protease (Table 7.2) was made up to 50 µl in assay buffer, combined with assay buffer (800 µl), and activated for 5 min (RT).

α_2M (Table 7.2), diluted in assay buffer to 50 µl, was added and incubated for 20 min at 37°C. Substrate solution (40 µl) was added and samples incubated for 1 h at 37°C. The assay was stopped with addition of 50% acetic acid (50 µl), and absorbance read at 405 nm.

Table 7.2. Quantities of cysteine proteases and α_2M used in the Bz-Pro-Phe-Arg-pNA assays.

Cysteine protease	Active Enzyme (pmol)[a]	α_2M (pmol)
Papain	10	0-20
Cathepsin L	5	0-10
Congopain	6	0-9
C2	20	0-40

[a] Amount of active enzyme determined by E-64 titration

7.2.3 Inhibition assay using azocasein as substrate

Azocasein is a derivative of casein where tyrosine and histidine residues have been coupled with diazotised sulfanilic acid, or sulfanilamide, in an alkaline medium. Proteolytic degradation of azocasein results in the release of peptides that are soluble in dilute trichloroacetic acid, whilst undigested fragments precipitate. The soluble peptides have an intense yellow colour that can be quantified by absorption at 366 nm (Barrett and Kirschke, 1981).

7.2.3.1 Reagents

Assay buffer [100 mM Tris-HCl buffer, pH 8, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 40 mM cysteine]. Tris (1.211 g) and Na_2EDTA (0.15 g) were dissolved in dH_2O (90 ml) and titrated to pH 8 with HCl. NaN_3 (0.02 g) was added and dissolved, and the volume made up to 100 ml with dH_2O. Just before use, cysteine.HCl (0.04 g) was added to assay buffer (5 ml).

0.1% (w/v) Brij 35. As per Section 2.9.1.

6% (w/v) Azocasein. Azocasein (3 g) was dissolved in dH_2O (50 ml) with gentle stirring at RT for about 1 h.

Azocasein/3 M urea solution. Urea (54 g) was dissolved in 6% (w/v) azocasein solution (50 ml) by stirring at 30°C. The volume was made up to 150 ml with assay buffer not containing cysteine.

Stopping reagent [5% (w/v) TCA]. Trichloroacetic acid (5 g) was dissolved in 100 ml dH_2O.

7.2.3.2 Procedure

The assay was performed in triplicate. Papain (200 pmol), diluted in 0.1% Brij to 200 µl, was activated in assay buffer (200 µl) for 5 min (RT). α_2M (0-400 pmol), diluted in 0.1% Brij to 150 µl was added, and incubated for 20 min at 37°C. Substrate was added (400 µl) and incubated in a water bath at 37°C for 1 h. The reaction was stopped by transfer of 200 µl into a microfuge tube containing 5% TCA (1 ml) and centrifuged (13 700 *g*, 5 min, RT). The absorbance of the supernatant was read at 366 nm.

7.3 Incorporation of oligopeptidase B into complexes with bovine α_2M

As previously discussed, oligopeptidase B does not interact with α_2M on its own. Therefore, to incorporate the oligopeptidase B into complexes with α_2M, it was necessary to activate α_2M by bait region cleavage with another protease, namely C2. The stoichiometry of the binding of insulin to human α_2M by this technique has been characterised (Chu *et al.*, 1991), and was used as a model in the present study. The maximum insulin incorporation occurred when two times molar excess of the protease was used in the reaction. The amount of insulin used in the reaction had to be in great excess for the covalent association to occur, with a maximum of 4 moles of insulin binding per 1 mole of α_2M, when 100 times molar excess of insulin was used. Based on this stoichiometry, it was estimated that at least 1 mole of oligopeptidase B would be bound to 1 mole of α_2M when 10 times molar excess of oligopeptidase B and 2 times

molar excess of C2 were used in the reaction. The procedure was first attempted on a small scale, but was later repeated on a large scale providing sufficient complexes for immunisation (Section 8.3).

7.3.1 Reagents

Congopain assay buffer [100 mM Bis-Tris buffer, pH 6, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 40 mM cysteine]. As per Section 2.9.1, with the omission of DTT, and addition of 0.035 g cysteine.HCl to buffer (5 ml) before use.

MEC buffer [100 mM sodium phosphate buffer, pH 7, 0.02% (w/v) NaN_3]. As per Section 2.12.2.1.

7.3.2 Procedure

Oligopeptidase B (37.5 nmoles) was combined with C2 (7.5 nmoles) in assay buffer (1.8 ml) and activated for 5 min at 37°C. Bovine α_2M (3.75 nmoles) was added and incubated for 1 h at 37°C. The mixture was loaded onto a Sephacryl S-300 HR column (as per Section 6.2.3.2) equilibrated with MEC buffer, and eluted with MEC buffer. Fractions of 5 ml were collected.

7.4 Results

7.4.1 Interaction of papain with α_2M

7.4.1.1 Inhibition assay using hide powder azure as substrate

A suitable incubation time for enzyme and α_2M was investigated by varying the incubation time of activated papain with a two times molar excess of α_2M, between 5 and 60 min at 37°C, before assaying for activity (Fig. 7.1). Inhibition was found to increase from 96.7% at 5 min to 98.6% at 20 min, after which it did not increase significantly. From this result it was decided that further incubation steps for α_2M and each of the cysteine proteases would be carried out for 20 min at 37°C.

It was found that α_2M was able to inhibit 27.4% of papain activity against hide powder azure at very low levels of 0.25 mol α_2M to 1 mol papain (Fig. 7.2). The trend observed

for the inhibition was such that as the amount of α_2M increased, so did the percentage inhibition, until the graphical representation reached a plateau at a 1.25 molar excess of α_2M. In this experiment, maximum inhibition (91.8%) was observed at a two times molar excess of α_2M.

Inhibition of papain by α_2M showed a bell shaped trend when percentage inhibition was plotted against pH (Fig. 7.3). The maximum inhibition occurred at pH 7, with 97.4%, and inhibition decreased noticeably below pH 6. The activity of papain also showed a bell shaped trend and peaked at pH 7.5. The activity of papain was more or less similar at pH 8 compared to pH 7, but the inhibition by α_2M was higher at pH 7 than at pH 8. Therefore it is likely that pH had a direct effect on the inhibitory properties of α_2M.

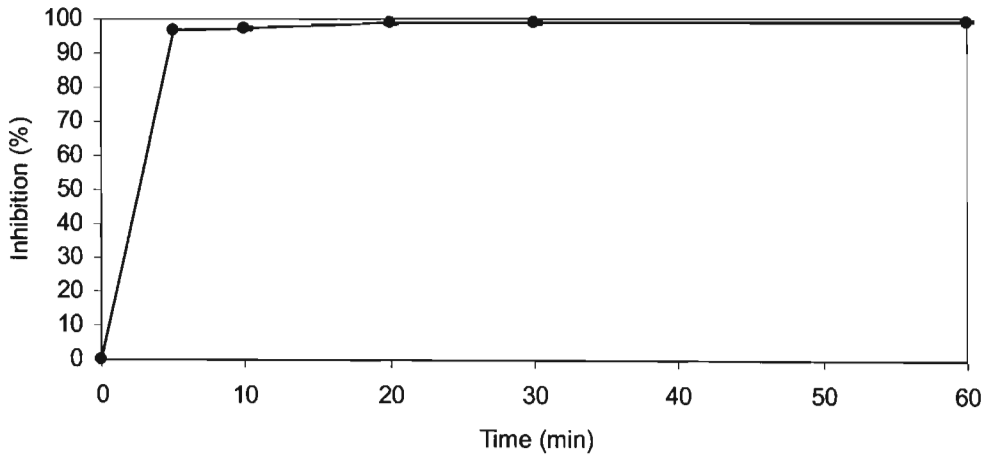

Figure 7.1. The effect of variation in the incubation time of papain with α_2M before assaying for activity against hide powder azure. Papain (10 pmol) was incubated with two times molar excess of α_2M (20 pmol) for 5-60 min at 37°C. Proteolytic activity was determined by the extent of hydrolysis of hide powder azure substrate compared to that of a papain control with no α_2M (100% activity), and expressed as a percentage inhibition. Error bars represent the mean ± SEM (n=3).

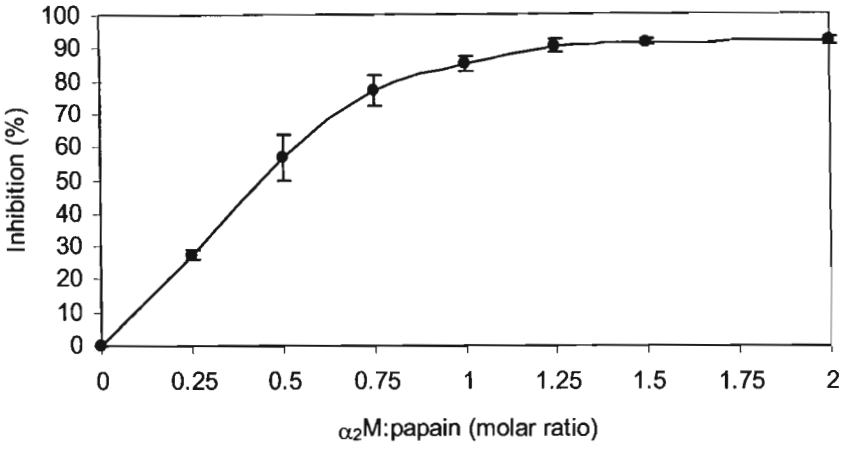

Figure 7.2. The effect of increasing amounts of α_2M on the activity of papain against hide powder azure. Different concentrations of α_2M were incubated with papain (10 pmol) at molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1 for 20 min at 37°C. Proteolytic activity was determined by the extent of hydrolysis of hide powder azure substrate compared to that of a papain control with no α_2M (100% activity), and expressed as a percentage inhibition. Error bars represent the mean ± SEM (n=3).

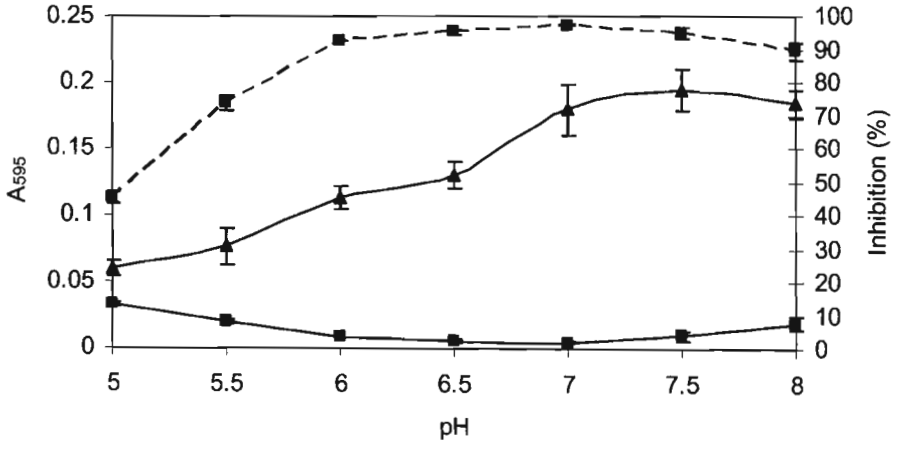

Figure 7.3. The effect of pH on the inhibitory action of α_2M on the activity of papain against hide powder azure. Papain (10 pmol) was incubated with (■) or without (▲) an equimolar amount of α_2M (10 pmol) in constant ionic strength buffers of various pH for 20 min at 37°C. Proteolytic activity was determined at each pH by the extent of hydrolysis of hide powder azure substrate compared to that of a papain control with no α_2M (100% activity), and expressed as a percentage inhibition. Absorbance at 595 nm is represented by the solid line, and percentage inhibition is represented by the broken line. Error bars represent the mean ± SEM (n=3).

7.4.1.2 Inhibition assay using azocasein as substrate

α_2M was able to inhibit papain activity against azocasein, albeit very slightly, at very low levels (0.25 mol α_2M to 1 mol papain), with inhibition of 5.9% (Fig. 7.4). The trend observed for the inhibition was again that as the amount of α_2M increased, so did the percentage inhibition, until the graphical representation reached a plateau, this time at an equimolar amount of α_2M. In this experiment, maximum inhibition (83.3%) was observed at a two times molar excess of α_2M.

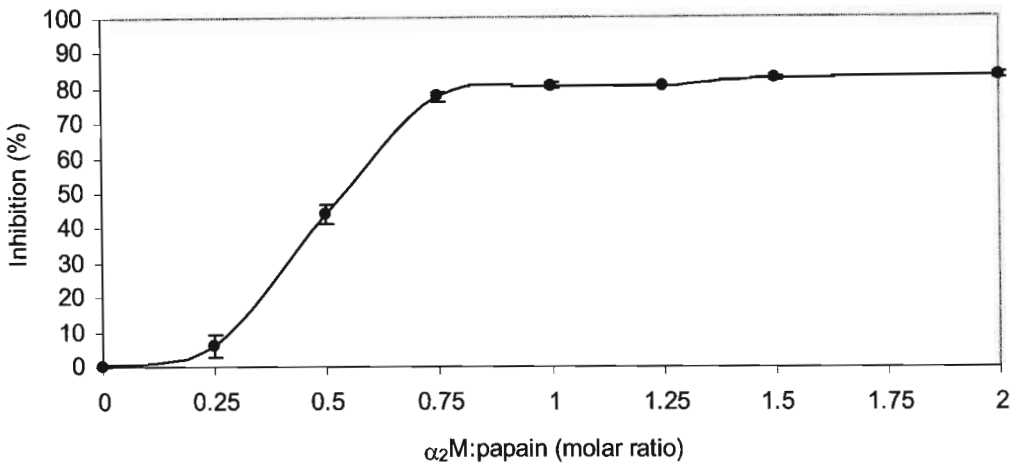

Figure 7.4. The effect of increasing amounts of α_2M on the activity of papain against azocasein. Different concentrations of α_2M were incubated with papain (200 pmol) at molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1 for 20 min at 37°C. Proteolytic activity was determined by the extent of hydrolysis of azocasein substrate compared to that of a papain control with no α_2M (100% activity), and expressed as a percentage inhibition. Error bars represent the mean ± SEM (n=3).

7.4.1.3 Inhibition assay using Bz-Pro-Phe-Arg-pNA as substrate

When the peptide nitroanilide substrate was used, no inhibition was observed (Table 7.3). In fact, an apparent increase in enzyme activity was observed with the addition of the inhibitor, α_2M.

Table 7.3. The effect of α_2M on papain activity against Bz-Pro-Phe-Arg-pNA.

Molar ratio (α_2M:Papain)	Activity (%)[a]
0.5	169.7 ± 2.78
1	173.1 ± 2.65
1.5	172.9 ± 0.80
2	177.1 ± 3.60

[a] Data are geometric mean ± SEM (n=3)

7.4.1.4 Non-denaturing PAGE analysis of the interaction between papain and α_2M

The effect of increasing the ratio of α_2M to papain was illustrated by non-denaturing PAGE (Fig. 7.5). For α_2M to papain molar ratios of between 0.25:1 and 1.25:1 (lanes 2-6), the α_2M occurred as a single band of higher mobility than in the absence of papain, illustrating that α_2M was completely transformed to the "fast" form. Therefore, all of the α_2M at these ratios was interacting with papain. For the α_2M:papain ratio of 1.5:1 (lane 7), some of the α_2M was still in the "slow" form, whilst most had interacted with the papain and was transformed to the "fast" form. For the α_2M:papain ratio of 2:1 (lane 8), most of the α_2M was in the "slow" form, whilst a small proportion had interacted with papain and was transformed to the "fast" form.

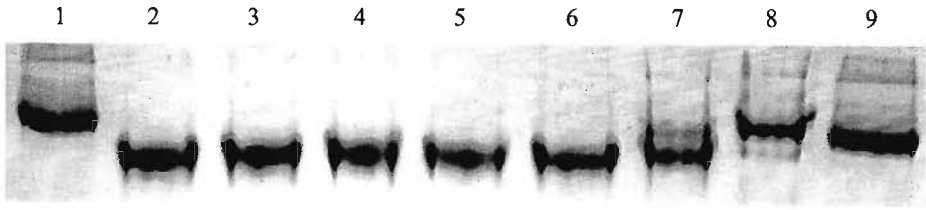

Figure 7.5. Non-denaturing PAGE analysis of the interaction between α_2M and papain. Papain was activated with 100 mM $NaPO_4$ buffer, pH 6.5, 4 mM Na_2EDTA, 40 mM cysteine, and incubated with α_2M for 20 min. Samples were diluted with treatment buffer and subjected to electrophoresis on a 5% gel. Lanes 1 and 9, 10 μg α_2M; lanes 2-8, α_2M (10 μg) was incubated with papain (1.5-0.2 μg) (molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, 2:1 respectively). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

7.4.1.5 Reducing SDS-PAGE analysis of the interaction between papain and α_2M

When papain was activated, combined with α_2M and subjected to reducing SDS-PAGE conditions, no protein bands were visible on the gel, even with silver staining (Fig. 7.6). The only protein evident was a band at the dye front.

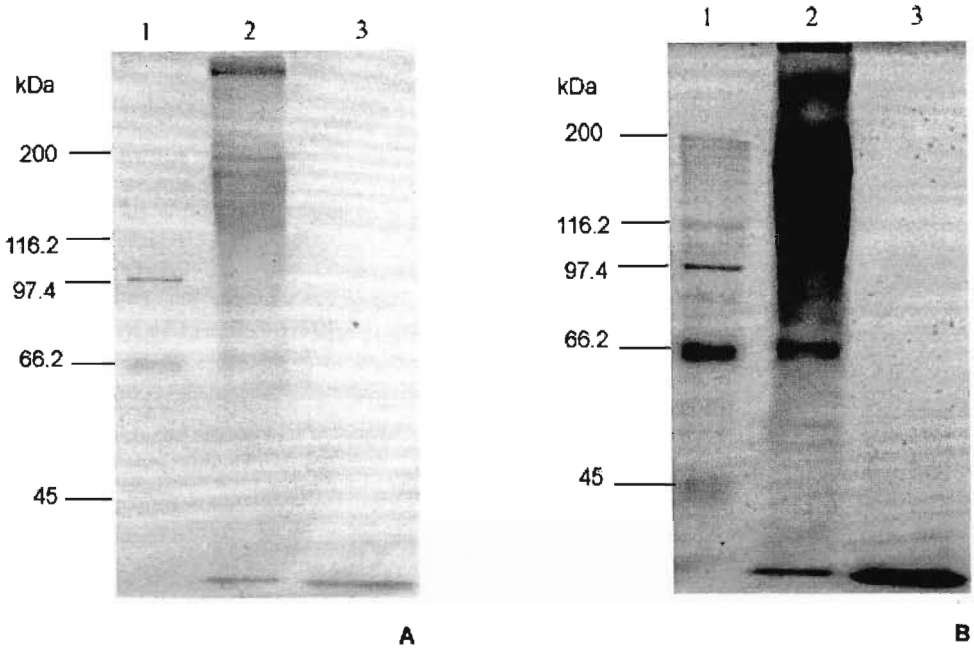

Figure 7.6. Reducing SDS-PAGE of α_2M after reaction with activated papain. Papain (645 ng) was activated with 100 mM $NaPO_4$ buffer, 4 mM Na_2EDTA, pH 6.5, 8 mM DTT, and incubated with α_2M (10 μg) for 20 min. The proteins were incubated in reducing treatment buffer for 30 min at 37°C before SDS-PAGE on 7.5% acrylamide gels. Lane 1, Bio-Rad molecular weight markers consisting of myosin (200 kDa), β-galactosidase (116.2 kDa), phosphorylase b (97.4 kDa), bovine serum albumin (66.2 kDa), and ovalbumin (45 kDa); lane 2, α_2M (10 μg); lane 3, papain-α_2M complex (10.645 μg). Proteins were stained with Coomassie blue R-250 as per Section 2.6.1 (panel A), followed by silver staining as per Section 2.6.2 (panel B).

7.4.2 Interaction of cathepsin L and α_2M

7.4.2.1 Inhibition assay using hide powder azure as substrate

α_2M was able to inhibit 61.3% of cathepsin L activity against hide powder azure at very low levels of 0.25 mol α_2M to 1 mol cathepsin L (Fig. 7.7). As the amount of α_2M increased, so did the percentage inhibition, until the graphical representation reached a plateau at a 0.75 mol α_2M to 1 mol cathepsin L, after which, the inhibition decreased slightly. In this experiment, maximum inhibition (97.5%) was observed at an equimolar amount of α_2M.

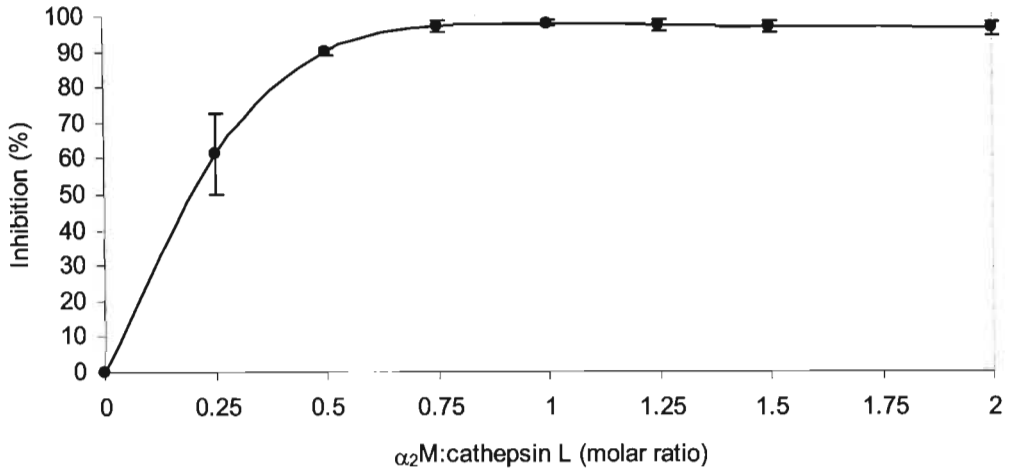

Figure 7.7. The effect of increasing amounts of α_2M on the activity of cathepsin L against hide powder azure. Different concentrations of α_2M were incubated with cathepsin L (26 pmol) at molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1 for 20 min at 37°C. Proteolytic activity was determined by the extent of hydrolysis of hide powder azure substrate compared to that of a cathepsin L control with no α_2M (100% activity), and expressed as a percentage inhibition. Error bars represent the mean ± SEM (n=3).

7.4.2.2 Inhibition assay using Bz-Pro-Phe-Arg-pNA as substrate

When the peptide nitroanilide substrate was used, no inhibition was observed (Table 7.4). In fact, an apparent increase in enzyme activity was observed with the addition of the inhibitor, as seen with papain (Table 7.3), but to a lesser degree than papain.

Table 7.4. The effect of α_2M on cathepsin activity against Bz-Pro-Phe-Arg-pNA.

Molar ratio (α_2M:Cathepsin L)	Activity (%)[a]
1	114.0 ± 10.03
2	100.2 ± 4.32

[a] Data are geometric mean ± SEM (n=3)

7.4.2.3 Non-denaturing PAGE analysis of the interaction between cathepsin L and α_2M

The effect of increasing the ratio of α_2M to cathepsin L was illustrated by non-denaturing PAGE (Fig. 7.8). For α_2M to cathepsin L molar ratios of 0.25:1 and 0.5:1 (lanes 2 and

3), the α_2M occurred as a single band of higher mobility than in the absence of cathepsin L, illustrating that α_2M was completely transformed to the "fast" form. Therefore, all of the α_2M at these ratios was interacting with cathepsin L. For α_2M:cathepsin L ratios of 0.75:1 (lane 4) and 1:1 (lane 5), some of the α_2M was still in the "slow" form, whilst most had interacted with the cathepsin L and was transformed to the "fast" form. At α_2M:cathepsin L ratios of 1.25:1 (lane 6), "slow" and "fast" α_2M appear to occur in equal proportions. For α_2M:cathepsin L ratios of 1.5:1 (lane 7) and 2:1 (lane 8), most of the α_2M was in the "slow" form, whilst a small proportion had interacted with cathepsin L and transformed to the "fast" form.

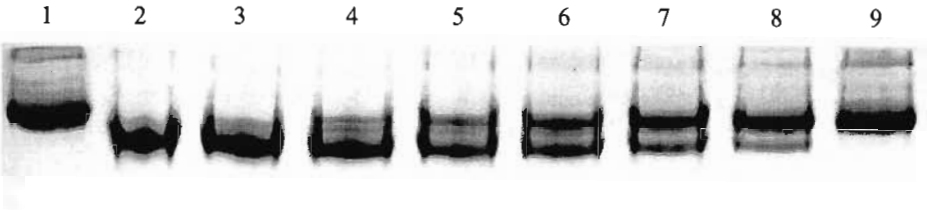

Figure 7.8. Non-denaturing PAGE analysis of the interaction between α_2M and cathepsin L. Cathepsin L was activated with 340 mM Na acetate, 60 mM acetic acid, 4 mM Na_2EDTA, pH 5.5, 40 mM cysteine, and incubated with α_2M for 20 min. Samples were diluted with treatment buffer and subjected to electrophoresis on a 5% gel. Lanes 1 and 9, 10 μg α_2M; lanes 2-8, α_2M (10 μg) was incubated with cathepsin L (1.5-0.2 μg) (molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, 2:1 respectively). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

7.4.2.4 Reducing SDS-PAGE analysis of the interaction between cathepsin L and α_2M

When cathepsin L was activated, combined with α_2M and subjected to reducing SDS-PAGE conditions, a number of protein bands were visible on the Coomassie stained gel (Fig. 7.9, lane 3), which was emphasised by silver staining (Fig. 7.9, lane 3). The bands indicated by the arrows are significant because they have estimated molecular weights of 95 and 85 kDa, which are the sizes of the fragments produced when the bait region of α_2M has been cleaved (Salvesen and Enghild, 1993).

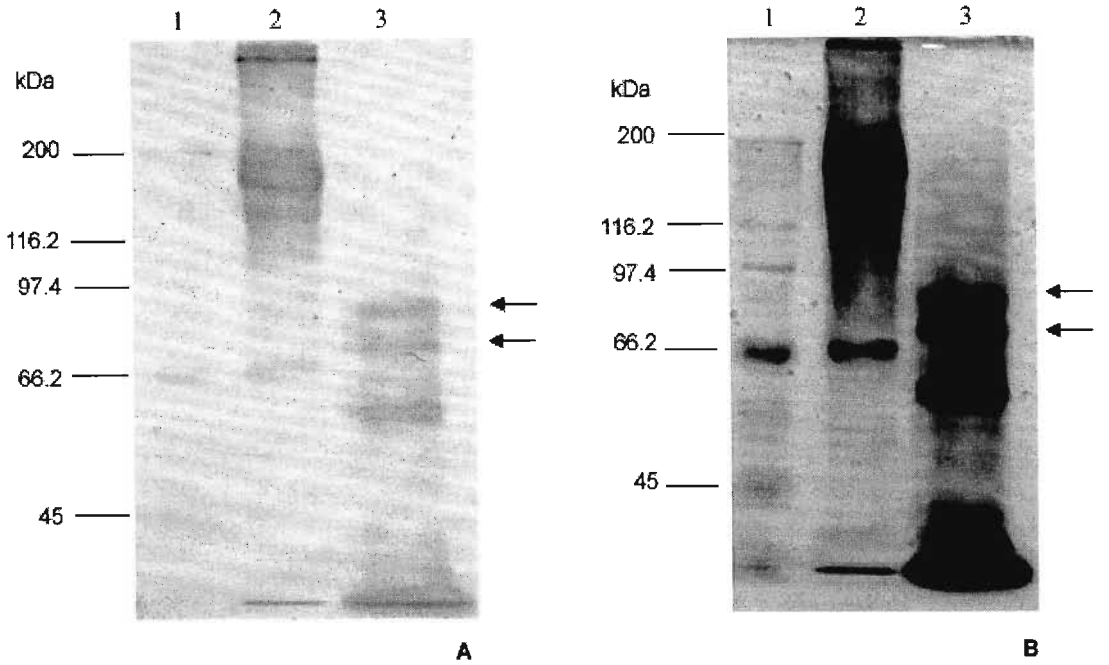

Figure 7.9. Reducing SDS-PAGE of α_2M after reaction with activated cathepsin L. Cathepsin L (1.5 μg) was activated with 340 mM Na acetate, 60 mM acetic acid, 4 mM Na_2EDTA, pH 5.5, 8 mM DTT, and incubated with α_2M (10 μg) for 20 min. The proteins were incubated in reducing treatment buffer for 30 min at 37°C before SDS-PAGE on 7.5% acrylamide gels. Lane 1, Bio-Rad molecular weight markers (as per Fig. 7.6); lane 2, α_2M (10 μg); lane 3, cathepsin L-α_2M complex (11.5 μg). Proteins were stained with Coomassie blue R-250 as per Section 2.6.1 (panel A), followed by silver staining as per Section 2.6.2 (panel B).

7.4.3 Interaction between congopain and α_2M

7.4.3.1 Inhibition assay using Bz-Pro-Phe-Arg-pNA as substrate

As mentioned, the recombinant enzyme congopain was in short supply, and was not very active, therefore, only the highly sensitive assay with the Bz-Pro-Phe-Arg-pNA substrate was feasible. Unlike assays with papain and cathepsin L, α_2M was found to inhibit congopain activity against Bz-Pro-Phe-Arg-pNA slightly, with 15.1% inhibition at equimolar amounts of α_2M, and 13.9% inhibition at 1.5 times molar excess of α_2M (Table 7.5).

Table 7.5. The effect of α_2M on the activity of congopain against Bz-Pro-Phe-Arg-pNA.

Molar ratio (α_2M:congopain)	Inhibition (%)[a]
1	15.1 ± 0.43
1.5	13.9 ± 0.63

[a] Data are geometric mean ± SEM (n=3)

7.4.3.2 Non-denaturing PAGE analysis of the interaction between congopain and α_2M

The effect of congopain on α_2M was illustrated by non-denaturing PAGE (Fig. 7.10). At an α_2M:congopain molar ratio of 1:1 (lane 2), the α_2M was mostly converted to the "fast" form, as the higher mobility band is darker than the upper band of "slow" α_2M. Whereas, for a molar ratio of 1.5:1 (lane 3), only a small proportion of the α_2M has been converted to the "fast" form.

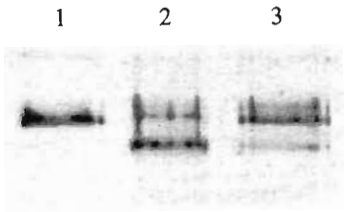

Figure 7.10. Non-denaturing PAGE analysis of the interaction between α_2M and congopain. Congopain was activated with 100 mM Bis-Tris buffer, 4 mM Na_2EDTA, pH 6, 8 mM DTT, and incubated with α_2M for 20 min. Samples were diluted with treatment buffer and subjected to electrophoresis on a 5% gel. Lanes 1 and 9, 500 ng α_2M; lane 2, α_2M (500 ng) was incubated with congopain (27.7 ng) (molar ratio of 1:1); lane 3, α_2M (500 ng) was incubated with congopain (13.7 ng) (molar ratio of 1.5:1). Proteins were silver stained as per Section 2.6.2.

7.4.3.3 Reducing SDS-PAGE analysis of the interaction between congopain and α_2M.

When congopain was activated, combined with α_2M and subjected to reducing SDS-PAGE, a number of protein bands were visible on the Coomassie stained gel (Fig 7.11A, lane 3), and emphasised by silver staining (Fig. 7.11B, lane 3). The bands indicated by the arrows are significant because they have estimated molecular weights of 95 and 85 kDa, which are the sizes of the fragments produced when the bait region of α_2M has been

cleaved (Salvesen and Enghild, 1993). A band is also evident at a molecular weight just higher than that of the native α_2M monomer, which may be the receptor recognised form of α_2M in complex with the enzyme. A band is also evident at the approximate molecular weight expected for the dimer of α_2M (360 kDa), probably in complex with the enzyme.

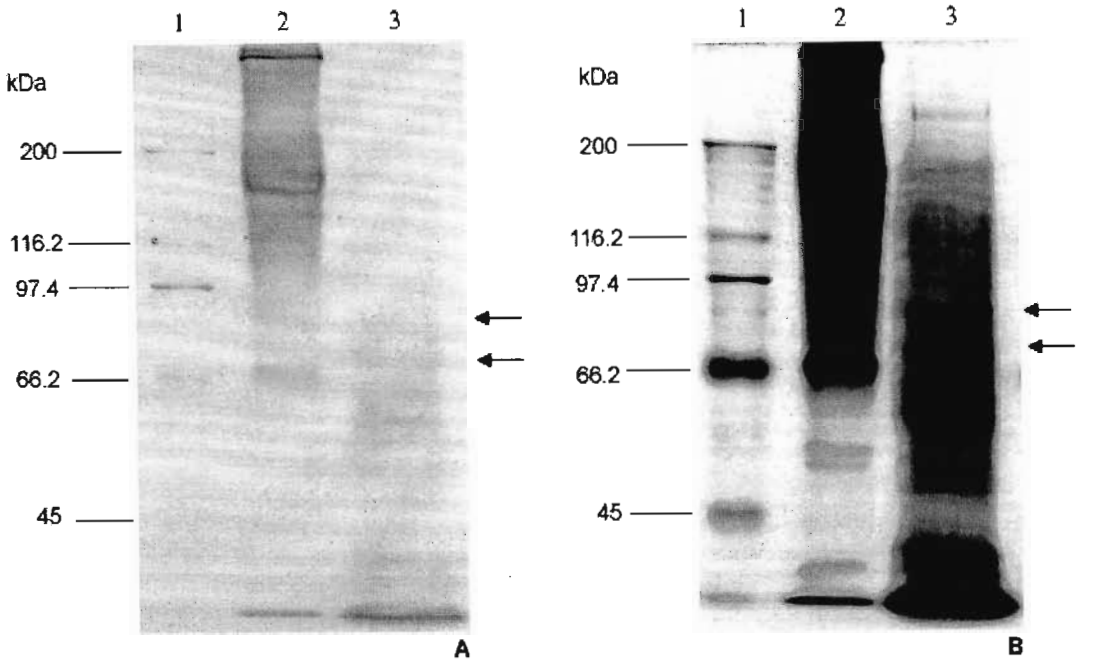

Figure 7.11. Reducing SDS-PAGE of α_2M after reaction with activated congopain. Congopain (1.5 μg) was activated with 0.1 M Bis-Tris buffer, 4 mM Na_2EDTA, pH 6, 8 mM DTT, and incubated with α_2M (10 μg) for 20 min. The proteins were incubated in reducing treatment buffer for 30 min at 37°C before SDS-PAGE on 7.5% acrylamide gels. Lane 1, Bio-Rad molecular weight markers (as per Fig. 7.6); lane 2, α_2M (10 μg); lane 3, congopain-α_2M complex (11.5 μg). Proteins were stained with Coomassie blue R-250 as per Section 2.6.1 (panel A), followed by silver staining as per Section 2.6.2 (panel B).

7.4.4 Interaction between the recombinant congopain catalytic domain, C2, and α_2M

7.4.4.1 Inhibition assay using hide powder azure as substrate

α_2M was able to inhibit 24.2% of C2 activity against hide powder azure at very low levels of 0.25 mol α_2M to 1 mol C2 (Fig. 7.12). As the amount of α_2M increased, so did the percentage inhibition, until the graphical representation reached a plateau at an

equimolar amount of α_2M. In this experiment, maximum inhibition (99.2%) was observed at a 1.5 times molar excess of α_2M.

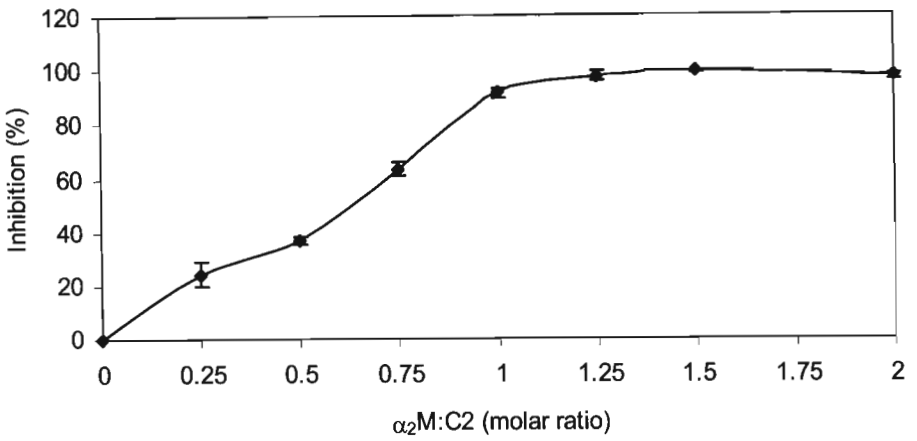

Figure 7.12. The effect of increasing amounts of α_2M on the activity of C2 against hide powder azure. Different concentrations of α_2M were incubated with C2 (100 pmol) at molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1 for 20 min at 37°C. Proteolytic activity was determined by the extent of hydrolysis of hide powder azure substrate compared to that of a C2 control with no α_2M (100% activity), and expressed as a percentage inhibition. Error bars represent the mean ± SEM (n=3).

7.4.4.2 Inhibition assay using Bz-Pro-Phe-Arg-pNA as substrate

Like congopain, inhibition of C2 by α_2M was evident when the peptide nitroanilide substrate was used (Fig. 7.13). α_2M was able to inhibit C2 activity against Bz-Pro-Phe-Arg-pNA, though only slightly, at very low levels (0.25 mol α_2M to 1 mol C2), with inhibition of 7.7%. The trend observed for the inhibition was again that as the amount of α_2M increased, so did the percentage inhibition. In this experiment, the graphical representation reached a plateau at 0.75 mol α_2M to 1 mol C2, and inhibition declined as α_2M was added above equimolar amounts. Maximum inhibition (44.9%) was observed at an equimolar amount of α_2M, which is much higher than 15.1% for congopain (Table 7.5).

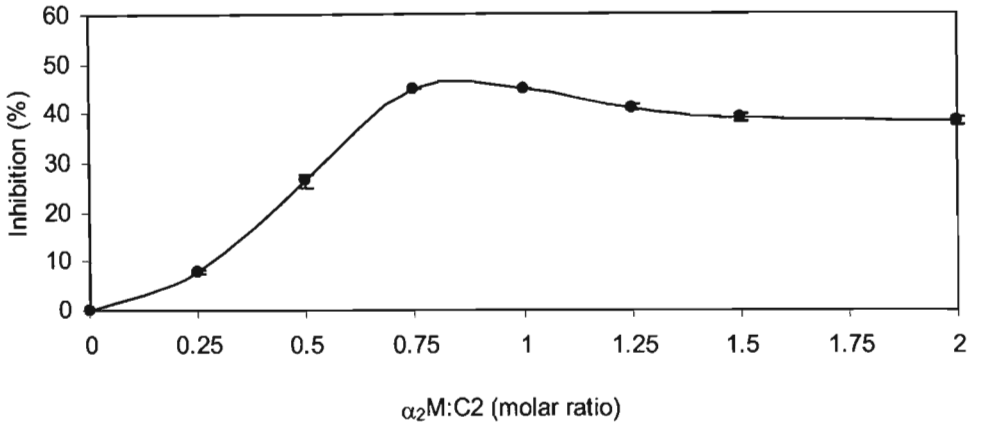

Figure 7.13. The effect of increasing amounts of α_2M on the activity of C2 against Bz-Pro-Phe-Arg-pNA. Different concentrations of α_2M were incubated with C2 (20 pmol) at molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, and 2:1 for 20 min at 37°C. Proteolytic activity was determined by the extent of hydrolysis of Bz-Pro-Phe-Arg-pNA substrate compared to that of a C2 control with no α_2M (100% activity), and expressed as a percentage inhibition. Error bars represent the mean ± SEM (n=3).

7.4.4.3 Non-denaturing PAGE analysis of the interaction between C2 and α_2M

The effect of increasing the ratio of α_2M to C2 was illustrated by non-denaturing PAGE (Fig. 7.14). For α_2M to C2 molar ratios of 0.25:1 and 0.5:1 (lanes 2 and 3), the α_2M occurred as a single, higher mobility band, illustrating that α_2M was completely transformed to the "fast" form. Therefore, all of the α_2M at these ratios was interacting with C2. For α_2M:C2 ratios of 0.75:1 (lane 4), some of the α_2M was still in the "slow" form, whilst most had interacted with the C2 and transformed to the "fast" form. At α_2M:C2 ratios of 1:1 (lane 5), 1.25:1 (lane 6), and 1.5:1 (lane 7), "slow" and "fast" α_2M appeared to occur in equal proportions. For α_2M:C2 ratios of 2:1 (lane 8), most of the α_2M was in the "slow" form, whilst a small proportion had interacted with C2 and transformed to the "fast" form.

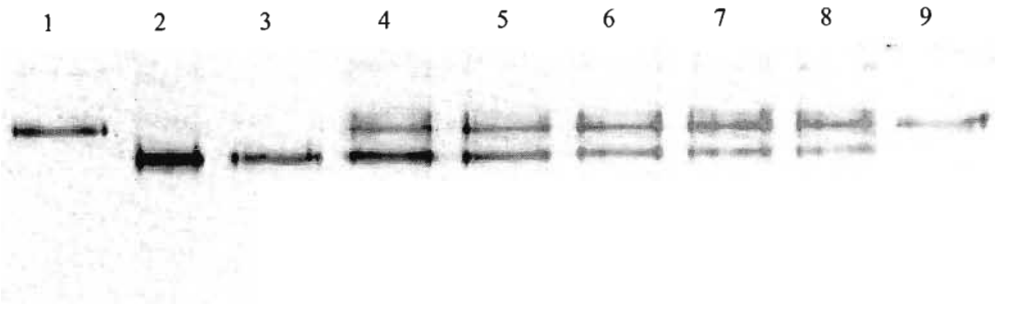

Figure 7.14. Non-denaturing PAGE analysis of the interaction between α_2M and C2. C2 was activated with 100 mM Bis-Tris buffer, 4 mM Na_2EDTA, pH 6, 8 mM DTT, and incubated with α_2M for 20 min. Samples were diluted with treatment buffer and subjected to electrophoresis on a 5% gel. Lanes 1 and 9, 500 ng α_2M; lanes 2-8, α_2M (500 ng) was incubated with C2 (91-11 ng) (molar ratios of 0.25:1, 0.5:1, 0.75:1, 1:1, 1.25:1, 1.5:1, 2:1 respectively). Proteins were silver stained (Section 2.6.2).

7.4.4.4 Reducing SDS-PAGE analysis of the interaction between C2 and α_2M

When C2 was activated, combined with α_2M and subjected to reducing SDS-PAGE, a number of protein bands were visible on the Coomassie stained gel (Fig 7.15A, lane 3), emphasised with silver staining (Fig. 7.15B, lane 3). The bands indicated by the arrows are significant because they have estimated molecular weights of 95 and 85 kDa, which are the sizes of the fragments produced when the α_2M bait region has been cleaved (Salvesen and Enghild, 1993). A band is also evident at a molecular weight just higher than that of the native α_2M monomer, which may be the receptor recognised form of α_2M in complex with the enzyme.

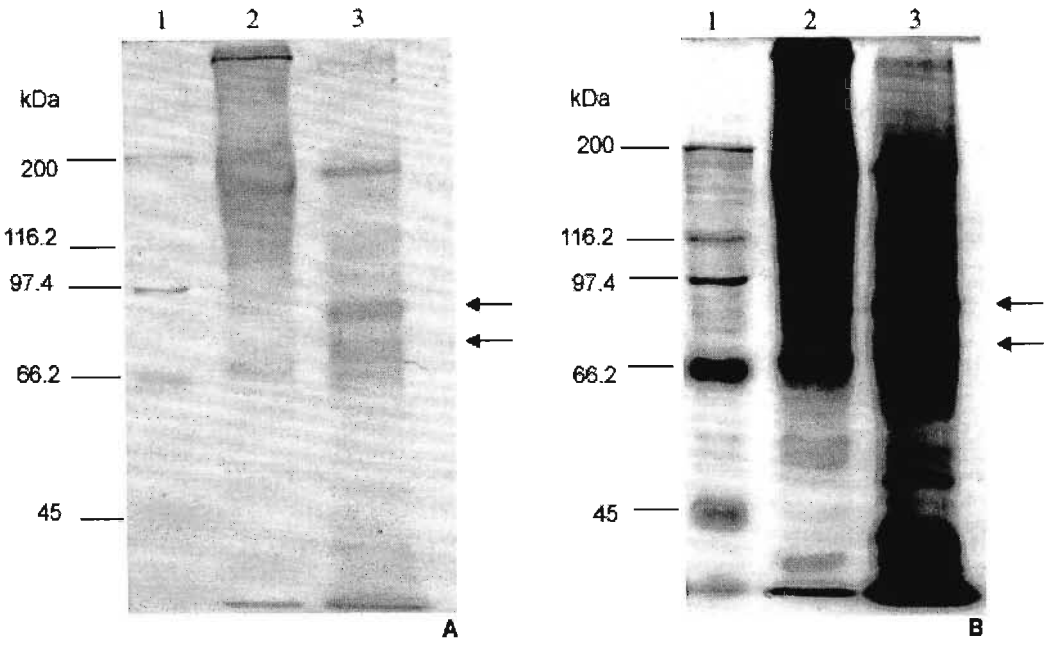

Figure 7.15. Reducing SDS-PAGE of α_2M after reaction with activated C2. C2 (372 ng) was activated with 100 mM Bis-Tris buffer, 4 mM Na_2EDTA, pH 6, 8 mM DTT, and incubated with α_2M (10 μg) for 20 min. The proteins were incubated in reducing treatment buffer for 30 min at 37°C before SDS-PAGE on 7.5% acrylamide gels. Lane 1, Bio-Rad molecular weight markers (as per Fig. 7.6); lane 2, α_2M (10 μg); lane 3, C2-α_2M complex (10.372 μg). Proteins were stained with Coomassie blue R-250 as per Section 2.6.1 (panel A), followed by silver staining as per Section 2.6.2 (panel B).

7.4.5 Conjugation of oligopeptidase B to bovine α_2M

When bovine α_2M was incubated with C2 and recombinant oligopeptidase B, either rOPC or rOPV, and subjected to Sephacryl S-300 HR gel filtration, the mixture was resolved into four peaks (Fig. 7.16). The two high molecular weight peaks were analysed by SDS-PAGE (result not shown) and found to have identical protein banding patterns. Therefore, the two high molecular weight peaks were pooled. Further analysis by SDS-PAGE (Fig. 7.17) and western blotting (Fig. 7.18) was carried out to immunogenically confirm the presence of α_2M, C2 and oligopeptidase B. Reducing SDS-PAGE of the complexes revealed two major bands, approximately 70 kDa and 85 kDa in size, with some faint bands observed between 37 and 25 kDa.

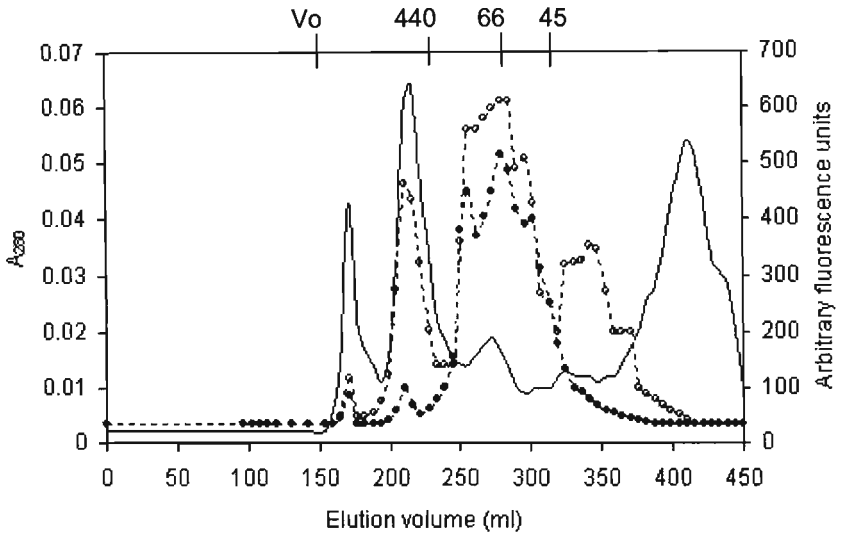

Figure 7.16. Molecular exclusion chromatography of α_2M combined with C2 and oligopeptidase B on Sephacryl S-300 HR. Sephacryl S-300 HR (25×840 mm, 25 ml.h^{-1}) was equilibrated with MEC buffer (100 mM sodium phosphate buffer, pH 7, 0.02% NaN_3). Sample (4 ml) was loaded, and protein eluted with MEC buffer. The molecular weights of the calibration proteins are indicated at the top in kDa. The elution profile is representative of both rOPC and rOPV. A_{280} (—), enzyme activity against Z-Arg-Arg-AMC (-●-), enzyme activity against Z-Phe-Arg-AMC (-○-).

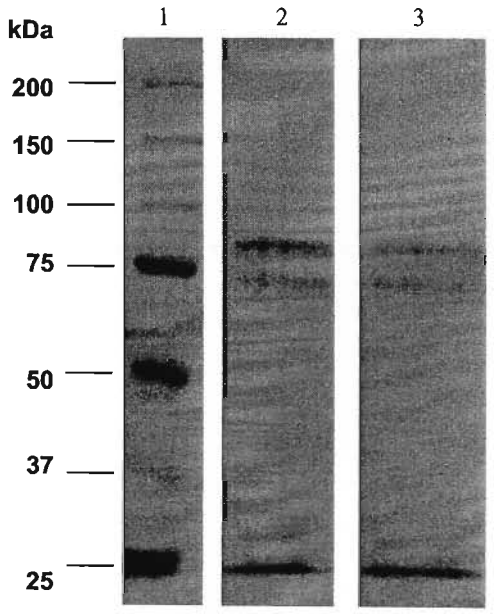

Figure 7.17. Reducing SDS-PAGE of complexes formed by the combination of C2, oligopeptidase B and α_2M. Complexes were reduced and electrophoresed on a 7.5% polyacrylamide gel. Lane 1, Bio-Rad precision plus markers; lane 2, complexes formed with rOPC (5 μg); lane 3, complexes formed with rOPV (5 μg). Proteins were stained with Coomassie blue R-250 (Section 2.6.1).

Probing the complexes with anti-α_2M antibody revealed that α_2M was present in the form of the two major bands, as observed by SDS-PAGE at 70 and 85 kDa (Fig. 7.18A). Probing the complexes with affinity purified anti-rOPV antibodies revealed the presence of oligopeptidase B (Fig. 7.18B). Oligopeptidase B may have become dissociated from α_2M through reduction since it appeared at approximately 80 kDa. Probing the complexes with affinity purified anti-peptide antibody (directed against the N-terminus of C2) revealed the presence of C2 at the same sizes as the α_2M bands, as well as at about 21 kDa.

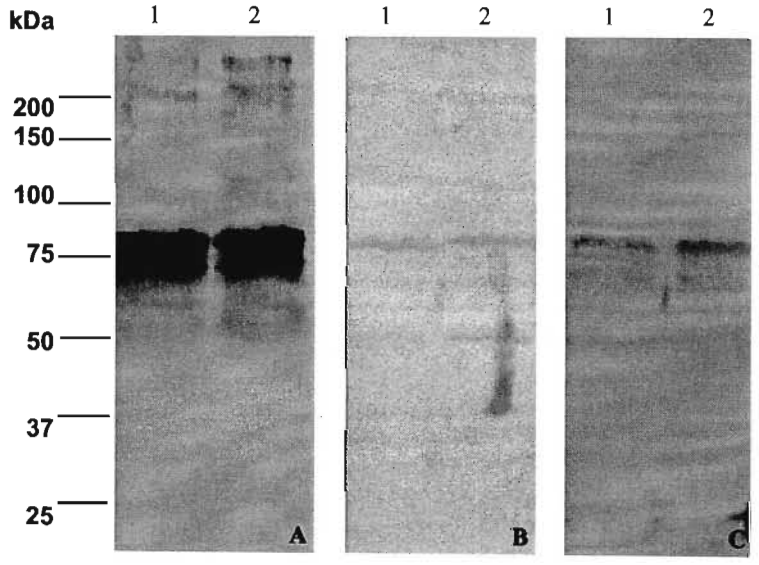

Figure 7.18. Western blotting of complexes formed by the combination of C2, oligopeptidase B and α_2M. Complexes were electrophoresed on a 7.5% acrylamide gel after reduction, and electroblotted onto a nitrocellulose membrane. Lane 1, complexes formed with rOPV (5 μg); lane 2, complexes formed with rOPC (5 μg). The membrane was incubated with (A) affinity purified chicken anti-bovine α_2M antibody, (B) affinity purified chicken anti-rOPV antibody (5 μg/ml; Section 8.2), or (C) affinity purified anti-C2 peptide antibody (75 μg/ml), followed by HRPO-conjugated rabbit anti-chicken IgY. Bands were developed with 4-chloro-1-naphthol/H_2O_2.

7.5 Discussion

Bovine α_2M interacted with papain, sheep liver cathepsin L, congopain and C2. The hydrolysis of one or more of the substrates tested by all these enzymes was inhibited by α_2M. In support of this evidence, the enzyme-inhibitor interaction was also observed by PAGE (conversion of "slow" to "fast" form) and reducing SDS-PAGE (presence of bait region-cleavage fragments).

It was found in the present study that α_2M inhibited 96.7% of the hydrolysis of hide powder azure by papain after five minutes of incubation. The greatest inhibition occurred after 20 min of incubation, so the standard time of 20 min was applied to all incubation steps for α_2M and proteases. α_2M is reported to react with proteases anywhere between 5 min (Mitsuda *et al.*, 1993) to 24 h (Ramos *et al.*, 2002). It would have been desirable to optimise the incubation step for all of the individual proteases used, but this assay was found to be too expensive in terms of enzyme for this to be practical.

The effect of pH on the inhibitory capacity of α_2M was investigated by measuring the proteolytic activity of papain on hide powder azure. Hide powder azure proved to be a sensitive substrate that accurately reflected the trapping of the enzyme within α_2M. It was necessary to have a control sample with no inhibitor at each pH value tested, to take into account the effect of pH on the activity of papain. The optimum pH for inhibition by bovine α_2M was found to be pH 7, with a broad optimal range of pH 6-8. Since α_2M functions in plasma (Barrett, 1981), it would be expected to function optimally at the physiological pH of 7.4, which is supported by the findings in the present study. To accurately assess the optimum pH for inhibition by α_2M, this assay would need to be repeated for the other enzymes in the present study. If the same effect of pH on inhibition by α_2M was seen for enzymes with different pH optima, it would suggest that the effect of pH on the enzyme's activity had not influenced the result. Unfortunately, in the scope of this study, this was not possible due to the large amount of enzyme required for this experiment. Papain showed its greatest activity at pH 7.5, with its optimum range falling between pH 7 and 8.

Of the three substrates used to assay for inhibition by α_2M, hide powder azure revealed the greatest inhibition. This is because the substrate is too large to gain access to the enzyme's active site within the α_2M complex (Barrett, 1981). The maximum inhibition shown by azocasein (for papain activity) was 83.3%. Therefore azocasein was apparently able to gain some access to the enzyme's active site. Because of this, and due to the low sensitivity of the substrate, azocasein was not used in further assays. The fact that no inhibition was shown by Bz-Pro-Phe-Arg-pNA for papain and cathepsin L suggests that the substrate had complete access to the enzyme's active site. Therefore, Bz-Pro-Phe-Arg-pNA is not a good substrate to assay for inhibition of papain or cathepsin L by α_2M. For some reason, Bz-Pro-Phe-Arg-pNA experienced restricted access to the active site of congopain, and to a greater extent to that of C2, when in the α_2M complex. A similar observation was made for cruzipain when assayed for inhibition by human α_2M using Bz-Pro-Phe-Arg-pNA (Ramos *et al.*, 2002).

Inhibition assays for the other proteases tested with hide powder azure as substrate also showed very high levels of inhibition by α_2M, with maximum values of 97.5% for cathepsin L and 99.2% for C2. Also seen in these assays was a marked inhibition of enzyme activity even at very low levels of α_2M (0.25 mol α_2M to 1 mol enzyme), with 27.4% for papain, 61.3% for cathepsin L and 24.2% for C2. The values for papain and C2 were similar but the inhibition of cathepsin L was more than double that of the other proteases tested.

Bovine α_2M appeared totally transformed from the "slow" to "fast" form by the addition of 1.25 molar excess of papain, as seen by non-denaturing PAGE. This was also shown by the hide powder azure assay where the inhibition plateaus at 1.25 molar excess of papain. The azocasein assay showed a plateau at equimolar ratios of α_2M:protease. This is an indication that the molar binding ratio between papain and α_2M is between 1 to 1.25 mol α_2M to 1 mol papain. In the same respect, other molar binding ratios were 0.5 to 0.75 mol α_2M to 1 mol cathepsin L, less than 1 mol α_2M to 1 mol congopain, and 1 mol α_2M to 1 mol C2. Based on these findings it would appear that 1 mol bovine α_2M was

able to trap more or less 1 mol of papain, congopain and C2, and between 1.3 to 2 moles of cathepsin L.

When active papain was reacted with α_2M and electrophoresed under reducing conditions, the proteins appeared to have been completely degraded. Other experiments showed that the enzyme is trapped within the molecule which most likely occurs by cleavage of the bait region. That is, under the conditions of non-denaturing PAGE the α_2M had appeared as a tetramer, and papain activity had been inhibited towards large, but not small, substrates. It thus appears that the papain-α_2M complex was unstable under the reducing SDS-PAGE conditions. For the reaction of α_2M with cathepsin L, congopain and C2, reducing SDS-PAGE resulted in a number of bands. Bands of 85 and 95 kDa indicate that the bait region of α_2M was cleaved by the enzymes (Salvesen and Enghild, 1993). The precise cleavage sites of these enzymes could be determined by N-terminal amino acid sequencing of these bands. N-terminal amino acid sequencing of the other bands formed would help to determine where in the α_2M molecule they originated from and perhaps further explain the interaction between α_2M and the enzymes.

Oligopeptidase B is unable to interact with α_2M due to the restriction of access of large molecules to its active site, thus preventing bait region cleavage. In the present study, however, oligopeptidase B was complexed to bovine α_2M when the bait region was cleaved with C2. C2 was also complexed to α_2M during this process. This illustrates how two antigens can be incorporated into α_2M. The drawback of using this co-incubation to complex antigen with α_2M is potential proteolytic degradation of antigen. A certain degree of degradation of oligopeptidase B (or α_2M) is likely to have occurred in the present study, due to the presence of the low molecular peak in the MEC elution profile of the complexes. Additionally, large amounts of non-reactive antigen, e.g. oligopeptidase B, are required to ensure coupling.

SDS-PAGE of the multi-component α_2M complexes showed fewer bands than was typically observed for the interaction of α_2M with cysteine proteases. This was because

C2 was present in excess to facilitate maximum complex formation, and thus, nearly all of the α_2M was cleaved by the protease. Complexes of α_2M, C2 and oligopeptidase B resolved into two peaks when analysed by molecular exclusion chromatography. This indicates that there were two subgroups of complexes, with differing molecular weights, each consisting of C2 and oligopeptidase B. The multi-component complexes may have formed 'dimers' of some kind. It is possible that two α_2M molecules could be covalently linked to a single oligopeptidase B molecule, which would result in a complex of very high molecular weight. The final peak that eluted from the Sephacryl S-300 HR column used to assess complex formation, of low molecular weight, did not show activity against Z-Phe-Arg-AMC or Z-Arg-Arg-AMC. As mentioned, it is likely that this peak was formed by protein fragments that resulted from degradation of oligopeptidase B or α_2M by the excess C2.

From the investigation into the interaction of C2 with bovine α_2M it was found that active C2 cleaves the bait region of bovine α_2M. This results in inhibition of C2 by trapping of the protease within the α_2M tetramer. C2 and bovine α_2M appear to interact in an equimolar ratio. This information was used to prepare complexes of C2 with bovine α_2M and rabbit α_2M for the production of anti-C2 antibodies in rabbits. Additionally, complexes of bovine α_2M, C2 and oligopeptidase B were used to immunise rabbit to assess the potential of a multi-component vaccine (Chapter 8).

CHAPTER 8
IMMUNOCHEMICAL STUDIES ON CONGOPAIN AND OLIGOPEPTIDASE B

8.1 Introduction

This chapter describes a series of separate, but related immunochemical studies on the catalytic domain of congopain, C2, and recombinantly expressed oligopeptidase B from *T. congolense* and *T. vivax*. The first section of this chapter involved the description of raising antibodies against the recombinant enzymes rOPC and rOPV for preliminary assays and as protein purification and detection aids. For this purpose, chickens were chosen as experimental animals. Large quantities of antibody are transferred to the egg yolk to provide immunity for the developing chicks (Polson *et al.*, 1980). Since eggs can be collected daily, antibody production is easily monitored. This route of antibody production is also preferred from an animal welfare point of view since bleeding is not necessary to harvest the antibodies.

An innovative strategy in the campaign against trypanosomosis is the formulation of an anti-disease vaccine. That is, a vaccine not expected to affect the survival of the parasite but instead designed to neutralise pathogenic factors. Since it is probable that congopain plays a role in the pathology of trypanosomosis, and antibody-mediated inhibition of this enzyme may contribute to mechanisms of trypanotolerance (Authié *et al.*, 2001), immunisation of susceptible cattle with congopain might confer a degree of resistance to the disease. As discussed in Section 1.3.7, it is likely that α_2M plays a role in targeting antigens to APCs as well as enhancing antigen processing and presentation by MHC class II molecules to $CD4^+$ T cells (Chu *et al.*, 1994). Also, since α_2M had been found to enhance immunogenicity of proteins to levels comparable with Freund's adjuvant, α_2M might be used in place of an adjuvant in a vaccine against trypanosomosis.

As discussed in Chapter 4, the C-terminal domain of congopain is highly immunogenic but unlikely to elicit antibodies that inhibit the enzyme. For this reason, the enzyme was

expressed in a truncated form, consisting of the propeptide and catalytic domain. The propeptide was included as it plays an essential role in the correct folding and secretion of expressed cathepsins (Vernet *et al.*, 1990). The inactive zymogen was processed into the mature enzyme at acidic pH. It was considered necessary to use catalytically active C2 to induce inhibitory antibodies by its conformational epitopes (Boulangé *et al.*, 2001).

C2 has been used in a cattle immunisation trial by Authié *et al.* (2001). There was no effect on the development of infection or acute anaemia. However, immunised cattle maintained or gained weight during infection and exhibited less severe anaemia and leukopaenia during the chronic phase of the disease. Also, immunised cattle developed prominent IgG responses to trypanosomal antigens such as VSG, which is typical of trypanotolerance. From this study it appeared that congopain may be involved in the mechanism of trypanosome induced immunosuppression.

Free antigen usually disperses rapidly from local tissues surrounding the site of injection. An important function of adjuvants is to counteract this by providing a long-lived reservoir of antigen (Roitt, 1997). Aluminium hydroxide (alum), a mineral salt adjuvant, and Freund's adjuvant, a mineral oil emulsion adjuvant, can both be used to create a depot of antigen. Alum operates by adsorbing proteins, where a protein with a lower pI will adsorb to the positively charged aluminium ion more strongly than a protein with a higher pI (Nicklas, 1992). Freund's adjuvant operates by creating a stable water-in-oil emulsion with aqueous antigen solution. Alum is generally a weaker adjuvant than Freund's adjuvant, but generally induces only mild inflammatory responses and is widely used in humans. Freund's adjuvant, especially complete adjuvant containing mycobacteria, causes chronic inflammatory responses and may result in the formation of chronic granulomas, sterile abscesses and tissue necrosis (Claassen *et al.*, 1992).

For the present investigation, rabbits were the most affordable and manageable mammalian system available. Bovine α_2M was used since the C2 vaccine is intended for use in cattle. Rabbit α_2M was also used in preliminary studies to test the efficiency of a "self" protein for antigen delivery, as bovine α_2M would be a "self" protein in cattle. It

was hypothesised that a "self" protein may be more efficient at eliciting an immune response towards the antigen, as the immune system would not be misdirected against the delivery system itself. In study I, the titres of antibodies raised against C2 complexed to these two proteins, and against free C2 in Freund's adjuvant, were compared. The effect of the antibodies, raised in the three different ways, on the activity of both C2 and congopain was also compared to determine whether the antibodies inhibit congopain activity.

Oligopeptidase B is another pathogenic factor of trypanosomosis which probably degrades host peptide hormones in the bloodstream. It has also been strongly implicated as a virulence factor in African trypanosomes, since it is released into the host circulation by dead or dying parasites, where it retains catalytic activity (Morty *et al.*, 2001). A second study was conducted in rabbits to assess the viability of using α_2M as an antigen delivery system for congopain and covalently bound oligopeptidase B, simultaneously. Since oligopeptidase B does not cleave the bait region of α_2M, another protease is required to activate α_2M, and also becomes bound to α_2M during the process. The method of complexing oligopeptidase B to α_2M therefore presented the opportunity to target two trypanosomal pathogenic factors, and C2 was selected to cleave the bait region of α_2M. In study II, the levels of antibodies raised against C2 and oligopeptidase B, when complexed to bovine α_2M, were compared to those obtained by immunising with the free proteins in alum. The effect of the antibodies on the activity of C2, congopain, rOPC, rOPV and native oligopeptidase B from *T. b. brucei*, *T. congolense* and *T. vivax* was assessed.

8.2 Production of antibodies in chickens

Chickens were used to produce antibodies against recombinant oligopeptidase B (from *T. congolense*, rOPC, or *T. vivax*, rOPV).

8.2.1 Preparation of immunogen

The recombinant enzymes produced in the present study were purified from an *E. coli* expression system, and thus may be contaminated with endotoxins. Gram negative

bacteria such as *E. coli* have lipopolysaccharide (LPS) in the outer membrane of their cell wall. LPS is referred to as endotoxin because it is bound to the bacterium and is released when the microorganism lyses (Prescott *et al.*, 1999). The toxic component of LPS is the lipid portion, called lipid A. In brief, endotoxin can provide problems in immunisation by activating complement and triggering an inflammatory response, as well as being toxic. Sensitive methods have been developed for the detection and removal of endotoxin. In the present study, endotoxin was removed from rOPC and rOPV using an endotoxin affinity resin called EndoTrap® (Profos AG, Germany), according to manufacturer's specifications.

8.2.1.1 Reagents

Sterile PBS, pH 7.2. As per Section 3.3.3.1.

8.2.1.2 Procedure

Recombinant oligopeptidase B from *T. congolense* and *T. vivax* (50 μg per immunisation) in Freund's adjuvant was used to immunise chickens over a 6 week period, as per Section 3.3.3.

8.2.2 Affinity purification of antibodies

For affinity purification of antibodies produced against recombinant oligopeptidase B, affinity matrices were prepared with purified fusion protein, which was relatively easy to obtain in large quantities. Prior to affinity purification, antibody was treated with lysate prepared from bacteria expressing GST, as described below, to adsorb any anti-GST antibodies that may be present. Affinity purification was carried out as described in Section 2.13.

8.2.2.1 Reagents

MEC buffer [100 mM sodium phosphate buffer, pH 7, 0.02% (m/v) NaN_3]. As per Section 2.12.2.1.

8.2.2.2 Procedure

Fusion-protein, i.e. GST-OPC and GST-OPV, was purified by glutathione agarose chromatography as described in Section 4.4.3, without cleavage by thrombin. Protein (5 mg) was dialysed against MEC buffer overnight at 4°C (3 changes), and coupled to Aminolink® resin as in Section 2.12.1.2

GST protein was expressed in *E. coli* JM109 transformed with pGEX4T1, i.e. non-recombinant plasmid, as described for recombinant plasmids in Section 4.4.2. After expression, cells were centrifuged (2 000 *g*, 10 min, 4°C), and resuspended in MEC buffer (40 ml per litre of culture). Triton X-100 was added to 0.1% (v/v). The suspension was sonicated (4 × 30 s), and clarified by centrifugation (10 000 *g*, 10 min, 4°C). Lysate (5 ml) was added to a pool of antibodies (anti-rOPC or anti-rOPV IgY; 60 ml), and stirred for 3 h at RT. The solution was clarified by centrifugation (10 000 *g*, 10 min , 4°C), and affinity purified as normal (Section 2.13).

8.3 Production of antibodies in rabbits

Antibodies were raised in rabbits in two separate experiments to compare different adjuvant systems. The first experiment, study I, involved the immunisation of rabbits (A, B, F, R; Table 8.1) with C2, either coupled to bovine or rabbit α_2M, or in free form, with or without adjuvant. The second experiment, study II, involved the immunisation of rabbits (AlC, AlV, MaC, MaV; Table 8.1) with C2 and oligopeptidase B (either rOPC or rOPV), in complex with bovine α_2M, or in free form with adjuvant.

8.3.1 Preparation of immunogen

8.3.1.1 Reagents

Activation buffer (100 mM Bis-Tris buffer, pH 6, 2 mM Na_2EDTA, 40 mM cysteine).
Bis-Tris (2.092 g) and Na_2EDTA (0.075 g) were dissolved in dH_2O (90 ml), titrated to pH 6 with HCl, and the volume made up to 100 ml with dH_2O. Cysteine.HCl (0.04 g) was added to buffer (5 ml) before use.

Sterile PBS, pH 7.2. As per Section 3.3.3.1.

8.3.1.2 Procedure

C2 (80 μg) was activated in activation buffer for 5 min, combined with a 1.5 times molar excess of either rabbit or bovine α_2M (3.222 mg), and incubated for 1 h at 37°C. Subsequently, the samples were divided into 4 equal aliquots, each containing the equivalent of 20 μg C2.

Table 8.1. Description and dosage of the different immunogens and adjuvants used for the immunisation of rabbits.

			Total immunogen (mg)		
Name	Description	Adjuvant	C2	Oligopeptidase B	α_2M
Study I					
A	C2	None	0.04	n/a	n/a
B	C2-α_2M complex	Bovine α_2M	0.04	n/a	1.6
F	C2	FCA/FIA	0.04	n/a	n/a
R	C2-α_2M complex	Rabbit α_2M	0.04	n/a	1.6
Study II					
AlC	C2 and rOPC	Aluminium hydroxide	0.04	0.132	n/a
AlV	C2 and rOPV	Aluminium hydroxide	0.04	0.132	n/a
MaC	C2-rOPC-α_2M complex	Bovine α_2M	0.04	0.132	1.5
MaV	C2-rOPV-α_2M complex	Bovine α_2M	0.04	0.132	1.5

C2-α_2M (rabbit or bovine) complexes were each made up to 2 ml with sterile PBS for immunisation. C2 (20 μg) was made up to 1 ml with sterile PBS, and emulsified in a 1:1 ratio with Freund's adjuvant for immunisation (complete adjuvant for the initial immunisation and incomplete adjuvant for the booster immunisations). For immunisation with C2 without adjuvant, C2 (20 μg) was made up to 2 ml with sterile PBS.

C2-oligopeptidase B-bovine α_2M complexes, prepared as described in Section 7.3, were each made up to 2 ml with sterile PBS for immunisation. C2 (20 μg) and oligopeptidase

B (rOPC or rOPV; 66 μg) were made up to 1 ml with sterile PBS and combined in a 1:1 ratio with Alhydrogel®, an aluminium hydroxide adjuvant.

Rabbits were immunised by subcutaneous injection at multiple sites on the back, each rabbit receiving a total of 1 ml; that is, an equivalent of 10 μg C2 per immunisation, and 33 μg oligopeptidase B per immunisation where applicable (Table 8.1). Rabbits were bled from the mid-ear artery. Non-immune serum was collected before immunisation. Test bleeds were performed at weeks 3, 6, 9 and 11. Rabbit B1 was not bled at week 3. Rabbits were boosted at weeks 2, 4, and 6.

8.4 Western blotting evaluation of antibody production

8.4.1 Reagents

As per Section 2.7.1.

8.4.2 Procedure

Proteins (C2, rOPC or rOPV) separated by Laemmli SDS-PAGE (Section 2.4) were electroblotted onto nitrocellulose (200 mA, 16 h), and treated as per Section 2.7.2. Membranes were incubated with chicken IgY (25 μg/ml in 0.5% BSA-PBS, Section 2.7.1), followed by HRPO-conjugated rabbit anti-chicken IgG (diluted 1:12 000 with 0.5% BSA-PBS, Section 2.7.1). Alternatively, membranes were incubated with rabbit IgG (50-150 μg/ml in 0.5% BSA-PBS, Section 2.7.1), followed by HRPO-conjugated goat anti-rabbit IgG (diluted 1:15 000 with 0.5% BSA-PBS, Section 2.7.1). Reaction was developed with 4-chloro-1-naphthol substrate.

8.5 Inhibition of enzymatic activity by specific antibodies

As discussed in Section 3.3.7, antibodies directed against enzymes can inhibit, activate or have no effect on an enzyme's activity. For an anti-disease vaccine to be successful, antibodies must inhibit the activity of their corresponding pathogenic factor *in vivo*. This assay was used to assess the ability of antibodies to inhibit congopain and/or oligopeptidase B *in vitro*. In the assay for congopain activity, the substrate Z-Phe-Arg-AMC was used, and Z-Arg-Arg-AMC was used to assay for oligopeptidase B activity.

The substrates themselves are barely fluorescent, but upon proteolytic cleavage, 7-amino-4-methylcoumarin is liberated and can be quantified by its intense fluorescence (Barrett and Kirschke, 1981). This substrate was suitable due to its high sensitivity. Lima bean trypsin inhibitor was included in assay with rabbit antibodies to inhibit the intrinsic activity of rabbit IgG against the synthetic substrates, which is probably due to serum kallikrein contamination (Colman and Bagdasarian, 1976).

8.5.1 Reagents

Assay buffer [200 mM sodium phosphate buffer, pH 7.2, 4 mM Na_2EDTA, 0.1% (v/v) Tween, 8 mM DTT]. As per Section 3.3.7.1.

1 mM Z-Arg-Arg-AMC stock solution. As per Section 3.3.7.1.

All other reagents as per Section 2.9.1.1.

8.5.2 Procedure

The inhibition of enzyme activity by specific antibodies was assayed in a stopped-time format with serial two-fold dilutions of rabbit IgG or chicken IgY from 1 mg/ml. All assays were performed in duplicate.

C2 (80 ng), congopain (100 ng), rOPC (20 ng), rOPV (20 ng), or native oligopeptidase B (100 ng) diluted to 75 µl in 0.1% Brij, was combined with 75 µl of IgG or IgY, diluted with assay buffer without DTT (containing 40 µg/ml lima bean trypsin inhibitor in assays with IgG), to yield final antibody concentrations of 1 mg/ml, 500 µg/ml, 250 µg/ml and 125 µg/ml. The samples were incubated for 15 min at 37°C. Aliquots (50 µl) were removed from the incubation mixture and combined with assay buffer containing 8 mM DTT (25 µl) in a microplate, and activated for 1 min at 37°C. Substrate was added (25 µl) and the microplate was incubated for 5 min at 37°C. Fluorescence (emission at 460 nm with excitation at 360 nm) was read with a 7620 microplate fluorometer from Cambridge Technology (UK). Inhibition was expressed as a percentage of the activity in the presence of non-immune antibody at the same concentration.

8.6 Results

8.6.1 Production of antibodies in chickens

8.6.1.1 ELISA evaluation of chicken anti-oligopeptidase B antibodies

Specific antibodies were produced against rOPC and rOPV in each of the chickens that were immunised, as shown by ELISA (Fig. 8.1). For both rOPC and rOPV, high levels of antibodies were detected in one out of the two chickens, and moderate levels of antibodies were detected in the second. Antibody production appeared to peak between weeks 8 and 12 of the immunisation protocol. After affinity purification of the pooled antibodies, recognition of the recombinant proteins was enhanced, since a high signal was acquired in ELISA at very low antibody concentrations (Fig. 8.2). The anti-rOPC and anti-rOPV antibodies were assessed for their ability to recognise certain immunogenic epitopes of oligopeptidase B. Peptides OpBTc1-7 (Chapter 3) were coated onto ELISA plates and probed with antibodies against the whole recombinant proteins in ELISA (Fig. 8.3). IgY preparations from week 9 were used in this assay since they were established as having high specific antibody levels (Fig. 8.1). Anti-rOPC antibodies reacted strongly with peptide OpBTc3, and weakly with OpBTc1. Anti-rOPV antibodies reacted strongly with peptide OpBTc1, and weakly with OpBTc3. No signal was apparent in ELISA for any of the other peptides.

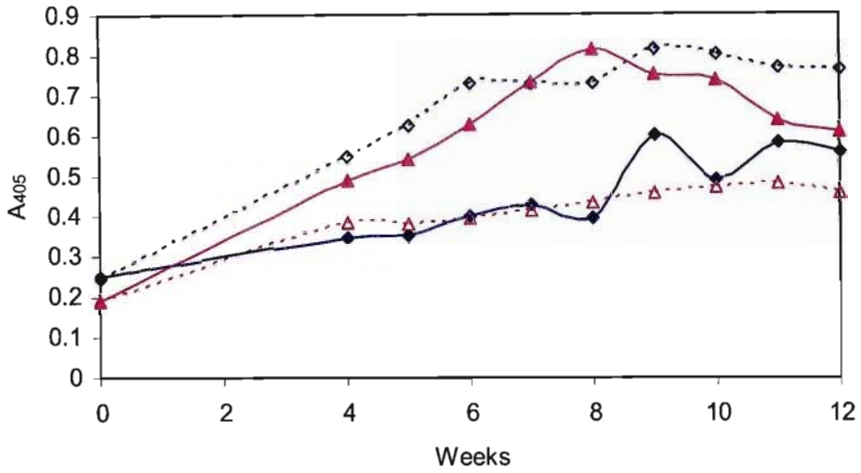

Figure 8.1. ELISA of anti-rOPC and anti-rOPV antibodies in IgY isolated from immunised chickens. rOPC or rOPV were coated onto ELISA plates (Section 2.8.2) and incubated with IgY primary antibody raised against the respective immunogens, from weeks 4-12 at 100 µg/ml (in BSA-PBS, 100 µl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 3.13. Absorbance readings at 405 nm of IgY from chickens immunised with rOPC (◆;◇) or rOPV (▲;△) represent the average of duplicate experiments. IgY from chicken 1 is represented by a solid line, while chicken 2 is represented as a broken line.

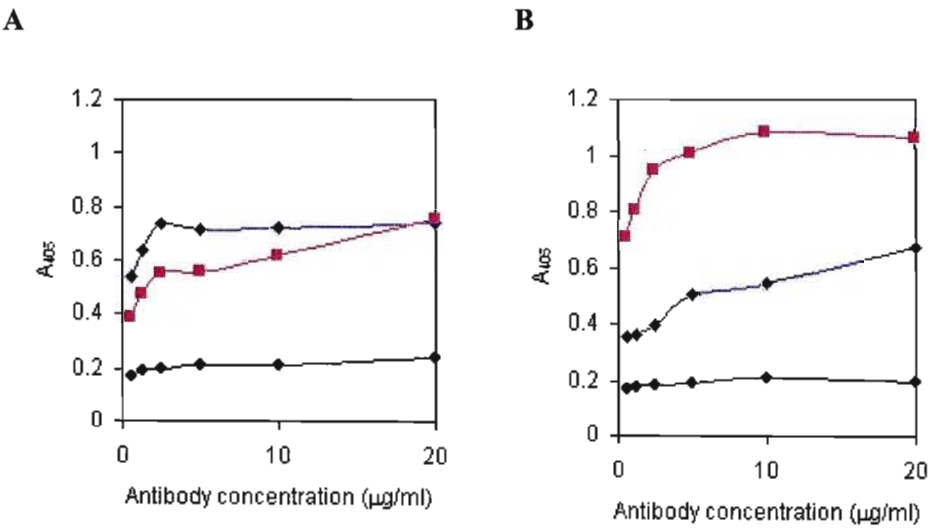

Figure 8.2. ELISA of recognition of rOPC and rOPV by affinity purified antibodies. Recombinant oligopeptidase B (A: rOPC; B: rOPV) was coated onto an ELISA plate (Section 2.8.2) and affinity purified IgY diluted serially from 20 µg/ml (in BSA-PBS, 100 µl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 3.13. Absorbance readings at 405 nm of affinity purified IgY raised against rOPC (◆) or rOPV(■), and non-immune IgY (●) represent the average of duplicate experiments.

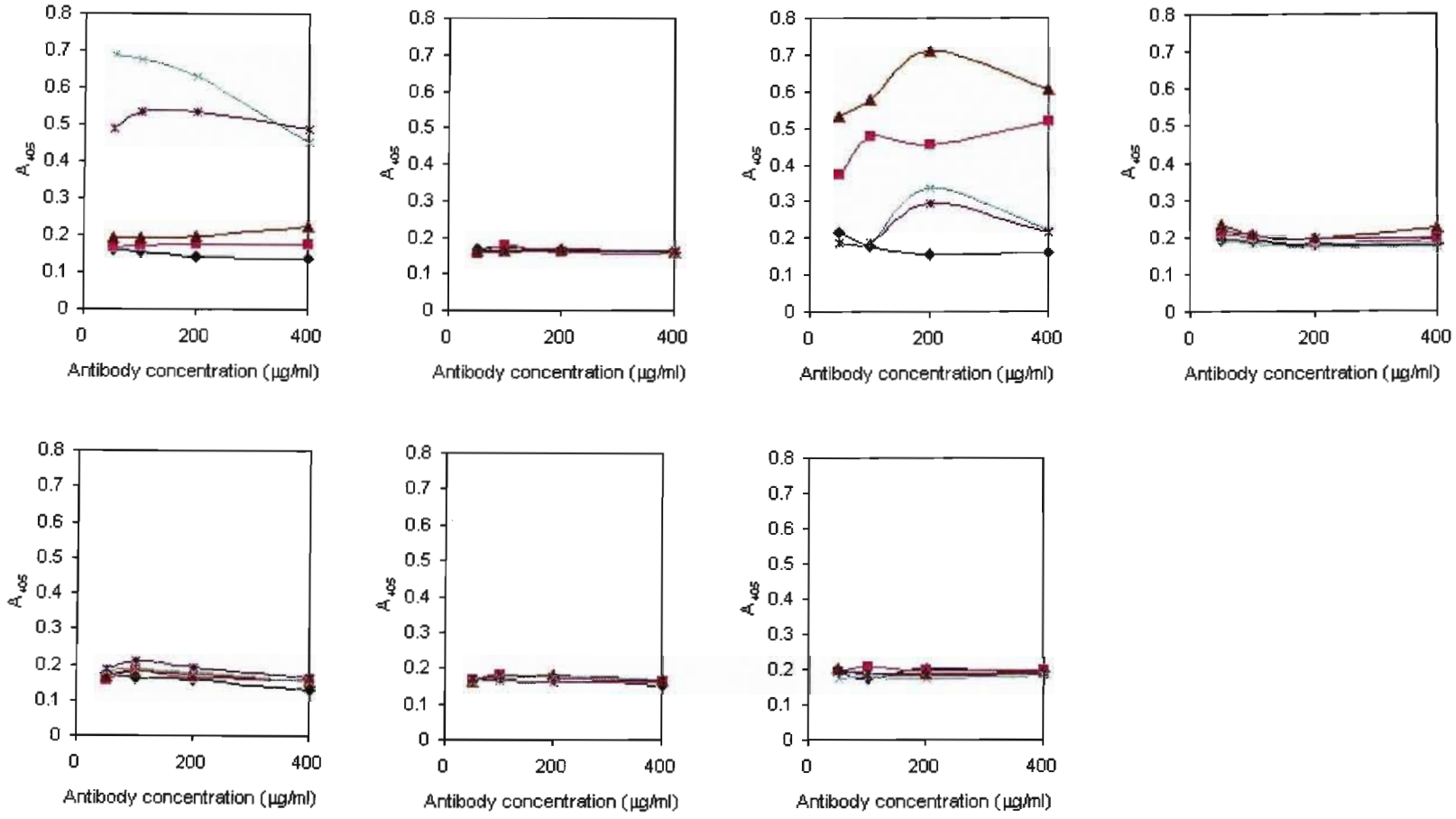

Figure 8.3. Recognition of peptides by anti-oligopeptidase B antibodies. Peptides were coated onto an ELISA plate (Section 2.8.2) and week 9 IgY from chickens immunised with rOPC or rOPV diluted serially from 400 µg/ml (in BSA-PBS, 100 µl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 3.13. Absorbance readings at 405 nm of week 9 anti-rOPC IgY from chicken 1 (■) and chicken 2 (▲); and week 9 anti-rOPV IgY from chicken 1 (×), and chicken 2 (✳); and non-immune IgY (◆) represent the average of duplicate experiments. Panels A-G show plates coated with peptides OpBTc1-7, as labelled.

8.6.1.2 Western blotting evaluation of antibodies raised in chickens

The antibodies that were raised in chickens against rOPC and rOPV were assessed for their ability to recognise denatured rOPC and rOPV by western blotting (Fig. 8.4). Both proteins were recognised by their respective antibodies, with a band apparent at approximately 80 kDa. Cross-reactivity between the antibodies was also observed, with rOPC being recognised by anti-rOPV antibodies and vice versa. A second band at approximately 66 kDa was also apparent in western blotting of rOPC.

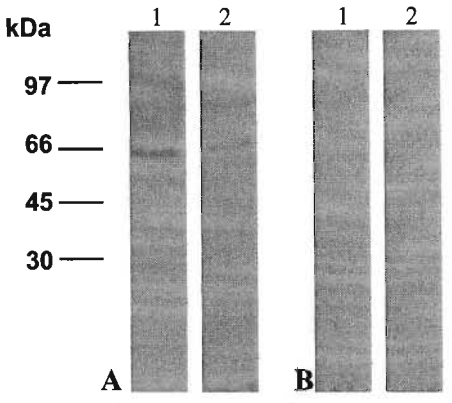

Figure 8.4. Western blotting of rOPC and rOPV to determine recognition of the denatured protein by antibodies. Protein, rOPC (A; 2 μg) and rOPV (B; 2 μg), was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with the following IgY: blot 1, anti-rOPC (25 μg/ml); blot 2, anti-rOPV(25 μg/ml). Secondary antibody was HRPO-conjugated rabbit anti-chicken IgY. Reaction was developed with 4-chloro-1-naphthol/H_2O_2.

Anti-oligopeptidase B antibodies were used to probe parasite lysates by western blotting, to assess their recognition of native oligopeptidase B. Probing of *T. congolense* lysate with anti-rOPC antibodies resulted in a band at approximately 80 kDa, corresponding to oligopeptidase B, and a prominent band at approximately 50 kDa (Fig. 8.5, lane 1). Anti-rOPV antibodies did not recognise oligopeptidase B, but did detect a band at approximately 65 kDa (lane 2). The anti-peptide antibodies against OpBTc1 (lane 3) and OpBTc3 (lane 4) recognised a band at approximately 80 kDa, corresponding to oligopeptidase B, and anti-OpBTc3 antibodies also showed several other bands. Probing of *T. vivax* lysate with the anti-rOPC antibodies resulted in a very light band at approximately 65 kDa (Fig. 8.6, lane 1), whereas anti-rOPV antibodies recognised the same band with a stronger signal, as well as a band at approximately 80 kDa (lane 2). Anti-OpBTc1 antibodies recognised a band at about 80 kDa, as well as a band at about

120 kDa (lane 3), whereas anti-OpBTc3 antibodies recognise multiple bands on the blot, including one at about 80 kDa (lane 4).

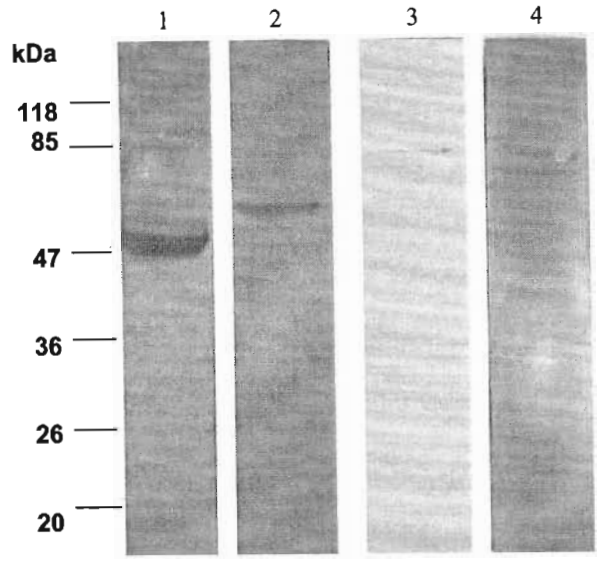

Figure 8.5. Western blotting of *T. congolense* parasite lysate with anti-oligopeptidase B antibodies. *T. congolense* IL3000 lysate (80 µl) was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The membrane was incubated with the following IgY: blot 1, anti-rOPC (50 µg/ml); blot 2, anti-rOPV (50 µg/ml); blot 3, affinity purified anti-OpBTc1 peptide (10 µg/ml); blot 4, affinity purified anti-OpBTc3 peptide (10 µg/ml). Bands were detected as per Fig. 8.4.

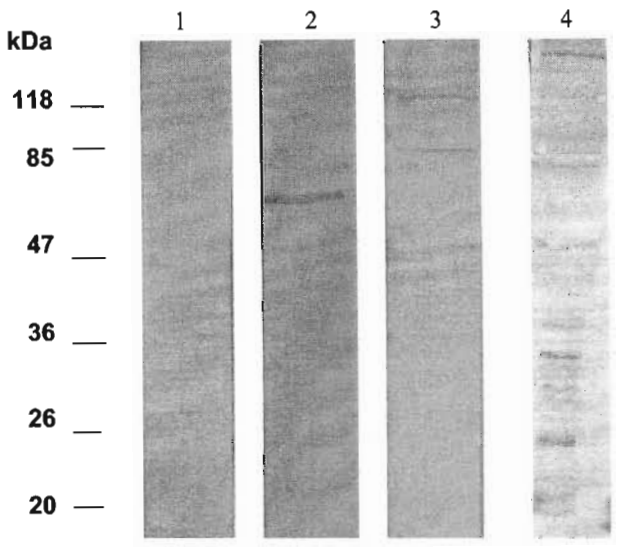

Figure 8.6. Western blotting of *T. vivax* parasite lysate with anti-oligopeptidase B antibodies. *T. vivax* IL4186 lysate (20 µl) was electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. The membrane was incubated with the following IgY: blot 1, anti-rOPC (50 µg/ml); blot 2, anti-rOPV (50 µg/ml); blot 3, affinity purified anti-OpBTc1 peptide (10 µg/ml); blot 4, affinity purified anti-OpBTc3 peptide (10 µg/ml). Bands were detected as per Fig. 8.4.

8.6.1.3 Inhibition of oligopeptidase B activity by antibodies raised in chickens against recombinant oligopeptidase B

Anti-oligopeptidase B antibodies were strongly inhibitory towards the peptidolytic activity of their respective enzymes (Fig. 8.7). At week 4 of antibody production, anti-rOPC antibodies were not inhibitory towards rOPC (A and B) since the relative activity against Z-Arg-Arg-AMC was unaffected. But, by week 5, inhibition began to increase, and was at a maximum (up to 84% inhibition by anti-rOPC-1, and 87% inhibition by anti-rOPC-2) at weeks 9 and 10. Inhibition of rOPV activity by anti-rOPV was more efficient than for anti-rOPC antibodies in the early weeks of antibody production (C and D). Inhibition was at a maximum (up to 88% inhibition by anti-rOPC-1, and 87% inhibition by anti-rOPC-2) between weeks 8 and 10.

After affinity purification of the anti-oligopeptidase B antibodies, their ability to inhibit the activity of the recombinant oligopeptidases was reassessed (Table 8.2). Inhibition of the enzymes by their respective antibodies was slightly enhanced after affinity purification, with a maximum of 88.7 % inhibition of rOPC and 91.5% inhibition by rOPV. Cross-reactivity of the inhibition was also evident, with anti-rOPC antibodies inhibiting up to 78% of rOPV activity, and anti-rOPV antibodies inhibition up to 86.4% of rOPC activity.

Table 8.2. Inhibition of recombinant oligopeptidase B activity by affinity purified antibodies

		Inhibition (%)[a]	
Enzyme	Concentration of antibody (mg/ml)	Anti-rOPC	Anti-rOPV
rOPC	1.000	n.d.[b]	86.4
	0.500	n.d.	82.5
	0.250	88.7	78.2
	0.125	85.6	68.5
rOPV	1.000	n.d.	91.5
	0.500	n.d.	89.3
	0.250	76.0	89.4
	0.125	78.0	84.9

[a] Activity was expressed as a percentage relative to the non-immune control (0% inhibition)
[b] n.d: Not determined due to low concentration of antibody samples

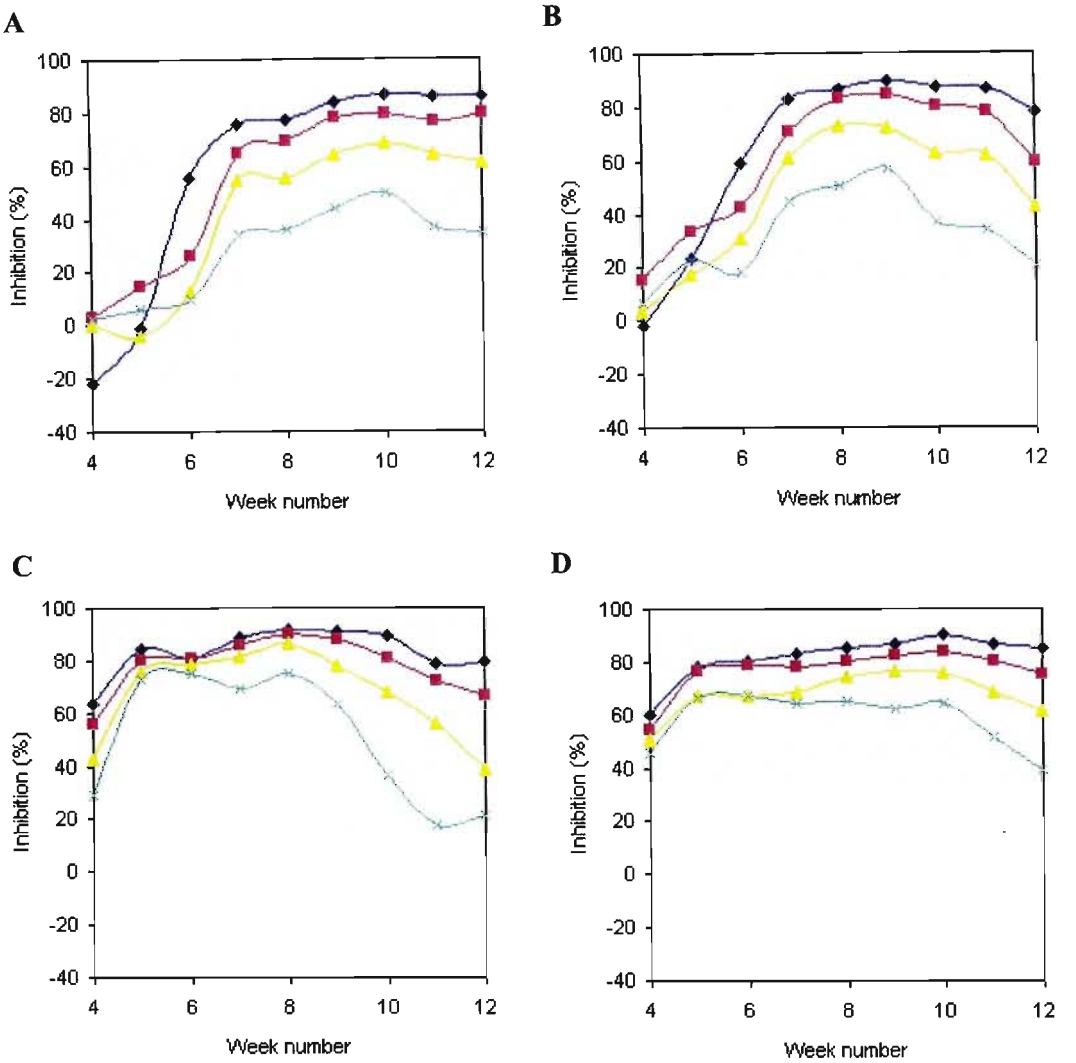

Figure 8.7. Inhibition of recombinant oligopeptidase B activity by varying concentrations of anti-oligopeptidase B IgY from weeks 4 to 12 in the immunisation protocol. Peptidolytic activity of recombinant oligopeptidase B with (A) anti-rOPC-1, (B) anti-rOPC-2, (C) anti-rOPV-1, and (D) anti-rOPV-2 IgY at 1 mg/ml (◆), 0.5 mg/ml (■), 0.25 mg/ml (▲), and 0.125 mg/ml (×) was determined by the extent of hydrolysis of Z-Arg-Arg-AMC and expressed as a percentage inhibition relative to that of an oligopeptidase B control with non-immune antibody (0% inhibition). Each plot represents the mean of two experiments. A and B, rOPC activity; C and D, rOPV activity.

8.6.1.4 Inhibition of native oligopeptidase B activity by affinity purified antibodies raised in chickens against recombinant oligopeptidase B

The effect of affinity purified anti-oligopeptidase B antibodies on partially purified preparations of native oligopeptidase B (isolated in Chapter 4) from *T. b. brucei*, *T. congolense* and *T. vivax* was assayed (Table 8.3). Affinity purified anti-rOPC antibodies strongly inhibited the peptidolytic activity of each of the three native enzymes, with the maximum inhibition of 78.2% against *T. congolense* oligopeptidase B. Anti-rOPV antibodies were also inhibitory, though to a lesser degree, towards the activity of all three enzymes, with a maximum inhibition of 48.1% against *T. b. brucei* oligopeptidase B.

Table 8.3. Inhibition of native oligopeptidase B activity by affinity purified antibodies

		Inhibition (%)[a]		
Antibody	Concentration (mg/ml)	*T. b. brucei*	*T. congolense*	*T. vivax*
Anti-rOPC	0.250	77.8	78.2	73.8
	0.125	62.7	66.4	63.4
Anti-rOPV	0.250	48.1	40.4	34.0
	0.125	45.0	26.5	29.6

[a] Activity was expressed as a percentage relative to the non-immune control (0% inhibition)

8.6.2 Production of antibodies in rabbits

8.6.2.1 Evaluation of rabbit anti-C2 antibodies by ELISA (study I)

Anti-C2 antibodies were present in the IgG from rabbits B2, F1, F2 and R1 at week 3 (Fig. 8.8, A). IgG from rabbits A1, A2 and R2 gave a signal in ELISA that was equivalent to the non-immune IgG. The highest antibody levels were present in the IgG of F2, whilst F1 and B2 showed low levels (see Table 8.1 for explanation of immunogens). Antibody levels for B1 are not shown for week three because the rabbit was not bled. At week 6, anti-C2 antibodies were present in the IgG from all of the rabbits, except for A2, which only rose above the non-immune signal at the highest antibody concentration (Fig. 8.8, B). Very high levels of antibody were shown for rabbits F2 and F1, whereas low levels were shown by rabbits A1 and R2. At week 9, the same trend in antibody levels as for week 6 was apparent, though the levels for A1 and R2 were lower since their signals were less discernable from that of the non-immune antibody (Fig. 8.8, C).

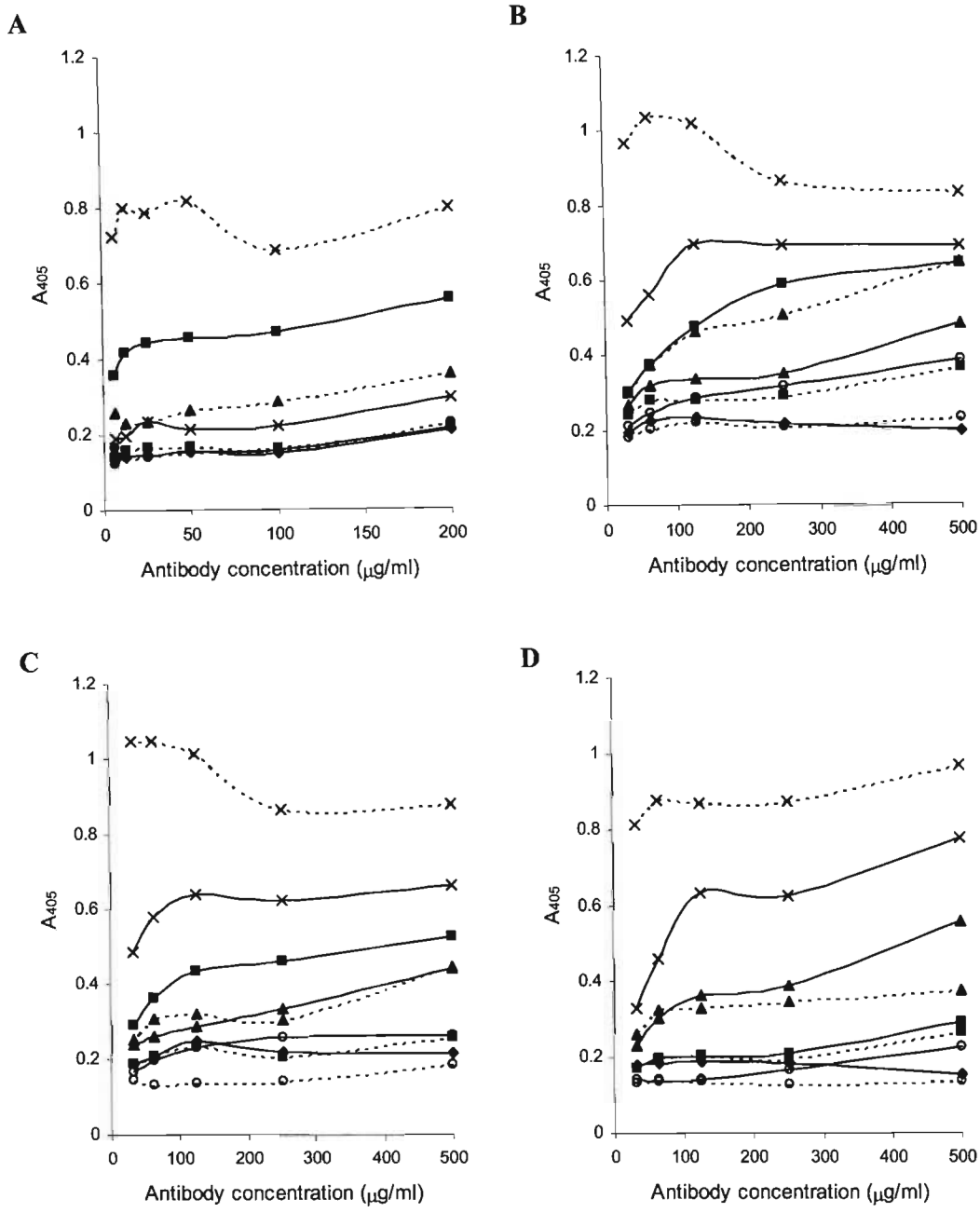

Figure 8.8. ELISA of anti-C2 antibodies produced at weeks 3 (A), 6 (B), 9 (C), and 11 (D) by rabbits immunised with C2 alone, or in combination with different adjuvants (study I). C2 was coated onto ELISA plates (Section 2.8.2) and rabbit IgG diluted serially from 200 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). Goat anti-rabbit-HRPO secondary antibodies (in BSA-PBS, 120 μl per well, 1 h, 37°C) and ABTS/H_2O_2 (150 μl per well) were used as the detection system. Absorbance readings at 405 nm of IgG from A1 (○; solid line), A2 (○; broken line), B1 (▲; solid line), B2 (▲; broken line), F1 (×; solid line), F2 (×; broken line), R1 (■; solid line), and R2 (■; broken line), and non-immune IgG (◆) represent the average of duplicate experiments.

At week 11, F2 and F1 still maintained the highest anti-C2 antibody levels whereas the levels for R1 had dropped to become level with R2. The anti-C2 antibody levels for B1 also showed an increase at week 11. Similar to all the other weeks tested, A2 did not show any detectable amounts of anti-C2 antibodies (Fig. 8.8, D). In summary, both rabbits immunised with C2 using Freund's adjuvant showed consistently high antibody levels throughout the immunisation schedule, although F1 showed a slower antibody response than F2. Rabbits immunised with C2 alone showed a very poor antibody response. Rabbits immunised with C2 complexed to either bovine or rabbit α_2M showed a very low antibody response.

Recognition of native congopain by anti-C2 antibodies collected over the 12 week immunisation period was assayed in an ELISA using a single antibody concentration of 100 μg/ml (Fig. 8.9). The anti-C2 antibodies showed good recognition of native congopain. The highest antibody levels were observed for weeks 3 and 6, after which antibody levels declined. Generally, these results agree with those shown in Fig. 8.8 for the recognition of C2 in an ELISA, except that there appears to be slightly better recognition of native congopain by antibodies from rabbits immunised with C2 without any adjuvant.

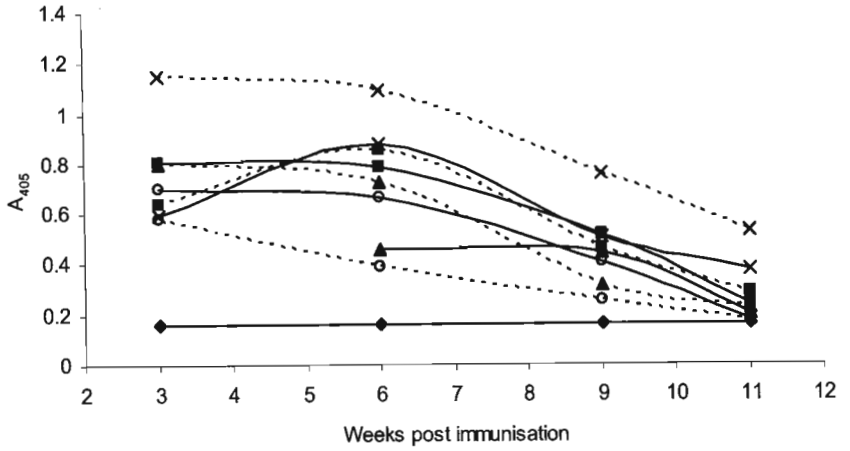

Figure 8.9. Recognition of congopain by anti-C2 antibodies produced at weeks 3, 6, 9, and 11 by rabbits immunised with C2 alone, or in combination with different adjuvants (study I). Congopain was coated onto ELISA plates (Section 2.8.2) and incubated with rabbit IgG at 100 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 8.8. Absorbance readings at 405 nm of IgG A1 (○; solid line), A2 (○; broken line), B1 (▲; solid line), B2 (▲; broken line), F1 (×; solid line), F2 (×; broken line), R1 (■; solid line), and R2 (■; broken line), and non-immune IgG (◆) represent the average of two experiments.

8.6.2.2 Evaluation of rabbit anti-C2 antibodies by ELISA (study II)

An ELISA to determine recognition of C2 by IgG from all of the rabbits that were immunised was conducted at a single concentration of 20 μg/ml over the entire 16 week immunisation schedule (Fig. 8.10). The highest antibody levels were observed for those rabbits that were immunised with free C2 (and rOPC) in alum (AlC1 and AlC2). The next highest antibody response was observed for those rabbits that were immunised with free C2 (and rOPV) in alum (AlV1 and AlV2). Moderate levels of antibodies were produced against C2 in rabbits immunised with C2-oligopeptidase B-α_2M complexes, and their levels were about the same as those observed for AlV1. Antibody production peaked at different times for individual rabbits. Generally, antibody levels were still high in all rabbits at week 16, although levels appeared to be decreasing in those rabbits immunised with proteins in alum.

A similar ELISA was conducted to assess recognition of congopain by anti-C2 IgG (Fig. 8.11). Again, the highest antibody levels were observed where free proteins were immunised in alum. IgG from rabbits immunised with C2-oligopeptidase B-α_2M

complexes showed poor recognition of congopain and were barely detectable above the background of the non-immune IgG. In summary, immunisation with C2 in alum resulted in the production of much higher levels of anti-C2 antibodies.

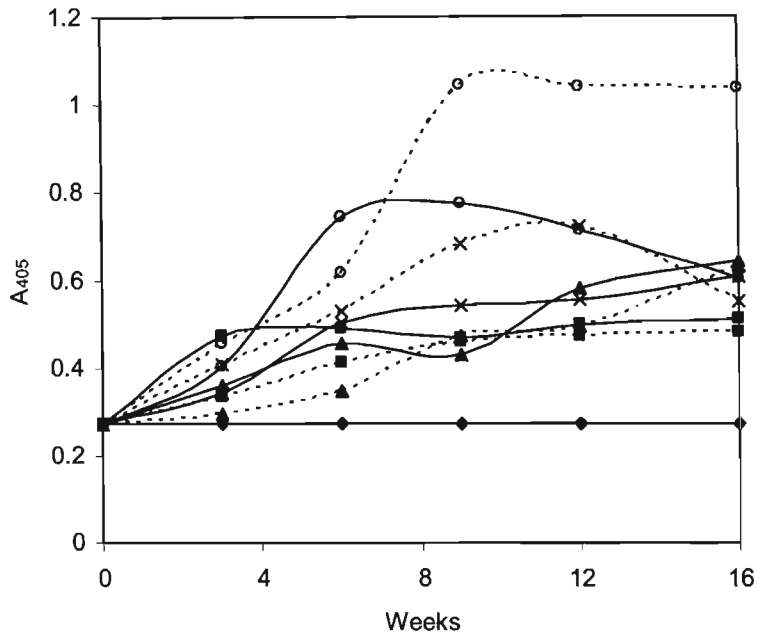

Figure 8.10. Recognition of C2 by rabbit IgG produced at weeks 3, 6, 9, 12 and 16 by rabbits immunised with C2 and oligopeptidase B in alum, or complexed to α_2M. C2 was coated onto an ELISA plate (Section 2.8.2) and incubated with rabbit IgG at 20 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 8.8. Absorbance readings at 405 nm of IgG AlC1 (○; solid line), AlC2 (○; broken line), MaC1 (▲; solid line), MaC2 (▲; broken line), AlV1 (×; solid line), AlV2 (×; broken line), MaV1 (■; solid line), and MaV2 (■; broken line), and non-immune IgG (◆) represent the average of two experiments.

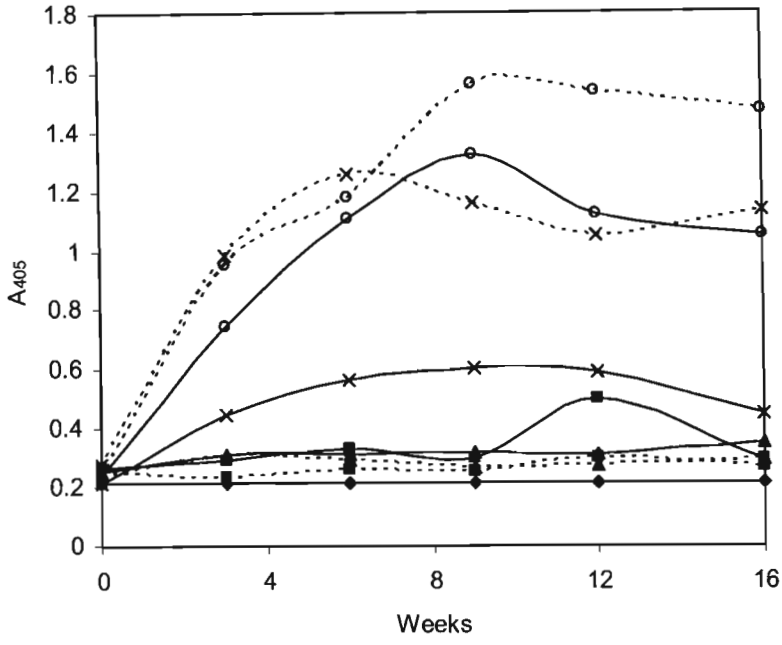

Figure 8.11. Recognition of congopain by rabbit IgG produced at weeks 3, 6, 9, 12 and 16 by rabbits immunised with C2 and oligopeptidase B in alum, or complexed to α_2M. Congopain was coated onto an ELISA plate (Section 2.8.2) and incubated with rabbit IgG at 20 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 8.8. Absorbance readings at 405 nm of IgG AlC1 (○; solid line), AlC2 (○; broken line), MaC1 (▲; solid line), MaC2 (▲; broken line), AlV1 (×; solid line), AlV2 (×; broken line), MaV1 (■; solid line), and MaV2 (■; broken line), and non-immune IgG (◆) represent the average of two experiments.

8.6.2.3 Evaluation of rabbit anti-oligopeptidase B antibodies by ELISA (study II)

ELISAs to determine recognition of rOPC and rOPV by IgG from the rabbits that were immunised with rOPC or rOPV (and C2) were conducted at a single concentration of 20 μg/ml over the entire 16 week immunisation schedule (Fig. 8.12). Levels of anti-rOPC antibodies appeared to be similar in IgG from rabbits that were immunised with rOPC (either in alum, or in complex with C2 and α_2M) (Fig. 8.12, A). Antibody levels appeared to peak at either week 12 or 16. High anti-rOPV antibody levels were observed in IgG from rabbit AlV2, which was immunised with rOPV (and C2) in alum (Fig. 8.12, B). Antibody levels were similarly lower in the IgG from the remaining 3 rabbits. Anti-rOPV levels were generally still increasing by week 16. In summary, anti-oligopeptidase B antibody levels were similar in all rabbits, regardless of the adjuvant type.

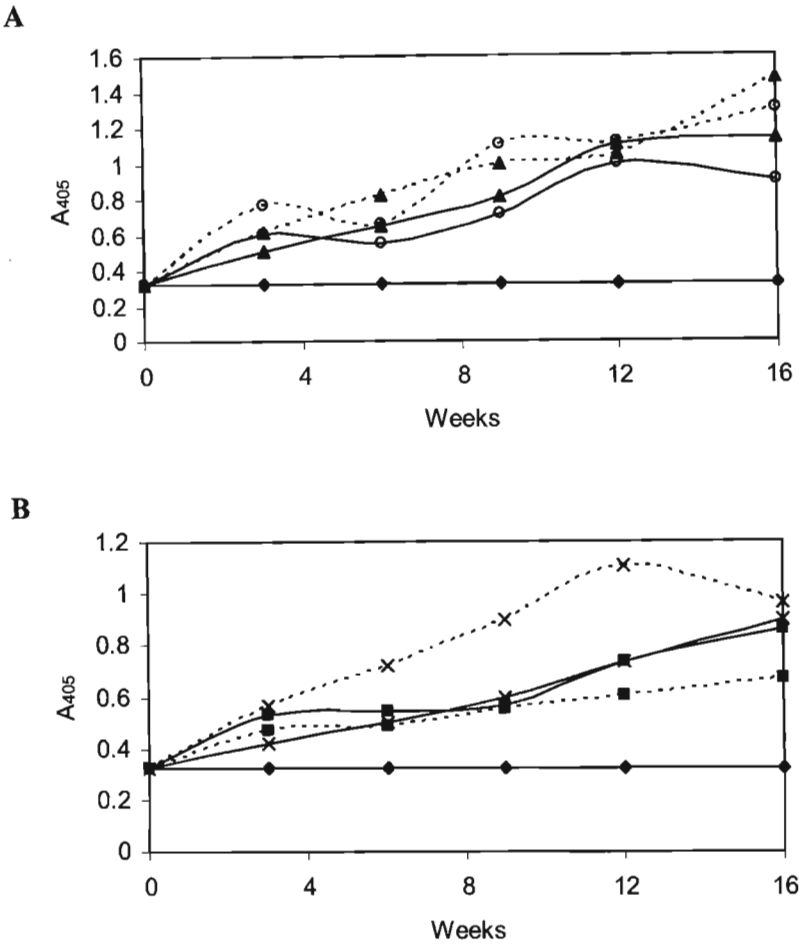

Figure 8.12. Recognition of rOPC and rOPV by rabbit IgG produced at weeks 3, 6, 9, 12 and 16 by rabbits immunised with C2 and oligopeptidase B in alum, or complexed to α_2M. Protein, (A) rOPC, (B) rOPV, was coated onto ELISA plates (Section 2.8.2) and incubated with rabbit IgG at 20 μg/ml (in BSA-PBS, 100 μl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 8.8. Absorbance readings at 405 nm of IgG AlC1 (○; solid line), AlC2 (○; broken line), MaC1 (▲; solid line), MaC2 (▲; broken line), AlV1 (×; solid line), AlV2 (×; broken line), MaV1 (■; solid line), and MaV2 (■; broken line), and non-immune IgG (◆) represent the average of two experiments.

8.6.2.4 Evaluation of rabbit anti-α_2M antibodies by ELISA (study II)

An ELISA was conducted to observe the trend of anti-α_2M antibody production in those rabbits that were immunised with α_2M-C2-oligopeptidase B complexes (Fig. 8.13), using a single concentration of IgG (20 μg/ml) over the immunisation period of 16 weeks. Anti-α_2M antibody levels were very high in each of the four rabbits, peaking rapidly at week 6. After week 6, anti-α_2M levels declined steadily up to week 16.

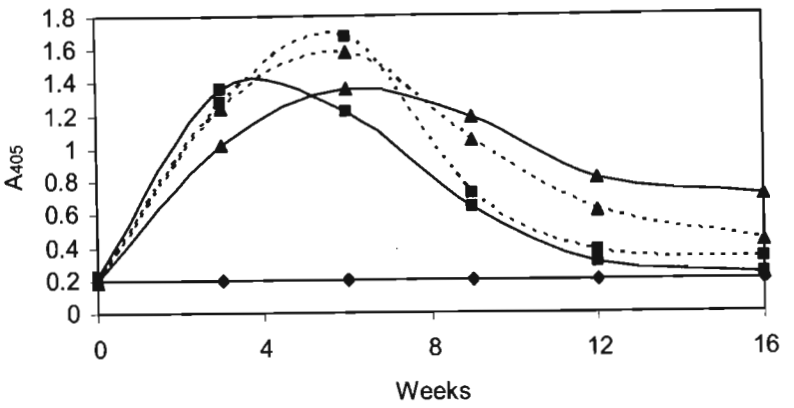

Figure 8.13. Recognition of α_2M by rabbit IgG produced at weeks 3, 6, 9, 12 and 16 by rabbits immunised with C2 and oligopeptidase B complexed to α_2M. α_2M was coated onto an ELISA plate (Section 2.8.2) and incubated with rabbit IgG at 20 µg/ml (in BSA-PBS, 100 µl per well, 2 h, 37°C). The ELISA was developed as described in Fig. 8.8. Absorbance readings at 405 nm of IgG MaC1 (▲; solid line), MaC2 (▲; broken line), MaV1 (■; solid line), and MaV2 (■; broken line), and non-immune IgG (◆) represent the average of two experiments.

8.6.2.5 Western blotting evaluation of antibodies raised in rabbits

The proteins that were used for immunisation purposes in the present study were electroblotted to determine whether the antibodies raised in rabbits were able to recognise the denatured form of the antigen. A representative positive result for western blotting is shown in Fig. 8.14B. C2, both reduced and non-reduced, showed two major bands by western blotting. The first band corresponded to the C2 monomer at 27 kDa (reduced; lane 2) and 22 kDa (non-reduced; lane 3), and the second band occurred at approximately 50 kDa. The two oligopeptidases, rOPC (lane 4) and rOPV (lane 5), also showed two major bands by western blotting, at approximately 80 kDa and 60 kDa.

Of the antibodies produced in study I, IgG from rabbits F1 and F2 (at 100 µg/ml) showed recognition of both reduced and non-reduced C2, whereas IgG from other rabbits showed no recognition. For study II, C2 was recognised by IgG from rabbits AlC1 and AlC2 at 50 µg/ml, as well as AlV2 and MaC1 at 100 µg/ml. rOPC was recognised by IgG from rabbits AlC1 at 50 µg/ml and AlC2 at 75 µg/ml, while rOPV was recognised by IgG from rabbit AlV2 at 75 µg/ml. IgG from other rabbits was tested up to 150 µg/ml but did not show recognition.

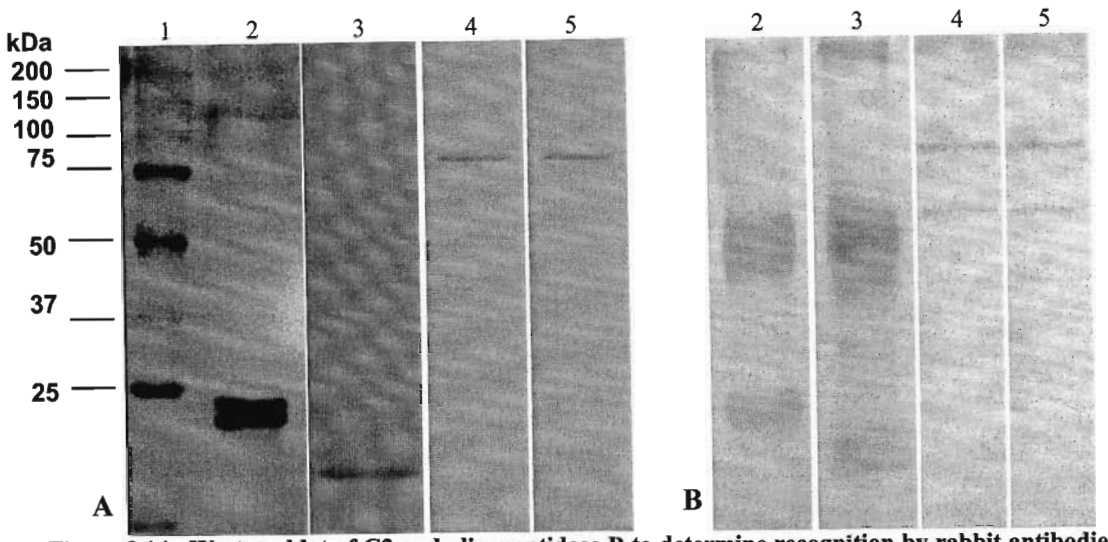

Figure 8.14. Western blot of C2 and oligopeptidase B to determine recognition by rabbit antibodies. Proteins were electrophoresed on a 10% SDS-PAGE gel (A) and electroblotted onto nitrocellulose membrane (B). Lane 1, Bio-Rad Precision Plus markers; lane 2, reduced C2 (5 μg); lane 3, non-reduced C2 (5 μg); lane 4, reduced rOPC (2 μg); lane 5, reduced rOPV (2 μg). (A) Proteins were stained with Coomassie blue R-250 (Section 2.6.1). (B) Transferred protein was incubated with IgG from rabbits, immunised with C2 alone or in combination with oligopeptidase B (as per Table 8.1), at between 50-150 μg/ml. Secondary antibody was HRPO-conjugated goat anti-rabbit IgG. Bands were developed with 4-chloro-1-naphthol/H_2O_2.

8.6.2.6 Inhibition of C2 activity by rabbit IgG (study I)

In the experiment for inhibition of C2 activity by varying concentrations of week 3 anti-C2 IgG, the greatest inhibition was observed for rabbit B2 (Fig. 8.15, A). This inhibitory effect on C2 activity against the substrate was titrated out as antibody concentration was decreased. Other IgG showed a small amount of activation, i.e. enhancement, of C2 activity, except for A1 and A2 which showed some inhibition at higher IgG concentrations. With week 6 IgG, all rabbits IgG showed inhibition of C2 activity (Fig. 8.15, B). This effect was titrated out with decreasing antibody concentration, and was highest with IgG from rabbits R1 and R2 (immunised with C2 complexed to rabbit α_2M; 50.3% by R1 at 1 mg/ml), followed by B1 and B2 (immunised with C2 complexed to bovine α_2M).

When testing the inhibition of C2 activity by varying concentrations of week 9 anti-C2 IgG, R1 showed the best inhibition at all concentrations of IgG, with a maximum of 55.6% at 1 mg/ml (Fig. 8.15, C). R2 showed better inhibition at 500 and 250 μg/ml than

at 1 mg/ml. B2 and F2 showed no inhibition, except for 1.2% for F2 at 250 μg/ml. IgG from A2, B2 and F2 enhanced the activity of C2 towards the substrate. In the experiment for inhibition of C2 activity by varying concentrations of week 11 anti-C2 IgG, all the IgG resulted in inhibition, except for A1 and A2 which generally showed activation of C2. (Fig. 8.15, D). All IgG showed the concentration dependent trend as seen for week 6. The best inhibition was by B2 which achieved 64.7%, followed closely by R2 at 61.2% inhibition.

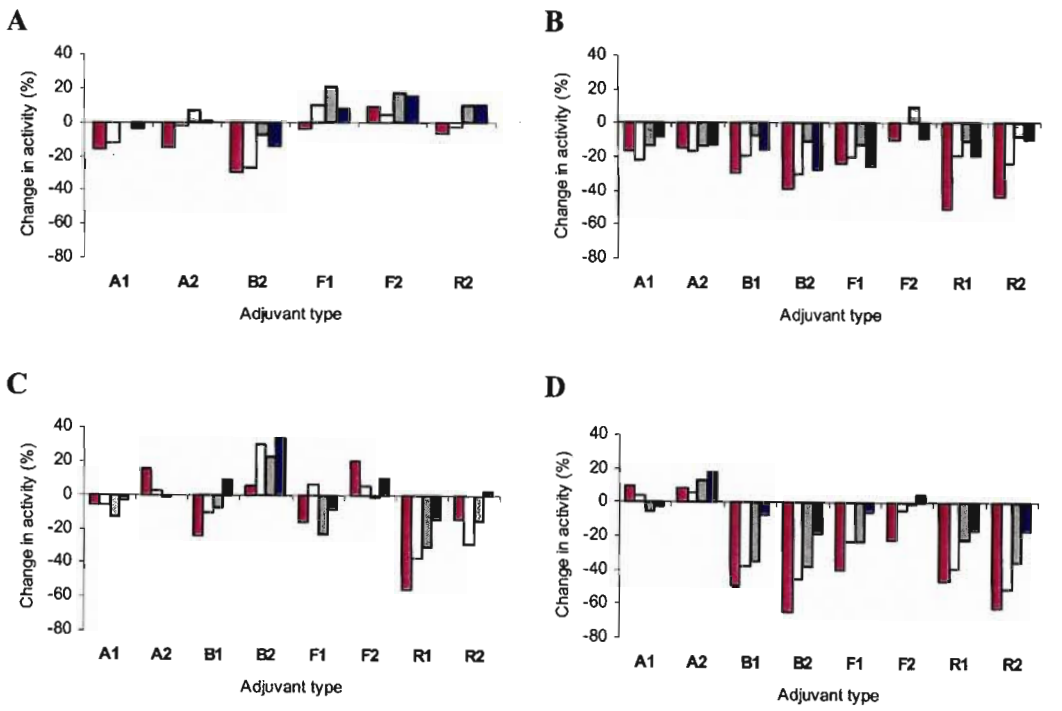

Figure 8.15. Inhibition of C2 by varying concentrations of anti-C2 antibodies from weeks 3, 6, 9 and 11 in the immunisation protocol (study I). Proteolytic activity of C2 in the presence of IgG from A1, A2, B1, B2, F1, F2, R1, and R2, at 1 mg/ml (■), 0.5 mg/ml (□), 0.25 mg/ml (■) and 0.125 mg/ml (■), at weeks 3 (A), 6 (B), 9 (C), and 11 (D), was determined by the extent of hydrolysis of Z-Phe-Arg-AMC and expressed as a percentage change in activity compared to that of a C2 control with non-immune antibody (0%). Each plot represents the mean of two experiments.

8.6.2.7 Inhibition of congopain activity by rabbit anti-C2 antibodies (study I)

In the experiment for inhibition of congopain activity by varying concentrations of week 6 anti-C2 IgG, all but F2 and A2 showed inhibition of congopain activity at 1 mg/ml (Fig. 8.16). F2 did not inhibit congopain activity at any concentration, whereas F1 showed inhibition at all concentrations. R1 and B1 were not inhibitory at 500 and 250 µg/ml. The best inhibition of congopain was by R2, which was 36.2% at 1 mg/ml.

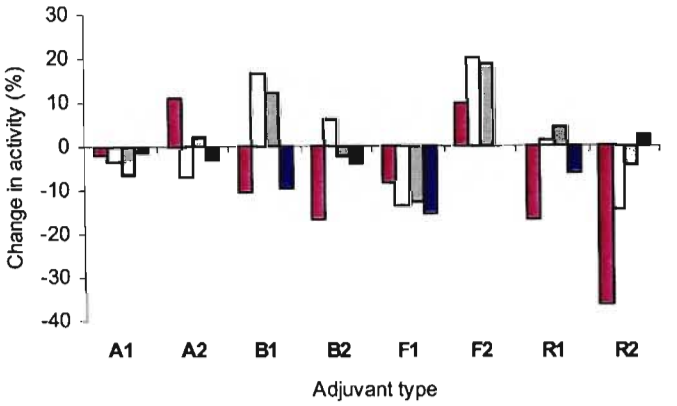

Figure 8.16. Inhibition of congopain by varying concentrations of anti-C2 antibodies from week 6 in the immunisation protocol. Proteolytic activity of congopain in the presence of IgG from A1, A2, B1, B2, F1, F2, R1, and R2, at 1 mg/ml (■), 0.5 mg/ml (□), 0.25 mg/ml (▣) and 0.125 mg/ml (▪), was determined by the extent of hydrolysis of Z-Phe-Arg-AMC and expressed as a percentage change in activity compared to that of a congopain control with non-immune antibody (0%). Each plot represents the mean of two experiments.

8.6.2.8 Inhibition of C2 activity by rabbit IgG (study II)

In the experiment where inhibition of C2 activity against Z-Phe-Arg-AMC by week 3 rabbit IgG preparations was tested, all preparations at all concentrations (except for AlC1 at 1 mg/ml and 0.125 mg/ml, and MaV1 at 0.125 mg/ml) resulted in inhibition (Fig. 8.17, A). The greatest inhibition was 43.8 % by MaC1 IgG at 0.5 and 0.25 mg/ml. The next best inhibition was by AlV1 and AlV2, followed by AlC2 and MaC2. AlC1, MaV1 and MaV2 showed relatively poor inhibition of C2 activity.

When testing the inhibition of C2 activity against Z-Phe-Arg-AMC by week 6 rabbit IgG, the best inhibition was observed at 0.5 mg/ml of IgG in each case (Fig. 8.17, B). The best inhibition was observed for MaC1 (36.8% at 0.5 mg/ml), followed closely by AlV1 and MaC2. The least inhibition was observed for AlC1 and AlC2. Relatively strong inhibition of C2 activity was observed with week 9 rabbit IgG, where IgG from all rabbits was inhibitory (Fig, 8.17, C). The greatest inhibition resulted with IgG from MaV1 (54.7% at 1 mg/ml), followed closely by IgG from MaC2, MaC1 and AlV1. The least amount of inhibition resulted with IgG from AlC1.

IgG from week 12 showed generally the least inhibition of C2 activity of all the weeks (Fig. 8.17, D). The greatest amount of inhibition was observed for MaV2 (36.9% at 0.5 mg/ml), followed by MaC2, AlV1 and AlV2. The greatest inhibition by week 16 IgG was by MaV2 and MaC1, with 46.1% inhibition at 0.5 mg/ml (Fig. 8.17, E). IgG from AlC2 showed the poorest inhibition of C2 activity. Overall, IgG from MaC1 was consistently the most inhibitory towards C2 activity, whilst AlC1 and AlC2 consistently showed the least inhibition.

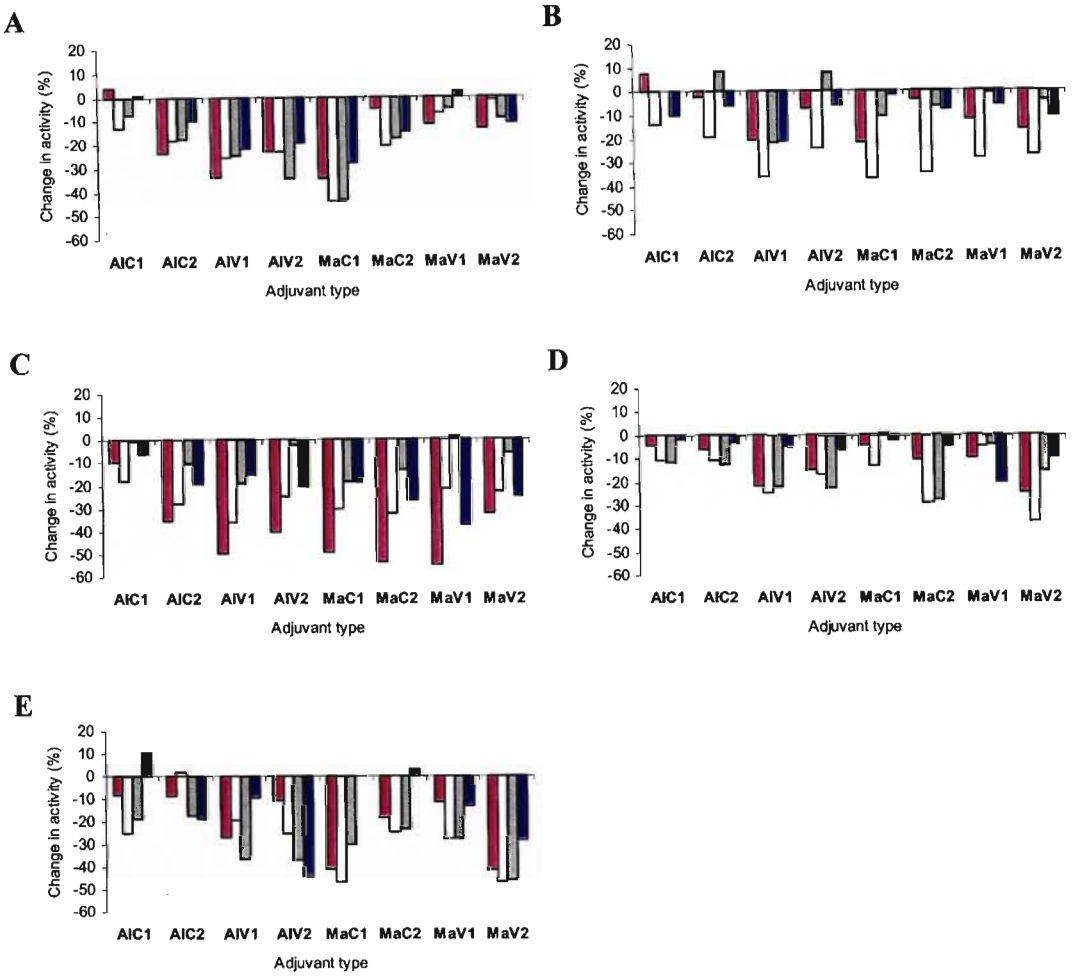

Figure 8.17. Inhibition of C2 by varying concentrations of anti-C2 antibodies from weeks 3, 6, 9, 12 and 16 in the immunisation protocol (study II). Proteolytic activity of C2 in the presence of IgG from AlC1, AlC2, AlV1, AlV2, MaC1, MaC2, MaV1and MaV2 at 1 mg/ml (■), 0.5 mg/ml (□), 0.25 mg/ml (▩) and 0.125 mg/ml (■), at weeks 3 (A), 6 (B), 9 (C), 12 (D), and 16 (E), was determined by the extent of hydrolysis of Z-Phe-Arg-AMC and expressed as a percentage change in activity compared to that of a C2 control with non-immune antibody (0%). Each plot represents the mean of two experiments.

8.6.2.9 Inhibition of congopain activity by rabbit IgG (study II)

In the experiment for inhibition of congopain activity by varying concentrations of week 9 anti-C2 IgG, a very low degree of inhibition was observed in general (Fig. 8.18). The best inhibition appeared to occur with the use of AlV1 (20% inhibition at 0.5 mg/ml) and AlC2, where all concentrations of antibody resulted in inhibition. For the other preparations of IgG, the levels of inhibition fluctuated between the different

concentrations of antibody. But, some degree of inhibition of congopain activity was observed for all IgG for at least one concentration.

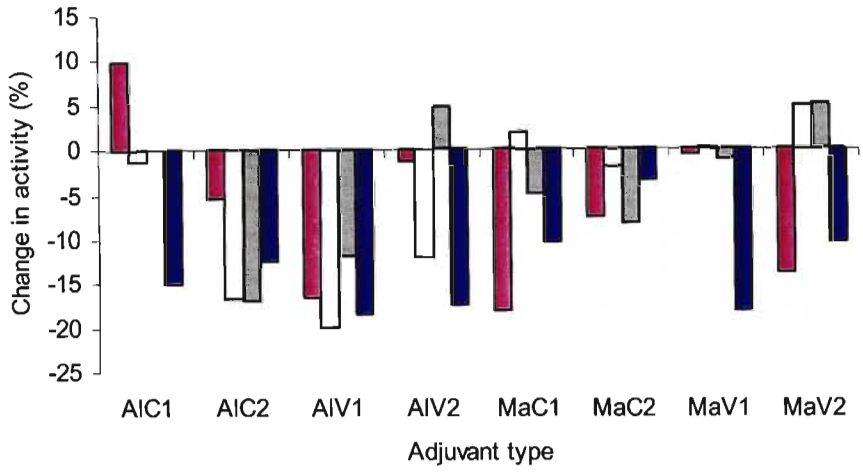

Figure 8.18. Inhibition of congopain by varying concentrations of anti-C2 antibodies from week 9 in the immunisation protocol (study II) Proteolytic activity of congopain with IgG from AlC1, AlC2, AlV1, AlV2, MaC1, MaC2, MaV1and MaV2 at 1 mg/ml (■), 0.5 mg/ml (□), 0.25 mg/ml (■) and 0.125 mg/ml (■) was determined by the extent of hydrolysis of Z-Phe-Arg-AMC and expressed as a percentage change in activity compared to that of a congopain control with non-immune antibody (0%). Each plot represents the mean of two experiments.

8.6.2.10 Inhibition of oligopeptidase B activity by rabbit IgG (study II)

IgG from rabbits that were immunised with rOPC or rOPV (with alum, or in complex with C2 and α_2M) was used in assays to assess their inhibitory effect towards their respective enzymes. IgG from rabbits AlC1 and AlC2 were strongly inhibitory towards the activity of rOPC at every week tested (Fig. 8.19). The maximum inhibition of rOPC activity was 100% by week 3 IgG from AlC2 at 1 mg/ml (Fig. 8.19, A). IgG from MaC1 and MaC2 mostly enhanced the activity of rOPC by up to 31.5% at 1 mg/ml by week 12 IgG from MaC2.

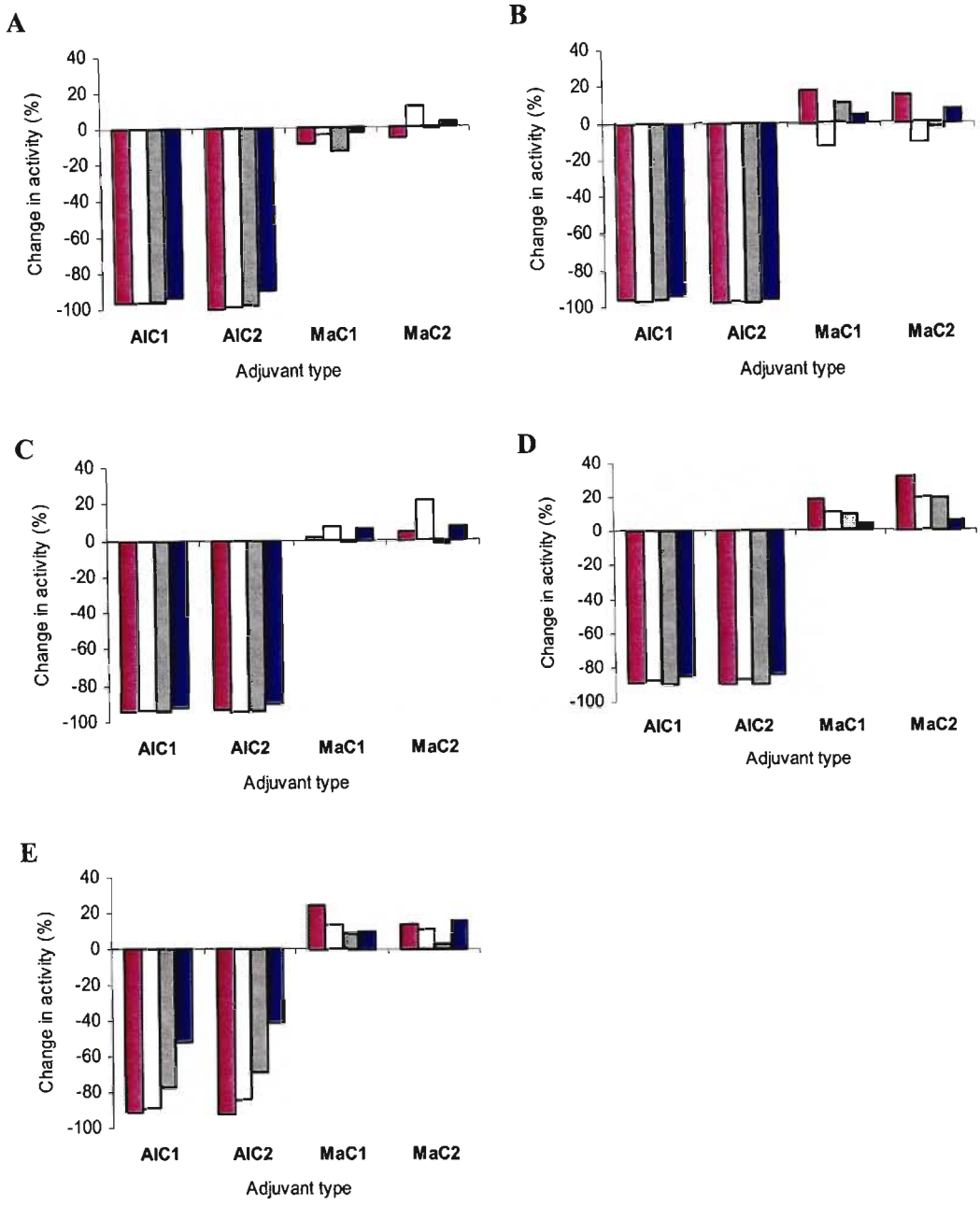

Figure 8.19. Inhibition of rOPC by varying concentrations of anti-rOPC antibodies from weeks 3, 6, 9, 12 and 16 in the immunisation protocol (study II). Proteolytic activity of rOPC with IgG from AlC1, AlC2, MaC1 and MaC2 at 1 mg/ml (■), 0.5 mg/ml (□), 0.25 mg/ml (▣) and 0.125 mg/ml (▣), at weeks 3 (A), 6 (B), 9 (C), 12 (D), and 16 (E), was determined by the extent of hydrolysis of Z-Arg-Arg-AMC and expressed as a percentage change in activity compared to that of a rOPC control with non-immune antibody (0%). Each plot represents the mean of two experiments.

IgG from rabbits AlV1 and AlV2 were strongly inhibitory towards the activity of rOPV at every week tested (Fig. 8.20), though not as strongly as observed for rOPC by AlC1

and AlC2. The maximum inhibition of rOPV activity was 99% by week 6 IgG from AlV2 at 0.5 mg/ml (Fig. 8.20, B). IgG from MaV1 and MaV2 mostly enhanced the activity of rOPV by up to 36.8% by week 16 IgG from MaV1, although some inhibition was observed for week 3 IgG (Fig. 8.20, A).

IgG from week 9 in the immunisation protocol was further assayed for cross-reactivity of inhibition (Fig. 8.21). IgG from AlV1 and AlV2 was found to inhibit rOPC activity to a small degree (maximum of 27.6% inhibition by AlV1 at 1 mg/ml), whereas MaV1 and MaV2 activated the enzyme (Fig. 8.21, A). AlC1 and AlC2 IgG were found to inhibit rOPV activity quite strongly, with a maximum of 76.2% inhibition by AlC1 at 1 mg/ml (Fig. 8.21, B). MaC1 and MaC2 had a mild activating effect on rOPV activity.

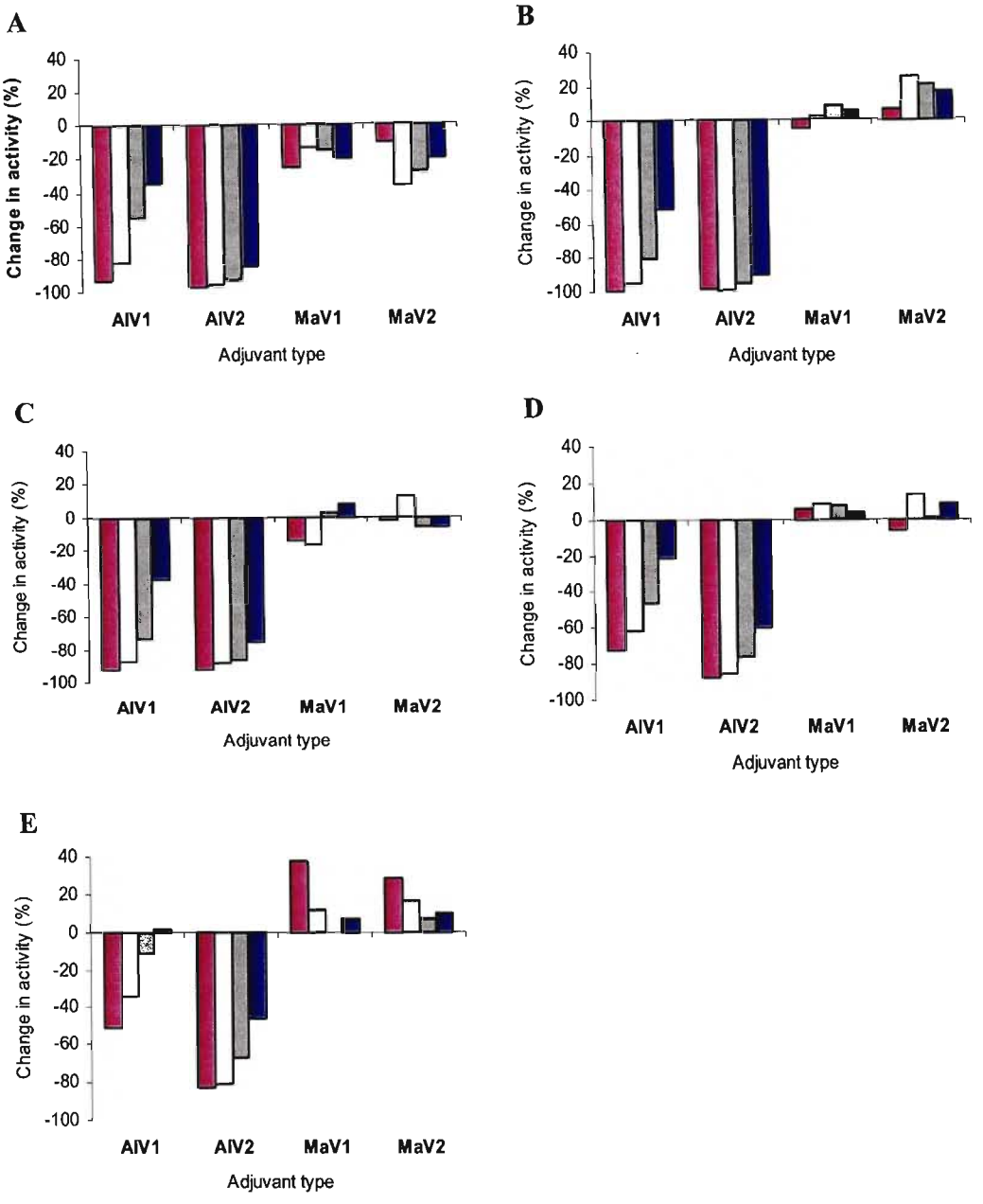

Figure 8.20. Inhibition of rOPV by varying concentrations of anti-rOPV antibodies from weeks 3, 6, 9, 12 and 16 in the immunisation protocol (study II). Proteolytic activity of rOPV with IgG from AlV1, AlV2, MaV1 and MaV2 at 1 mg/ml (■), 0.5 mg/ml (□), 0.25 mg/ml (■) and 0.125 mg/ml (■), at weeks 3 (A), 6 (B), 9 (C), 12 (D), and 16 (E), was determined by the extent of hydrolysis of Z-Arg-Arg-AMC and expressed as a percentage change in activity compared to that of a rOPV control with non-immune antibody (0%). Each plot represents the mean of two experiments.

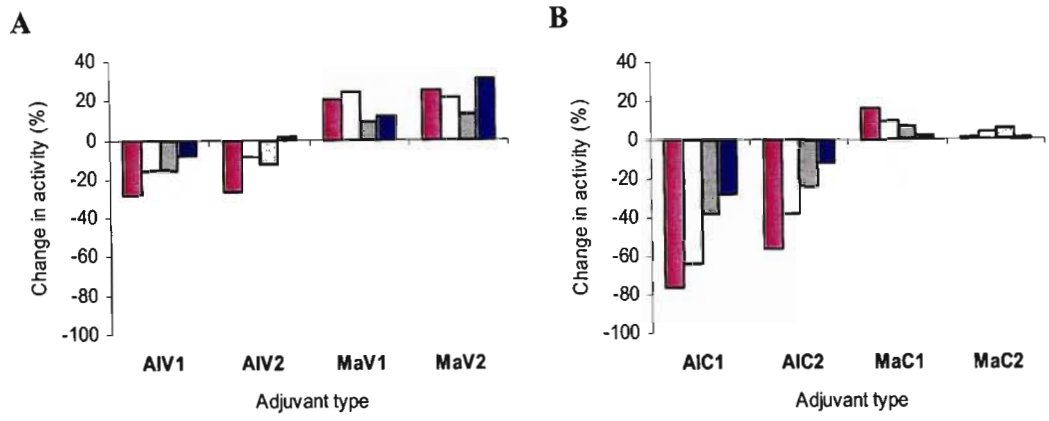

Figure 8.21. Cross-reactivity of inhibition of rOPC and rOPV by varying concentrations of anti-oligopeptidase B antibodies from week 9 in the immunisation protocol (study II). Proteolytic activity of rOPC (A) or rOPV (B) with IgG from AlV1, AlV2, MaV1 and MaV2, or AlC1, AlC2, MaC1 and MaC2 at 1 mg/ml (■), 0.5 mg/ml (□), 0.25 mg/ml (■) and 0.125 mg/ml (■) was determined by the extent of hydrolysis of Z-Arg-Arg-AMC and expressed as a percentage change in activity compared to that of an oligopeptidase B control with non-immune antibody (0%). Each plot represents the mean of two experiments.

8.6.2.11 Inhibition of native oligopeptidase B activity by rabbit IgG (study II)

IgG from MaC1, MaC2, MaV1 and MaV2 had very little effect on the activity of native oligopeptidase B from *T. congolense* and *T .vivax*, at both week 6 (Fig. 8.22, A) and week 9 (Fig. 8.22, B). These same antibodies had an activating effect on oligopeptidase B from *T. b. brucei* at week 6, but similarly little effect at week 9. The greatest inhibition was observed for all three enzymes by AlC1 and AlC2, at both weeks 6 and 9. AlV1 and AlV2 were also inhibitory to all three enzymes, although to a lesser extent.

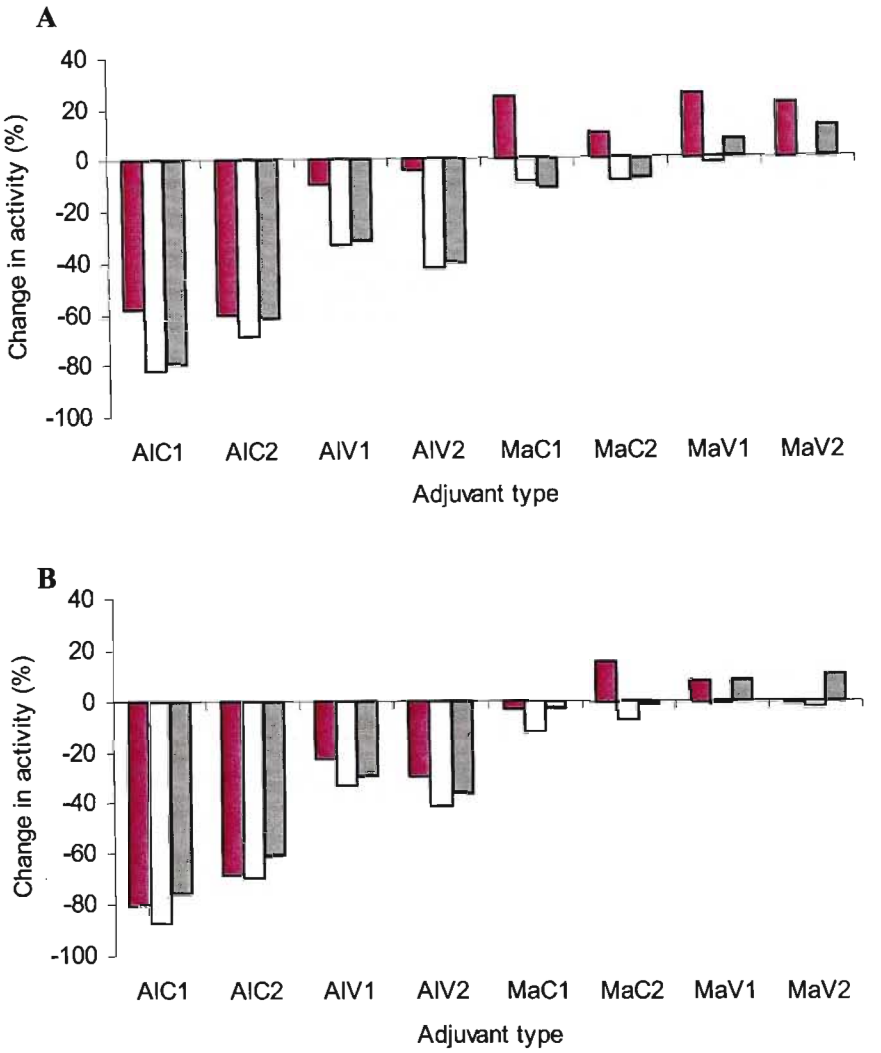

Figure 8.22. Inhibition of native oligopeptidase B by anti-oligopeptidase B antibodies from weeks 6 and 9 in the immunisation protocol (study II). Proteolytic activity of oligopeptidase B isolated from *T. b. brucei* (■), *T. congolense* (□), and *T. vivax* (■) with week 6 (A) or week 9 (B) IgG from AlC1, AlC2, AlV1, AlV2, MaC1, MaC2, MaV1, and MaV2 at 1 mg/ml was determined by the extent of hydrolysis of Z-Arg-Arg-AMC and expressed as a percentage change in activity compared to that of an oligopeptidase B control with non-immune antibody (0%). Each plot represents the mean of two experiments.

8.7 Discussion

Antibodies were produced in chickens against the recombinant proteins rOPC and rOPV. The results of this study were an initial indication that oligopeptidase B was a good immunogen, as high levels of specific antibodies were produced in experimental animals. A large amount of pure protein was required to prepare affinity matrices for the affinity purification of these antibodies. For this reason, uncleaved fusion protein (GST-OPC or GST-OPV) was immobilised to Aminolink® as an alternative to purified rOPC or rOPV, since larger amounts of this could be expressed and purified. A drawback of this was the possibility of co-purification of anti-GST antibodies that may have resulted through contamination of the immunogen. To counter this, anti-GST antibodies were adsorbed out by combining antibody preparations with an *E. coli* lysate containing expressed GST. The main purpose of affinity purifying these antibodies was for use in the immunoaffinity purification of the native enzymes. When using polyclonal antibodies for this purpose it is highly desirable that they be affinity purified (Harlow and Lane, 1999).

An ELISA was conducted to determine if any of the peptides selected through epitope mapping of *T. congolense* oligopeptidase B (Chapter 3) would be recognised by the chicken antibodies produced against rOPC and rOPV. This would indicate whether antibodies produced against the whole protein were directed specifically against these predicted epitopes. Peptides OpBTc1 and 3 were identified in this manner as being true epitopes of both rOPC and rOPV. Peptide OpBTc1, corresponding to amino acid residues 20-37, was found to be located on the surface of the catalytic region (Figs. 3.11A and 3.12A). Peptide OpBTc3, corresponding to amino acid residues 124-142, was found to be located on the surface of the β-propeller domain (Figs. 3.11C and 3.12C). These ELISAs to determine recognition of the peptides only gave a weak signal, even if left to develop for long periods of time. This could be explained by the fact that the individual peptides represent a single epitope and the chicken IgY consisted of a polyclonal response to many epitopes. A more conclusive study would need to be performed, using more peptides representing the entire repertoire of epitopes on oligopeptidase B.

Western blotting evaluation of chicken antibodies produced against rOPC and rOPV confirmed that the specific antibodies recognised their denatured antigen. Cross-reactivity between the oligopeptidase B from *T. congolense* and *T. vivax* and their antibodies was also observed, which was expected due to the high degree of similarity between the amino acid sequences (Section 3.4). The band at approximately 66 kDa for rOPC that was also recognised by the antibodies was considered to be a degradation product of rOPC.

Lysates of *T. congolense* and *T. vivax* parasites were probed with anti-rOPC and anti-rOPV IgY, as well as anti-peptide antibodies against OpBTc1 and OpBTc3 that were described in Chapter 3. Oligopeptidase B was detected in the *T. congolense* lysate by anti-rOPC antibodies, as well as both anti-peptide antibodies. The most specific antibody was anti-OpBTc1 which showed no cross-reactivity with any other trypanosomal proteins. Oligopeptidase B was detected in the *T. vivax* lysate by anti-rOPV IgY, and both anti-peptide antibodies. Again, anti-OpBTc1 antibodies showed high specificity, but a protein of high molecular weight also gave a signal in the western blot. Anti-OpBTc3 showed almost no specificity, cross-reacting with multiple trypanosomal proteins.

Anti-rOPC and anti-rOPV antibodies, produced in chickens using Freund's adjuvant, inhibited up to 89% and 91.7% of the peptidolytic activity of rOPC and rOPV respectively. Most importantly, these antibodies also inhibited 73.8% to 77.8% (anti-rOPC IgY), and 34% to 48.1% (anti-rOPV IgY) of the peptidolytic activity of native oligopeptidase B from *T. b. brucei*, *T. congolense*, and *T. vivax*, exhibiting cross-reactivity across the three species. These results were promising when considering the use of oligopeptidase B in an anti-disease vaccine. This encouraged the production of anti-oligopeptidase B antibodies in a mammalian system, and the investigation of the use of α_2M as an adjuvant for oligopeptidase B.

The antibody inhibition assays in the present study were conducted at a pH close to that of physiological pH, to attempt to mimic the environment in which these antibodies

would encounter the enzyme *in vivo*. In other studies where the inhibition of enzymes by antibodies has been assayed, pH conditions were used that were more suitable to the enzyme, rather than the antibodies. For example, Coetzer *et al.* (1991) used a pH of 6 for cathepsin L, and Troeberg *et al.* (1997) used a pH of 8 for trypanopain. In inhibition assays of this kind the pH used is typically a compromise between the optimum pH for both enzyme activity and antibody stability. In the present study, however, the focus was to generate antibodies that would inhibit the activity of the enzyme oligopeptidase B (and congopain in parallel studies) when encountered by the antibodies *in vivo*, as this would have application to a vaccine. Conditions of physiological pH were therefore used in order to produce relevant results.

Two separate studies were undertaken to evaluate different adjuvants for the production of antibodies against C2 and oligopeptidase B in a mammalian system. In study I, rabbits were immunised with equal amounts of C2, either alone, in Freund's adjuvant, or complexed with bovine or rabbit α_2M. This was done to assess the importance of the type of adjuvant on the inhibitory capacity of the antibodies produced. Antibodies specific to C2 were present in the sera of all the rabbits immunised with C2, as determined by ELISA. The highest titres of these antibodies were apparent in the sera obtained 6 weeks after the initial immunisation. The specific week at which the antibody levels peaked could not be accurately pinpointed since serum was not collected every week. The amount of C2 injected per immunisation was 10 μg, which is below 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 in a total amount of 30 μg over a six week period using Freund's adjuvant (Troeberg *et al.*, 1997). Also, oligopeptidase B from *T. b. brucei* has been used to immunise chickens in a total amount of 50 μg over an eleven week period using Freund's adjuvant (Morty *et al.*, 1999a). Based on the success of these immunisations and the limited supply of antigen, the amount of 40 μg C2 over a period of 6 weeks was decided upon.

Rabbits immunised using C2 in Freund's adjuvant showed high antibody levels that were sustained over the 11 week period of study I. Rabbits immunised with C2 alone showed levels of antibodies that were barely detectable. Rabbits immunised with C2-α_2M complexes (both rabbit and bovine α_2M) started off at week 3 with antibody levels that were comparable with those obtained using Freund's adjuvant, but these levels were not sustained. An exception was B1, immunised with C2-bovine α_2M complexes, which showed sustained antibody levels until week 11. Interestingly, B1 also showed more sustained anti-bovine α_2M antibody levels over the 11 week period than B2. This compares well with a study by Chu *et al.* (1994) where human α_2M complexed to hen egg lysozyme (HEL) produced antibody responses in rabbits to HEL that were comparable to those produced using HEL mixed with Freund's adjuvant. In the same study, a similar result was obtained for the same antigen complexed to rabbit α_1M, and it was found that antibody titres were not sustained in rabbits immunised with HEL-α_2M. Complexing to both human α_2M and rabbit α_1M also drastically enhanced the antibody response to HEL when compared to immunisation with HEL alone. This compares well with the results obtained in the present study.

Importantly, the anti-C2 antibodies produced in rabbits in study I were able to recognise congopain in ELISA. These antibodies generally also showed inhibitory properties towards both C2, and the whole congopain molecule. A general trend was observed in the results of the assays for inhibition of C2 and congopain by anti-C2 antibodies. This trend was that rabbits immunised with rabbit α_2M complexed with C2 produced antibodies that gave the best enzyme inhibition, followed closely by rabbits immunised with bovine α_2M complexed with C2. Rabbits immunised with C2 mixed with Freund's adjuvant produced antibodies that were only mildly inhibitory at best, and sometimes resulted in enhancement of enzyme activity. Rabbits immunised with C2 alone produced antibodies that were either weakly inhibitory or often enhanced enzyme activity. In conclusion, whilst immunisation using Freund's adjuvant resulted in a quantitatively better antibody production, immunisation using α_2M (both rabbit and bovine) resulted in qualitatively superior antibody production (i.e. antibodies that inhibited C2 activity). The

present study represents the first time that antibodies were raised using α_2M where the protease used to cleave the bait region was also the immunogen.

A second study (study II) was undertaken in rabbits, immunising animals with both trypanosomal antigens, C2 and oligopeptidase B. Since oligopeptidase B does not cleave the bait region of α_2M, another protease was required to activate α_2M, also becoming bound to α_2M. This presented the opportunity to target two trypanosomal pathogenic factors, and C2 was selected to cleave the bait region of α_2M. The use of the adjuvant alum was compared to complexation of the two antigens with bovine α_2M. Alum was chosen as an alternative adjuvant since Freund's had been assessed in study I, and shown to produce antibodies that only weakly inhibited C2 activity and antibody production was not sustained. Bovine α_2M was used in study II due to its availability, and since little difference had been found between its use and that of rabbit α_2M in study I.

Antibodies produced in study II were assessed for their ability to recognise C2, congopain and either rOPC or rOPV in ELISA. In general, immunisation of rabbits with free C2 combined with alum produced higher levels of anti-C2 antibodies. These antibodies were also able to recognise the full length protein congopain. The levels of antibodies produced in rabbits against oligopeptidase B did not appear to depend on the use of alum or α_2M as adjuvants. Generally, antibody levels throughout study II appeared to be higher than study I. This was apparent since ELISAs in study II could be conducted at much lower primary antibody concentrations, and a prozone effect, i.e. steric hindrance of antibody binding, was observed at antibody concentrations that were comparable to those used in study I (results not shown). This may be because two immunogens, and thus a higher combined dosage, was used in study II, resulting in better stimulation of the immune system. Study II was conducted over a 16 week period, and all antibody levels were shown to be sustained over the full 16 weeks. This contrasts with the results of study I where antibody levels appeared to decline steadily after week 6. Therefore, the use of alum does indeed lead to sustained antibody production. Alternatively, it was the use of two immunogens that led to better stimulation of the immune system, as described above, and hence a sustained production of antibodies,

since anti-C2 and anti-oligopeptidase B antibody production was also sustained where α_2M was used as an adjuvant. It was shown by ELISA that rabbits produced a strong antibody response to bovine α_2M. Interestingly, in study II, anti-bovine α_2M levels were shown to decline from week 6. High doses of antigen are reported to induce deletion and/or anergy of the antigen producing B-cells (Tobagus *et al.*, 2004). Whilst the similarities between bovine and rabbit α_2M were sufficient for bovine α_2M to bind to the rabbit α_2M receptors, there were strong enough differences for the bovine α_2M to be recognised as foreign after internalisation by APCs. These differences probably occurred mainly at the bait region, since it is well known that there are strong dissimilarities between species at this region.

In study II, all of the anti-C2 antibodies produced by rabbits were able to inhibit the activity of C2 against Z-Phe-Arg-AMC, though with varying degrees of efficiency. Generally, antibodies produced in rabbits immunised with C2-oligopeptidase B-α_2M complexes achieved slightly higher inhibition. And, at each week, the greatest inhibition of C2 activity was seen for antibodies from rabbits immunised with α_2M complexed antigens, confirming the findings of study I. Overall, the degree of inhibition by antibodies was slightly lower in study II than for study I, even though the levels of anti-C2 antibodies were apparently higher in study II. The highest amount of inhibition of C2 by antibodies for the whole of study I was 64.7% observed for rabbits immunised with C2 complexed to bovine α_2M. In study II, the highest amount of inhibition of C2 by antibodies was 46.1% overall, for rabbits immunised with C2 (and rOPV) complexed to bovine α_2M. This trend was also true for inhibition of congopain activity, whereby the greatest inhibition of congopain in study I was 36.2% and a maximum of 20% was observed in study II. The amount of C2 used for immunisation was the same in both studies, but the inclusion of the second immunogen, oligopeptidase B, may have had some impact on the levels of inhibition that were observed in study II. The differences in the degree of inhibition observed may also have been impacted on by the active concentration of C2 used in the assays. Enzyme used in study II was generally less active than in study I. Where C2 was less active, more total enzyme was used in the assay.

Therefore, antibodies could have been interacting to a greater extent with inactive enzyme in study II, leading to a lower apparent inhibition.

While the type of adjuvant used appeared to have no impact on the levels of anti-oligopeptidase B antibodies in rabbits, it greatly affected the inhibitory capacity of the antibodies towards oligopeptidase B. Rabbits immunised with C2-oligopeptidase B-α_2M complexes produced antibodies that slightly enhanced the activity of oligopeptidase B towards Z-Arg-Arg-AMC. Rabbits immunised with C2 and oligopeptidase B in alum produced antibodies that inhibited up to 100% of the activity of oligopeptidase B. These inhibitory antibodies were found to cross-react between rOPC and rOPV, as well as efficiently inhibit the activity of native oligopeptidase B from *T. b. brucei*, *T. congolense* and *T. vivax*. Interestingly, in rabbits, antibodies against rOPC showed better inhibition of its corresponding enzyme than rOPV, whereas the opposite was found when the preliminary study was undertaken in chickens.

The maximum inhibition of C2 activity against Z-Phe-Arg-AMC by anti-C2 antibodies was 64.7%, by week 11 IgG from a rabbit immunised with C2-bovine α_2M complexes (B2) in study I. This percentage inhibition may seem high in the context of such a small substrate but higher degrees of inhibition have been observed in other studies. For example, a maximum inhibition of 85% was obtained for inhibition of the activity of trypanopain, a cysteine protease from *T. b. brucei*, against Z-Phe-Arg-AMC by anti-trypanopain peptide IgY, although some enhancement of activity was observed, and rabbit anti-trypanopain peptide antibodies showed only enhancement of activity (Troeberg *et al.*, 1997). Much higher amounts of inhibition are observed for oligopeptidases, as seen in the present study. Comparably, a maximum inhibition of 92% was obtained for inhibition of oligopeptidase B from *T. b. brucei* activity against Z-Arg-Arg-AMC by specific IgY (Morty *et al.*, 2001).

Not all of the antibodies produced in the present study were able to recognise their respective denatured antigen by western blotting. Recognition did, however, appear to correlate to the strength of the antibody response and could probably have been improved

by the use of higher antibody concentrations. Western blotting of the anti-C2 antibodies produced in the present study showed a strong signal for a protein of about 50 kDa, under both reducing and non-reducing conditions. The monomeric form of C2 was also recognised but did not give as strong a signal as the higher molecular weight form, which interestingly was not visible by Coomassie staining. The 50 kDa form of C2 is unlikely to be a dimer, because it still occurs under reducing conditions. The recognition of a protein of this size by anti-C2 antibodies is common, however, and may in fact represent a covalently-joined multimeric form of C2 (Dr Alain Boulangé, University of KwaZulu-Natal, South Africa, personal communication).

In conclusion, antibodies were produced against C2 in all of the rabbits immunised. The antibodies had varied effects on the activity of both C2 and congopain against Z-Phe-Arg-AMC, but antibodies produced in rabbits immunised with C2 complexed to either rabbit or bovine α_2M generally showed the best inhibition of activity. In the context of a vaccine to neutralise congopain activity, immunisation with C2 complexed to α_2M seems a viable option since the antibodies produced were inhibitory towards the protease *in vitro*.

With respect to oligopeptidase B, alum was found to be an overwhelmingly better adjuvant than bovine α_2M, producing antibodies that inhibited up to 100% of oligopeptidase activity against Z-Arg-Arg-AMC. Using α_2M as an antigen delivery system for C2 and oligopeptidase B produced antibodies that were only marginally better at inhibiting the activity of C2 than antibodies produced using alum. In the context of a multi-component anti-disease vaccine, using free (i.e. not complexed with α_2M) C2 and oligopeptidase B in alum offers the best overall inhibition of enzyme activity. Alternatively, the possibility of using C2-α_2M complexes in conjunction with oligopeptidase B in alum should be investigated.

A study is currently underway in collaboration with Dr Luis Neves (Veterinary facility, Eduardo Mondlane University, Mozambique) where goats have been immunised with C2 complexed to bovine α_2M, to test the reproducibility of the results of the present study in

small livestock. Animals will be challenged by infection with *T. congolense* parasites to assess the ability of the anti-C2 antibodies raised against C2-α_2M complexes to inhibit the native enzyme *in vivo*, and hence reduce the pathogenic effects of the enzyme.

CHAPTER 9
GENERAL DISCUSSION

African trypanosomosis in cattle is a disease caused by tsetse-transmitted *T. congolense, T. vivax, and T. b. brucei.* The primary cause of death in cattle infected with these parasites is anaemia, but other pathologic effects are immunosuppression, tissue lesions, circulatory disturbances and lymphoid cell proliferation (Vickerman *et al.*, 1993). This disease is a severe constraint to animal husbandry in many parts of Africa.

There are currently three principal control strategies for trypanosomosis in cattle: trypanocidal drugs, trypanotolerant cattle, and tsetse control (McDermott and Coleman, 2001). A control strategy with the greatest probable impact would be vaccination. However, due to the antigenic variation of the parasite, development of a vaccine is proving to be a difficult task. Antigenic variation is the ability of the parasite to change the antigenic make-up of its surface proteins, called variable surface glycoproteins (VSG), and thereby evade the host's immune response. For this reason, a vaccine based on VSG would have to cover the entire repertoire of antigenic types, which is currently not feasible.

Our research group forms part of a collaborative endeavour to explore the feasibility of an alternative vaccine aimed at directing an immune response to parasite antigens that play a role in the pathology of the disease. This type of vaccine would probably not affect the survival of the parasite, but would neutralise pathogenic factors, and perhaps induce a state of trypanotolerance. Congopain, a cysteine protease of *T. congolense,* has been identified as having a probable pathogenic role in trypanosomosis (Authié *et al.*, 2001; Serveau *et al.*, 2003). Also, anti-congopain antibodies may contribute to mechanisms of trypanotolerance (Lalmanach *et al.*, 2002). Oligopeptidase B, a serine peptidase of all African trypanosomes, has been implicated as a therapeutic target (Morty, 1998; Morty *et al.*, 2000), and as a virulence factor in African trypanosomes (Morty *et al.*, 2001). Oligopeptidase B probably degrades host regulatory peptides resulting in serious consequences to the metabolic homeostasis of the host. For these reasons,

congopain and oligopeptidase B were chosen as candidates for investigation in the present study.

The catalytic domain of congopain, C2, and oligopeptidase B from *T. congolense* and *T. vivax* were recombinantly expressed and purified in the present study. Recombinant oligopeptidase B was enzymatically characterised. The interaction between congopain (and C2) and bovine α_2M was assessed and compared to other cysteine proteases, papain and cathepsin L. Complexes were prepared with C2 and α_2M through cleavage of the bait region of α_2M by C2 and trapping of the enzyme within the inhibitor's structure. Oligopeptidase B was also complexed to α_2M through cleavage of the bait region with C2. Complexes of C2 and α_2M, as well as complexes of oligopeptidase B, C2 and α_2M were used in immunochemical studies.

One of the main aims of the present study was to produce antibodies against congopain that would neutralise its proteolytic activity. Congopain has a C-terminal extension that is highly immunogenic, but considered unlikely to elicit antibodies that inhibit the enzyme. Therefore, C2, a truncated form of congopain which excluded the C-terminal extension, was used for immunisation. In study I, rabbits were immunised with small amounts of C2, a total of 40 μg over a six week period, either alone or using three different adjuvants: Freund's adjuvant, bovine α_2M, and rabbit α_2M. α_2M is a high molecular weight plasma glycoprotein that is capable of inhibiting proteases from all classes (Barrett and Starkey, 1973). When the bait region of α_2M is cleaved by a protease, the protease becomes trapped within the α_2M molecule. Once this transformation has taken place, receptor recognition sites become exposed on the α_2M molecule and it is rapidly taken up by APCs expressing the α_2M receptor, such as dendritic cells and macrophages. Complexing antigen to α_2M and subsequent delivery of antigen to APCs has been found to enhance immune responses (Chu *et al.*, 1994). α_2M was used in the present study to target C2 to APCs and enhance antigen processing and presentation.

In general, in study I, it appeared that immunisation with C2-rabbit α_2M complexes produced antibodies with the best inhibitory capacity towards C2 activity. Therefore, immunisation of rabbits with rabbit α_2M appeared to be the better option. Although it cannot be concluded from the present study that immunisation with the same species of α_2M as adjuvant produces 'better' antibodies, immunisation of cattle with C2-bovine α_2M complexes may produce 'better' antibodies than with C2 complexed with another species' α_2M.

The results obtained in the present study suggest that the direct uptake of C2 complexed with α_2M by APCs and presentation by MHC class II molecules to $CD4^+$ T cells resulted in antibodies that were better able to inhibit the activity of the enzyme than antibodies produced with the use of Freund's adjuvant. Perhaps this is because the three-dimensional conformation of the enzyme is maintained when it is trapped inside the α_2M molecule. With Freund's adjuvant, the free enzyme is exposed to denaturation by the very low pH of the adjuvant and receives no such protection as in the α_2M complexes. This would suggest that antibodies against conformational epitopes are required for enzyme inhibition, and that immunisation with Freund's adjuvant alone results in antibody production against linear epitopes. Where the inhibition of congopain and C2 activity by anti-peptide antibodies has been investigated, antibodies were generally found to enhance the activity of the enzyme (Mkhize, 2003). Therefore it would seem that antibodies generated against the linear epitopes of congopain (i.e. peptides) and C2 (i.e. with Freund's adjuvant) do not inhibit the enzyme, whereas antibodies produced against the conformational epitopes of C2 (i.e. complexed to α_2M) are capable of inhibiting the enzyme.

In study I, immunisation with C2 without adjuvant elicited a very poor antibody response, which demonstrates the need to use an adjuvant during immunisation. Anti-C2 antibody titres were sustained in rabbits immunised using Freund's adjuvant over the immunisation period, whereas anti-C2 antibody titres in rabbits immunised using C2-α_2M complexes were not. Freund's adjuvant has a depot effect, and thus the antigen was

retained in the tissues surrounding the injection site. These results suggest that α_2M had no such depot effect and as expected for "fast" α_2M, was rapidly removed from the tissues by cells expressing the α_2M receptor. Since the antigen was removed from the tissues in this case, there was no continuous stimulation of antibody production.

Although Freund's adjuvant is considered one of the most effective adjuvants today, it has many disadvantages and its use is very limited. Freund's adjuvant is a water-in-oil emulsion containing mineral oil, a surfactant (Arlacel A), and the complete adjuvant also contains killed mycobacteria (Singh and O'Hagan, 2003). Freund's complete adjuvant may cause a chronic inflammatory response at the site of injection that may be severe and painful to the animal. The inflammatory response may result in granulomas, sterile abscesses, and ulcerating tissue necrosis. Incomplete adjuvant can also result in abscesses and granuloma formation. Another disadvantage is the lifelong persistence of the mineral oil of Freund's adjuvant in the tissues of the animal (Roitt, 1997). Other adjuvants that have a depot effect include Ribi's adjuvant, Hunter's TiterMax, and aluminium salt adjuvants. Each of these adjuvants is less toxic than Freund's adjuvant and can produce equally efficient immune responses, depending on the antigen (Bennet *et al.*, 1992). It was therefore decided to evaluate alum as an alternative adjuvant in future studies.

Another main aim of the present study was to test the efficiency of α_2M as an adjuvant delivery system for oligopeptidase B. Since oligopeptidase B does not interact with α_2M on its own, a protease was required to cleave the bait region of α_2M to allow covalent interaction between the thioester of α_2M and adjacent lysine residues of oligopeptidase B. C2 was selected for this function since its interaction with α_2M had been analysed in the present study, and it would also form part of the complexes, allowing delivery of multiple antigens. Rabbits were immunised with C2 and oligopeptidase B (either rOPC or rOPV), in alum or complexed to bovine α_2M. Bovine α_2M was used because it was available in large quantities and its efficiency for antigen delivery in rabbits had been established in study I. Freund's adjuvant had been shown in the present study to be an undesirable alternative to the use of α_2M. Therefore, alum was used for comparison in study II,

because it is a widely used adjuvant with a depot effect, and because it has none of the disadvantages of Freund's adjuvant (Nicklas, 1992).

The use of alum as an adjuvant proved to be superior in terms of levels of anti-C2 antibodies. However, as was seen in study I, antibodies produced in rabbits immunised with α_2M-complexed antigen were better able to inhibit the activity of C2 *in vitro*. Therefore, based on the evidence of the present study, immunisation of rabbits with C2 complexed to α_2M, where the antigen is trapped inside the tetramer, results in antibodies that are better able to inhibit C2 activity than those antibodies raised with Freund's or alum. Antibodies raised against C2 with alum appeared to inhibit the activity of C2 with more consistent efficiency than that observed for Freund's adjuvant, although a direct comparison was not made. This offers support to the theory that non-linear, conformational epitopes are required for inhibition of C2 by antibodies, since alum has a pH between 6 and 7, and is not likely to denature the antigen.

The immunochemical findings for C2 do not appear to apply to an antigen that is covalently linked to α_2M, i.e. oligopeptidase B. Comparable levels of anti-oligopeptidase B antibodies were produced in rabbits, regardless of the adjuvant type. This finding is consistent with Chu *et al.* (1994) where α_2M and Freund's adjuvant were compared. However, the epitopes towards which these antibodies were directed must have been impacted on by the type of adjuvant, because their ability to inhibit the activity of oligopeptidase B activity differed greatly. Immunisation with alum resulted in the production of antibodies which inhibited oligopeptidase B activity to up to 100%; a result which was observed in all four rabbits. Immunisation with α_2M-complexes resulted in the production of antibodies which were unable to inhibit oligopeptidase B activity. In general, specific antibody levels were higher in study II and were sustained over a period of 16 weeks (last booster injection administered at week 6), regardless of the adjuvant. This may be because two antigens were used in study II, causing greater stimulation of the immune system than that observed for a single antigen.

For some reason, immunisation of rabbits with oligopeptidase B (together with C2) in alum produced antibodies able to completely inhibit oligopeptidase B activity against Z-Arg-Arg-AMC. A similar inhibitory efficiency was observed for chicken IgY raised against oligopeptidase B with Freund's adjuvant. The low pH of Freund's adjuvant would result in the denaturation of the enzyme prior to immunisation. Therefore, it would be expected that the 'inhibitory' epitopes of oligopeptidase B are linear. Although alum has a pH between 6 and 7 and would not denature the enzyme, it is highly possible that the C2 in the sample could have degraded the oligopeptidase B, also resulting in linear epitopes. Antigen delivery of a covalently attached antigen by α_2M does not elicit an antibody response to the 'inhibitory' epitopes of oligopeptidase B. It is possible that the covalent attachment of oligopeptidase B to α_2M may alter these 'inhibitory' epitopes.

Speculation that the 'inhibitory' epitopes of oligopeptidase B are linear suggests that inhibition of oligopeptidase B activity could be achieved with an anti-peptide antibody. Immunisation with a peptide may actually be a better alternative to immunisation with whole protein as it would eliminate the introduction of an active pathogenic factor into the host. Also, immunisation with recombinant oligopeptidase B produced in bacteria introduces the need for endotoxin removal prior to immunisation, adding to the cost and labour involved in the process. Identification of the 'inhibitory' epitopes could be accomplished by the use of multiple pin peptide scanning (pepscan) technology. This involves the synthesis of overlapping peptides spanning the entire sequence of the protein of interest. Peptides are synthesised on specially designed polystyrene pins in a 96-well microtitre plate format, which are then screened against sera or antibodies to identify linear B-cell epitopes (Sumar, 2001). The epitopes identified in this manner from anti-oligopeptidase B IgG or IgY that is inhibitory towards oligopeptidase B activity could be compared against those identified from anti-oligopeptidase B IgG that is not inhibitory (i.e. from rabbits immunised with α_2M complexes). Putative 'inhibitory' epitopes could be identified on the 3D model of oligopeptidase B to assess their proximity to the narrow hole leading to the active site of the enzyme. It is likely that the 'inhibitory' epitope is close to this area. Pepscan technology could also be applied to determine whether the 'inhibitory' epitopes of C2/congopain are linear or conformational.

Binding of antibodies to an enzyme is thought to affect the enzyme in two ways. The enzyme's active site may be occluded by the antibody, physically blocking the access of substrate to the active site. Also, the antibody may induce a conformational change at the active site, which may result in either a less active or more active conformation. 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 and congopain activity by antibodies was assessed with Z-Phe-Arg-AMC, which is a very small substrate. If occlusion of the active site was the cause of inhibition of the enzymes then it must have been very efficient in order to have blocked access to this small substrate. The effect of the antibodies on the activity of the enzyme could have been better investigated by using substrates of various sizes. With knowledge of the *in vivo* substrates of congopain a more physiologically relevant study could be undertaken. The extent of inhibition of oligopeptidase B activity was consistently higher than that observed for C2 and congopain in the present study. This is probably because access to the active site of oligopeptidase B is restricted by the presence of the β-propeller domain and the narrow entrance to the active site through this domain is probably easily blocked by the binding of antibodies.

In the present study, large amounts of active α_2M, from both bovine and rabbit plasma, were required. This study reports the first isolation of bovine and rabbit α_2M from plasma by the combination of protein fractionation by PEG 6 000, zinc chelate chromatography and molecular exclusion chromatography on Sephacryl S-300 HR. The affinity technique of zinc chelate chromatography has in the past been reserved for the isolation of human α_2M (Salvesen and Enghild, 1993) and has not been applied to other species, except ostrich α_2M (Van Jaarsveld *et al.*, 1994). To obtain a maximum amount of active α_2M, it was necessary to use a rapid isolation procedure. It was for this reason that these particular techniques were combined, to a high degree of success. That is, a high yield of α_2M was obtained and purified to electrophoretic homogeneity and high activity.

Before complexes of α_2M and C2 could be prepared, it was necessary to study the interaction between them. Other cysteine proteases, cathepsin L and papain, were used in the investigation for comparative purposes. Also, since the recombinant proteases C2 and congopain were in such short supply at the time of the assays, papain and cathepsin L were used for optimisation. Bovine α_2M was found to interact with and inhibit papain, sheep liver cathepsin L, congopain and C2. Therefore all of these proteases must have cleavage sites in the bait region of bovine α_2M. The fact that papain's action on α_2M resulted in a relatively unstable complex suggests that papain might also cleave α_2M outside the bait region. Of the four proteases, cathepsin L appeared to be most efficiently inhibited by bovine α_2M, and bovine α_2M appeared to be able to trap a larger amount of cathepsin L. However, more in-depth studies into the binding ratios between α_2M and the enzymes should be done. This could be done by radioactive labelling of the enzyme, as done with the antigens in previous studies. For example, Chu *et al.* (1991) measured insulin association with α_2M by reacting α_2M with ^{125}I-insulin and protease, separating the complexes from unbound material by electrophoresis, and quantitatively determining the amount of ^{125}I-insulin bound by autoradiography. Unfortunately, this technique was not readily available.

The accessibility of the active site of the protease in the α_2M complex to low molecular weight substrates differed between the individual cysteine proteases. Bz-Pro-Phe-Arg-pNA proved to have complete access to the active site of papain (23.4 kDa) and cathepsin L (28 kDa) in complex with α_2M. Bz-Pro-Phe-Arg-pNA was slightly restricted from the active site of congopain (33 kDa) in complex with α_2M, whilst moderately restricted from the active site of C2 (27 kDa) in complex with α_2M. C2 is a truncated form of congopain (i.e. congopain has the C-terminal extension), and perhaps the α_2M molecule was able to close tighter around the C2 molecule, thus restricting access of Bz-Pro-Phe-Arg-pNA to the enzyme's active site. But this does not follow since cathepsin L is of a similar size as C2 and papain is smaller than C2. Another noteworthy result was that whilst cathepsin L and C2 are of similar molecular weight, bovine α_2M was apparently

able to trap more cathepsin L than C2. Therefore, size of the protease does not seem to be a factor in these interactions.

In the present study, recombinant oligopeptidase B from *T. congolense* (rOPC) and *T. vivax* (rOPV), the major causative agents of African trypanosomosis, was expressed and purified for the first time. Whilst the oligopeptidase B ORF was successfully expressed in both *E. coli* and *P. pastoris*, it was only purified from the *E. coli* system. It would seem that no post-translational modifications of the proteins were necessary since the purified enzymes were active. A disadvantage of expressing oligopeptidase B in *E. coli* is that the proteins may be contaminated with endotoxin. Thus, for use in a vaccine, the proteins would need to be tested for the presence of endotoxin, and if necessary, undergo endotoxin removal. The ability to produce pure oligopeptidase B in relatively large amounts will aid in further study of the enzyme, including crystallisation of the enzyme. Knowledge of the 3D structure of trypanosomal oligopeptidase B would aid in obtaining greater understanding of the enzyme's mechanism as well as drug and inhibitor design.

The present study reports the first enzymatic characterisation of oligopeptidase B from *T. vivax*, which has yet to be completely purified in its native form. Native enzyme from *T. b. brucei*, *T. congolense* and *T. vivax* was partially purified in the present study and found to have K_m values very similar to those obtained for the recombinant enzymes for Z-Arg-Arg-AMC. Native oligopeptidase B activity was also inhibited by anti-oligopeptidase B IgY and IgG. Recombinant OPC and OPV were found to have typical oligopeptidase B behaviour, and thus were considered an accurate representation of their native counterparts. Recombinant OPV, like other trypanosomal oligopeptidases, was found to be highly active and stable at physiological pH and ionic strength, which lends support to its role as a pathogenic factor of trypanosomosis.

There was a notable difference in the kinetic constants observed in the present study and those reported for the various synthetic substrates that were assayed. Values of K_m tended to be approximately ten times higher than those reported for other salivarian trypanosomes (Morty *et al.*, 1999a; Morty *et al.*, 1999b). This difference carries across in

the conversion to values for k_{cat}/K_m, resulting in values that are a factor of ten times lower than those previously reported. The kinetic values obtained in the present study were more comparable with those reported for *T. cruzi* (Burleigh *et al.*, 1997).

A remarkable finding of the present study was the apparent preference of rOPV for Z-Gly-Gly-Arg-AMC over the other substrates tested, while still displaying a similar affinity to rOPC for Z-Arg-Arg-AMC. Recombinant OPV also had a high affinity for the substrate Z-Gly-Arg-Arg-AMC. It would seem that rOPV has specificity for Gly at the P3 position, where little specificity is shown by rOPC (and native oligopeptidase B from *T. b. brucei* and *T. congolense*) (Morty *et al.*, 1999a; Morty *et al.*, 1999b) and a preference for Gly over Arg at P2. Other differences observed in the enzymatic behaviour of rOPV include an increased sensitivity to high pH, decreased sensitivity to increasing ionic strength conditions, and a higher degree of activation by reducing agents. These differences in enzymatic activity may be exploited in a species specific diagnostic test.

An interesting observation in the present study was that rOPC and rOPV were not inhibited by the thiol-reactive, irreversible, inhibitors iodoacetamide, sodium iodoacetate and *N*-ethylmaleimide. This contradicts previous reports where native oligopeptidase B from *T. congolense* was sensitive to these inhibitors (Morty *et al.*, 1999a). A possible explanation for the findings of the present study came from analysis of the amino acid sequences of the enzymes from *T. congolense* and *T. vivax*, which were previously not available. Inhibition of *T. b. brucei* oligopeptidase B by thiol-reactive inhibitors had been attributed to the presence of a key cysteine residue (Cys^{256}) (Morty *et al.*, 2005b). This cysteine residue is not present in the amino acid sequences of *T. congolense* and *T. vivax* oligopeptidase B. The two cysteine residues found responsible for thiol-enhancement (Cys^{559} and Cys^{597}) (Morty *et al.*, 2005b) are found in all three enzymes at the same position, but the reason for their different degrees of activation by reducing agents remains unclear.

In conclusion, antibodies produced in rabbits against C2 using α_2M as an adjuvant were more inhibitory towards C2 activity than antibodies against C2 mixed with Freund's adjuvant or alum. Whilst initial anti-C2 antibody titres were comparable between rabbits immunised with C2-α_2M complexes and C2 mixed with Freund's adjuvant or alum, the use of an adjuvant enhanced the production of specific antibodies over time. Antibodies produced in rabbits against oligopeptidase B using alum as an adjuvant were more inhibitory towards oligopeptidase B activity than antibodies against oligopeptidase B complexed to α_2M and C2. Anti-oligopeptidase B antibody levels were comparable between rabbits immunised with antigens complexed to α_2M and free antigens in alum throughout the study. Overall, immunisation of cattle with C2 complexed to α_2M, and, separately, oligopeptidase B in alum, offers the most promise in the context of an anti-disease vaccine against trypanosomosis. However, immunisation of cattle with both free antigens in alum would be a slightly less effective, but simpler alternative. The findings of the present study are thus a step towards the development of an anti-disease vaccine for cattle trypanosomosis.

Congopain and oligopeptidase B are not the only molecules that contribute towards the pathology of trypanosomosis. A surface located acid phosphatase has also been identified in *T. congolense* (Tosomba *et al.*, 1996), and may contribute to pathogenesis by hydrolysing host phosphate esters, facilitating penetration of host tissues (Coetzer, 1998). The enzyme's location on the surface of the parasite makes it an attractive target for a vaccine. Also, analysis of the *T. b. brucei* data bank identified three carboxypeptidases, three oligopeptidases, and eight MMPs, four of which are expressed differentially in bloodstream and insect life cycle forms (Prof. Theo Baltz, University of Bordeaux, France, personal communication), and five metacaspases (Helms *et al.*, 2006). If other pathogenic factors are found to interact with α_2M, becoming trapped within its structure, complexation with α_2M prior to immunisation might enhance the production of inhibitory antibodies against the parasite proteases identified from the *T. b. brucei*, *T. congolense* and *T. vivax* genomes. This requires further investigation. A combination of these parasite proteases in complex with bovine α_2M may be the ideal anti-disease vaccine for cattle trypanosomosis, resulting in efficient neutralisation of multiple pathogenic factors.

Also, the use of alum as an adjuvant should be investigated for those pathogenic factors that do not interact with α_2M. Alum appears to be a more viable alternative to the use of Freund's adjuvant for the production of anti-C2 antibodies that inhibit congopain, and a more viable alternative to complexation with α_2M for production of inhibitory anti-oligopeptidase B antibodies.

The findings of this study might have far-reaching application to a number of vaccines. Immunisation using α_2M complexed to any parasitic protease involved in the pathogenesis of diseases may be the solution to the neutralisation of these pathogenic factors *in vivo*.

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Appendix 1

Epitope mapping of oligopeptidase B

The locations of the peptides selected as described in Chapter 3 using Predict7 are illustrated graphically in Figs. A1 to A5.

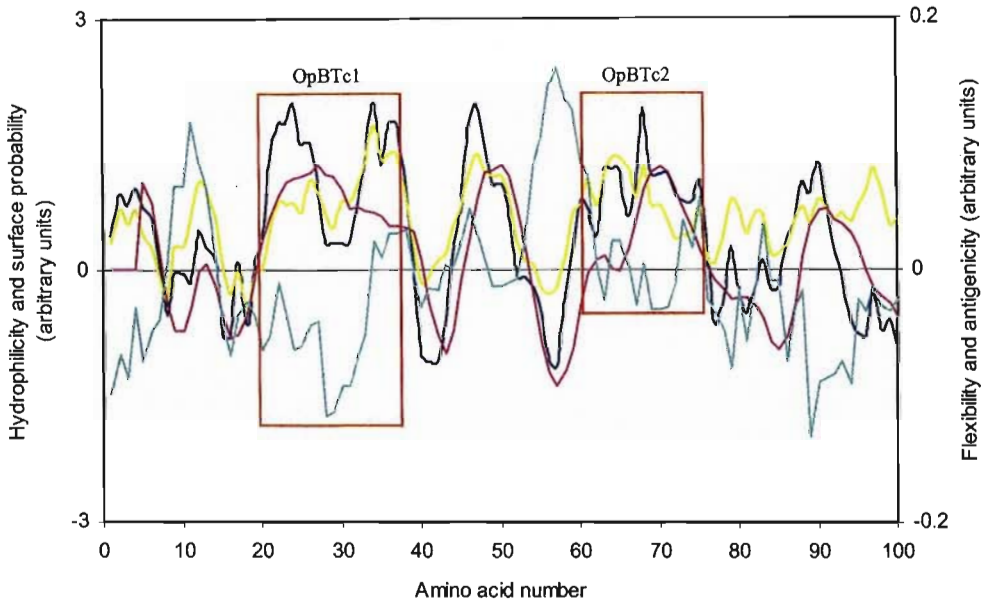

Figure A1. Amino acid residues 0-100 of oligopeptidase B from *T. congolense*. Hydrophilicity (—), surface probability (—), flexibility (—) and antigenicity (—), as predicted with Predict7, are depicted. Peptides OpBTc1 and OpBTc2 are highlighted with red boxes.

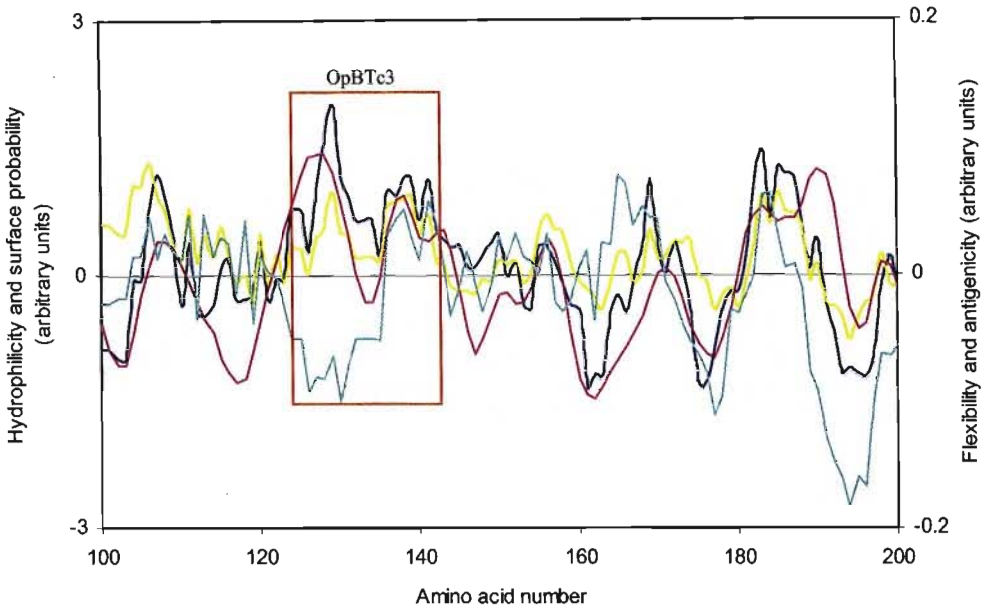

Figure A2. Amino acid residues 100-200 of oligopeptidase B from *T. congolense.* Hydrophilicity (—), surface probability (—), flexibility (—) and antigenicity (—), as predicted with Predict7, are depicted. Peptide OpBTc3 is highlighted with a red box.

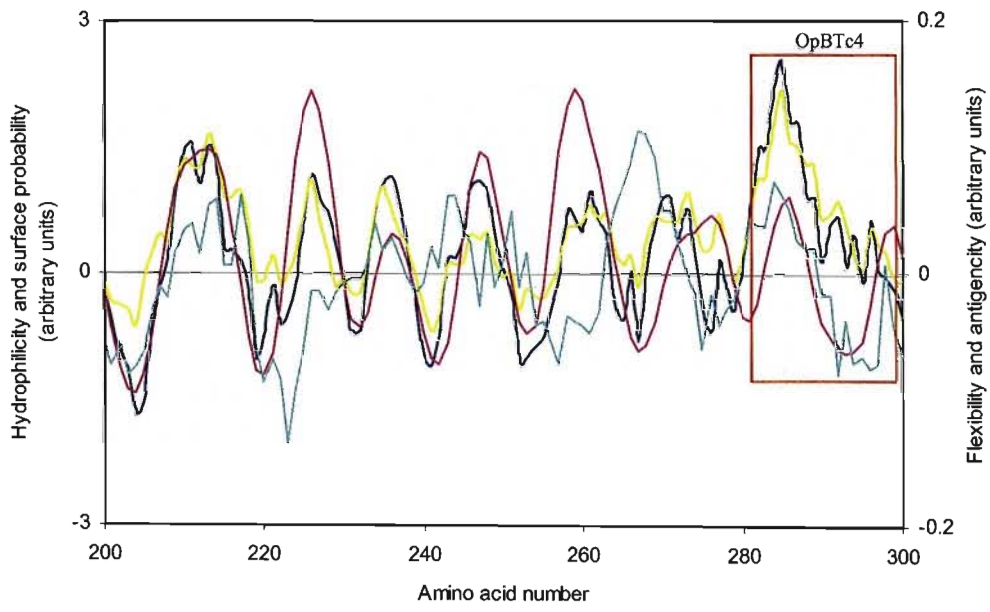

Figure A3. Amino acid residues 200-300 of oligopeptidase B from *T. congolense.* Hydrophilicity (—), surface probability (—), flexibility (—) and antigenicity (—), as predicted with Predict7, are depicted. Peptide OpBTc4 is highlighted with a red box.

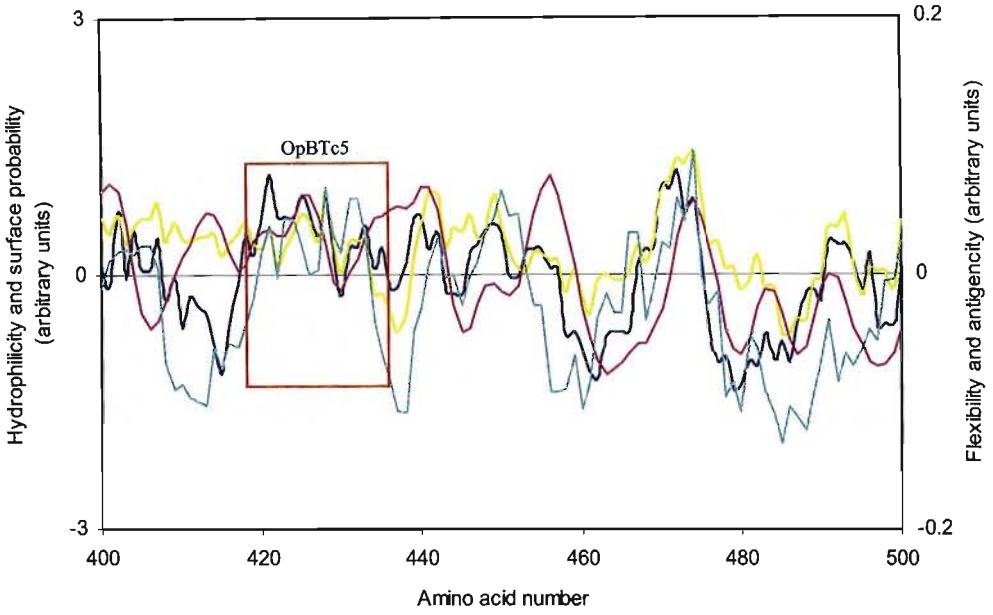

Figure A4. Amino acid residues 400-500 of oligopeptidase B from *T. congolense*. Hydrophilicity (—), surface probability (—), flexibility (—) and antigenicity (—), as predicted with Predict7, are depicted. Peptide OpBTc5 is highlighted with a red box.

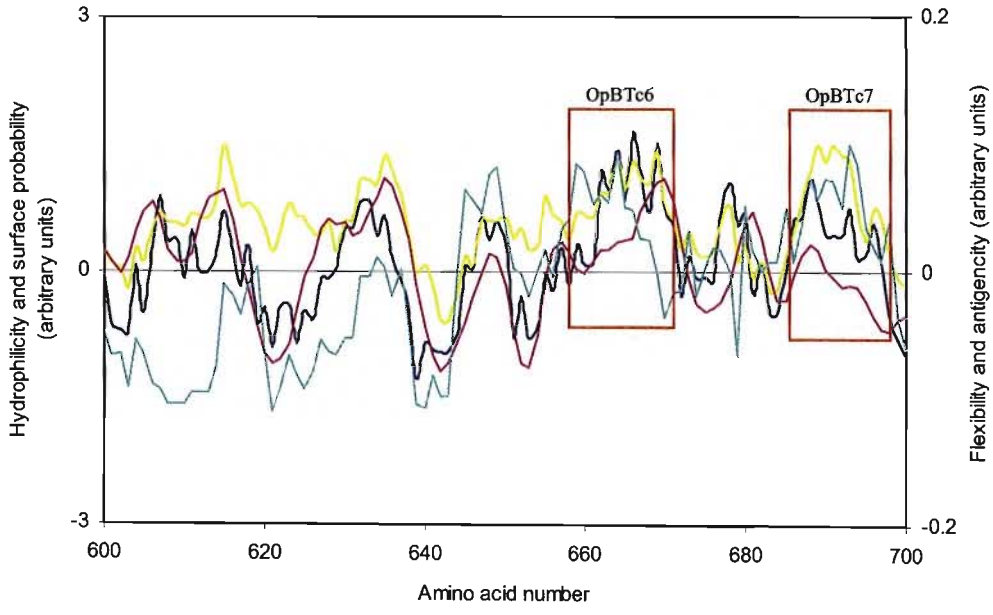

Figure A5. Amino acid residues 600-700 of oligopeptidase B from *T. congolense*. Hydrophilicity (—), surface probability (—), flexibility (—) and antigenicity (—), as predicted with Predict7, are depicted. Peptides OpBTc6 and OpBTc7 are highlighted with red boxes.

Appendix 2

Oligopeptidase B nucleotide sequences

The oligonucleotide sequences for oligopeptidase B from *T. congolense* and *T. vivax* are shown in Fig. A6 in an alignment prepared using CLUSTAL W, as described in Section 3.3.

```
T_congo         ATGACATCTGATCGCGGCCCCATCGCTGCGCACAAGCCCTATGAGGTGGTCTTTGGTAAA 60
T_vivax         ATGCCAGCTCCCTGCGGTCCGGTCGCGGAACAGCAGGATCATGAGGTAATCTTTGGGAAG 60
                *** ** **    **** **  **** *  **  **    *******  ******* **

T_congo         GTTGAGGGGGAGGACCGTGGGCCAAATCCGATGGATCCCCCACGCCGTCGCGTCGATCCC 120
T_vivax         GTTGAGGGTGAAGATCGCGGCCCAAATCCAATGGACCCCCCTCTGCGCCGTAATGACCCG 120
                ******** ** ** ** ** ******** ***** ***** *  ** **    ** **

T_congo         CTCTTTTGGCTCCGGGACGACTCCCGCACCAACCCAGACGTCCTCGCTCACCTTCACCTA 180
T_vivax         CTGTTTTGGCTTCGCGACGACAATCGCACGAATCCGAAGGTGCTTGCGCACCTGCATTTG 180
                ** ******** ** ******   ***** ** **  * ** ** ** ***** **  *

T_congo         GAGAGGGACTATTTCGAGAAGCGCACGGGGGACATTAAGGACCTGGCGGAGACGATCTAC 240
T_vivax         GAGAAGGCGTATTTTGAAGAGTGCACAGTGGATCTTAAGGACCTCTCTGAGACAGTGTAC 240
                **** **  ***** **  ** **** * ***  **********  * *****  * ***

T_congo         CAGGAGCATATTTCACACATTGAGGAAACTGACATGTCGCCTCCTTACACATACAACCGC 300
T_vivax         CAGGAACACCTCTCCCACATTCAGGAAACGGACATGTCTGCACCTTACGTACACGATAAC 300
                ***** **  * ** ****** ******* ********  * ******  * ** *   *

T_congo         TTTGTGTACTACACGCGCAGGGTGAAGGGACTGTCTTACAAGATCCATTGCCGCGTGCCA 360
T_vivax         TACATGTACTACAAGCGCGAGGTGAAGGGGCTCTCGTACAAAATACATTGCCGCGTACCC 360
                *   ********* ****  ********* ** ** ***** ** ********** **

T_congo         CTTGGCAAGGTGCCTGGCGAAGGCCCCGACGAGGAGATTGTGCTGGACGAGAACAAACTT 420
T_vivax         CTTGGAAAAACCCCCGG---ATCCAGTGACGAGCAGGTGGTTCTGGACGAGAACAAGCTA 417
                ***** **    ** **   *  *   ****** ** * ** ************** **

T_congo         GCAGAGGGAAAGGCATTTTGTGACGTGCGCTCGGTCGCCCCTGCTCCACCGGAACACATG 480
T_vivax         GCGGAGGGCAAAGCATTTTGTCACGTGCGCCAAGTGGAGCCAGCCCCGCCCAAGCACTCA 477
                ** ***** ** ********* ********   ** *  ** ** ** **  * ***

T_congo         CTGGTGGCGTACTCTGTTGACCACCTCGGAGACGAGTTGTACACGATTCAGTTTGTGGGG 540
T_vivax         CTGGTGGCTTATTCCGTGGATTACACTGGAAATGAGGTGTACACCATTCGCTTTGTTGGG 537
                ******** ** ** ** **  **   *** * *** ******* ****  ***** ***

T_congo         GATGCGTCGCCAGATAAGCTTGAGGGCACCACTGGGAGCATCATATGGGGTACCAACGCT 600
T_vivax         GATGATGTGCCAGACGTAGTGGAGGGGACGAATGGCCATATCGTATGGGGCCCTGACGGC 597
                ****    ******     * ***** ** * ***    *** *******  *  ***

T_congo         GAGTGCTTCTTCTACGTTACACCAGACTCTACAAAGCGAAGCAACAAGGTGTGGCGTCAC 660
T_vivax         GGGTGCTTCTTCTATACGACAAAGGATGCGGCGCAACGTGACTACAAGGTGTGGAGGCAT 657
                * ************    ***   **  *  *  * **   * *********** * **

T_congo         ATTATTGGCCAATCACAAAGCGAGGATGTGTGCCTTTACACAGACGATGACCCACTGTTC 720
T_vivax         GTGATTGGCCAGCAGCAGAGTGAGGATGTGTGTCTCTACACAGAGGTGGACGCCGTTTTT 717
                 * ********    ** ** ********** ** ******** *  *** *  * **

T_congo         TCTGTTGCTGCGTCCAAGTCTGGCGATGGCCATACGCTGCTAATTAGCTCCTCCTCCAGT 780
T_vivax         TCCGCATGTATGAGTAAGTCCGGTGATGGAAACACGTTGCTCATTACTTCCTCCTCCAGC 777
                ** *    *  *   ***** ** *****  * *** **** ****  **********

T_congo         GAAACCACTGAGCTTCACCTCTTGGATTTGCGGAAGGGCCTGAACAACACTACACTCGAG 840
T_vivax         GAGACAACAGAGGTCCACTTGCTCGATCTTCGGGGTGGTGTTGCGCACAATGAACTAGAA 837
                ** ** ** *** * *** *  * *** * ***   **  *     *** *  *** **
```

```
T_congo         GTTGTGCGGAAGCGGGAGAAGAACGTTCGATACGAGGTGGAGATGCACGGCACTGAGACA 900
T_vivax         ACAGTGCGCCCTCGGGAGAAAGGTGTGCGTTACGACGTGGCATTGCATGGTACCGACACC 897
                   *****    ********    ** ** ***** ****   **** ** ** ** **

T_congo         CTGGTGATACTAACAAACAAGGACAAATGTATTAACGGTAAGGTCGTGCTGGCTAAGCGG 960
T_vivax         TTGCTTGTGCTAACAAATAAAGATAAATGTATCAATGGACAGGTACTGATCGTACAGCGA 957
                 ** *  * ******** ** ** ******** ** **  ****  ** * *   ****

T_congo         GCTTCACCATCGGAGTGGACAAATGTTCTCGTACCGCATGACGACAAGGTTTTTATCGAT 1020
T_vivax         AGTGCTCCTTCTGACTGGACGCGTGTGTTGGTACCGCATAGCGAAGAGGTTTTCATTGAG 1017
                  * * ** ** ** *****   ***  * *********  ***  ******* ** **

T_congo         GATATTGCCGTATTCGCCAAGTTTGCTGTTCTCTCAGGACGGCGTGATGGACTGACACGC 1080
T_vivax         GAGATTGCCGTCTTCTCGACGTTTGCTGTGCTTGCCGGTCGGCGTGCAGGCTTGTCGCGA 1077
                ** ******** *** * * ********* **  * ** *******  **  ** * **

T_congo         GTCTGGACGGTTCAGGTGGGTCCAGATAATTGTTTCAGTGCCGGGACGCGTCGTGAGCTA 1140
T_vivax         ATTTGGACAATGCAGGTAGGACCGGATAACACTTTCAGCAATAGCTTGGTGAACGAGCTG 1137
                 * *****  * ***** ** ** *****   ******     *   *      *****

T_congo         CAGTTCGATGAGCCCGTTTTTACTGCCCACGTAATCACCTCACAAATGAAGACGTACGAC 1200
T_vivax         CAGTTGGACGAGCCTGTTTTCACAACTCATGTCGTTCTGTCTCAGATGCACATGTATAAC 1197
                ***** ** ***** ***** **  * ** **  *    ** ** *** * * ***  **

T_congo         ACATCATCGCTGCGTCTGGAGTATTCCTCCATGACCACTCCCACCACGTGGTTTGATGAG 1260
T_vivax         ACTGCCACGCTGCGGCTCACTTACTCTTCCATGACAACCCGCACCACATGGTACGATGTC 1257
                **  *  ******* **    ** ** ******** ** * ****** ****  ****

T_congo         GACTTGGCGAGTGGGAAGCGCACAGTCGTAAAAGTGTCCAAGGTGGGTGGCGGGTTTGAC 1320
T_vivax         CACGTTGCAGAAGGGGGCCGAACTCCCGTGAAGATACGCGAAGTCGGTGGTGGTTTCAAT 1317
                 ** * **    ***   ** **   *** **  *   * * ** ***** ** **  *

T_congo         TCGAAGAATTACGTTTGCCAACGGCGCCTTGCCACAGCACCCGACGGGACAACCATCCCC 1380
T_vivax         CCCGCACATTACGTCAGCAAGCGGCTGTTAGCCACCGCACCTGACGGAACAAAGATCCCC 1377
                 *     *******  ** * ****   * ***** ***** ***** ****  ******

T_congo         CTTTCGATTCTCTACGACGTTAGTCTTGACATGAAGAAGCCGCATCCGACGATGCTCTAC 1440
T_vivax         ATTTCTCTGGTTTATGATGCTGCCCTTGATCTTACGAAGCCCCACCCAACCATGTTGTAT 1437
                 ****  *  * ** ** * *   *****  * * ****** ** ** ** *** * **

T_congo         GGTTACGGTTCCTATGGGATTTGTGTTGAGCCGCAGTTCGACATCCGGTGCTTGCCGTAT 1500
T_vivax         GGCTATGGGTCCTATGGGATATGTGTGGAGCCAGAGTTCAACATTCAGTATCTTCCGTAC 1497
                ** ** ** ********** ***** *****  ***** **** * **   * *****

T_congo         GTGGACCGTGGTGTGATATACGCCATTGCACATGTGCGCGGTGGAGGAGAGATGGGCCGA 1560
T_vivax         GTTGACCGTGGTGTAATTTTCGCTATTGCCCATGTGCGCGGGGGTGGTGAGATGGGACGT 1557
                ** ********** ** * *** ***** ********** ** ** ******** **

T_congo         GCATGGTACGAGATCGGAGGGAAGTACTTGACAAAGCGCAACACCTTCATGGATTTTATT 1620
T_vivax         GAGTGGTATGAGCTGGGTGCCAAGTACCTAACGAAACGCAATACTTTTATGGACTTCATT 1617
                *  ***** *** * ** *  ****** * ** ** ***** ** ** ***** ** ***

T_congo         TCGTGTGCCGAGCATCTTATTTCGTCTGGTGTGACGACTCCCCCGCAGCTTGCCTGCGAG 1680
T_vivax         GCGTGTGCCGAGCACCTTATTTCAAGTGGTCTCACAACGCCCAACCAGCTGGCCTGTGAA 1677
                 ************* ********   **** * ** ** ***   ***** ***** **

T_congo         GGGCGTAGCGCCGGTGGTCTTCTGGTTGGCGCTGTACTAAATATGCGCCCTGACTTATTC 1740
T_vivax         GGACGTAGTGCTGGTGGGCTGTTAATTGGTGCTGTGCTGAACATGCGCCCAGATTTATTT 1737
                ** ***** ** ***** **  *  **** ***** ** ** ******** ** *****

T_congo         CGTGTCGCCGTTGCTGGCGTGCCGTTTGTTGACGTCATGACGACTATGTGTGACCCGAGC 1800
T_vivax         CATGTTGCTCTTGCTGGCGTGCCATTTGTCGATGTCATGACCACCATGTGTGACCCAACC 1797
                * *** **  ************* ***** ** ******** ** ********** * *

T_congo         ATTCCGCTCACAACGGGCGAGTGGGAGGAATGGGGGAACCCGAACGAATACAAATTCTTC 1860
T_vivax         ATACCACTCACAACATTTGAGTGGGAGGAGTGGGGCAACCCCAACGAGTACAAGTACTTT 1857
                ** ** ********    *********** ***** ***** ***** ***** * ***

T_congo         GACTATATGAACAGCTACAGTCCGATTGATAACGTGCGCCCGCAGGACTACCCGAACCTC 1920
T_vivax         GACTACATGAACAGTTACAGCCCAATTGATAATGTCCGGGCCCAGGCGTATCCTCACCTG 1917
                ***** ******** ***** ** ******** ** **  * ****  ** **  ****
```

```
T_congo         ATAATCCAAGCTGGACTGCACGATCCCCGCGTGGCATATTGGGAGCCAGCGAAGTGGGCC 1980
T_vivax         ATGATCCAGGCTGGATTGCATGATCCACGCGTGGCTTATTGGGAGCCAGTTAAGTGGGCG 1977
                ** ***** ****** **** ***** ******** *************  ********

T_congo         TCGAAGCTGCGGGAGCTCAAGACGGACAACAACGAGGTGTTGCTGAAGATGGACTTGGAC 2040
T_vivax         TCAAGGCTCCGTCAACTGAAGACTGACGGCAACGAGGTGCTTGTGAAGATGGACCTTGAC 2037
                ** * *** **  * ** ***** ***  ********** *  *********** * ***

T_congo         AGTGGCCACTTCTCCGCGAGTGATCGTTACAAGTACCTGCGAGAGCACGCCATACAACAG 2100
T_vivax         AGTGGTCACTTCTCCTCGGCTGACCGTTACAAGTACTGGAGGGAAATGGCAATTCAGCAG 2097
                ***** ********* **  *** ************  * * **    ** ** ** ***

T_congo         GCTTTTGTGTTGAAGCACCTTGGCGTGCGCCGGTTGCTGCGGCATTAA 2148
T_vivax         GCGTTTGTGCTGAAGCACCTGAACGTGCGCTGTCTTTTGCGGCGCTAG 2145
                ** ****** **********   ******* *  *  ******  **
```

Figure A6. Alignment of the nucleotide sequences of oligopeptidase B from *T. congolense* and *T. vivax*, prepared using CLUSTAL W. The sequences that are complimentary to the primers used for cloning are shown in blue, and the sequences that are complimentary to the primers used for colony PCR are shown in red.

Appendix 3

History of *T. vivax* IL4186

The *T. vivax* clone IL4186 originated from the isolate IL3769, isolated from a cow in Kapira town, Teso district, Uganda in 1968 (Edward Okoth, ILRI, Kenya, personal communication). It was passaged in goat, then sheep to become the stock IL V 13, and subsequently used to infect tsetse fly *Glossina morsitans morsitans*. These flies were used to infect rabbits, whose blood was passaged into mice, to give rise to the reference stock IL7e10 after extra passage through tsetse fly and goat to confirm infectivity (despite adaptation to mice). From stock IL7e10, the trypanosomes were cloned by two cyclic passages in mice (infection with a single trypanosome) to give rise to the clone IL4186. This clone is one of the 3-4 in existence that can grow steadily in rodents while maintaining their infectivity for flies and ruminants.