VIVAPAIN: A CYSTEINE PEPTIDASE FROM *Trypanosoma vivax*

PERINA VATHER
BSc (Hons) Biochemistry

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

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PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, from January 2008 to December 2009, under the supervision of Professor Theresa. H. T. Coetzer

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

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Perina Vather (candidate)

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Professor Theresa H. T. Coetzer (supervisor)

DECLARATION – PLAGIARISM

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2. This thesis has not been submitted for any degree or examination at any other university.

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ABSTRACT

African animal trypanosomosis is a devastating disease affecting livestock mainly found in sub-Saharan Africa. This disease is known as nagana and is transmitted by the trypanosome parasite from the tsetse fly vector to a mammalian host. There are three African trypanosomes namely *Trypanosoma vivax, T. congolense and T. brucei brucei* that are the causative agents responsible for this disease in African cattle. This disease is serious since it not only affects livestock but also has a negative impact on the sub-Saharan African economy. There is, therefore, a great demand for better control methods of the disease and suitable diagnostic methods.

Current control measures such as the use of trypanocidal drugs, tsetse fly eradication methods and trypanotolerant cattle have become inadequate. The defence mechanism of the trypanosome to continuously change its surface coat by a process of antigenic variation has made it impossible to produce a suitable vaccine. Therefore, chemotherapy is still one of the key approaches for control of this wasting disease. The long existence of the current trypanocidal drugs has allowed the development of drug resistance. The development of new chemotherapeutic drugs is focused on targeting the pathogenic factors such as parasite cysteine peptidases that contribute to the disease.

Vivapain is the main cysteine peptidase of *T. vivax* and shares high sequence identity with congopain, the main cysteine peptidase of *T. congolense*, which was previously shown to be a pathogenic factor contributing to trypanosomosis. Vivapain, thus, has potential as a target for chemotherapeutic drug design. Hence, the first part of this study involved the recombinant expression and enzymatic characterisation of vivapain for future production of new synthetic inhibitors for the use in new trypanocidal drugs.

The catalytic domain of vivapain (Vp) was recombinantly expressed in the *Pichia pastoris* yeast expression system and enzymatically characterised. The main finding from this study

was that Vp was only able to hydrolyse a substrate if the P_2 position was occupied by either a hydrophobic Phe or Leu residue. Vp was also found to be active close to physiological pH and was inhibited by the reversible cysteine peptidases, leupeptin, antipain and chymostatin and the irreversible cysteine peptidases L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane (E-64), iodoacetic acid (IAA) and iodoacetamide (IAN).

A further important aspect of controlling trypanosomosis is the diagnosis of the disease. Clinical, parasitological, molecular and serological techniques have been applied and used to diagnose trypanosomosis. One of the most promising serological techniques has proven to be the enzyme-linked immunosorbent assay (ELISA), more specifically the antibody and antigen detection ELISAs. The main requirement for this technique is a readily available and reproducible antigen such as that produced by recombinant expression.

While there are recombinant antigens that are available to be used to detect *T. congolense, T. brucei brucei* and even *T. evansi* infections, there are none available to detect *T. vivax* infections. In the second part of this study, a mutant inactive full length form of vivapain (ΔFLVp) was expressed in a bacterial expression system for the detection of *T. vivax* infections. Antibodies against this antigen were produced in both chickens and mice. Both the chicken IgY and mice sera were able to detect the recombinant ΔFLVp in western blots. The mice sera were also able to detect native vivapain in a *T. vivax* lysate, which is very promising for future use of the ΔFLVp antigen and the corresponding antibodies in diagnosis of *T. vivax* infections in sera of infected animals.

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LIST OF ABBREVIATIONS

2x YT	2 × yeast extract, tryptone
ΔFLVp	full length inactive mutant of vivapain
A_x	absorbance at x nm
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
AMC	7-amino-4-methylcoumarin
AMT	acetate-MES-Tris
BCA	bicinchoninic acid
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
Boc	butyloxycarbonyl
BSA	bovine serum albumin
C2	congopain catalytic domain
C-terminal	carboxy terminal
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
$[E]_0$	active enzyme concentration
E-64	L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
g	relative centrifugal force
HRPO	horseradish peroxidase
[I]	inhibitor concentration
IAA	iodoacetic acid
IAN	iodoacetamide
IgG	immunoglobulin G

IgY	immunoglobulin Y
ISG	invariant surface glycoprotein
k_{ass}	rate of complex association
K_{av}	availability constant
k_{cat}	turnover number
kDa	kilo-Dalton
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
MD	minimal dextrose
MEC	molecular exclusion chromatography
MeoSuc	methoxy-succinyl
MES	acetate-2(N-morpholino)ethanesulfonic acid
min	minute
N-terminal	amino terminal
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PSG	phosphate saline glucose
PCR	polymerase chain reaction
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
RT	room temperature (22 ± 2 ^{o}C)
[S]	substrate concentration
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
Suc	succinyl

$t_{½}$	half-life
TBS	tris buffered saline
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
Vp	catalytic domain of vivapain
YP	yeast extract, peptone
YPD	yeast extract, peptone, dextrose
Z	benzyloxycarbonyl

1. LITERATURE REVIEW

1.1 AFRICAN ANIMAL TRYPANOSOMOSIS

African animal trypanosomosis is a serious haemoparasitic endemic disease caused by the transmission of parasites known as trypanosomes from a tsetse fly vector, *Glossina species*, to a mammalian host. Tsetse-transmitted trypanosomosis is mainly found in sub-Saharan Africa distributed over an area of between 8 and 11 million km^2 (Hursey 2001; Krafsur 2003; Antoine-Moussiaux *et al.* 2009). The distribution of trypanosomes correlates to the distribution of tsetse flies in sub-Saharan Africa (Van den Bossche 2001). This location of tsetse flies is known as the tsetse fly belt (Fig. 1.1). African trypanosomes that affect cattle are *Trypanosoma congolense, T. vivax* and to a lesser extent *T. brucei brucei*, causing a disease known as nagana. The symptoms of nagana are anaemia, weight loss, decline in productivity and sometimes even death (Lalmanach *et al.* 2002). This disease affects approximately 46 million cattle and causes a staggering economic loss of US $4.5 billion annually for sub-Saharan Africa (Swallow 2000; Antoine-Moussiaux *et al.* 2009).

The control measures for African animal trypanosomosis include tsetse fly eradication, the use of trypanocidal drugs and livestock that have acquired the genetic trait of resistance to trypanosomosis (trypanotolerant cattle) (Naessens *et al.* 2002; Aksoy 2003; Holmes *et al.* 2004). Attempts to eradicate tsetse fly populations involve the use of trypanocidal sprays, which is a problem since it has a negative impact on the environment (Allsopp and Hursey 2004). The use of trypanotolerant cattle is a good approach; however, their small size and low productivity has led to farmers using them less often (Murray *et al.* 2004). The trypanocidal drugs used for African animal trypanosomosis include isometamidium chloride, homidium and diminazene aceturate. These very expensive drugs have been around for over five decades and the greatest problem associated with their use is the emergence of drug resistance (Anene *et al.* 2001).

With this in mind there is a great demand for new control measures. Chemotherapy is still the key approach and it is based on targeting the pathogenic factors that cause the disease (Antoine-Moussiaux *et al.* 2009). Peptidases, more importantly cysteine peptidases of the

parasite, have vital functions in trypanosomosis, including the invasion of host cells and tissues, the breakdown of mediators involved in the host immune response and the hydrolysis of host proteins in order to sustain the nutritional needs of the parasite (Rosenthal 1999). Since these cysteine peptidases contribute to the pathogenesis of the disease it is likely that they would be good targets for chemotherapeutic drugs (Robertson *et al.* 1996).

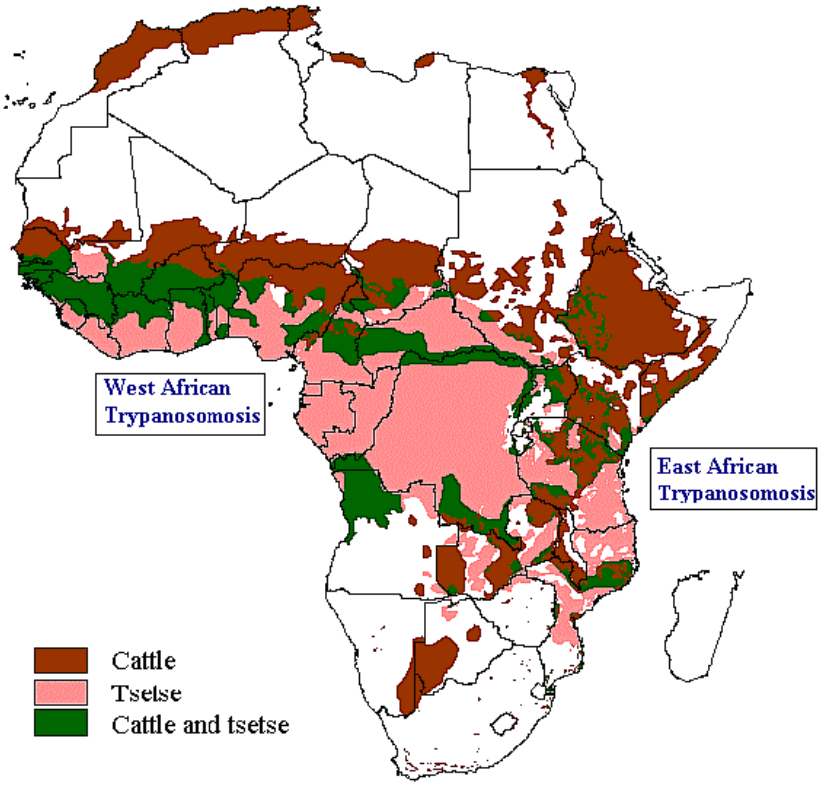


Figure 1.1 Illustration of the distribution of cattle and tsetse flies in Africa. Acquired from the University of Liverpool www.genomics.liv.ac.uk/tryps/index accessed on 17.12.08. The area occupied by the tsetse flies is known as the tsetse fly belt, which corresponds to the area of infected cattle.

1.2 TRYPANOSOMES

Trypanosomosis was first discovered by Sir David Bruce in 1894, who found a correlation between nagana, a disease affecting cattle, the tsetse fly and trypanosomes, while investigating the deaths of many cattle in the Zululand region of South Africa (Duggan

1977). *T. congolense, T. vivax* and *T. b. brucei* are the major causative agents of bovine trypanosomosis in sub-Saharan Africa (Lalmanach *et al.* 2002; Stevens and Brisse 2004). *T. vivax* and *T. evansi* are also transmitted mechanically by biting insects, such as tabanids and stomoxes, in areas outside the tsetse belt as well as in South and Central America and Asia (Nantulya 1990; Osório *et al.* 2008), while *T. equiperdium* is transmitted sexually and have a wider geographic distribution (Nantulya 1990; Brun *et al.* 1998). Infections by these trypanosome species are not confined to cattle since they infect a wide range of domestic animals such as horses, camels, donkeys, mules, water buffalo, pigs, goats and dogs (Nantulya 1990; Brun *et al.* 1998; Uilenberg *et al.* 1998; Stevens and Brisse 2004). African trypanosomes also affect humans, causing sleeping sickness or human African trypanosomosis. These parasites are *T. b. gambiense* found mainly in West Africa and *T. b. rhodesiense* located mainly in East Africa (Baltz *et al.* 1985; Barrett *et al.* 2003; Stevens and Brisse 2004). The direct effect of trypanosomosis on humans and cattle, is the death of more than 100 people and 10 000 cattle daily (Hursey 2001). The South American trypanosome known as *T. cruzi* is transmitted by triatomas, and causes Chagas disease in humans (Cazzulo *et al.* 1997). Dogs and cats have recently been shown to be associated in the transmission of *T. cruzi* (Gürtler *et al.* 2006)

1.3 CLASSIFICATION OF TRYPANOSOMES

The classification of trypanosomes has been based solely on medical and veterinary features, which is represented in Fig. 1.2. The unicellular trypanosome parasites belong to the order Kinetoplastida due to the presence of a kinetoplast at the base of the flagellum (Uilenberg *et al.* 1998; Stevens and Brisse 2004). This kinetoplast contains the mitochondrial DNA of the parasite (Vickerman 1985). The family Trypanosomatidae is

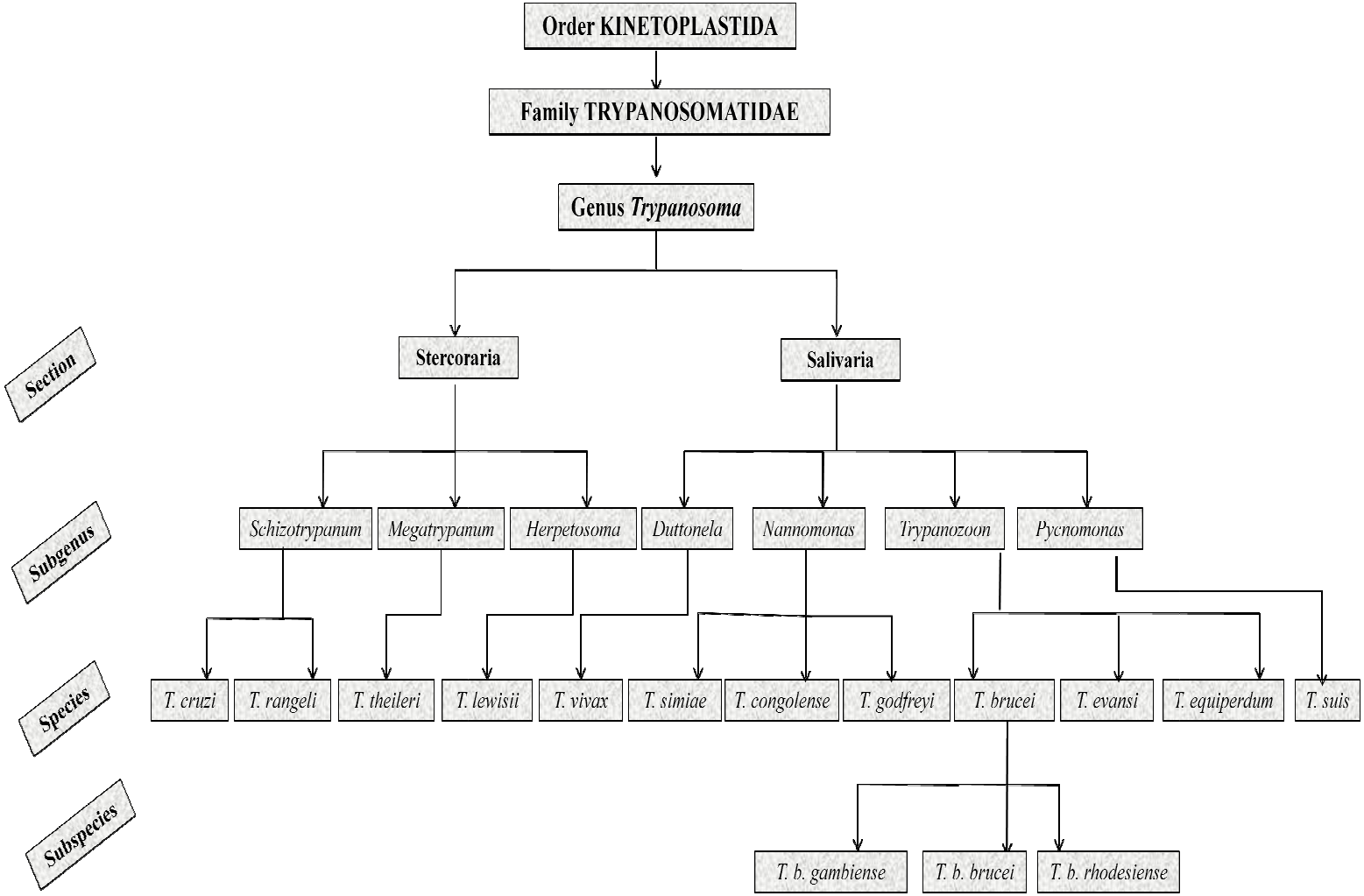


Figure 1.2 Classification of trypanosomes based on medical and veterinary significance (Stevens and Brisse 2004).

subdivided into *Trypanosoma* and *Leishmania* genera, which are classified according to their morphology and range of hosts the parasites infect (Momen 2001).

The *Trypanosoma* genus is further subdivided into two sections, namely the Stercoraria and Salivaria, based on how the parasites are transmitted from the insect vector to the mammalian host once the parasite has completed its cyclic development (Uilenberg *et al.* 1998). In the Stercoraria section, the metacyclic trypanosomes develop in the hindgut and are transmitted via the faeces of the insect vector. The Salivarian parasites develop into the metacyclic stage in the anterior part of the digestive tract of the tsetse fly and they are inoculated via the saliva into the mammalian host (Stevens and Brisse 2004). A striking feature of the salivarian species is that they contain and express variable surface glycoproteins (VSGs); therefore, they are able to change their surface coats by a process known as antigenic variation (Donelson 2003; Stevens and Brisse 2004). Salivaria are further divided into four subgenera namely, Duttonella, Nannomonas, Trypanozoon and Pycnomonas. The *Duttonella* genus has a principle species known as *Trypanosoma vivax* and a morphologically similar, but smaller species known as *T. uniforme. T. vivax* parasites mainly infect mammalian hosts in Africa and Latin America. The presence of a terminally situated kinetoplast in *T. vivax* is the distinguishing feature that separates the *Duttonella* genus from the Salivarian trypanosomes (Stevens and Brisse 2004).

1.4 BIOLOGY OF TRYPANOSOMES

1.4.1 Morphology of African trypanosomes

Trypanosomes are unicellular streamlined elongated organisms that vary in size from 8-50 µm, with *T. vivax* ranging between 18-31 µm (Uilenberg *et al.* 1998; Osório *et al.* 2008). These parasites are tapered on either end and they contain a highly organised microtubule cytoskeleton, which gives the parasite its shape (Fig. 1.3) (Uilenberg *et al.* 1998; Matthews 2005). The extremely flexible outer layer of the trypanosome cytoplasm is called the pellicle. This flexible pellicle allows for the free movement of the parasite but at the same time it also maintains the rigid structure of the trypanosome. The pellicle and the cytoplasm together form the undulating membrane (Uilenberg *et al.* 1998). The flagellar pocket is

situated at the posterior end of the parasite arising from the parabasal body. A single flagellum arises and it spans the length of the parasite. Since the flagellar pocket is the point of exit for the flagellum, it is known as the site of endo- and exocytosis (Matthews 2005). The parasite uses both the flagellum and the undulating membrane for mobility (Branche *et al.* 2006). There is only a single mitochondrion present, which is elongated and runs from the posterior to the anterior end of the trypanosome. The posterior end of the mitochondrion contains a specialised area, known as the kinetoplast that contains between 10-20% of the parasite's DNA (Matthews 2005). The structure of *T. vivax* differs from that of *T. congolense* since *T. vivax* contains a larger kinetoplast, free flagellum (3-6 µm in length) and its posterior end is more club-shaped and the anterior end tends to taper off (Fig. 1.4). *T. vivax* also contains a distinctive vibrating and quick movement, which can be used to diagnose the presence of the parasite in the host (Osório *et al.* 2008).

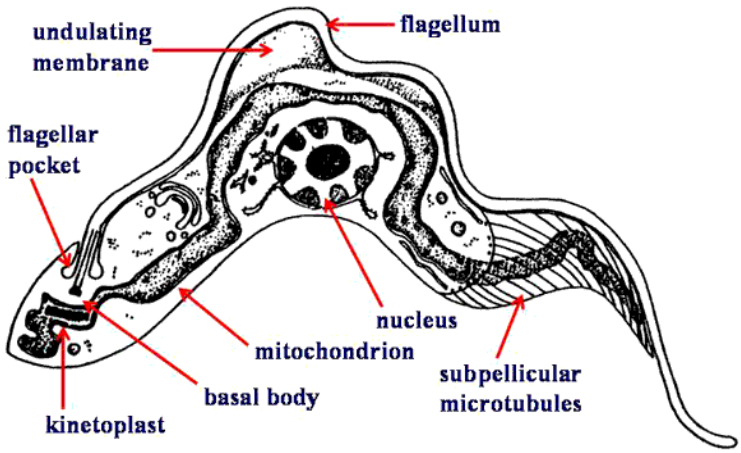


Figure 1.3 Diagrammatic representation of the morphology of a trypanosome. Acquired from www.tulane.edu/~wiser/protozoology/notes/kinet.html accessed on 24.10.09.

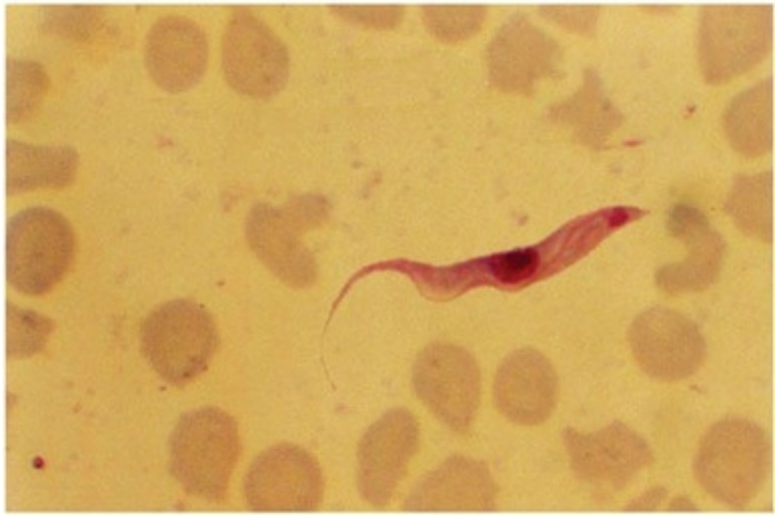


Figure 1.4 Blood smear of *T. vivax* (Osório *et al.* 2008). The *T. vivax* parasite contains a larger kinetoplast, and a free flagellum.

1.4.2 Genetic organisation

To date, the *T. brucei* genome has been completely sequenced (www.sanger.ac.uk/Project/T.brucei) (Berriman *et al.* 2005). Therefore the knowledge of *T. vivax* and *T. congolense* genomes are based on the information of the *T. brucei* genome (Melville *et al.* 2004). The *T. brucei* African trypanosome has a 35 Mb (3.5 x 10^7 bp) genome, which is approximately the same size as the genomes of *Plasmodium falciparum* (2.5-3.0 x 10^7 bp) (Dame *et al.* 1996), *Leishmania major* (3.2 x 10^7 bp) (Ivens *et al.* 2005) parasites. *T. cruzi* genome (4.3 Mb) is much smaller than the *T. brucei* genome (Agüero *et al.* 2000). The genome of the African trypanosome is divided into two sections. One section is within the nucleus and the other is within the kinetoplast (Donelson 2003; Melville *et al.* 2004). Two thirds of the nuclear genome has a single copy sequence and is thus relatively low in complexity while the remainder is made up of highly repetitive sequences (Melville *et al.* 2004). The nuclear genome is diploid, but the VSG genes, the VSG expression sites and the minichromosomes are not (El-Sayed *et al.* 2000). The kinetoplast contains two types of DNA circles, namely maxicircles and minicircles, with the latter keeping the maxicircles together in a network (Vickerman and Coombs 1999).

1.4.3 Antigenic variation

Trypanosomes are able to evade the immune system of their mammalian host by regularly changing their surface coat by a phenomenon known as antigenic variation (Borst and Rudenko 1994; Donelson 2003; McCulloch 2004). The entire cell surface of the blood stream and metacyclic trypanosomes; including the flagellum, are covered by the variant surface glycoprotein (VSG) coat (Barry and Carrington 2004). The role of this VSG coat is to protect the parasites from the host immune responses (Donelson 2003; McCulloch 2004). Each change in the VSG coat is known as a distinct variable antigen type (VAT) (Barry and Carrington 2004). This coat is highly immunogenic and results in high antibody titre in the mammalian host. Antibodies that were made against older VATs would be able to target these parasites; however, parasites with a new VAT will survive and can produce a new wave of parasitaemia. These different VATs do not all occur together but rather spread out throughout the infection and the switch from one VAT to the next is spontaneous. This event occurs in such a way that the new VAT is generated before the antibody attack on the trypanosomes of the old VAT occurs (Barry and Carrington 2004). This phenomenon of antigenic variation has therefore made it very hard to develop a VSG-based vaccine for trypanosomosis (Vickerman 1978).

1.4.4 Life cycle of trypanosomes

Trypanosomes are transmitted by both male and female tsetse flies of all 23 species of the *Glossina* genus (Uilenberg *et al.* 1998; Aksoy 2003). The transmission of the trypanosomes, such as *T. b. brucei, T. congolense* and *T. vivax*, by tsetse flies are cyclical (Fig. 1.5). However, mechanical transmission of *T. vivax and T. evansi* also occurs outside the tsetse belt of Africa by biting flies, such as tabanids (Vickerman *et al.* 1993; Uilenberg *et al.* 1998).

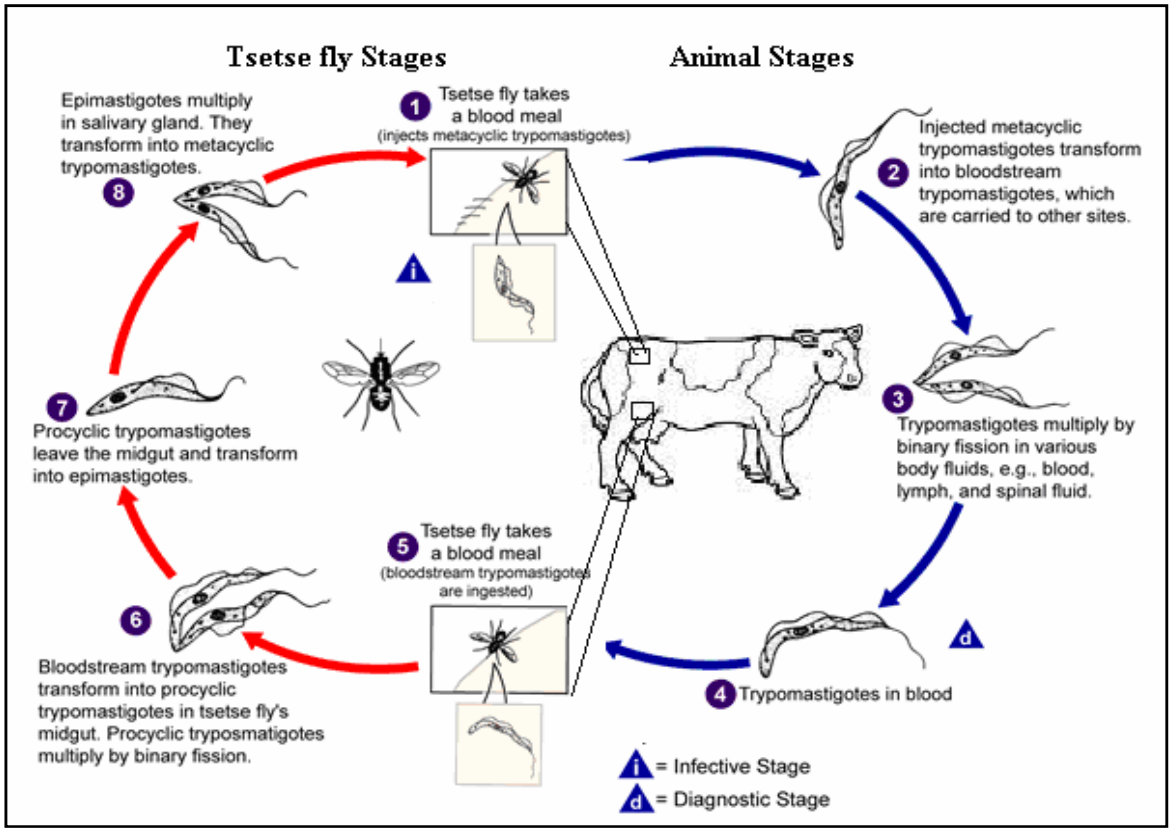


Figure 1.5 Life cycle of *T. brucei* showing the different morphological changes that occurs during the different stages in the vector and mammalian host. Adapted from pathmicro.med.sc.edu/lecture/trypanosomiasis.htm accessed on 24.10.09. The life cycle of *T. vivax* differs since the morphological changes in the vector only occur in the proboscis.

The life cycle of the trypanosome is divided into two phases, one of which occurs in the insect vector and the other in the mammalian host (Fig. 1.5) (Uilenberg *et al.* 1998). The life cycle begins when the tsetse fly takes a blood meal from an infected host as short stumpy non-diving trypomastigotes. During this time the parasite lacks the glycoprotein surface coat and is not infective to mammals (Baltz *et al.* 1985; Vickerman *et al.* 1988). The parasite migrates to the midgut of the fly, where it transforms into the procyclic trypomastigote form. The procyclics then travel anteriorly to a region of the midgut, where they begin to divide and increase in number. It is the migration of the trypanosomes from the midgut to its next destination that differs between *T. b. brucei* and *T. congolense* species. *T. b. brucei* migrates from the midgut to the salivary glands and *T. congolense* from the midgut to the proboscis of the tsetse fly where they transform into long slender

epimastigotes (Uilenberg *et al.* 1998; Aksoy 2003). Thereafter, the epimastigotes transform into the metacyclic form, where they regain their glycoprotein surface coat (Baltz *et al.* 1985). The metacyclics are the non-dividing infective form of the parasite (Aksoy 2003). The *T. vivax* life cycle differs from that of *T. b. brucei* and *T. congolense* since development occurs only in the proboscis of the tsetse fly with no events taking place in the midgut or the salivary glands of the vector (Matthews *et al.* 2004). The time period between the ingestion of the blood stream trypomastigotes from the mammalian host and the conversion into the metacyclics varies from three to five weeks for *T. b. brucei* and ten days for *T. congolense* and *T. vivax* (Vickerman et al. 1988).

When the fly is takes its next blood meal it injects the infective metacyclics into its host. The parasites get deposited at the site of infection into the host's dermal connective tissue, where the parasites divide by binary fission and develop into long slender trypomastigotes (Uilenberg *et al.* 1998). This leads to a local inflammatory reaction and a chancre develops in the skin at the site of infection (Vickerman 1985). The dividing trypanosomes can be found within the chancre, between collagen fibres and within dermal spaces. The parasites are then released into the host's bloodstream from lymph nodes via lymph vessels. While the parasites are in the bloodstream they differentiate into rapidly dividing long slender trypomastigotes, which migrate to different organs of the host via the bloodstream (Vickerman *et al.* 1988). A portion of the trypomastigotes transform into a short, stumpy form, which is infective for the tsetse fly and can be picked up by the next fly taking a blood meal from the host (Uilenberg *et al.* 1998).

1.5 PATHOGENESIS OF AFRICAN ANIMAL TRYPANOSOMOSIS

African trypanosomes are the cause of three disease states, which are; nagana (caused by *T. b. brucei*, *T. congolense* and *T. vivax* in cattle), surra (caused *T. evansi* in equines, water buffaloes and camels) and dourine (caused by *T. equiperdium* in horses) (Clair 1988). These pathogenic parasites can be divided into haematic and humoral trypanosomes, based on their distribution in tissues. The haematic trypanosomes, *T.*

congolense and *T. vivax*, are found in the blood vessels (intravascular) and the humoral trypanosomes, *T. b. brucei, T. evansi* and *T. equiperdium*, are present in the extravascular compartment and in tissues (Uilenberg *et al.* 1998; Lonsdale-Eccles and Grab 2002; Taylor and Authié 2004). The humoral trypanosomes are the causative agents of neurological disease since they can break through the blood-brain barrier (Antoine-Moussiaux *et al.* 2008). Even though these trypanosomes are located in different areas, anaemia is a principal symptom in both groups (Prowse 2005).

Interestingly, pathogenicity may differ within species of trypanosomes based on their geographical region. Some *T. vivax* trypanosomes from East Africa may cause an acute haemorrhagic disease in cattle, whereas *T. vivax* trypanosomes from West Africa may result in a milder non-haemorrhagic disease (Taylor and Authié 2004). There has also been evidence of a correlation between the biological vector species and the level of virulence of *T. vivax* isolates. The *G. pallipides* from central Kenya transmits a *T. vivax* isolate that causes acute disease and eventually leads to death in approximately one month in 70% of infected cattle, whereas the *G. fuscipes* transmits an isolate in Nyanza (a province located in the south west of Kenya), that causes a chronic infection and eventually leads to death in 100 to 160 days post infection (Osório *et al.* 2008).

In bovine trypanosomosis, *T. vivax* seems to be the most pathogenic followed by *T. congolense* and to a lesser extent *T. b. brucei* (Taylor and Authié 2004). Even though *T. vivax* is less virulent than *T. congolense* it is responsible for over 50% of mortalities in cattle (Prowse 2005). *T. congolense* and *T. b. brucei* have a relatively low parasitaemia when compared to *T. vivax,* which is usually 10^8 trypanosomes/mL of blood) (Taylor and Authié 2004).

During bovine trypanosomosis three successive stages in infection may occur, namely acute, stabilisation and chronic; however, death can occur at any stage. The initial stage is the acute phase of the disease, which may contain clinical signs such as enlarged lymph nodes and spleen, weakness, lethargy, abortion and reduced milk production. The acute phase is characterised by a continuous drop in the haematocrit value (packed red blood cell

volume, PCV), haemoglobin concentration and red blood cell numbers. Death of the animal may occur in the first weeks or months of the infection due to the acute phase. If cattle survive the acute phase, infection tends to stabilise after six to eight weeks, characterised by stabile PCV values, typical of the stabilisation phase. The animal then enters the third or chronic phase of infection during which the animal may develop cachexia, intermittent parasitaemia and may become stunted, wasted and infertile. This chronic infection may lead to the death of the animal by congestive heart failure due to prolonged anaemia, damage to the heart muscles and increased vascular permeability. The haemorrhagic *T. vivax* isolates from East Africa cause a hyperacute disease that shows symptoms of high parasitaemia, severe anaemia and haemorrhages. These cattle either die in two weeks or self-cure under favourable conditions after two months (Taylor and Authié 2004).

1.6 DIAGNOSIS OF AFRICAN ANIMAL TRYPANOSOMOSIS

African bovine trypanosomosis can be diagnosed by an array of techniques some of which are based on clinical, parasitological, molecular and serological methods. The key reason for diagnosis is to ensure application of the most appropriate therapeutic methods. Diagnostic techniques should be suitable for field diagnosis under resource poor conditions (Luckins 1992). The diagnostic techniques used need to give rapid diagnostic results, since the response time for cattle that are treated earlier on in the acute phase of infection is rapid as compared to cattle treated during the chronic phase of infection, during which time the treatment is slow and cattle may even die (Taylor and Authié 2004). Other reasons for diagnosis are to monitor tsetse fly control and eradication methods, and gain insight into the efficacy of chemotherapy and the development of trypanocidal drug resistance (Eisler *et al.* 2004).

Clinical diagnosis of African bovine trypanosomosis is usually the favoured technique for field conditions since it is exclusively based on a physical examination of the animal. However, the clinical signs of bovine trypanosomosis described in Section 1.5 are not specific for the disease and the results obtained could pertain to other tropical diseases

(Nantulya 1990). In some cases trypanocidal drugs are administered to the cattle that show the clinical signs and a positive response to the drugs confirms the diagnosis (Eisler *et al.* 2004).

Parasitological examination techniques involve viewing wet blood films as well as Giemsa-stained thick and thin fixed blood films with a light microscope. This technique varies in sensitivity and it is usually very low during the chronic phase of the disease (Magona *et al.* 2003; Eisler *et al.* 2004). Since there is a high level of morphological similarity between the trypanosome species, it is difficult to use this technique to diagnose the different trypanosomal species infections. The sensitivity of this technique was improved by haematocrit centrifugation, which results in three layers forming after centrifugation: a red blood cell layer, the buffy coat layer, which contains the white blood cells and the trypanosomes, and a plasma layer. This concentration method allows for the analysis of the trypanosomes in the buffy coat layer, which is a more sensitive method of detection, with as low as 2.5×10^2 *T. congolense*, 5×10^2 *T. vivax* and 5×10^3 *T. brucei* being diagnosed (Luckins 1992). The problem with this technique is that it is difficult to perform in the field because fresh blood, a haematocrit centrifuge and microscopes are needed. Therefore, more reliable, sensitive and specific techniques were developed. These methods were based on molecular techniques and serological immunological techniques.

A more recent molecular biology tools used for the diagnosis of bovine trypanosomosis is the polymerase chain reaction (PCR). PCR seems to be a promising technique since it allows for species-specific detection of the trypanosomes and it is a more sensitive technique (Solano *et al.* 1999; Desquesnes and Davila 2002). This technique is very costly since specialised equipment is needed.

Serological techniques include complement fixation, indirect haemagglutination, indirect fluorescent antibody test and enzyme-linked immunosorbent assay (ELISA). Complement fixation has been used to diagnose *T. equiperdium* in North America. This test is not very reliable since problems such as reagent preparation, standardisation and anticomplementary

activity in the sera (removal of complement from the sera) occurred (Eisler *et al.* 2004). The indirect haemagglutination test was used to diagnose *T. evansi* infection, but was unreliable in detecting *T. vivax* infections (Clarkson *et al.* 1971).

The indirect fluorescent antibody primary binding assay test was regarded as one of the most significant improvements for diagnostics. This test is used to directly measure the interaction between the antigen and the antibody. This was achieved by allowing the test sera (containing the antibody) to interact with the antigens fixed on a microscope slide. The antigens are fixed on a microscope slide using fixatives such as acetone and formalin (Nantulya 1990; Luckins 1992). The antibodies that bind to the antigen are then visualised by using anti-host species immunoglobulins conjugated to a fluorescent dye. The antigen and antibody interaction can be observed by using a fluorescence microscope. The indirect fluorescent antibody test has been shown to be very sensitive and specific in detecting trypanosome antibodies (Aquino *et al.* 1999). The problem with this method is that it is not species-specific and as a result there is high cross reactivity between the different trypanosome species. Another disadvantage of this technique is that it requires an expensive fluorescence microscope.

Two types of ELISAs that can be used for the diagnosis, namely; the antibody-detection ELISA (Hopkins *et al.* 1998; Rebeski *et al.* 1999) and the antigen-detection ELISA (Nantulya and Lindqvist 1989). The antibody-detection ELISA allows for the detection of specific antibodies in sera from trypanosome infected cattle. This particular test possesses the same problem of cross-reactivity between species. Therefore, in the diagnosis of bovine trypanosomosis antibodies have to be screened against all three trypanosome species; namely, *T. congolense, T. vivax, T. brucei*, to increase the sensitivity (Eisler *et al.* 2004). One of the drawbacks of this test is that the presence of the antibodies does not confirm an active infection. This is due to the fact that the antibodies may persist in the mammalian host much longer than the infectious agent. Antibodies may be present in the host between 6-13 months after the infection has cleared (Luckins 1992; Van den Bossche *et al.* 2000). Another disadvantage is that it usually takes 8-12 days for antibody detection in the host,

therefore, this could lead to a false negative result (Greiner *et al.* 1997). For these reasons the antibody detection test has been used more as epidemiological tools for mapping and quantifying the prevalence and risk of trypanosomosis rather than for diagnostics (Eisler *et al.* 2004).

An alternative approach to the antibody-detection ELISA is known as the antigen-detection ELISA. This technique involves detecting the presence of the parasite antigens in the blood of the infected cattle. This results in a good indication of an active infection (Nantulya 1990). Modifications of the antigen-detection ELISA allowed for the detection of antigens in the host within 10-14 days of *T. congolense* and *T. evansi* infection. These antigens disappeared within 21 days of administering trypanocidal drugs after diagnosis (Rae and Luckins 1984). In this sandwich ELISA method, the wells of a 96 well polystyrene microtitre plate are coated with polyclonal antibodies made against crude recombinant antigen. The test serum is added and the antigen present in the sera binds to the antibody. The antibody-antigen interaction is detected using the same antibody, used to coat the plate, conjugated with an enzyme in the presence of a suitable substrate (Eisler *et al.* 2004).

The major problem for both the antibody and the antigen-detection ELISA is the lack of availability of recombinant trypanosome antigens for the detection and production of anti-trypanosome antibodies. Thus far, the ELISAs for both antibody and antigen detection have relied on native antigens prepared from whole parasite lysate; therefore, this had made it difficult to optimise and standardise the tests (Greiner *et al.* 1997; Eisler *et al.* 2004; Tran *et al.* 2009).

1.7 CONTROL OF AFRICAN ANIMAL TRYPANOSOMOSIS

1.7.1 Vector control

Trypanosomosis is dependent on the transmission of the trypanosome from a single insect vector; therefore, the key form of control would be to target the vector (Aksoy 2003). The control of trypanosomosis has relied heavily on the control of the tsetse fly vector due to the absence of suitable vaccines and affordable drugs (Aksoy 2003; Donelson 2003;

Holmes *et al.* 2004). The main strategies for vector control include; ground and aerial insecticide spraying, sterile insect technique and the latest development of the bait technique, which involves applying insecticide on live bait such as cattle (Allsopp 2001; Allsopp and Hursey 2004; Vale and Torr 2004).

The insecticide spraying approach seemed to be very successful; however, the major problem was the negative impact of the insecticides on the environment. The chemical compounds that are used in the successful eradication of tsetse flies are organochlorides such as dichlorodiphenyltrichloroethane (DTT) and dieldrin and the endosulfan group of compounds, such as the synthetic pyrethroids (Allsopp and Hursey 2004). Even though insecticidal spraying has been able to eradicate the tsetse fly vectors, there is the problem of the tsetse flies reinvading the area. Due to this problem, care has to be taken to ensure complete elimination of the tsetse fly population in that area or protect the area that has been cleared (Allsopp 2001).

The sterile insect technique involves the release of sterile male flies into the wild population. These insects are sterilised by irradiation. With time, this procedure leads to a drastic decrease in the number of fertile males, which leads to a decrease in the number of successful matings. As the sterile flies are continually introduced into the wild, the tsetse fly population may slowly become eliminated. This technique was successful in eradicating the screwworm fly in the USA, the Mediterranean fruit fly and *G. austeni* from Zanzibar (Aksoy 2003). An organisation known as the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC) had introduced a large-scale tsetse control project based on the sterile insect technique and insecticide treated cattle (Torr *et al.* 2005). This approach to eradicate tsetse flies over the large area occupied by tsetse flies in sub-Saharan Africa raise some doubt as to the feasibility of the project (Rogers and Randolph 2002).

1.7.2 Trypanotolerant Cattle

Trypanotolerant cattle breeds have developed the genetic capacity to manage the effects of trypanosomosis (Naessens *et al.* 2002). These cattle still develop trypanosomal infections;

however, they are able to limit parasitaemia and control anaemia (Lalmanach *et al.* 2002). *Bos taurus*, which includes the N'Dama, Mutura and the Dahomey cattle breeds, are trypanotolerant. These cattle are able to survive in areas that have a high infestation with tsetse flies and are mainly found in West and Central Africa (Authié 1994; Murray *et al.* 2004). Zebu (*Bos indicus)* are susceptible to trypanosomosis and would die in the areas of high tsetse challenge (Authié 1994). *Bos taurus* cattle, in particular the N'Dama are much smaller in size compared to the Zebu and as a consequence have lower productivity and are not used as much as the larger, more susceptible cattle. For this reason, trypanotolerant cattle only make up one third of the cattle present in the tsetse infected areas of Africa (Murray *et al.* 2004).

1.7.3 Vaccines

There is a demand for vaccine development due to the problems of drug resistance and toxicity of prophylactic drugs. The greatest obstacle for vaccine development is the phenomenon of antigenic variation (Borst and Rudenko 1994; Donelson 2003; McCulloch 2004). A new approach that has gained popularity over the past two decades is the development of an anti-disease vaccine that targets the pathogenic factors that are the causative agents of the disease, rather than the parasite itself (Playfair *et al.* 1990; Antoine-Moussiaux *et al.* 2009). The concept of an anti-disease vaccine was first developed and is currently being worked on to combat malaria (Playfair *et al.* 1990; Authié 1994; Matuschewski 2006; Schofield 2007). Vaccination with pathogenic factors leads to the production of antibodies against these factors that reduce the symptoms of the disease. These pathogenic factors are either actively secreted when the parasite is alive or released upon death of the parasite (Tizard *et al.* 1978). One of the major pathogenic factors associated with trypanosomes are the parasites' proteolytic enzymes; more importantly the cysteine peptidases, which can potentially become components for an anti-disease vaccine (Sajid and McKerrow 2002; Antoine-Moussiaux *et al.* 2009).

1.7.4 Chemotherapy

Chemotherapy is still the most widely used form of control for bovine trypanosomosis in the 37 Sub-Saharan countries dominated by the endemic disease (Geerts and Gryseels 2000; Holmes *et al.* 2004). The chemotherapeutic control of trypanosomosis relies on the use of three trypanocidal drugs that have been available on the market for over five decades; namely, isometamidium chloride (Samorin®), homidium (bromide and chloride) (Novidium®) and diminazene aceturate (Berenil®). Isometamidium chloride is most commonly used as a prophylactic and both homidium and diminazene aceturate are used as therapeutic agents. Homidium also has partial prophylactic properties (Anene *et al.* 2001; Holmes *et al.* 2004). Only thirty five million doses of the drugs are used in Africa each year with an estimated 50-70 million cattle at risk (Geerts and Gryseels 2000).

The greatest concern and risk of using these drugs is the development and spread of drug resistance, rendering the drugs ineffective. In 13 out of the 37 countries where trypanosomosis is prevalent, signs of resistance to one or more of the drugs have been reported (Geerts and Gryseels 2000; Holmes *et al.* 2004). Another downfall of using the current drugs is that they are provided to African countries by European pharmaceutical countries at great cost to the end-user. For this reason, there are a few generic forms of the drugs available in Africa but they are of poorer quality. This leads to a decrease in confidence in trypanocidal drugs, which may prevent farmers from using the drugs (Holmes *et al.* 2004).

There is thus an urgent need for the development of cheap, novel and effective drugs (McKerrow 1999; Caffrey *et al.* 2000). One of the key chemotherapeutic targets are the cysteine peptidases since they play important roles in the parasite and the pathogenesis of the disease (Antoine-Moussiaux *et al.* 2009).

During the last decade studies have shown that synthetic peptidyl inhibitors of cysteine peptidases improve the health of infected animals, reduce and even cure *Plasmodium*, *Leishmania* and *Trypanosoma* infections (McKerrow *et al.* 1999). Therefore,

characterisation of the substrate specificity of cysteine peptidases would assist in producing a synthetic peptide inhibitor.

The cysteine peptidase inhibitors that have potential as trypanocidal agents are the irreversible peptide inhibitors with vinyl sulfones and the peptidyl ketones, namely; chloromethylketones, diazomethylketones and fluromethylketones groups attached (Troeberg *et al.* 1999; Anene *et al.* 2001; Vicik *et al.* 2006). In vitro studies showed that these inhibitors were trypanocidal at low micromolar concentrations against blood stream forms of *T. b. brucei* with trypanopain-Tb being the major target (Troeberg *et al.* 1999). The chloromethylketones, Z-Phe-Phe-CMK was the most trypanocidal methylketone with an IC_{50} value of 3.6 μM whereas the diazomethylketone, Z-Phe-Ala-CHN_2, killed the blood stream forms of *T. b. brucei* (Scory *et al.* 1999; Troeberg *et al.* 1999). An important feature of these diazomethylketones was the fact that they were non-toxic to mammalian cells (Scory *et al.* 1999). These peptidyl diazomethylketones were also inhibitory to congopain (trypanopain-Tc) from *T. congolense.* The inhibitors that worked the best were Z-Leu-Leu-Met-CHN_2, Z-Leu-Met-CHN_2 and Z-Leu-Lys-CHN_2. Inhibitors became trypanocidal after incubation for 24-48 h with the cultured *T. congolense* parasites (Mbawa *et al.* 1992).

Fluromethylketones Z-Ala-Phe-CH_2F and Z-Ala-Imidazoyl-norvalyl-CH_2F lysed trypomastigote forms of *T. cruzi* and blood stream forms of *T. b. brucei* but at high concentrations (Ashall *et al.* 1990). Incubation of *T. cruzi* (amastigotes) with Z-Phe-Ala-CH_2F and Z-Phe-Arg-CH_2F prevented their transformation to trypomastigotes (Harth *et al.* 1993). Diazomethylketone inhibitors used at lower concentrations were able to prevent differentiation of *T. cruzi* epimastigotes to trypomastigotes (Bonaldo *et al.* 1991).

Vinyl sulfones were introduced as irreversible inhibitors due to their low toxicity to the mammalian host (Caffrey *et al.* 2000). Troeberg *et al.* (1999) demonstrated that the vinyl sulfone methylpiperazin-urea-Phe-homoPhe-VS killed 50% *T. b. brucei* parasites in culture, while Engel *et al.* (1998) showed that the Mu(morpholine urea)-Phe-homoPhe-VS was able to prevent cell division of *T. cruzi* epimastigotes resulting in the death of the parasites after five days (Engel *et al.* 1998).

Trypanopain-Tb was also inhibited by a group of reversible inhibitors, i.e. chalcones, acyl hydrazides and related amides. Micromolar concentrations of these inhibitors were able to reduce the growth of bloodstream forms of *T. b. brucei* in culture (Troeberg *et al.* 2000).

In vivo studies were also performed using inhibitors such as the vinyl sulfones and diazomethylketones. The vinyl sulfone, *N*-Me-Pip-Phe-homoPhe-VS, proved to be a promising inhibitor for *T. cruzi* infections (Engel *et al.* 1998; McKerrow *et al.* 1999). *T. b. brucei* infected mice were treated with the diazomethylketone, Z-Phe-Ala-CHN_2 from day three post-infection. This resulted in a decrease in parasitaemia to points where they were undetectable (Scory *et al.* 1999).

A study on the dipeptidyl inhibitors, Z-Phe-Tyr-CHO, Z-Phe-Tyr(OtBu)-CHN_2 and 1-NapSO2-Ile-Trp-CHO, against trypanopain from *T. b. brucei* (Nkemgu *et al.* 2003) showed that they were more effective than the inhibitors described by Troeberg *et al* (1999) and Scory *et al.* (1999). The IC_{50} values of these inhibitors were between 18-126 nM which was much lower than the IC_{50} of the commercially available trypanocidal drugs such as suramin, at 130- 360 nM and diminazene aceturate at 20-100 nM (Nkemgu *et al.* 2003).

An important question raised regarding the activity of cysteine peptidase inhibitors on the parasite is whether the inhibitory effect is due to the inhibition of the targeted cysteine peptidase or whether it is due to unrelated effects (McKerrow *et al.* 1999). A number of studies provided evidence that the inhibitors were acting on the target cysteine peptidase. The first study showed that despite the chemistry of the inhibitor, the same morphological deformities were present in the parasite e.g. when *T. cruzi* was reacted with vinyl sulfones and fluromethylketones, both inhibitors led to an accumulation of the unprocessed cruzipain in the Golgi apparatus, which changed the morphology of the organelle and decreased the normal trafficking of the cysteine peptidase to the lysosomes (Engel *et al.* 1998; McKerrow 1999). Another type of study was performed which involved tagging the

inhibitor with biotin, which proved that trypanopain was the target cysteine peptidase (McKerrow *et al.* 1999; Troeberg *et al.* 1999).

The design of these synthetic inhibitors based on targeting cysteine peptidases could be a promising chemotherapeutic application; however, care must be taken when choosing an inhibitor against the cysteine peptidases since the mammalian host affected by trypanosomosis also contains their own cysteine peptidases. For this reason, the inhibitor chosen should be able to differentiate between the mammalian and parasite targets and have a low toxicity in the host (Pink *et al.* 2005). Inhibitors such as vinyl sulfones and diazomethylketones could be used since they were shown to have low toxicity to the host (Caffrey *et al.* 2000). Studies have shown that the vinyl sulfones were not toxic to infected hosts such as mice, rats and even dogs. A possible explanation for this is that the host cysteine peptidases are present in higher concentrations than those of the parasites, therefore there is no evidence of toxicity in the host (McKerrow 1999). Even though parasite cysteine peptidases may be homologous to their mammalian hosts they have distinctive structural and biochemical properties such as pH optimum and stability, domain extensions (C-terminal extensions), diverse substrate specificity and cellular location (Sajid and McKerrow 2002). These differences could be advantageous when designing an inhibitor.

1.8 PEPTIDASES

1.8.1 Characterisation of proteolytic enzymes

Proteolytic enzymes or peptidases are a collection of enzymes that have the ability to catabolise proteins and polypeptides by cleavage of the peptide bonds giving rise to peptides and free amino acids (North *et al.* 1990; Rosenthal 1999). The active site (catalytic site) of the enzyme is often located in a groove that is found at the surface of the enzyme or buried slightly. The substrate binding sites are located near the active site and their properties contribute to substrate specificity. The substrate binding sites have a specific geometry which allows the substrate to fit into the binding groove. The specific

geometry can be explained using the nomenclature of Schechter and Berger (1967), where the binding sites adjacent to the active site are numbered S_1, S_2, S_n from the active site towards the amino-terminus and S_1', S_2', S_n' from the active site towards the carboxy-terminus (Fig. 1.6). The scissile bond is the peptide bond hydrolysed by the enzyme. The corresponding substrate positions are numbered P_1, P_2, P_n N-terminal to the scissile bond and P_1', P_2', P_n' C-terminal to the scissile bond (Fig. 1.6) (Schechter and Berger 1967). Even though only a single peptide bond is cleaved by the enzyme, the presence of a number of amino acid residues on either side of the scissile peptide bond is important for the hydrolysis to occur (Sajid and McKerrow 2002).

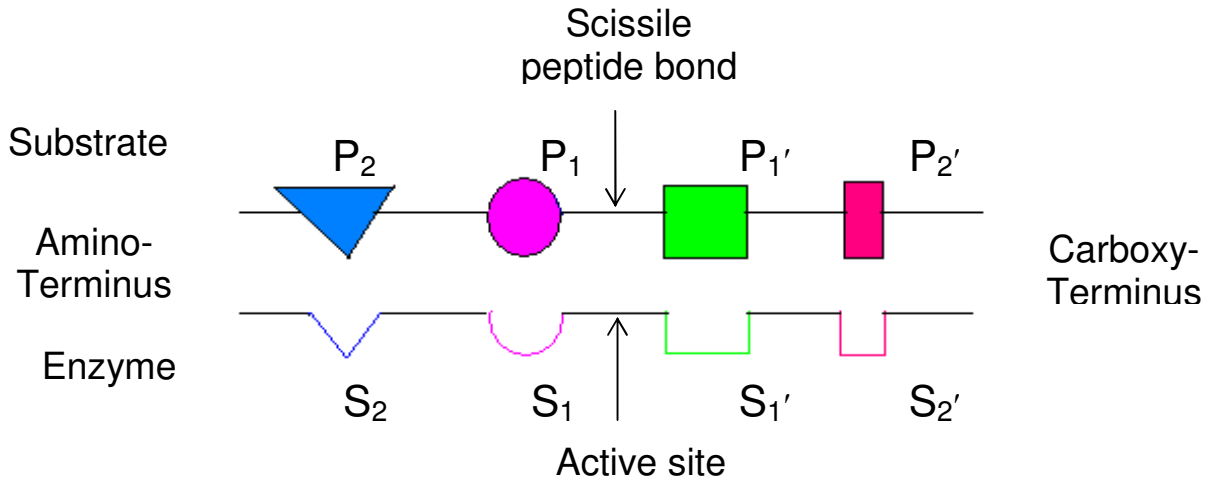


Figure 1.6 Representation of the nomenclature for the action of an enzyme on a substrate. (Adapted from Schechter and Berger, 1967). S_1, S_2, S_n and P_1, P_2, P_n are on the amino side of the scissle bond on the enzyme and substrate, respectively.

Proteolytic enzymes either hydrolyse the protein within the polypeptide chain (endopeptidase activity) or from the amino or carboxyl end (exopeptidase activity) known as an amino- or carboxypeptidases (Sajid and McKerrow 2002).

Based on the active site residues, six classes of peptidases are distinguished, i.e. serine, threonine, aspartate, metallo, glutamic and cysteine peptidases (Table 1.1) (Rawlings and Barrett 1999; Sajid and McKerrow 2002; Rawlings *et al.* 2008). Some peptidases may only hydrolyse particular amide bonds, while others only hydrolyse a certain peptide bond that is found in a specific protein. Peptidases may even be able to hydrolyse many peptide bonds that have similar sequences or conformations.

Table 1.1 Six main classes of peptidases divided according to their catalytic mechanisms. [Adapted from: (Barrett 1994; Rosenthal 1999; Rawlings et al. 2008)]

Peptidase	Active site residues	Example
Aspartate	Asp^{33}, Asp^{213}	Digestive enzymes, intracellular acid peptidases, extracellular regulatory peptidases, pepsin a, HIV I peptidase
Cysteine	Cys^{25}, His^{159}, Asn^{175}	Papain, cathepsins B and L, congopain, vivapain, cruzipain, trypanopain
Glutamic	Gln^{107}, Glu^{190}	Scytalidoglutamic peptidase
Metallo	His^{387}, Glu^{388}, His^{391}, Glu^{410}, Tyr^{476}	Aminopeptidases, Matrix metallopeptidases
Serine	Asp^{102}, Ser^{195}, His^{57}	Trypsin, chymotrypsin, oligopeptidase B
Threonine	Thr^{9}	Proteasome

1.8.2 General mechanism of hydrolysis by cysteine peptidases

The active site of all cysteine peptidases contains a catalytic triad of Cys^{25}, His^{159}, and Asn175. The presence of a cysteine residue with its thiol group in the active site is important for the hydrolysis of the substrate. The thiol group becomes deprotonated by the presence of the His residue in the active site. This mechanism improves the nucleophilicity of the thiol-group. The sulfhydryl (-SH) group of the Cys side chain interacts with the

imidazole present in the His residue, giving rise to a thiolate-imidazolium charged diad. The thiolate cysteine attacks the carbonyl carbon of the scissile bond present in the substrate forming a tetrahedral intermediate (Fig. 1.7). This complex becomes stabilised by the presence of the Asn in the active site. The presence of a conserved Glu in the proximity of the active site creates an oxyanion hole, creating an electrophilic centre, which leads to further stabilisation of the tetrahedral intermediate during the process of hydrolysis. The tertrahedral intermediate is transformed into an enzyme-substrate thiol ester with the release of the C-terminal section of the substrate by a process known as acylation. The enzyme-substrate thiol ester is hydrolysed by water forming a second tertrahedral intermediate. This intermediate undergoes deacylation resulting in a free enzyme and an N-terminal section of the substrate (Lecaille *et al.* 2002; Sajid and McKerrow 2002; Barrett and Rawlings 2004). Trypanosomal cysteine peptidases have a wide range of substrate specificities, which provides an advantage for developing inhibitors for the enzymes, but it can also be a disadvantage because it makes it difficult to develop inhibitors that will be specific for particular peptidases (Lalmanach *et al.* 1998).

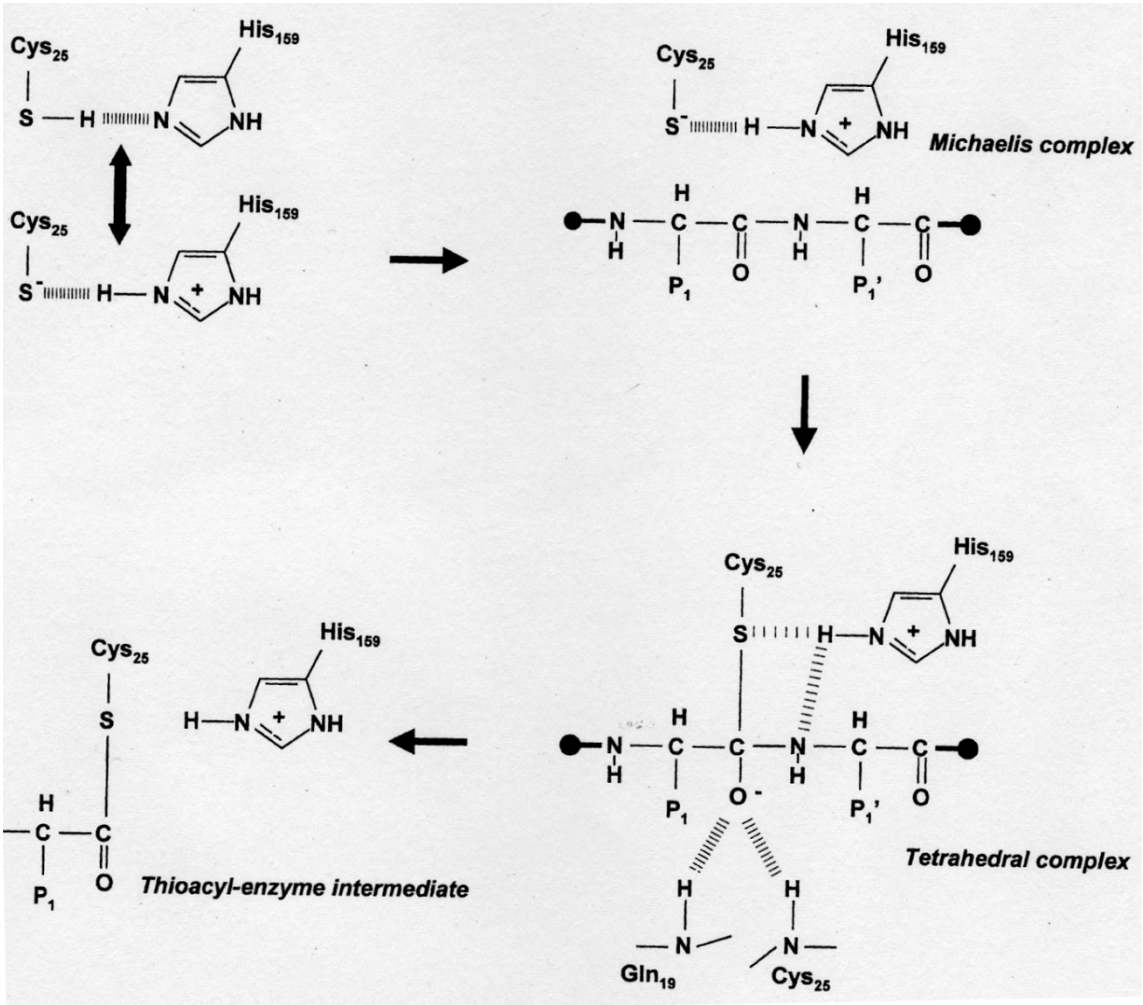


Figure 1.7 Scheme representing the steps involved in the catalysis of cysteine peptidases (Dunn 2001)

1.8.3 Classification of cysteine peptidases

There are over 400 types of cysteine peptidases, many of which are responsible for important functions in and survival of parasites. They are also believed to be involved in the disease process (Mottram *et al.* 2003). These cysteine peptidases can be divided into 10 clans, which include: CA, CD, CE, CF, CH, CL, CM, CN, CO and C- (Rawlings *et al.* 2008). Cysteine peptidases belong to the papain superfamily of peptidases. They belong to *Clan CA* and subfamily *C1A* (Barrett and Rawlings 2004). This group of cysteine peptidases is the most widely studied (Barrett and Rawlings 2004; Caffrey and Steverding 2009). The family contains papain (the plant cysteine peptidase) and the mammalian lysosomal cysteine peptidases: cathepsins B, C, K, L and S; parasite cysteine peptidases:

congopain (*T. congolense*), cruzipain (*T. cruzi*), falcipains (*Plasmodium falciparum*) and CPA, CPB and CPC (*Leshmania mexicana*). The C1 family is characterised by the presence of a Cys^{25}, His^{159} and Asn^{175} (papain numbering) catalytic triad in the active site. All clan CA cysteine peptidases are irreversibly inhibited by L-trans-Epoxysuccinyl-leucylamido (4-guanidino) butane (E-64) and their substrate specificity is determined by binding to the S2 pocket (Sajid and McKerrow 2002; Barrett and Rawlings 2004; Rosenthal 2004; Caffrey and Steverding 2009). All the members of this family have very similar sequences and a common mechanism for hydrolysis (Fig. 1.7) (Barrett and Rawlings 2004). The majority of the cysteine peptidases are lysosomal enzymes, they often act at an acidic pH. However, there are some members of the papain family that act extracellularly (Rosenthal 1999).

1.8.4 Parasitic cysteine peptidases

A wide range of cysteine peptidases have been found in protozoan parasites namely, *P. falciparum* (Rosenthal 2004), *T. cruzi* (Martinez *et al.* 1991), *T. congolense* (Authié *et al.* 1992; Mbawa *et al.* 1992; Mendoza-Palomares *et al.* 2008), *L. mexicana* (Sanderson *et al.* 2000), *Entamoeba histolytica* (Quintas-Granados *et al.* 2009; Youn 2009), *Fasciola hepatica* (Prowse *et al.* 2002; Beckham *et al.* 2009) and *Toxoplasma gondii* (Youn 2009). The cysteine peptidases are associated with assisting the parasite in pathogenesis of the disease by differentiation at the different stages of the life cycle, invading the host by parasite migration through tissue barriers and altering the host immune defence, degrading haemoglobin and other blood proteins and activation of inflammation (Chagas *et al.* 1997; McKerrow *et al.* 2006). The diverse functions of the peptidases in the parasite could be due to the fact that they have a unique nucleophilicity, i.e. they are able to hydrolyse many different substrates and they are stable in different biological environments (Sajid and McKerrow 2002).

The trypanosomal cysteine peptidases include congopain from *T. congolense,* trypanopain from *T. b. brucei,* cruzipain from *T. cruzi* and vivapain from *T. vivax.* These cysteine peptidases are expressed mainly during the parasite life cycle stages that are involved in infecting the mammalian host. This leads to alterations of the host's immune defences and

cysteine peptidases are, therefore, regarded as immunodominant antigens (Chagas *et al.* 1997). The cysteine peptidase activity of *T. congolense, T. b. brucei* and *T. vivax* are confined to lysosomal organelles. The cysteine peptidases of *T. congolense* and *T. cruzi* have also been found on the cell surface; with the cysteine peptidases of *T. cruzi* also located in flagellar pocket (Mbawa *et al.* 1991a; Cazzulo *et al.* 1997). Substrate specificity is determined by the P_2/S_2 interaction. The cysteine peptidases prefer large aromatic residues in P_2 (Lalmanach *et al.* 2002; Lecaille *et al.* 2002). These lysosomal cysteine peptidases have broad substrate specificity and mostly prefer an acidic pH (Rosenthal 1999). However, parasite peptidases are more stable at neutral pH, whereas the mammalian lysosomal cysteine peptidases are relatively unstable at neutral pH (Sajid and McKerrow 2002). The pH optimum of lysosomal cysteine peptidases may also be affected by the assay conditions used, where the pH optima were shown to be higher in acetate-4-morpholineethane sulfonic acid (MES)-Tris buffers of constant ionic strength (Dehrmann *et al.* 1995).

1.8.5 Primary structure of trypanosomal cysteine peptidases

Congopain (Serveau *et al.* 2003), trypanopain (Troeberg *et al.* 1999) and cruzipain (Cazzulo *et al.* 1997) are cathepsin L-like cysteine peptidase of *T. congolense*, *T. b. brucei* and *T. cruzi*, respectively. All three peptidases are synthesised in an inactive precursor form. Each enzyme has a pre-region, pro-region and a catalytic domain. The pre-region is usually hydrophobic and serves as a signal peptide (North *et al.* 1990). The pro-region may contribute to a number of functions. It can assist in protein folding because this region can take on the role of an intramolecular chaperone. This region may also regulate peptidase activity since it can act as an endogenous inhibitor and may even act as a signal that enables the peptidase to travel to its intracellular location (Sajid and McKerrow 2002).

These three enzymes also contain an unusual C-terminal extension that is not present in their mammalian homologues (Authié *et al.* 2001). The C-terminal extension of cruzipain and congopain is made up of 130 amino acid residues and the C-terminal extension of trypanopain is 110 amino acids in length. The C-terminal extension is connected to the

catalytic domain of the enzyme via sequence motifs that either contain threonine, proline or serine residues, which may form a flexible region that could be easily accessible to proteolytic cleavage (Robertson *et al.* 1996). Congopain's C-terminal extension is linked to the catalytic domain by a stretch of proline residues, while that of cruzipain is linked to the catalytic domain by several threonine residues (Chagas *et al.* 1997) (Fig. 1.8). The function of the C-terminal extension is unknown. However, it could potentially play a role in altering the host's immune defences since this region is extremely immunogenic. Recombinant forms of *T. cruzi* and *T. b. brucei* cysteine peptidases expressed in *Escherichia coli*, that did not have the C-terminal extension, were active which proved that the presence of the C-terminal extension is not vital for enzyme activity (Robertson *et al.* 1996).

A cathepsin B-like cysteine peptidase was identified in *T. congolense* (Mendoza-Palomares *et al.* 2008). There are 13 genes coding for the peptidase, which are grouped into three evolutionary groups. The groups include; TcoCBc1 to TcoCBc5, TcoCBc6 and TcoCBs7 to TcoCBs13. The first two groups have the conventional Cys, His, Asn catalytic triad and the third group has Ser, Xaa, Asn in the active site, where Xaa could be any amino acid. The structure of cathepsin B-like cysteine peptidase contains an occluding loop, signal peptide, pro-peptide region and catalytic domain but the trypanosomal cathepsin B-like cysteine peptidases do not contain a C-terminal extension as do their cathepsin L-like counterparts (Mendoza-Palomares *et al.* 2008).

Sequence alignment (Fig. 1.8) reveals that vivapain has a high sequence identity to trypanopain and congopain. Therefore, the enzyme characteristics of trypanopain and congopain should theoretically be a good indication of those of vivapain. The red box in the sequence alignment indicates the N-terminus of the mature enzyme for all three peptidases. The C-terminal extension for the enzymes is indicated by the blue boxes. The C-terminal domain of trypanopain and congopain are proline rich and the C-terminal domain of vivapain is threonine rich. The pink boxes indicate the amino acids that are part of the catalytic domain; Cys^{25}, His^{159} and Asn^{175} (papain numbering). There are multiple genes

coding for congopain (Lalmanach *et al.* 2002) and even cruzipain (Mottram *et al.* 1998) that are arranged in tandem array; however, this is not the case for vivapain.

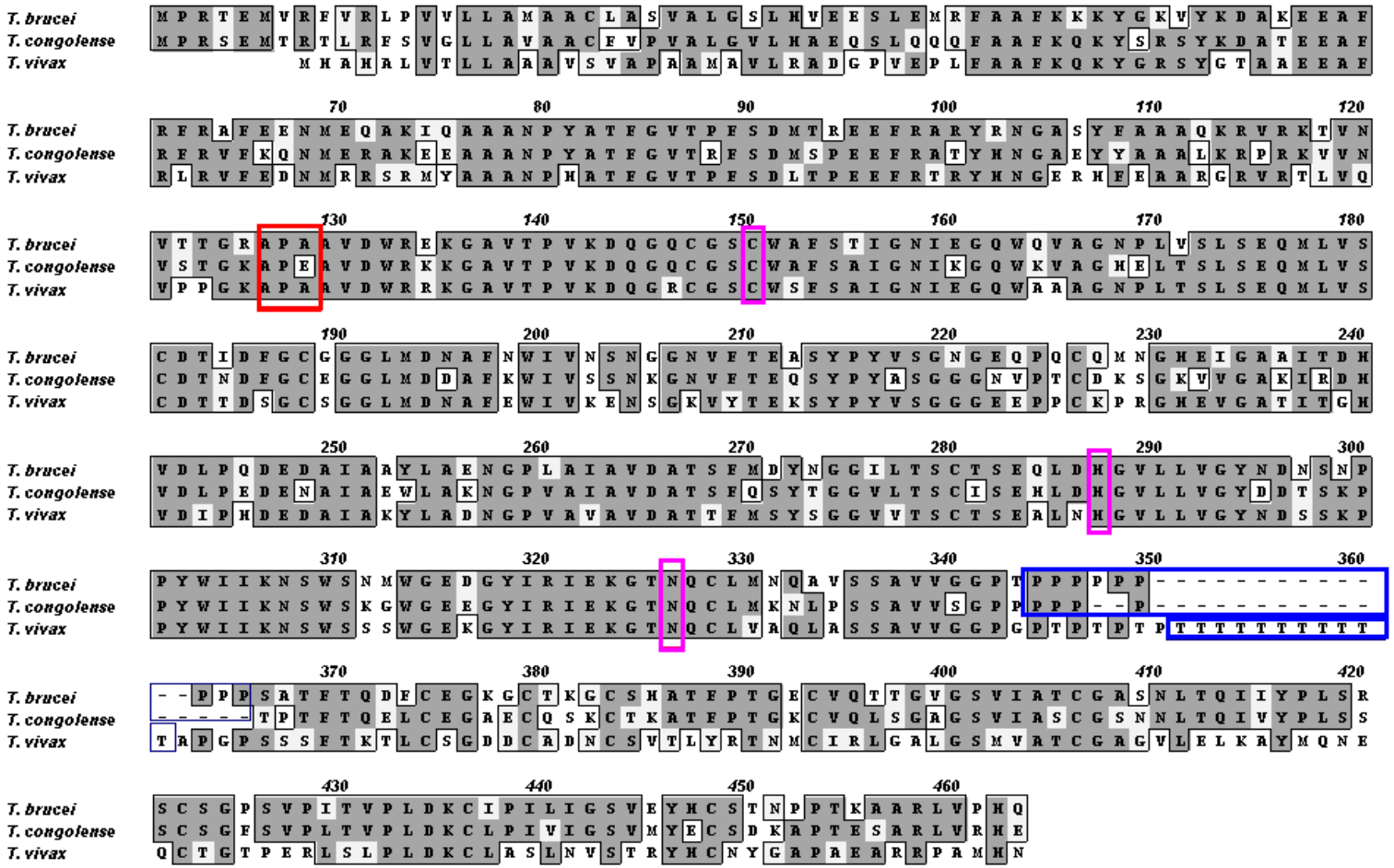


Figure 1.8 The sequences of trypanopain, congopain and vivapain. Representing sequence identity (grey boxes), the beginning of the mature enzyme (red box), the amino acids present in the catalytic site; C, H, N (pink boxes) and the C-terminal extension (blue boxes). (Adapted from Nicola Biteau, University Victor Segalen, Bordeaux, France).

1.9 OBJECTIVES OF THE PRESENT STUDY

In the present study a cysteine peptidase, vivapain, from *T. vivax* was studied in order to develop chemotherapeutic drugs and a diagnostic assay for *T. vivax* induced trypanosomosis . Both the catalytic domain of vivapain (Vp) and the full length mutant form of vivapain (ΔFLVp) were examined. The reason for studying vivapain is that it could potentially be a pathogenic factor contributing to the disease caused by *T. vivax*. This assumption can be made since the cysteine peptidase, congopain from *T. congolense*, which has a high sequence identity to vivapain (Fig. 1.8), was shown to be a pathogenic factor in trypanosomosis (Authié *et al.* 2001). The key reason for pursuing this study is that *T. vivax* is responsible for over 50% of mortality rates in cattle (Prowse 2005) and vivapain could be a potential pathogenic factor. To date, there has been no published data on the expression and characterisation of Vp; hence, congopain was used as a positive control in the experiments.

This study can be divided into two main parts. The first part involved the recombinant expression of the catalytic domains of vivapain (Vp) and congopain (C2) in a yeast expression system. The recombinant proteins were then purified and extensive enzymatic characterisation studies were performed (Chapter 2). This information will, in future, be useful for the potential development of a new chemotherapeutic drug based on synthetic inhibitors. The second part of the study involved the cloning and expression of a full length mutant form of vivapain, termed (ΔFLVp) in both yeast and bacterial expression systems and antibodies against ΔFLVp were made in both chickens and mice for future use in diagnostic tests (Chapter 3). The basis for the production of the recombinant ΔFLVp was to provide a vital tool that could be used for diagnostic tests in *T. vivax* infections. All the results obtained for this study are summarised in a general discussion (Chapter 4).

2. RECOMBINANT EXPRESSION AND ENZYMATIC CHARACTERISATION OF THE CATALYTIC DOMAIN OF VIVAPAIN (Vp)

P. Vather[1], A. F. V. Boulangé[1, 2] and T. H. T. Coetzer[1].

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

ABSTRACT

Trypanosoma vivax is one of the three main trypanosomes responsible for African bovine trypanosomosis. Vivapain is the cathepsin L-like cysteine peptidase of *T. vivax* and is believed to be a promising target for chemotherapeutic drug design. The main aim of this study was to express and enzymatically characterise the catalytic domain of vivapain (Vp) in order to gain information for future work in chemotherapeutic drug design. Vp was successfully recombinantly expressed in a *Pichia pastoris* yeast expression system and purified by three phase partitioning (TPP) and molecular exclusion chromatography (MEC). The catalytic domain of congopain (C2) was also expressed in the *P. pastoris* yeast expression system for use as a positive control since there has been no published work on the characterisation of Vp. Vp was expressed as a glycosylated active form, represented as a double band on SDS-PAGE gel at 29 and 33 kDa. The recombinant Vp was active against a range of synthetic substrates, which was comparable to other parasite cysteine peptidases. An interesting finding was that the substrate specificity of Vp was primarily based on the presence of a hydrophobic residue in P_2. The best substrates for hydrolysis by Vp were Z-Phe-Arg-AMC and D-Val-Leu-Lys-AMC. Vp was also inhibited by the classical cysteine peptidase inhibitors such as the reversible leupeptin, chymostatin and antipain and the irreversible E-64, iodoacetic acid and iodoacetamide. Vp has a pH optimum of 7 when assayed in constant ionic strength buffers. The information obtained from the present study could potentially assist future drug design for trypanosomosis caused by *T. vivax*.

2.1 INTRODUCTION

African bovine trypanosomosis is a deadly disease which is causing an enormous strain of US$ 45 billion on the sub-Saharan African economy (Antoine-Moussiaux *et al.* 2009). *Trypanosoma vivax* seems to be the most pathogenic parasite causing trypanosomosis in African livestock (Taylor and Authié 2004). This parasite is also responsible for over 50% of mortality rates in cattle (Prowse 2005). Therefore, there is a great demand for the control of this disease especially the infections caused by *T. vivax.*

Current control measures include the use of trypanocidal drugs, attempts to eradicate tsetse fly populations and the use of trypanotolerant cattle. These methods have become insufficient (Naessens *et al.* 2002; Aksoy 2003; Allsopp and Hursey 2004; Holmes *et al.* 2004; Murray *et al.* 2004). The trypanocidal drugs currently available for bovine trypanosomosis are isometamidium chloride, homidium and diminazene aceturate. These drugs are not as effective as they used to be since parasites have developed drug resistance because the drugs have been in use for over five decades and there is thus a need to develop new drugs (Anene *et al.* 2001).

Chemotherapy is still the principal approach for the control of trypanosomosis. This approach is purely based on targeting the pathogenic factors contributing to the disease in the hopes of identifying new synthetic inhibitors for the production of new drugs. The parasite peptidases, especially the cysteine peptidases from parasites such as *P. falciparum* (Rosenthal 2004), *T .cruzi* (Martinez *et al.* 1991), *T. congolense* (Authié et al. 1992; Mbawa et al. 1992), *L. mexicana* (Sanderson *et al.* 2000) and *Fasciola hepatica* (Prowse 2005), seem to be a good option as targets for drug design since they play essential roles in the parasite. They also contribute to and assist in the survival of the parasite (McKerrow 1999).

Since *T. vivax* contributes significantly to the number of infected cattle in Africa, there is a need to study the cysteine peptidase associated with this parasite in the hope that it could become a potential drug target. This particular cysteine peptidase has been termed vivapain. Vivapain has a high sequence identity to congopain and trypanopain (Fig 1.8, Section

1.8.5) and vivapain is likely to be a pathogenic factor in *T. vivax* since congopain acts as a pathogenic factor in *T. congolense* (Authié *et al.* 2001) .

To date, there has been no published work on the recombinant expression and characterisation of vivapain and, consequently, congopain was used as a positive control in the present study. The most important aspect of characterising the cysteine peptidase is determining substrate specificity since it can be used to discover anti-peptidase inhibitors, which could be lead compounds for the development of anti-disease drugs. This is a good approach since many cysteine peptidase inhibitors can disrupt the life cycle of the parasite without leading to the production of major side effects in the host (Troeberg *et al.* 1999; Caffrey *et al.* 2000; Serveau *et al.* 2003).

In the present study, the catalytic domains of vivapain (Vp) and congopain (C2) were recombinantly expressed in a *Pichia pastoris* yeast expression system. This was followed by purification of the two recombinant proteins. The enzymatic characteristics of both Vp and C2 were studied extensively. These studies involved testing substrate specificity using peptides with different amino acid residues in P_1-P_3 and kinetics of the effect of reversible and irreversible inhibitors on the hydrolysis of two peptide substrates by Vp and C2.

2.2 MATERIALS AND METHODS

2.2.1. Materials

***P. pastoris* expression:** A glycerol stock of *P. pastoris* containing recombinant pPic9-Vp was obtained from Professor Theo Balts (University Victor Segalen, Bordeaux, France). A glycerol stock of *P. pastoris* containing recombinant pPic9-C2 was obtained from Dr Alain Boulangé (University of KwaZulu-Natal, Pietermaritzburg).

Purification and quantification: Sephacryl S-300 was purchased from Sigma (Munich Germany). BCA™ Protein Assay kit (Pierce, Rockford, IL, USA). Nunc-Immuno™ 96-well plates and the Nunc Black 96-well were purchased from Nunc Intermed (Denmark). The plates were read in a FLUORStar Optima Spectrophotometer (BMG Labtech, Germany).

Antibodies: Polyclonal antibody raised in rabbits against the bacterial-expressed central domain of congopain and the Chicken anti rabbit IgY Horse radish peroxidase (HRPO) conjugate (Dr Alain Boulangé, University of KwaZulu-Natal).

Peptide substrates and inhibitors: The peptide substrates H-D-Val-Leu-Lys-7-amino-4-methylcoumarin (AMC), H-D-Ala-Leu-Lys-AMC, succinyl (Suc)-Leu-Tyr-AMC, Suc-Ala-Phe-Lys-AMC, H-Ala-Phe-Lys-AMC, H-Pro-Phe-Arg-AMC, methoxy-succinyl (MeoSuc)-Asp-Tyr-Met-AMC**,** butyloxycarbonyl (Boc)-Leu-Arg-Arg-AMC, Boc-Leu-GLy-Arg-AMC, H-Leu-AMC, H-Gly-AMC were obtained from Bachem (King of Prussia, PA, USA). Benzyloxycarbonyl (Z)-Phe-Arg-AMC, Z-Gly-Pro-Arg-AMC, Z-Gly-Gly-Arg-AMC, Z-Arg-AMC, Z-Arg-Arg-AMC, Z-Gly-Arg-Arg-AMC, Z-Ala-Arg-Arg-AMC, L-*trans*-epoxysuccinyl-leucylamido(4-guanidino)butane (E64), leupeptin, chymostatin, antipain, bestatin, ethylenediaminetetra-acetic acid (EDTA), pepstatin A, iodoacetic acid (IAA), iodoacetamide (IAN), phenylmethylsulfonylfluoride (PMSF), N-tosyl-L-lysyl chloromethylketone (TLCK) and N-tosyl-L-phenylalanyl chloromethylketone (TPCK) were obtained from Sigma (Munich, Germany)

2.2.2 Expression of Vp (catalytic domain of vivapain) and C2 (catalytic domain of congopain) in *Pichia pastoris*

The *P. pastoris* strain GS115 glycerol stocks containing pPic9-Vp and pPic9-C2 were streaked on yeast extract peptone dextrose (YPD) plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose (glucose) and 20 g/l bacteriological agar] containing ampicillin (50 µg/mL). A single colony from the YPD plate was inoculated into 100 mL YPD liquid medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose (glucose)] containing ampicillin (50 µg/mL) to prevent contamination of the cultures. The YPD culture was grown at 30^{o}C (in a 1 L baffled flask) in an orbital shaking incubator for two to three days. The culture was transferred into a 2 L baffled flask and the culture volume was made up to 1 L with buffered complex glycerol medium (BMGY) [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6.5, and 1.34% (w/v) yeast nitrogen base (YNB)]. The culture was grown for a further two days at 30^{o}C in a shaking

incubator until an OD_{600} of 2-6 was reached. The BMGY medium was used since the combination of yeast extract and peptone in the medium allow for an increase in the biomass of cells and better cell growth. Cells were harvested by centrifugation (2000 *g*, 10 min, 4°C) and resuspended in 500 mL of buffered minimal medium (BMM) [100 mM potassium phosphate buffer, pH 6.5, 1.34% (w/v) YNB, 0.00004% (w/v) biotin and 0.5% (v/v) methanol] The BMM culture was placed in 2 L baffled flasks and incubated at 30°C in a shaking incubator for four to six days. Methanol (0.5%) was added daily to the cultures in order to maintain induction of expression of the protein of interest. The cells were harvested by centrifugation (5000 *g*, 10 min, 4°C) once again, however this time the supernatant was retained since the *P. pastoris* strain used secretes the protein being expressed into the medium. The supernatant was stored in plastic bottles at -20°C until further use.

2.2.3 Purification of Vp and C2

Three phase partitioning (TPP)

Three phase partitioning (TPP) was the initial method used for purification and concentration of Vp and C2. This method was conducted according to Pike and Dennison (1989). The Vp and C2 supernatants (500 mL) were thawed and filtered through Whatman No 1 filter paper. The pH of the respective supernatants was lowered to pH 4.2 with phosphoric acid in order to process the peptidases into mature proteins. Tertiary butanol [30% (v/v)] was added to the culture supernatant and both Vp and C2 were precipitated out of solution with ammonium sulfate $(NH_4)_2SO_4$ [40% (w/v) of the total volume i.e. culture supernatant and tertiary butanol]. The amount of $(NH_4)_2SO_4$ to be used was determined by optimising TPP using 10, 20, 30 and 40% $(NH_4)_2SO_4$ on a crude supernatant sample of Vp to which 30% (v/v) tertiary butanol had been added. Thereafter, the mixture was centrifuged (6000 *g*, 10 min, 4°C) in a swing out rotor. The centrifugation led to the successful separation of the three layers: a tertiary butanol layer, a precipitated protein layer and an aqueous layer. The precipitated protein layer for Vp and C2 was retained and dissolved in phosphate buffered saline (PBS) dialysis buffer [100 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl and 137 mM NaCl, pH 7.2]. The proteins were then dialysed in a

10 kDa cut-off dialysis bag (Thermo Scientific Snakeskin® pleated dialysis tubing) overnight at 4^{o}C against PBS, pH 7.2 in order to eliminate any residual tertiary butanol and $(NH_4)_2SO_4$. The dialysed protein samples were concentrated by dialysis against solid polyethylene glycol (PEG) (M_r 20 000) until the volume in the dialysis bag was brought down to 5 mL. Analysis of the C2 sample after TPP revealed that the protein was pure, therefore no further steps of purification were required.

Molecular exclusion chromatography (MEC)

The Vp sample was further purified by molecular exclusion chromatography (MEC) using a Sephacryl-300 (Sigma) HR column (25 x 840 mm; flow rate of 25 mL/ hour, 4^{o}C). The resin used has a fractionation range of 10 to 1500 kDa (Dennison 1999). The column was equilibrated with one column volume of MEC buffer [100 mM sodium acetate buffer, pH 7.0, 0.02% (w/v) NaN_3). Thereafter, the column was calibrated with blue dextran (2000 kDa), phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (34.3 kDa), and soybean trypsin inhibitor (25 kDa). The proteins (12.5 mg each) were dissolved and made up to 5 mL with MEC buffer. The determination of the void volume (V_o) of the column was based on the elution volume (V_e) of blue dextran. Therefore, from this, the availability constant K_{av} can be calculated by using the following equation:

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

Where, K_{av} = availability constant

V_e = elution volume

V_o = void volume

V_t = total column volume (Dennison 1999)

The K_{av} values were then subsequently used to produce a plot of K_{av} vs log M_w (results not shown), which was used to approximate the Mw of the eluted proteins. The Vp sample obtained after TPP (5 mL) was applied to the column and eluted using the MEC buffer. The

amount loaded on a MEC column should always be between 2 and 5% of the total column volume. Eluted fractions were analysed by measuring their absorbance at A_{280} as well as their activity against a synthetic substrate, Z-Phe-Arg-AMC.

2.2.4 Recombinant protein quantification

2.2.4.1 Quantification of Vp

The purified Vp sample was quantified by two methods; namely, BCA™ Protein Assay kit (Pierce, Rockford, IL, USA) and by direct comparison of the Vp sample against known concentrations of BSA samples on a reducing SDS-PAGE gel. The BCA test was performed by combing reagent B with reagent A in a 1:20 ratio. The working reagent (200 µl) was mixed with bovine serum albumin (BSA) standards (0.025 – 2 mg/mL) and placed in a Nunc® 96 well microtitre plate. A dilute purified Vp sample was treated in the same manner. The plate was covered and incubated at 37°C for 30 min. This test was based on the presence of bicinchoninic acid in the working reagent, which interacts with the proteins to produce a purple coloured product. The absorbance of each sample was measured at 562 nm. A standard curve of A_{562} vs BSA standard concentrations was constructed (Fig. 2.1). This was used to determine the unknown concentration of the Vp sample.

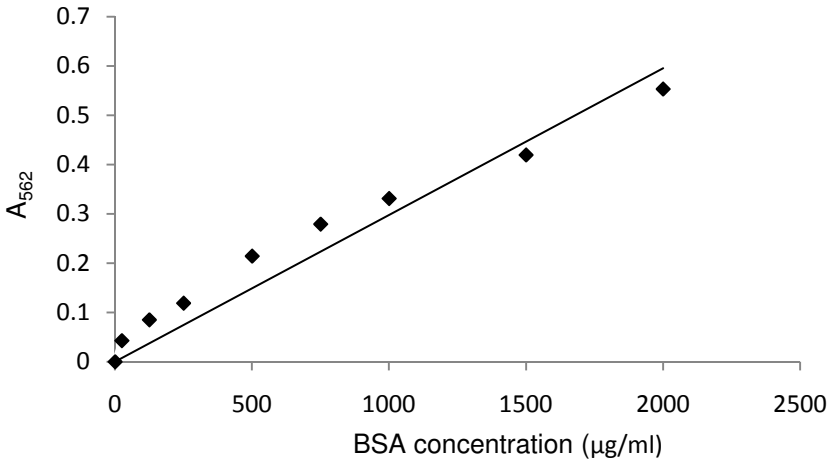


Figure 2.1 Standard curve of absorbance (A_{562}) vs BSA concentrations for determination of protein concentration. Bovine serum albumin (BSA) standards (0.025 – 2 mg/mL) were used to construct a standard curve for the BCA assay. The equation of the trend line is y=0.0003x, with a correlation coefficient of 0.940. Microsoft excel 2007 was used to construct the plot.

The second method of Vp quantification involved running equal volumes of BSA standards (0.025 – 2 mg/mL) and a purified Vp sample of unknown concentration on a 12.5% reducing SDS-PAGE gel. The protein bands were visualised by staining the gel with Coomassie blue R-250 [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid] followed by destaining in destain solution I [50% (v/v) methanol, 10% (v/v) acetic acid] overnight and then in destain solution II [5% (v/v) methanol, 7% (v/v) acetic acid] to allow for complete destaining.. The concentration of the Vp was estimated by comparing the protein band intensity of the BSA standards with the Vp protein band (Fig. 2.2).

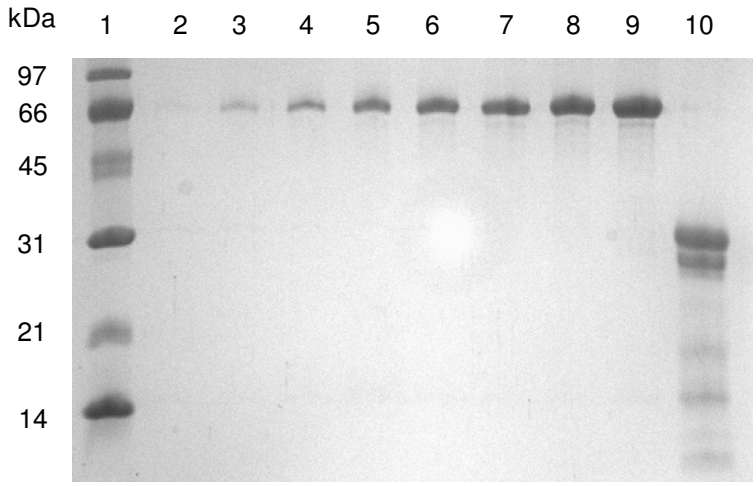


Figure 2.2 Analysis by 12.5% SDS-PAGE for the estimation of the concentration of Vp by direct visualisation. The BSA standards (0.025 – 2 mg/mL) were resolved on the gel alongside the Vp sample of unknown concentration. Lane 1, molecular weight marker; lanes 2-9, BSA standards (0.025 – 2 mg/mL) (2.5 µl); lane 10, purified Vp sample (2.5 µl). The samples loaded on the gel were mixed with an equal volume of reducing treatment buffer and boiled for 2 minutes. The gel was stained with Coomassie blue R-250.

2.2.4.2 Quantification of C2

The purified C2 was quantified by a method described by Bradford (1976), known as the Bradford dye-binding assay. This method is based on the binding of Coomassie Brilliant Blue G-250 dye to a protein sample. This interaction leads to a change in the absorbance maximum of the dye from 365 to 595 nm that is used to determine the concentration of a sample of unknown concentration (Bradford 1976). A micro assay was performed to quantify the purified C2 sample. Briefly, this method involved combining the dye reagent (950 µl) [0.01% (w/v) Coomassie Brilliant Blue G-250, 88% (v/v) phosphoric acid and 99.5% (v/v) ethanol] with BSA standards (50 µl), ranging from 1-5 ng. The reaction mixture was left to stand for approximately two minutes after inversion. The C2 sample was diluted and treated under the same conditions as the BSA standards. The absorbance at 595 nm was read for each sample. A standard curve of A_{595} vs BSA protein standards was constructed (Fig. 2.3). The concentration of C2 was determined by using the equation generated by the linear regression analysis of the standard curve and the absorbance at 595 nm obtained for the C2 sample.

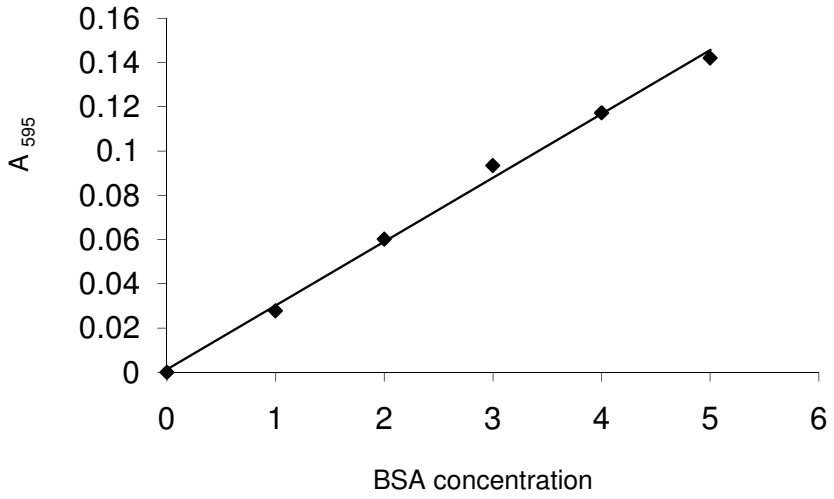


Figure 2.3 Bradford standard curve of absorbance (A_{595}) vs BSA standard concentrations for determination of unknown protein concentration. Bovine serum albumin (BSA) standards (1-5 ng) were used to construct a standard curve for the Bradford micro assay. The concentration of the C2 sample was determined by using the standard curve. The equation of the trend line is y=0.0289x + 0.0012, with a correlation coefficient of 0.9964. Microsoft excel 2007 was used to construct the plot.

2.2.5 SDS-PAGE gel analysis of proteins

Analysis of recombinant protein samples were performed by a method described by Laemmli (1970). This was achieved by using a discontinuous Tris-Glycine buffer and gel system. Samples were combined with an equal volume of reducing treatment buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol, pH 6.8]. The SDS in this buffer binds tightly to the protein sample resulting in a negatively charged rod-like protein complex. Disulfide bonds are reduced by 2-mercaptoethanol allowing the resulting subunits to also bind to SDS, which opens the protein structure even further. Once an electric field passes through the system a separation of the protein takes place based solely on the size of the protein and/or subunits. The proteins are first resolved through a stacking gel with small pore sizes in a 500 mM Tris-HCl buffer, pH 6.8 and thereafter through a running gel of large pore sizes in a 1.5 M Tris-HCl buffer, pH 8.8. Electrophoresis was conducted at 18 mA per gel for approximately 1.5 - 2 hours using a tank buffer [250 mM Tris-HCl buffer, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS]. The

relative migration distance of proteins on a gel has an inversely proportional relationship with the molecular weight of the protein. A standard curve of log M_w of proteins of known molecular weight vs distance travelled (relative mobility) was constructed (Fig. 2.4). The curve generated was used to determine the molecular weight of the protein of unknown size.

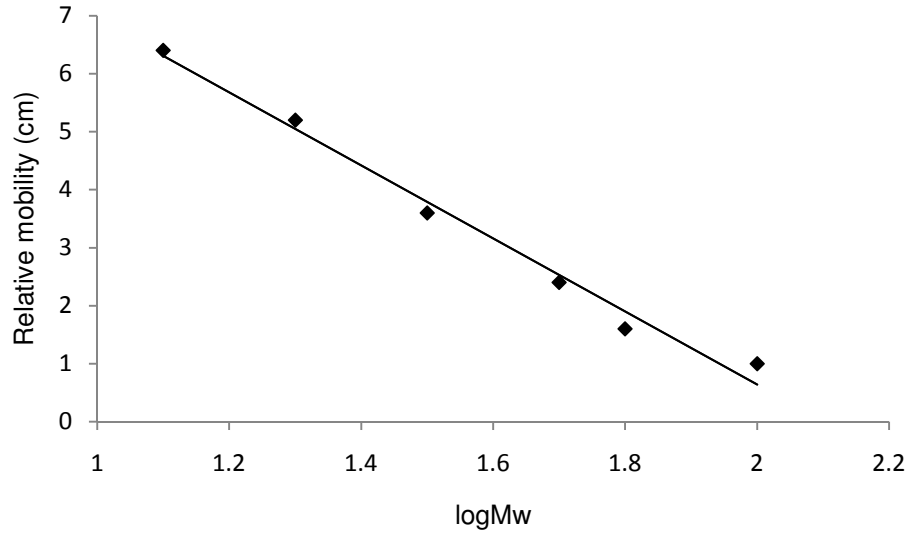


Figure 2.4 Standard curve of log Mw against relative mobility for the estimation of protein Mw by SDS-PAGE. The low range marker is made up of phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21Da) and lysozyme (14 kDa). The equation of the trend line is y= -6.3012x + 13.239, with a correlation coefficient of 0.9864. Microsoft excel 2007 was used to construct the plot.

Following electrophoresis, proteins were visualised by incubating the gel in a Coomassie blue R-250 stain for four hours followed by destaining in destain solution I overnight and then in destain solution II to allow for complete destaining. The detection of the Coomassie blue stain ranges from approximately 50-100 ng of protein. When a more sensitive protein detection was required, a silver stain method was used as described by Blum *et al.* (1987). This detection method limit is approximately 1-10 ng of protein. The silver staining method has to be carried out in extremely clean glassware to decrease the chance of background staining and all the steps must be carried out on an orbital shaker. Briefly, the silver staining method involves incubating the gel, after electrophoresis, in 100 mL fixing

solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) formaldehyde] for 1 hour or overnight at room temperature (RT). The gel was incubated in 50% (v/v) ethanol (3 x 20 min). This was followed by soaking of the gel in pretreatment solution [0.4% (w/v) $Na_2S_2O_3.5\ H_2O$] for 1 min. The gel was washed in distilled water (3 x 20 sec). This was followed by soaking the gel in impregnation solution [0.2% (w/v) $AgNO_3$, 0.75% (v/v) 37% formaldehyde] for 20 min. Once again, the gel was washed in distilled water (3 x 20 sec) and, thereafter, incubated in developing solution [6% (w/v) Na_2CO_3, 0.5% (v/v) 37% formaldehyde, 2% (v/v) pretreatment solution (0.4% (w/v) $Na_2S_2O_3.5\ H_2O$)] until the first protein band became evident. Once this occurred, the gel was immediately placed in distilled water until sufficient bands appeared. The development of the bands was stopped by placing the gel in a stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] for 10 min. The gel was washed in 50% (v/v) methanol and stored in polythene zip-seal bags.

2.2.6 Western Blot analysis of proteins

Western blots were carried out in order to confirm the expression of the recombinant proteins. Proteins were separated on a Laemmli SDS-PAGE gel and transferred from the SDS-PAGE gel onto a nitrocellulose membrane, using a wet blotter (BioRad Hercules, CA, USA) in the presence of blotting buffer [45 mM Tris-HCl buffer, pH 8.3, 173 mM glycine and 0.1% (w/v) SDS] for 16 hours at 200 mA (Towbin *et al.* 1979; Gershoni and Palade 1982). Following transfer of the proteins, the nitrocellulose membrane was stained with Ponceau S [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. Once the protein bands became visible, the positions of the molecular weight markers were marked with a pencil. Thereafter, the nitrocellulose membrane was destained with distilled water. The unoccupied sites on the nitrocellulose membrane were blocked with low fat milk powder solution [5% (w/v) low fat milk powder in Tris buffered saline (TBS) (20 mM Tris-HCl buffer, pH 7.4, 200 mM NaCl) for one hour at RT. The membrane was subsequently washed with TBS (3 x 5 minutes) and incubated in primary antibody diluted in 0.5% (w/v) BSA-TBS for two hours at RT. This was followed by washing the membrane in TBS (3 x 5 minutes). The membrane was then incubated in enzyme labelled secondary antibody diluted in 0.5% (w/v) BSA-TBS for one hour at RT. Lastly, the membrane was washed in TBS (3 x 5 minutes)

and placed in substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.0015% (v/v) hydrogen peroxide (H_2O_2), 0.1% (v/v) methanol, in TBS]. The blot was left in the dark until the appearance of protein bands became evident.

2.2.7 Enzymatic characterisation

2.2.7.1 Gelatin SDS-PAGE analysis of Vp and C2

This method involved running a standard Laemmli 12.5% SDS-PAGE gel with modifications as described by Heussen and Dowdle (1980). These modifications include running the samples under non-reducing conditions i.e. the samples are combined with an equal volume of non-reducing treatment [125 mM Tris-HCl buffer pH 6.8, 4% (w/v) SDS and 20% (v/v) glycerol] and a gelatin solution [1% (w/v)] is added to make the 12.5% SDS-PAGE running gel buffer (Heussen and Dowdle 1980). After electrophoresis the running gel is soaked in two changes of Triton X-100 solution [2.5% (v/v)] over a one hour period at RT to remove the SDS, which allows for the renaturation of the recombinant protein. The gel is subsequently incubated in congopain assay buffer [100 mM Bis-Tris buffer, pH 6.5, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3 and 8 mM DTT] for three hours at 37^{o}C. The gel was then stained with amido black solution [0.1% (w/v) amido black, 30% (v/v) methanol, 10% (v/v) glacial acetic and 60% (v/v) distilled water] for one hour at RT and destained in 30% (v/v) methanol, 10% (v/v) glacial acetic and 60 % (v/v) distilled water until clear bands became evident, which is an indication of proteolytic activity of the recombinant peptidase.

2.2.7.2 Effect of endoglycosidase H (Endo H) on Vp

The action of Endo H on Vp was conducted according to Caffrey *et al.* (2001). Vp (50 µl), 5 M NaCl (1.5 µl), 5% (w/v) SDS and 10% of 2-mercaptoethanol were combined and boiled for 5 minutes. The preparation was allowed to cool and 0.5 M sodium citrate buffer (pH 5.5); (6 µl) was added and the preparation was divided into two. Endo H (500 U) was added to one of the preparations and the other was used as a control. The two preparations were incubated at 37^{o}C for 3 hours. Thereafter an equal volume of reducing treatment

buffer was added to each preparation and boiled for 5 minutes. The samples were electrophorised on 12.5% reducing SDS-PAGE gel as described by Section 2.2.5.

2.2.7.3 Active site titration of Vp and C2

Active site titration with the irreversible inhibitor, E-64, was conducted on Vp and C2 according to Barrett *et al.* (1982) in order to determine the active concentration of both cysteine peptidases. The enzyme (25 µl) [1 µM diluted in 0.1% (w/v) Brij-35] was incubated with E-64 [0.1 – 1 µM diluted in 0.1% (w/v) Brij-35] in the presence of congopain assay buffer (50 µl) at 37^{o}C for 30 minutes. The DTT was added to the buffer just before use. Thereafter, 10 µl aliquots of the incubated mix were combined with 25 µl of Z-Phe-Arg-AMC synthetic substrate solution [20 µM, diluted in distilled water]. This step was performed in triplicate for each E-64 concentration. The fluorescence (activity of the enzyme against the substrate) was measured (excitation, Ex_{360nm} and emission, Em_{460nm}) using a FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany). The active concentration of the enzyme was determined by plotting fluorescence against the E-64 concentrations (0.1-1 µM).

2.2.7.4 Effect of pH on both Vp and C2

The determination of the pH optimum for Vp and C2 using constant ionic strength acetate-MES-Tris (AMT) buffer [100 mM Na-acetate, 200 mM Tris-HCl, 100 mM MES, 8 mM DTT, 4 mM Na_2EDTA] was conducted according to Ellis and Morrison (1982). The AMT buffer was aliquoted and titrated from pH 4 to 9 with either HCl or NaOH. The enzyme [1.5 ng active enzyme (25 µl) diluted in 0.1 % (w/v) Brij-35] was incubated separately with AMT buffers of pH 4-9 (50 µl) for 15 minutes at 37^{o}C. This was performed in triplicate for each buffer (pH 4-9). The enzyme and buffer mix (75 µl) and Z-Phe-Arg-AMC (25 µl) [20 µM, diluted in distilled water] was placed in black Nunc® 96 well microtitre plate and the fluorescence was measured (Ex_{360nm}, Em_{460nm}) using a FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany). The optimum pH of the recombinant protein was determined by plotting fluorescence against the pH.

2.2.7.5 Determination of kinetic constants and parameters

AMC standard curve

In order to quantify the amount of product formed in the hydrolysis of synthetic peptide by the enzyme, an AMC calibration curve was constructed. A range of AMC concentrations (50 µl) [5 x 10^{-3} – 15 µM] were incubated with pre-warmed congopain assay buffer (50 µl) and the fluorescence (Ex_{360nm}, Em_{460nm}) measured using a FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany). The standard AMC curve was constructed from a plot of AMC concentrations versus fluorescence (Fig. 2.5). The slope of this curve was used to convert the fluorescence units acquired from the synthetic substrate assays and inhibitor assays into concentration.

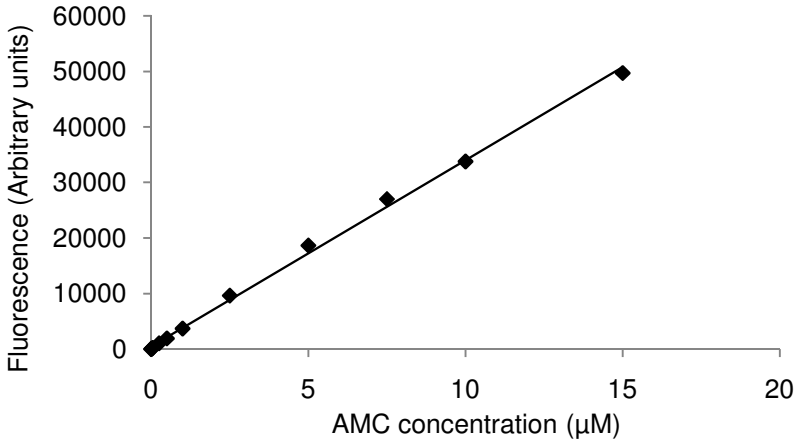


Figure 2.5 Standard curve relating the amount of AMC to fluorescence (Ex_{360nm}, Em_{460nm}). The equation of the trend line is given by y = 3.356x + 445.06, with a correlation coefficient of 0.9978. Microsoft excel 2007 was used to construct the plot.

Peptidolytic specificity

The substrate specificity of a particular enzyme is analysed by measuring the following parameters: Michaelis-Menten constant, K_m, and the maximum velocity of an enzyme catalysed reaction, V_{max}. K_m indicates the affinity of the enzyme for the substrate and it is independent of enzyme concentration (Palmer 1995). The lower the K_m the more the enzyme prefers the substrate. V_{max} describes the maximum number of substrate particles that can be converted to products per molecule of enzyme per unit time. V_{max} is dependent on enzyme concentration. When an active site titrant such as E-64 for cysteine peptidases is

available, the active amount of enzyme can be determined. This value is known as $[E]_o$. When this value is known, V_{max} can be converted to k_{cat}, which is the turnover number of the enzyme (Palmer 1995). The relationship between these values are represented in the following equation:

$$k_{cat} = \frac{V_{max}}{[E]_o}$$

(Salvesen and Nagase 1989)

The apparent second order rate constant, k_{cat} / $K_{m,}$ describes the catalytic efficiency of the reaction. Therefore, it is used to describe the specificity of the enzyme for the substrate. This value can be used to compare different substrates for a particular enzyme.

The kinetic constants of a reaction can be determined from single-substrate-enzyme catalysed reactions. This determination is based on measuring the initial velocity (v_o) over a range of substrate concentrations [S]. The K_m, V_{max}, [S] and v_o are related by the Briggs and Haldane revised Michaelis-Menten equation:

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

(Briggs and Haldane 1925)

V_{max} and K_m, are determined from the direct plot of v_o versus [S].

The hydrolysis of the synthetic fluorogenic substrate by Vp and C2 were performed using the same method. Briefly, the enzyme [1.5ng (0.56 nM) active Vp and (0.56 nM) active C2] was made up to 25 µl with Brij [0.1 % (w/v)] and activated in congopain assay buffer (50 µl) for 10 min at 37^{o}C. Thereafter, substrate (25 µl) (ranging from 2.5 – 50 µM final concentration) was added. The fluorescence (Ex_{360nm}, Em_{460nm}) was monitored continuously for 10 min using the FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany). The initial steady state velocity (v_o) was determined over the range of substrate concentrations. The kinetic constants V_{max} and K_m were determined by hyperbolic

regression analysis of the kinetic data using the Hyper32© software data package (1991-2003, J.S. Easterby, University of Liverpool, UK).

50 mM Substrate stock solutions: Z-Phe-Arg-AMC (3.25 mg), H-D-Val-Leu-Lys-AMC (1.03 mg), H-D-Ala-Leu-Lys-AMC (2.44 mg), Suc-Leu-Tyr-AMC (2.76 mg), Suc-Ala-Phe-Lys-AMC (3.68 mg), H-Ala-Phe-Lys-AMC (2.61 mg), H-Pro-Phe-Arg-AMC (2.88 mg), Z-Gly-Pro-Arg-AMC (3.28 mg), Z-Gly-Gly-Arg-AMC (3.08 mg), Z-Arg-AMC (2.51 mg), Z-Arg-Arg-AMC (3.11 mg), Z-Gly-Arg-Arg-AMC (3.33 mg), Z-Ala-Arg-Arg-AMC (3.40 mg), Meosuc-Asp-Tyr-Met-AMC (3.49 mg), Boc-Leu-Arg-Arg-AMC (3.69 mg), Boc-Leu-Gly-Arg-AMC (3.01 mg), H-Leu-AMC (1.44 mg), H-Gly-AMC (1.57 mg) were dissolved separately in DMSO (100 µL).

1 mM Working substrate solutions: 50 mM stock solutions (20 µL) was dissolved in distilled water (980 µL). Substrates were diluted from this 1 mM working solution with distilled water to four times the appropriate final concentration, which allows for the dilution in the assay.

Assay for the effect of reversible inhibitors on Vp and C2

The inhibition kinetic constant (K_i) was determined for reversible inhibitors according to Salvesen and Nagase (1989). Firstly, the uninhibited rate of substrate hydrolysis (v_o) was determined. Briefly, the enzyme [0.15ng (0.056 nM) active Vp and (0.056 nM) active C2] was made up to 25 µl with 0.1 % (w/v) Brij and incubated with 50 µl congopain assay buffer for 10 min at 37°C to allow for activation of the enzyme. Substrate (25 µl) of 20 µM Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC (final concentration) was added. This substrate concentration was used since it allowed for less than 5% hydrolysis, which is vital since this ensures that the substrate concentration is kept constant. The fluorescence (Ex_{360nm}, Em_{460nm}) was monitored continuously for 5 min using the FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany). Inhibitor was added at a concentration range at least 20-fold molar excess over the enzyme concentration. The volume of inhibitor added did not exceed 5% of the total assay volume. Fluorescence

(Ex_{360nm}, Em_{460nm}) was monitored continuously using the FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany) until a new steady state velocity was obtained in the presence of the inhibitor (v_i). Under these conditions the apparent inhibition constant in the presence of substrate ($K_{i(app)}$) is given by the following equation:

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

(Salvesen and Nagase 1989)

$1/K_{i(app)}$ is the slope from the plot of v_o/v_i against [I].

The true equilibrium inhibitor constant K_i was be determined from the following equation:

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

Assay for the effect of irreversible inhibitors on Vp and C2

The effect of irreversible inhibitors on Vp and C2 activity was determined under pseudo first-order conditions, where the inhibitor concentration is 50 times greater than the enzyme concentration ([I] > 50[E]) (Salvesen and Nagase, 1989). Briefly, the enzyme [0.15ng (0.056 nM) active Vp (1.56 µl) and (0.056 nM) active C2 (1 µl)] was made up to 97.5 µl with 1 % (w/v) Brij and activated with 1.95 mL congopain assay buffer. The inhibitor (880 µl; the volume added allowed for the final concentration to be 50 x > enzyme concentration) was added to buffered enzyme solution and incubated at 37^{o}C. Aliquots (75 µl) were removed at 5 min time intervals for 1 hour and the residual activity was determined against 25 µl of substrate (20 µM final concentration of Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC). The k_{obs} was determined from a plot of the ln (natural logarithm) of residual enzyme activity against time.

The slope of this plot gives -k_{obs} (pseudo first-order rate constant), which allows for the calculation of k_{ass} from the following relationship,

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

(Salvesen and Nagase 1989),

where [I] is the inhibitor concentration. The time required for the free enzyme to decrease by 50% (half-time, $t_{1/2}$) during the reaction of the enzyme with the irreversible inhibitor is given by:

$$t_{1/2} = \frac{0.693}{k_{\text{ass}}\,[\text{I}]}$$

(Salvesen and Nagase 1989)

2.3 RESULTS

2.3.1 Expression of the catalytic domains of vivapain (Vp) and congopain (C2)

Vp and C2 were recombinantly expressed in *P. pastoris* by methanol induction. Fig. 2.6 A and 2.6 B show the recombinant expression supernatant of Vp on day two and day five of expression and of C2 on day five of expression. Vp was observed as two bands at approximately 29 kDa and 33 kDa (Fig. 2.6 A, lanes 2 and 3). C2 also has two bands at approximately 27 kDa and 33 kDa (Fig. 2.6 B, lane 2). The reason for the 33 kDa C2 band could be that not all of the C2 was processed into the active form of C2

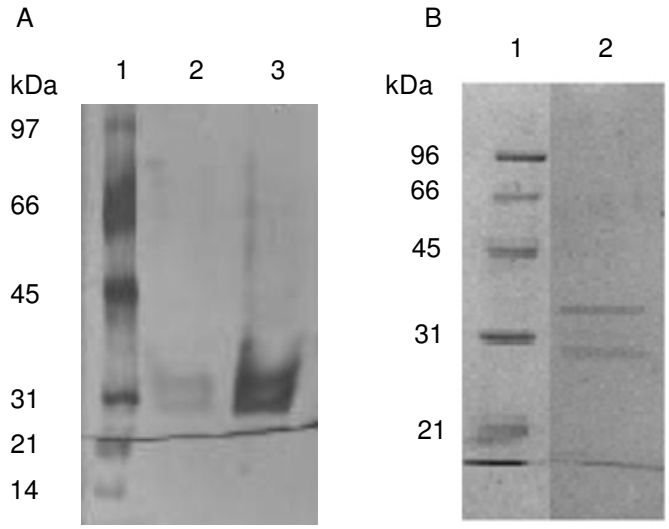


Figure 2.6 Analysis by 12.5% SDS-PAGE of the expression of Vp and C2 in *P. pastoris*. Panel A, silver stained gel showing the expression of Vp after day two and day five. Lane 1, molecular weight markers; lane 2, supernatant of culture from day 2 of expression (25 µl); lane 3, supernatant of culture from day 5 of expression (25 µl). Panel B, Coomassie stained gel showing the expression of C2 on day five. Lane 1, molecular weight markers; lane 2, supernatant of culture from day 5 of expression (25 µl). Expression for both Vp and C2 were induced with 0.5% (v/v) methanol in BMM. Each of the Vp and C2 supernatants were mixed with an equal volume of reducing treatment buffer and boiled for 2 minutes prior to loading.

2.3.2 Purification of Vp and C2

Three phase partitioning (TPP) was used as the initial step to both concentrate and purify the recombinantly expressed proteins from the culture supernatant. TPP was optimised on a crude supernatant of Vp and the optimal amount of $(NH_4)_2SO_4$ was determined to be 40% (results not shown). The arrows in Fig 2.7 panel A indicate two prominent bands present in each lane corresponding to Vp at 29 kDa and 33 kDa (Fig 2.7, lanes 2 and 3). TPP was also performed on the C2 culture supernatant using 40% $(NH_4)_2SO_4$. Once again, the protein precipitated efficiently out of the supernatant which can be seen by the prominent band present in panel B (Fig 2.7, lane 2). The 33 kDa band that was present in the C2 culture supernatant before TPP (Fig 2.6 B, lane 2) has disappeared after TPP; however, the 33 kDa band that was present in the Vp culture supernatant before TPP (Fig 2.6 A, lane 2) is still present after TPP and at the same intensity as the 29 kDa band. This 33 kDa band could potentially be glycosylated.

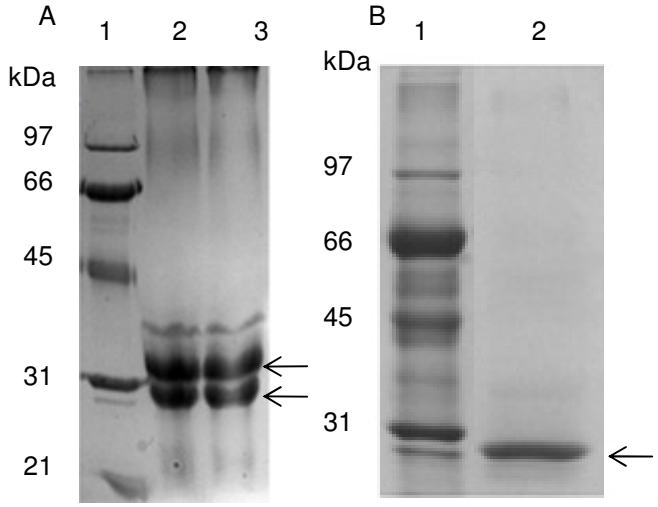


Figure 2.7 Analysis by 12.5% SDS-PAGE of the precipitation of Vp and C2 from the culture supernatant using TPP. Panel A: Precipitation of Vp from the expressed culture supernatant using 40% $(NH_4)_2SO_4$. Lane 1, molecular weight markers; lanes 2 and 3, different Vp fractions after TPP, dialysis and concentration (10 µl). Panel B: Precipitation of C2 from the expressed culture supernatant using 40% $(NH_4)_2SO_4$. Lane 1, molecular weight markers; lane 2, C2 fractions after TPP, dialysis and concentration (10 µl). The samples loaded on the respective gels were mixed with an equal volume of reducing treatment buffer and boiled for 2 minutes. The gel was stained with Coomassie blue R-250. The arrows indicate the precipitated protein bands representing Vp (Panel A) and C2 (Panel B).

A western blot was performed in order to verify that Vp and C2 were expressed (Fig. 2.8). Both Vp and C2 were detected using rabbit anti-central domain of congopain IgG. This antibody is specific for C2; however, it had a good chance of detecting Vp because of the high sequence identity between Vp and C2 (Fig. 1.8, Section 1.8.5). The antibody recognised bands at 29 and 33 kDa (lanes 2 and 3), which correspond to the sizes of the two bands present in Vp and it also detected C2 at 27 kDa (lane 4) that was used as a positive control. The blot also shows multiple contaminating bands in lanes 2 and 3 that are larger in size compared to the bands expected to be Vp.

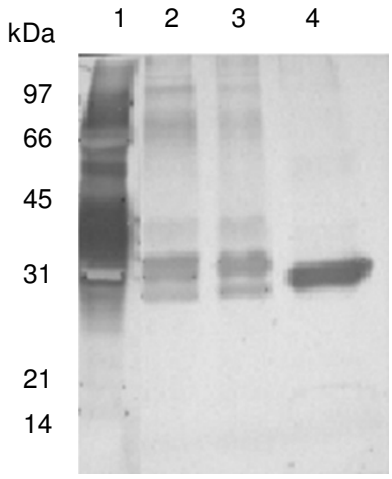


Figure 2.8 Western blot analysis of Vp and C2 expression. Proteins were electrophoresed on a 12.5% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with rabbit anti-central domain of congopain IgG (1: 1000). Secondary antibody was goat anti-rabbit HRPO-conjugated (1: 2000). Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, molecular weight markers; lane 2 and 3, Vp (10 µg) after TPP and lane 4, of C2 after TPP (5 µg).

The SDS-PAGE gel (Fig 2.7 A, lanes 2 and 3) and the western blot (Fig 2.8, lanes 3 and 4) showed the presence of higher molecular weight contaminants in the Vp sample after TPP. Vp was further purified using Sephacryl S-300 molecular exclusion chromatography (MEC). Two elution peaks were observed after the MEC purification (Fig 2.9). The first peak corresponds to the higher molecular weight contaminants. The second peak corresponds to Vp. This was further verified by performing an activity assay using the substrate Z-Phe-Arg-AMC. This showed that the proteins eluting in the first peak have no activity in contrast to the proteins in the second peak which showed Vp activity. A silver stain, which was performed on the fractions present in the second peak (Fig. 2.10) revealed two bands corresponding to the expected size of Vp at 29 and 33 kDa. These fractions were also active against the substrate and were pooled and concentrated by ultrafiltration.

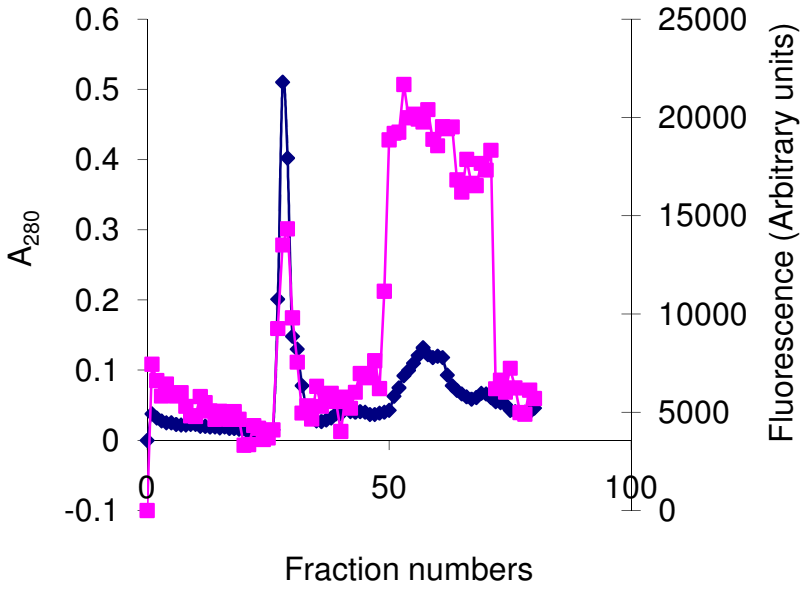


Figure 2.9 Purification of Vp by molecular exclusion chromatography using Sephacryl-300. The column has dimensions of 25 x 840mm with a flow rate of 25ml/hour. Fractions (5 mL) were collected. The absorbance at A_{280} was taken (■) as well as the activity against Z-Phe-Arg-AMC (■).Microsoft excel 2007 was used to construct the plot.

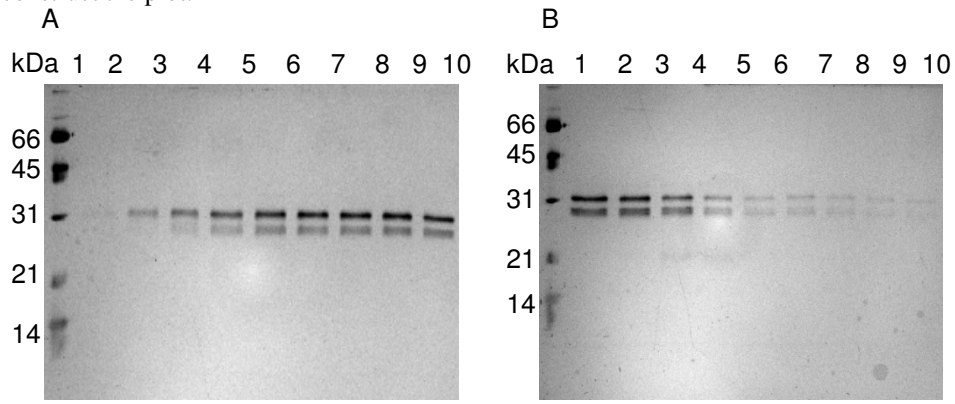


Figure 2.10 Silver stained 12.5% reducing SDS-PAGE gel indicating fractions eluted during MEC of Vp. Panel A: lane 1, molecular weight markers; lanes 2-10, MEC fractions 50-58. Panel B: lane 1, molecular weight markers; lanes 2-10, MEC fractions 59-67.

The western blot (Fig. 2.11) represents the progression of purification of Vp, which shows that the majority of the contaminants present after TPP were successfully eluted in the first peak of the MEC purification (Fig. 2.9) This is evident when comparing the Vp bands present in lanes 3 (after TPP) and 4 (after MEC) (Fig. 2.11 A), which show that most of the

A B

contaminating bands present after TPP are not present after MEC. The primary antibody used for the western blot was rabbit anti-central domain of congopain IgG. Because of the congopain and vivapain amino acid sequence identity (Fig. 1.8 Chapter 1), the western blot verifies that the bands present at 29 and 33 kDa are in fact Vp.

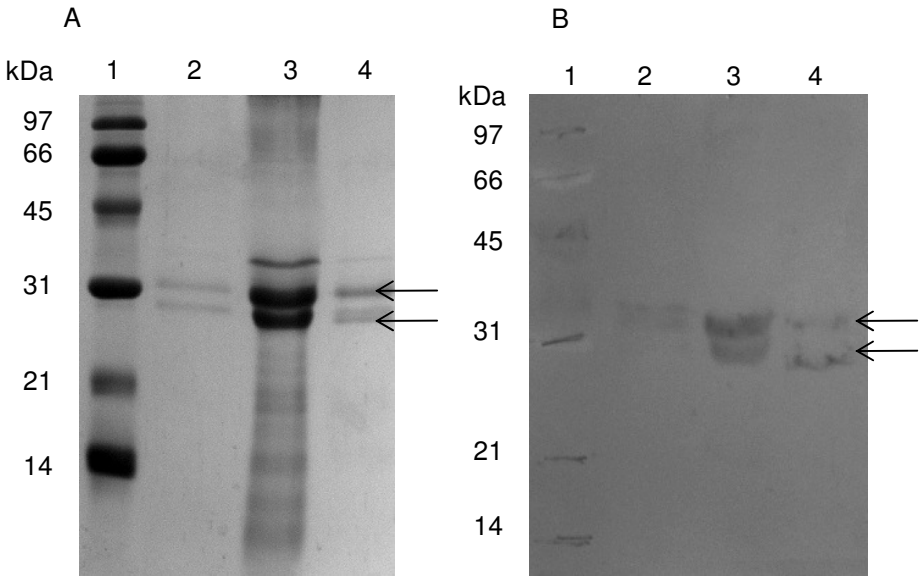


Figure 2.11 Analysis of the purification of the Vp after TPP and MEC. Panel A: 12.5% Reducing SDS-PAGE and panel B: western blot. Lane 1, molecular weight markers; lane 2, culture supernatant before purification (10 µl); Lane 3, Vp after TPP (10 µl) and Lane 4, Vp after MEC (10 µl). For panel A: the 12.5% SDS-PAGE gel each sample was mixed with an equal volume of reducing treatment buffer and boiled for 2 minutes and the gel was stained with Coomassie blue R-250. For panel B: proteins were electrophoresed on a 12.5% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with rabbit anti-central domain of congopain IgG (1: 1000). Secondary antibody was goat anti-rabbit HRPO-conjugated (1:2000). Reaction was developed with 4-chloro-1-naphthol/H_2O_2.The arrows indicate the bands representing vivapain in lanes 2-4 in both A and B.

The purification of Vp and C2 is represented in Table 2.1. The activity of Vp was measured before and after TPP and after MEC and the activity of C2 was only measured before and after TPP, since C2 did not have any contaminating bands after TPP (Fig. 2.7 B). The activity was determined by assaying the hydrolysis of Z-Phe-Arg-AMC. Therefore the values for activity were calculated from the total activity obtained from the hydrolysis of the substrate. The specific activity of Vp increased 22 -fold after TPP and a further 18-fold after MEC. The Vp yield after TPP and MEC was 95% and 143% respectively. The specific activity of C2 increased 8-fold after TPP and the yield was 162%.

Table 2.1 Purification of Vp (A) and C2 (B). Activity was measured on Vp samples before and after TPP as well as after MEC and on C2 samples before and after TTP. Activity was measured by analysing the hydrolysis of each sample against Z-Phe-Arg-AMC.

A)

Step	Volume (ml)	Concentration (mg/ml)	Total Protein (mg)	Total Activity (units)	Specific Activty (units/mg)	Purification (fold)	Yield (%)
Supernatant	730	1.3	949	35166	37.06	1	100
TPP	8	5.2	41.6	33421	803.39	21.68	95
MEC	0.6	5.9	3.54	50401	14238	384	143

B)

Step	Volume (ml)	Concentration (mg/ml)	Total Protein (mg)	Total Activity (units)	Specific Activty (units/mg)	Purification (fold)	Yield (%)
Supernatant	100	0.06	5.6	31153	5563	1	100
TPP	3	0.38	1.14	50478	44279	7.9	162

2.3.3 Enzymatic characterisation of Vp

In order to assess the activity of the recombinantly expressed Vp and C2 against a protein substrate, both the purified samples were electrophoresed on a 12.5% SDS-PAGE gel containing 1% (w/v) gelatin under non-reducing conditions. The digestion of the gelatin by the Vp and C2 samples corresponds to the respective positions of their protein bands on the gel (Fig. 2.12). The bands in each lane associated with Vp are at approximately at 35 kDa and 50 kDa (Fig. 2.12, lanes 2 and 3). The area where C2 digested the gelatin is at approximately 39 kDa (Fig. 2.12, lane 4).

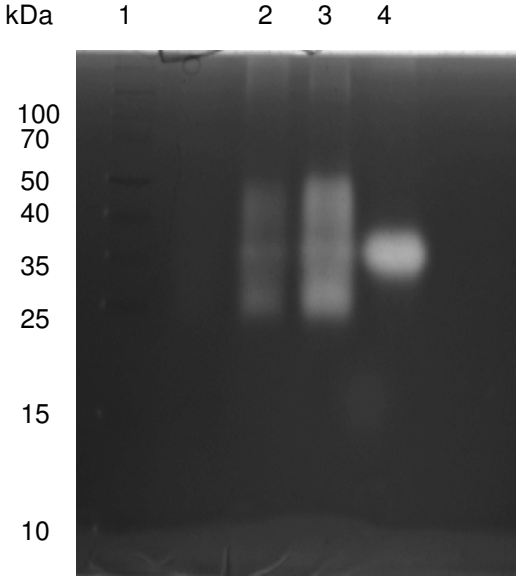


Figure 2.12 Analysis by non-reducing 12.5% SDS-PAGE gel containing 1% gelatin showing the endoproteolytic activity of Vp and C2. Proteins were not boiled and reduced. Lane 1, Spectra™ Multicolour Broad Range Protein Ladder; lanes 2 and 3, Vp 0.5 µg and 1 µg; lane 4, C2 (5 µg). (B) Gels were stained with Amido black.

Vp was observed on both the reducing (Fig. 2.6 A, 2.7 A, 2.10 A and B and 2.11 A) and non-reducing (Fig 2.12) SDS-PAGE gels as a double band. The presence of the two bands suggests that vivapain could be glycosylated. This was investigated by conducting endoglycosidase H (Endo H) digestion of Vp, which cleaves glycosylated areas of a protein and assessing the cleavage products on SDS-PAGE (Fig. 2.13). In comparison to the control where Endo H was omitted from the incubation mixture that showed two bands at

29 kDa and at 33 kDa (lane 3), the 33 kDa band disappeared in the presence of Endo H (Fig. 2.13, lane 2) indicating that Vp is glycosylated.

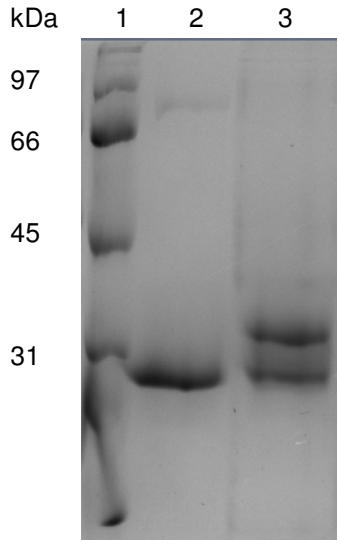


Figure 2.13 Analysis by a reducing 12.5% SDS-PAGE of endoglycosidase H treatment of Vp. Lane 1, molecular weight markers; lane 2, Vp (15 µg) treated with 500 units endoglycosidase H; lane 3, control-Vp without endoglycosidase H (15 µg). The Vp samples were boiled with 2-mercaptoethanol before the addition of endoglycosidase H since the enzyme is only able to work on the reduced sample. Vp and Endo H were incubated for 3 hours at 37^{o}C. The gel was stained with Coomassie blue R-250.

Active site titration was carried out on the recombinant purified Vp and C2 by using E-64 as the active site titrant. This gives an indication of the concentration of the active enzyme present from the total concentration of Vp and C2. Both preparations of Vp (Fig. 2.14) and C2 (results not shown) were shown to be 20% active, which translates into active concentrations of 1180 µg/mL for Vp and 400 µg/mL for C2.

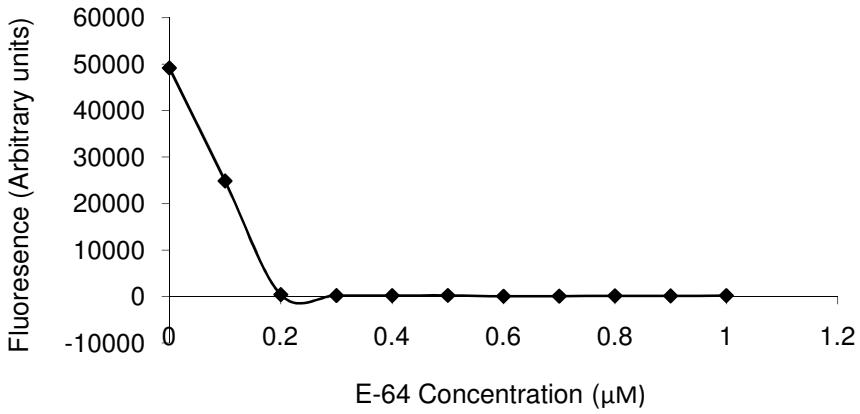


Figure 2.14 Percentage of active vivapain in the presence of differing concentrations of E-64. A constant amount of vivapain (1 μM) was reacted with different concentrations of E-64 (ranging from 0.1-1 μM) and the hydrolysis of Z-Phe-Arg-AMC was measured. Microsoft excel 2007 was used to construct the plot.

The pH optima for maximal activity of Vp and C2 were determined in the presence of a constant ionic strength AMT buffer ranging from pH 4-9. The pH profiles for Vp and C2 are shown in (Fig. 2.15). The activity of Vp against Z-Phe-Arg-AMC was optimal at pH 7.0. Vp is active over a narrower pH range (from pH 6-9) than C2. The pH optimum for C2 is 6.5. Further assays were hence carried out at pH 6.5 for both Vp and C2.

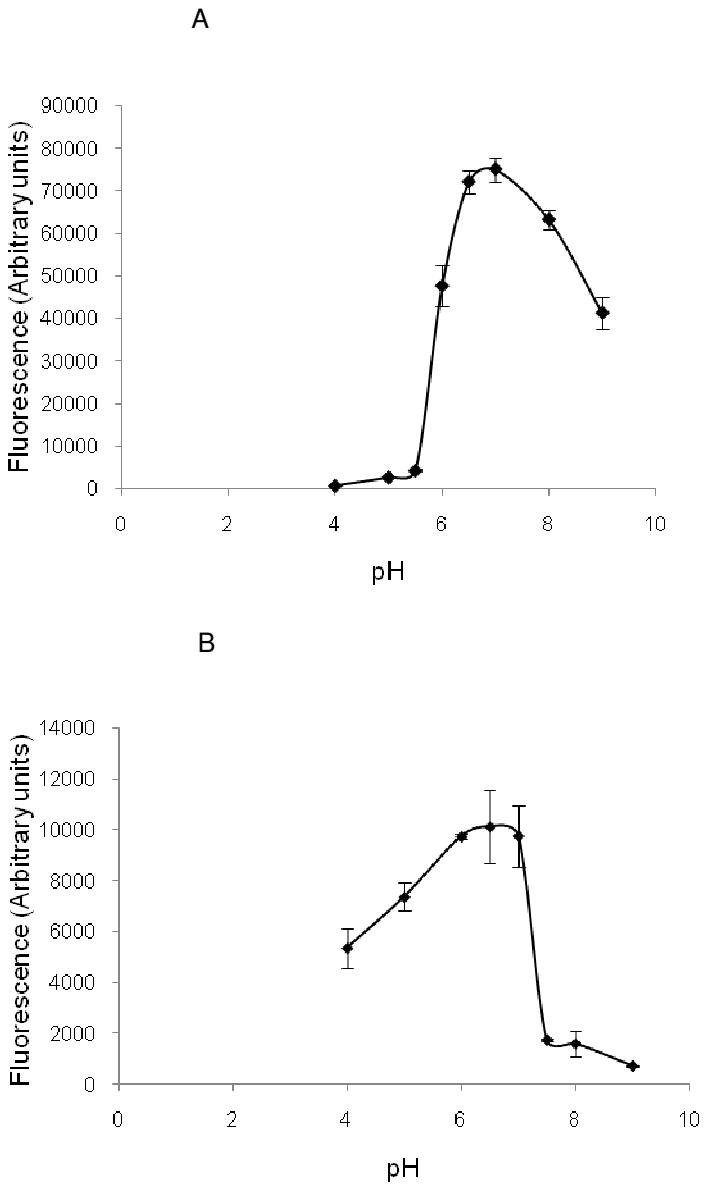


Figure 2.15 Effect of pH on Vp (A) and C2 activity (B) against Z-Phe-Arg-AMC. Vivapain (1.5 ng active enzyme) and C2 (1.5 ng active enzyme) were used and their activity against Z-Phe-Arg-AMC was tested at 37°C. The AMT buffers of constant ionic strength was used over a pH range from pH 4-9. The error bars represent the mean fluorescence ± SD (n=3). Microsoft excel 2007 was used to construct the plot.

Both the recombinantly expressed purified Vp and C2 were reacted with an array of endopeptidase and exopeptidase synthetic substrates. The kinetic constants, namely K_m, k_{cat} and k_{cat}/K_m are represented in Table 2.2. Vp and C2 have relatively low K_m (9.61 µM and

6.78 μM, respectively) and a high k_{cat}/K_m (22.57 $s^{-1}mM^{-1}$ and 149.59 $s^{-1}mM^{-1}$, respectively) for hydrolysis of Z-Phe-Arg-AMC.

Table 2.2 Kinetic constants for the hydrolysis of synthetic substrates by purified recombinant Vp and C2. The various substrate concentrations used ranged from 2.5-50 μM with 1.5 ng enzyme. All incubation steps were carried out at 37°C. Assays were performed in congopain assay buffer [100 mM Bis-Tris, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. The K_m values were calculated based on the AMC standard curve (y=3.356x) from the means of triplicate values. K_m values and the Michaelis-Menten plots were calculated using the Hyper® 32 software (J.S Easterby).

Substrate	Enzyme	[a] K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($s^{-1}mM^{-1}$)
Z-Phe-Arg-AMC	Vp	9.61	0.22	22.57
	C2	6.78	1.01	149.59
H-D-Val-Leu-Lys-AMC	Vp	14.23	0.55	38.61
	C2	1.78	2.36	1324.96
H-D-Ala-Leu-Lys-AMC	Vp	14.97	0.33	21.74
	C2	1.55	2.75	1772.17
Suc-Leu-Tyr-AMC	Vp	186.1	0.04	0.22
	C2	728.5	0.90	1.24
Suc-Ala-Phe-Lys-AMC	Vp	83.03	0.01	0.14
	C2	131.5	0.48	3.66
H-Ala-Phe-Lys-AMC	Vp	88.06	0.05	0.52
	C2	33.26	1.19	35.82
H-Pro-Phe-Arg-AMC	Vp	61.63	0.11	1.81
	C2	17.92	1.37	76.31

No activity was detected against Z-Gly-Pro-Arg-AMC, Z-Gly-Gly-Arg-AMC, Z-Arg-AMC, Z-Arg-Arg-AMC, Z-Gly-Arg-Arg-AMC, Z-Ala-Arg-Arg-AMC, Meosuc-Asp-Tyr-Met-AMC**,** Boc-Leu-Arg-Arg-AMC, Boc-Leu-Gly-Arg-AMC, H-Leu-AMC, H-Gly-AMC, after one, two, three and four hours of incubation at 37°C.

[a] The Michaelis-Menten plots are included in Appendix 1 for Vp and Appendix 2 for C2

This shows that Z-Phe-Arg-AMC is a good substrate for Vp and C2; however, results show that it is not the best substrate. The best substrate for Vp is H-D-Val-Leu-Lys-AMC. Vp has a K_m of 14.23 μM and a k_{cat}/K_m of 38.61 $s^{-1}mM^{-1}$ when reacted with H-D-Val-Leu-Lys-AMC. Even though the K_m is higher than for the hydrolysis of Z-Phe-Arg-AMC by Vp, a comparison of the k_{cat}/K_m values (catalytic efficiency constants) determines the best substrate for a particular enzyme. Since the reaction between Vp and H-D-Val-Leu-Lys-AMC yields a higher k_{cat}/K_m value, it is the better substrate. The results also show that D-Ala-Leu-Lys-AMC is also a good substrate for Vp. The best substrates for C2 are H-D-Val-Leu-Lys-AMC and H-D-Ala-Leu-Lys-AMC since the K_m values are low (1.78 μM and 1.55 μM respectively) and their k_{cat}/K_m are exceptionally high (1324.96 $s^{-1}mM^{-1}$ and 1772.17 $s^{-1}mM^{-1}$ respectively). It is also interesting to note that both these cysteine peptidases, Vp and C2, have endopeptidase and exopeptidase activity as shown by the hydrolysis of Z-Phe-Arg-AMC, which has a N-terminal blocking group and H-D-Val-Leu-Lys-AMC and H-D-Ala-Leu-Lys-AMC, which do not have blocking groups. The following substrates: Z-Gly-Pro-Arg-AMC, Z-Gly-Gly-Arg-AMC, Z-Arg-AMC, Z-Arg-Arg-AMC, Z-Gly-Arg-Arg-AMC, Z-Ala-Arg-Arg-AMC, MeoSuc-Asp-Tyr-Met-AMC, Boc-Leu-Arg-Arg-AMC, Boc-Leu-Gly-Arg-AMC, H-Leu-AMC, H-Gly-AMC, were not hydrolysed by Vp or C2.

The inhibition of recombinant Vp and C2 by competitive, low molecular weight reversible inhibitors is shown in Table 2.3. Both the enzymes seemed to follow the same trend when they reacted with the inhibitors in the presence of either Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC. The best inhibitor for both recombinant Vp and C2 was leupeptin since the lowest K_i value was obtained for this inhibitor when comparing it to the values obtained for the other inhibitors. There is no significant difference between the reactions of chymostatin and antipain with the recombinant Vp in the presence in D-Val-Leu-Lys-AMC and C2 in the presence of Z-Phe-Arg-AMC. Chymostatin seems to be a better inhibitor than antipain for Vp in the presence of Z-Phe-Arg-AMC since a lower K_i value is obtained. Chymostatin is the better inhibitor of the two for the reaction with recombinant C2 in the presence of D-Val-Leu-Lys-AMC since a lower K_i is obtained. Other

low molecular weight reversible inhibitors such as bestatin, EDTA and pepstatin A did not inhibit either Vp or C2.

Table 2.3 Inhibition of recombinant Vp and C2 by competitive reversible inhibitors. Assays were performed in congopain assay buffer [100 mM Bis-Tris, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. All incubation steps were at 37°C. The K_i values were calculated based on the AMC standard curve (y=3.356x) from the means of triplicate values.

	Vp		C2	
	Z-Phe-Arg-AMC	D-Val-Leu-Lys-AMC	Z-Phe-Arg-AMC	D-Val-Leu-Lys-AMC
Inhibitor	K_i (μM) (x10^3)	K_i (μM) (x10^3)	K_i (μM) (x10^3)	K_i (μM) (x10^3)
Leupeptin	4.46	4.35	5.45	102.1
Chymostatin	9.77	18.07	9.62	240
Antipain	13.32	11.4	8.5	434.9

No inhibition was observed with bestatin (10 μM), EDTA (10 mM) or pepstatin A (1 μM)

The inhibition of recombinant Vp and C2 by competitive, low molecular weight irreversible inhibitors is shown in Table 2.4. The reaction of both Vp and C2 with IAA and IAN shows very similar kinetic values in the presence of either Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC. The action of IAA and IAN on Vp seems to better in the presence of D-Val-Leu-Lys-AMC since a higher k_{ass} values of 17.86 $mM^{-1}.s^{-1}$ and 12.5 $mM^{-1}.s^{-1}$ are obtained and the $t_{1/2}$ values are much lower for these reactions. The action of IAA on C2 seems to better in the presence of Z-Phe-Arg-AMC since a higher k_{ass} value of 14.29 $mM^{-1}.s^{-1}$ is obtained and the $t_{1/2}$ value is lower. IAN seems to be the better inhibitor

for C2 in the presence on D-Val-Leu-Lys-AMC due to the higher k_{ass} value of 16.08 $mM^{-1}.s^{-1}$ is and $t_{1/2}$ is much lower (1593 s) for this reaction. The low molecular weight irreversible inhibitors such as PMSF, TLCK and TPCK did not inhibit Vp or C2.

Table 2.4 Inhibition of recombinant Vp (A) and C2 (B) by competitive irreversible inhibitors. All incubation steps were at 37°C. Assays were performed in congopain assay buffer [100mM Bis-Tris, pH 6.5, 4mM EDTA, 0.02% (w/v) NaN_3, 8mM DTT]. The k_{ass} values were calculated based on the AMC standard curve (y=3.356x) from the means of triplicate values.

A	Vp			
	Z-Phe-Arg-AMC		D-Val-Leu-Lys-AMC	
	k_{ass} [a] ($mM^{-1}.s^{-1}$)	$t_{1/2}$ (s)	k_{ass} [a] ($mM^{-1}.s^{-1}$)	$t_{1/2}$ (s)
IAA [b]	10.71 ± 0.01	2311	17.86 ± 5.04	1386
IAN [b]	7.14 ± 0.01	3466	12.5 ± 7.58	1980
B	C2			
	Z-Phe-Arg-AMC		D-Val-Leu-Lys-AMC	
	k_{ass} [a] ($mM^{-1}.s^{-1}$)	$t_{1/2}$ (s)	k_{ass} [a] ($mM^{-1}.s^{-1}$)	$t_{1/2}$ (s)
IAA [b]	14.29 ± 0.01	1732	10.72 ± 5.06	2309
IAN [b]	10.72 ± 5.06	2309	16.08 ± 2.52	1539

No inhibition was observed with PMSF, TLCK and TPCK

[a] Standard deviation

[b] 28 nM inhibitor concentration

2.4 DISCUSSION

In the present study, the catalytic domain of vivapain (Vp) was expressed in a yeast expression system. This decision was based on the fact that the catalytic domain of congopain (C2) had been previously expressed at high functional levels in the methylotrophic *Pichia pastoris* yeast expression system (Boulangé A, University of KwaZulu-Natal, personal communication). The use of the methylotrophic *P. pastoris* yeast expression system had become very popular due to the simplicity of the expression technique (Cereghino and Cregg 2000). This system is also able to express high levels of foreign proteins either extracellularly or intracellularly. Most importantly, this system is capable of producing multiple eukaryotic post translational modifications; namely: disulfide bond formation, glycosylation, protein folding and proteolytic processing (Cereghino and Cregg 2000; Macauley-Patrick *et al.* 2005). The occurrence of the post translational modifications obviates the need for the renaturation of the protein after expression (Aloulou *et al.* 2006). Another key feature of this system, for extracellular expression, is that most *P. pastoris* expression vectors contain a *Saccharomyces cerevisiae* MAT-α-prepro signal peptide sequence, which directs the expressed protein out of the yeast cells and into the culture medium. This serves as an initial step for purification (Cereghino and Cregg 2000; Cereghino *et al.* 2002). The methylotrophic *P. pastoris* yeast expression system has become a highly successful expression system for the expression of many heterologous proteins such as cathepsin B from *L. major* (Chan *et al.* 1999), rhodensain, a cysteine peptidase from *T. b. rhodesiense* (Caffrey *et al.* 2001), cathepsin B-like cysteine peptidase from *T. congolense* (Mendoza-Palomares *et al.* 2008) and multiple isoforms of cathepsin B-like cysteine peptidases from *Trichobilharzia regent* (Dvořák *et al.* 2005).

The catalytic domain of vivapain (Vp) was successfully expressed as an active mature enzyme resulting in a double band corresponding to 29 kDa and 33 kDa on reducing SDS-PAGE. These bands were also recognised by the polyclonal antibody raised in rabbits against bacterial-expressed central domain of congopain (Boulangé *et al.* 2001). Since the bacteria-expressed congopain was not re-natured before antibody production it is expected that the serum will recognise mainly linear epitopes. Therefore, it was likely that Vp would

be detected by this antibody especially due to the high sequence identity between congopain and vivapain (Fig. 1.8, Section 1.8.5). The recombinant Vp sample is observed as a double band on a reducing/non-reducing SDS-PAGE gel since the peptidase is glycosylated. This was confirmed by endoglycosidase H (Endo H) analysis. In the presence of Endo H, the 29 kDa band remained and the 33 kDa band disappeared. Therefore, it can be deduced that the 29 kDa band represents the non-glycosylated form, while the 33 kDa form is glycosylated. An interesting finding was that the double band was still observed in the Vp sample before and after activation at pH 4.2, which suggests that the mature enzyme has a glycosylation site. This was verified by using a prediction program; NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc), which showed that Vp has a possible glycosylation site at Asn^{288} in the mature region of the protein. This feature of Vp is unlike that of cruzipain and rhodensain where the pro-enzyme of these peptidases are glycosylated with glycosylation sites present at Asn^{113} for cruzipain (Eakin *et al.* 1993) and at Asn^{295} in rhodensain (Caffrey *et al.* 2001). Therefore, it can be concluded that the Vp that is present in the *P. pastoris* supernatant does not obtain the pro-enzyme and both the glycosylated and unglycosylated mature enzyme is present before and after activation at pH 4.2.

The catalytic domain of congopain (C2) was also successfully expressed as shown by the presence of a 27 and a 33 kDa band on reducing SDS-PAGE. This double band was only present in the *P. pastoris* expression supernatant and upon activation at pH 4.2 the 33 kDa band disappeared, which indicates that this was the pro-enzyme form of C2. The mature enzyme form of C2 has a M_r of 27000 (Boulangé *et al.* 2001). This band was detected by the polyclonal antibody raised in rabbits against the bacterial-expressed central domain of congopain.

The digestion of gelatin at both 35 and 50 kDa for Vp and at 39 kDa for C2 showed that both peptidases have endoproteolytic activity. This further validates the activity of both the glycosylated and unglycosylated forms of Vp. The reason why the bands of digestion are at a higher M_r than expected is that the gelatin gel is electrophoresed under non-reducing conditions. By analogy of C2, that it is known to be a dimer (Boulangé A, University of

KwaZulu-Natal, personal communication), the activity displayed by Vp at 35 and 50 kDa suggest that Vp is also a dimer.

The initial purification step used for both Vp and C2 was three phase partitioning (TPP). This time saving technique is also cheap and easy to perform. The TPP technique as described by Pike and Dennison (1989) for the purification of cathepsin L from sheep liver was very successful in purifying and concentrating Vp and C2. In fact this technique was so successful for the purification of C2 that no further purification techniques had to be performed due to the purity of the sample after TPP. Molecular exclusion chromatography (MEC) was used to remove the higher molecular weight contaminating bands present in the Vp sample after TPP resulting in a homogenous 29 and 33 kDa preparation of Vp. The yield of Vp obtained after TPP and MEC and of C2 after TPP was greater than 100%. A similar trend was reported by Dennison and Lovrien (1997) for *Bacillus subtilis* peptidase, *Saccharomyces cerevisiae* invertase and *Candida cylindracea* lipase where yields for these proteins were 300, 100 and 900% respectively after purification. The unusually high yield could be due to the fact that inhibitors in the culture supernatants were being removed during the purification steps (Dennison and Lovrien 1997; Sharma and Gupta 2001) This could be a possible explanation for the high yield obtained for Vp and C2 after purification.

The synthetic substrates revealed that Vp preferred the basic residues, Arg and Lys, are preferred in P_1 only when P_2 is occupied by Leu or Phe since Z-Arg-Arg-AMC, Z-Gly-Arg-Arg-AMC, Z-Ala-Arg-Arg-AMC, Boc-Leu-Arg-Arg-AMC and Z-Arg-AMC are not hydrolysed. Neither Vp nor C2 tolerate other hydrophobic residues in P_2 as shown by the absence of hydrolysis of Z-Gly-Pro-Arg-AMC, Z-Gly-Gly-Arg-AMC and Boc-Leu-Gly-Arg-AMC. Papain, mammalian cathepsin L, cruzipain from *T. cruzi* and falcipain from *Plasmodium falciparum* also prefer a hydrophobic residue in P_2 (Brömme *et al.* 1989; Cazzulo *et al.* 1990; Wijffels *et al.* 1994; Serveau *et al.* 1996; Shenai *et al.* 2000; Salvati *et al.* 2001). The presence of either the smaller hydrophobic Ala or Val in P_3 also proved to be preferred by Vp and C2 as evident by a 10-fold increase in k_{cat}/K_m as compared to the presence of other residues in P_3. Consequently, the best substrates for Vp and C2 are Z-Phe-Arg-AMC, H-D-Val-Leu-Lys-AMC and H-D-Ala-Leu-Lys-AMC. Peptides with a

hydrophilic Tyr in P_1, though a Leu is present in P_2, were poor substrates for both Vp and C2. Substrates such as H-Ala-Phe-Lys-AMC and Suc-Ala-Phe-Lys-AMC, which contain a basic amino acid Lys in P_1, hydrophobic Phe in P_2 and the hydrophobic Ala in P_3 were poorly hydrolysed by Vp. This shows that when Lys is in P_1 and Ala in P_3, the preferred amino acid in P_2 is Leu instead of Phe. When Leu is in P_2 (H-D-Ala-Leu-Lys-AMC) the hydrolysis by Vp yields a k_{cat}/K_m that is approximately 42-fold higher than when Phe is in P_2 (H-Ala-Phe-Lys-AMC). Suc-Ala-Phe-Lys-AMC is also poorly hydrolysed by C2. It is also interesting to note that C2 is able to hydrolyse H-Pro-Phe-Arg-AMC whereas Vp hydrolyses this substrate poorly. Another important feature to note is that both Vp and C2 have endopeptidase and exopeptidase activity since they are able to hydrolyse Z-Phe-Arg-AMC, which has a N-terminal blocking group and H-D-Val-Leu-Lys-AMC and H-D-Ala-Leu-Lys-AMC, which do not have blocking groups. There was no activity against H-Leu-AMC and H-Gly-AMC, which suggests that Vp and C2 has no aminopeptidase activity. This was further borne out by the lack of inhibition by the aminopeptidase inhibitor, bestatin. H-Leu-AMC is a key synthetic substrate for a leucyl aminopeptidase from *Leishmania amazonesis, L. donovani* and *L. major* (Morty and Morehead 2002).

The K_m of 6.78 μM obtained for the hydrolysis of Z-Phe-Arg-AMC by C2 is comparable to the K_m values for native congopain of 1.5 μM (Chagas *et al.* 1997), 4.4 μM (Mbawa *et al.* 1992) and 7.3 μM (Authié *et al.* 1992). The K_m obtained for the hydrolysis of Z-Phe-Arg-AMC, H-D-Val-Leu-Lys-AMC and H-D-Ala-Leu-Lys-AMC by Vp are 9.61 μM, 14.23 μM and 14.97 μM, respectively. The K_m obtained for the hydrolysis of Z-Phe-Arg-AMC, H-D-Val-Leu-Lys-AMC and H-D-Ala-Leu-Lys-AMC by native trypanopain from *T. b. brucei* was 1.2 μM, 25 μM and 27 μM, respectively (Troeberg *et al.* 1996), which are comparable to the values obtained for Vp. The K_m obtained for the hydrolysis of Z-Phe-Arg-AMC by native and recombinant falcipain-2 from *P. falciparum* were 17.3 μM and 9.13 μM, respectively (Shenai *et al.* 2000), 1.8 μM and 0.96 μM for native and recombinant cruzipain from *T. cruzi* (Cazzulo *et al.* 1990; Serveau *et al.* 1996), 2.8 μM for mammalian cathepsin L (Brömme *et al.* 1989), 46 μM for cathepsin L-like cysteine

peptidase from *Fasciola hepatica* (Wijffels *et al.* 1994), and 14 μM for a cysteine peptidase from *L. infantum* (Salvati *et al.* 2001).

Both Vp and C2 were inhibited by the peptide aldehydes leupeptin, antipain and chymostatin. The action of these inhibitors on the enzyme involves the interaction of the aldehyde group from the inhibitor with the thiolate group of the enzyme. This interaction forms a tetrahedral hemithioacetate (Otto and Schirmeister 1997). Since both Z-Phe-Arg-AMC and H-D-Val-Leu-Lys-AMC were good substrates for Vp, it was decided to test all the inhibitors in the presence of either substrate. Leupeptin seems to be the better inhibitor for Vp and C2 in the presence of Z-Phe-Arg-AMC and H-D-Val-Leu-Lys-AMC. This is evident from the low K_i values for leupeptin as compared to those obtained for chymostatin and antipain. This result is expected since the aldehyde group of leupeptin (N-acetyl-leucyl-leucyl-arginal is present on the Arg residue in position P_1 (the arginal group is essential for inhibition) (Umezawa 1976) and it was deduced from the synthetic substrate assays that Vp prefers an Arg residue in P_1. A comparable K_i value of 1.12×10^{-3} μM was obtained for the inhibition of papain with leupeptin (Schultz *et al.* 1989). Chymostatin seems to be a better inhibitor for Vp hydrolysis of Z-Phe-Arg-AMC than antipain, whereas there was no significant difference for C2. There were no comparable differences in the K_i values for the inhibition of Vp hydrolysis of H-D-Val-Leu-Lys-AMC, but chymostatin seems to be the better inhibitor for the hydrolysis of this substrate by C2. In most cases, chymostatin seems to be the better inhibitor which is not expected since, like leupeptin, antipain also has an aldehyde group on the Arg residue in position P_1 (Umezawa 1976) and the aldehyde group of chymostatin is present on the Phe residue in position P_1 (Burleigh *et al.* 1997). The metallo-peptidase inhibitor, EDTA, and the aspartic peptidase inhibitor, pepstatin A, did not inhibit Vp or C2. Studies have shown that both native and recombinant cysteine peptidases are also inhibited by these inhibitors; leupeptin, antipain and chymostatin. Native trypanopain from *T. b. brucei* (Troeberg et al. 1996), native cruzipain from *T. cruzi* (Cazzulo *et al.* 1990) are inhibited by leupeptin, antipain and chymostatin. Native congopain from *T. congolense* (Mbawa *et al.* 1992), native and recombinant falcipain-2 from *P. falciparum* (Shenai *et al.* 2000) and recombinant cruzipain from *T. cruzi* (Eakin et

al. 1992) were shown to be inhibited by leupeptin. A cathepsin L-like cysteine peptidase from *Fasciola hepatica* (Wijffels *et al.* 1994) was inhibited by leupeptin and antipain.

Both Vp and C2 were inactivated by the cysteine peptidase specific irreversible inhibitors; iodoacetic acid (IAA), iodoacetamide (IAN) and E-64. E-64 also inhibits the cysteine peptidases cathepsins B, H and L and papain (Barrett *et al.* 1982) and it is the *trans*-L-(*S*,*S*)-epoxysuccinic acid reactive group present in E-64 that is responsible for the inhibition (Otto and Schirmeister 1997). In this study, E-64 was used as the active-site titrant, which gave an indication of the amount of active enzyme present in both the Vp and C2 preparations. For both Vp and C2, 20% of the total peptidase concentration was enzymatically active. E-64 was also shown to inhibit native congopain from *T. congolense* (Authié *et al.* 1992; Mbawa *et al.* 1992; Chagas *et al.* 1997), native and recombinant falcipain-2 (Shenai *et al.* 2000), native and recombinant cruzipain (Cazzulo *et al.* 1990; Eakin *et al.* 1992) and a cathepsin L-like cysteine peptidase from *F. hepatica* (Wijffels *et al.* 1994).

Both IAA and IAN are sulfhydryl alkylating reagents. These inhibitors bind covalently to the active site Cys sulfhydryl group (Dickens 1933).The inhibition kinetic values obtained for IAA and IAN inhibition of the hydrolysis of either Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC by Vp and C2 were very similar. When comparing the action of IAA on Vp in the presence of either Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC, better inhibition seems to be in the presence of D-Val-Leu-Lys-AMC since a higher k_{ass} value was obtained and the $t_{1/2}$ value for this reaction is almost two times less than that of the reaction in the presence of Z-Phe-Arg-AMC. This means that it takes a shorter time for the free enzyme concentration to decrease by 50% in the presence of D-Val-Leu-Lys-AMC. A similar trend is evident for the reaction of IAN on Vp in the presence of either Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC, where the k_{ass} value in the presence of D-Val-Leu-Lys-AMC is higher and the $t_{1/2}$ value is almost two times less. For the action of IAA on C2 in the presence of either Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC very similar k_{ass} values are obtained; however, the $t_{1/2}$ value is almost one and half times less than in the presence of Z-Phe-Arg-AMC. The k_{ass}

values for the reaction of IAN on C2 in the presence of D-Val-Leu-Lys-AMC is higher than in the presence of Z-Phe-Arg-AMC and the $t_{1/2}$ value obtained in the presence of D-Val-Leu-Lys-AMC is almost two times less. Literature has shown that papain is also inhibited by IAA (Light *et al.* 1964; Husain and Lowe 1968) and IAN (Blumberg and Ogston 1957). IAA was also shown to inhibit the cathepsin L-like cysteine peptidase from *F. hepatica* (Wijffels *et al.* 1994). The low molecular weight irreversible serine peptidase inhibitors PMSF, TLCK and TPCK did not inhibit Vp or C2.

The pH optimum obtained for C2 of 6.5, is comparable to the value of pH 6 obtained from earlier studies for native congopain (Mbawa *et al.* 1992). The pH optimum obtained for Vp was 7 and almost no activity remained at pH 4, which is not expected since it is a lysosomal peptidase. The pH optimum obtained for native trypanopain from *T. b. brucei* was pH 5.7 (Troeberg *et al.* 1996), and similar near basic pH optima were reported for native and recombinant falcipain-2 (pH 6 and pH 5, respectively) (Shenai *et al.* 2000), native cruzipain (pH 6) (Serveau *et al.* 1996), a cathepsin L-like cysteine peptidase from *F. hepatica* (pH 7.5) (Wijffels *et al.* 1994), mammalian cathepsin L (pH 6) (Brömme *et al.* 1989) and for papain (pH 7.0) (Wijffels *et al.* 1994). Therefore, these cysteine peptidases could possibly be contributing to the pathology of the diseases due to their pH optima being so close to physiological pH.

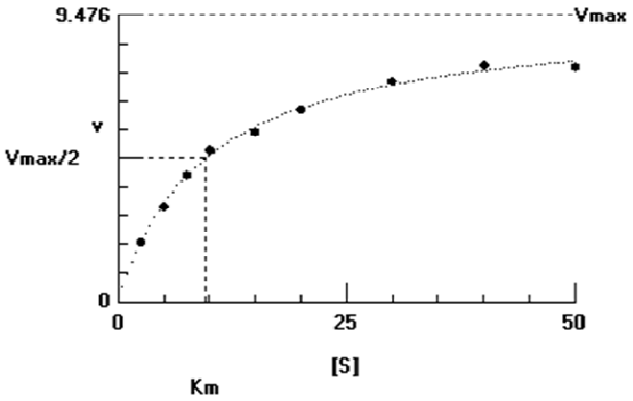
In this study, Vp was successfully expressed in the *P. pastoris* yeast expression system and purified using TPP and MEC. Substrate assays revealed that Vp preferred the hydrophobic amino acid residues Phe and Leu in P_2 and a smaller hydrophobic residue in P_3. Only when P_2 is occupied by Phe or Leu, a basic residue is preferred in P_1. Vp was also reversibly inhibited by leupeptin, chymostatin and antipain and irreversibly inhibited by IAA and IAN in the presence of Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC. Vp showed an optimum for catalytic activity at pH 7.0. The information required for the synthesis of synthetic peptide inhibitors lies in understanding which amino acid residues are preferred in P_1, P_2 etc. Therefore, future work would be based on using the information obtained from this study to construct synthetic peptide inhibitors with chemical groups, such as vinyl sulfones and the

peptidyl ketones attached and testing the effects of these irreversible inhibitors on Vp and *T.vivax* grown in culture.

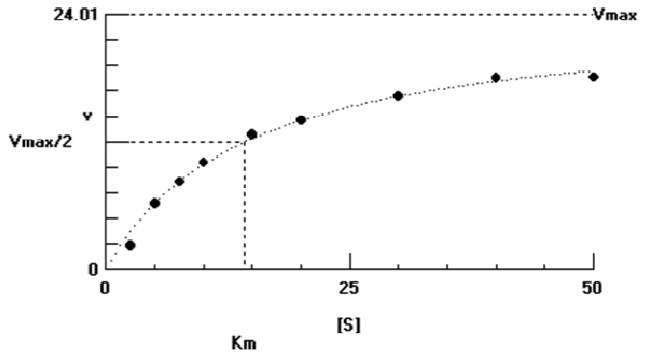
Appendix 1: Michaelis-Menten curves for Vp

The plots generated by the Hyper® 32 software program appears to give an incorrect V_{max}, however the V_{max} value was verified by the GraphPad Prism® softeware program aswell. The Hyper® 32 software program does not allow for the addition of units to the axes. The units of v is $\mu M.s^{-1}$ and of [S] is µM

Z-Phe-Arg-AMC



H-D-Val-Leu-Lys-AMC



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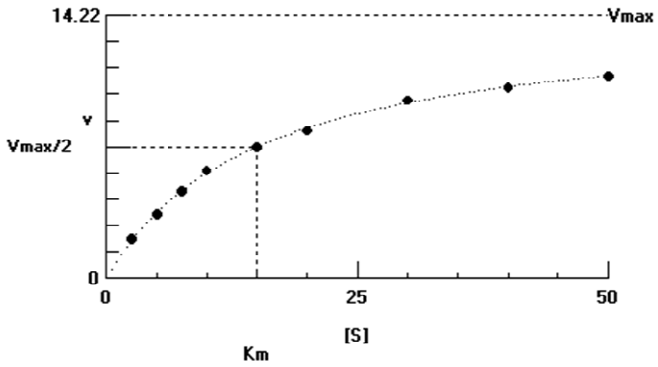
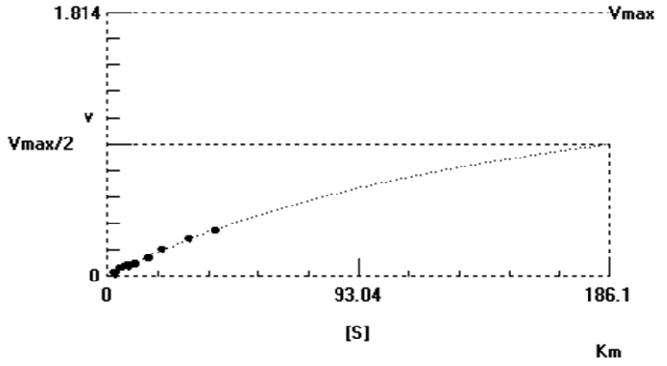


Suc-Leu-Tyr-AMC



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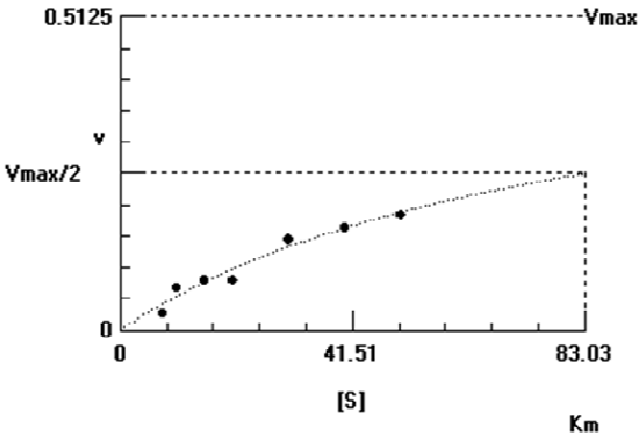

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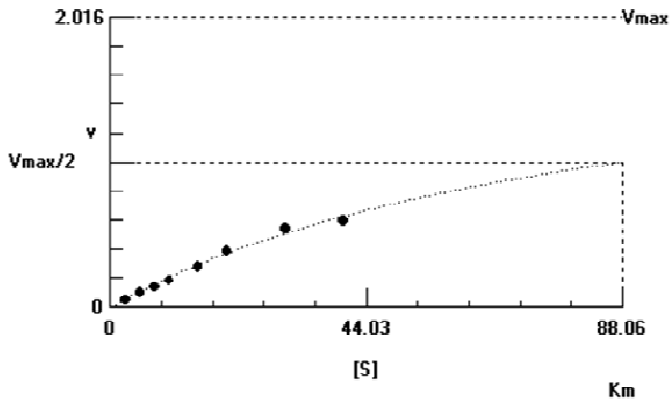
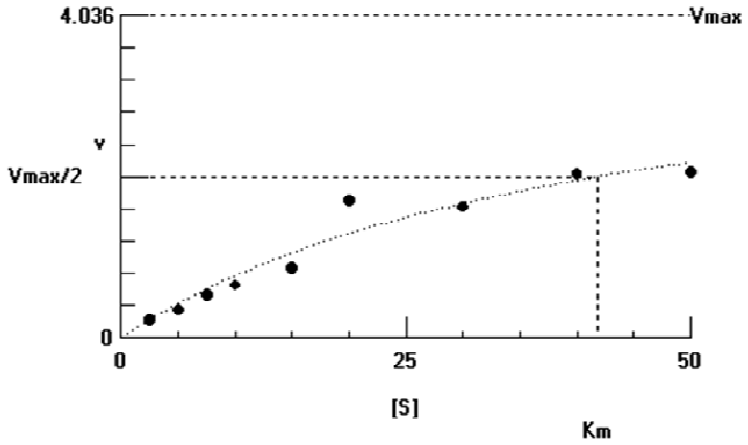


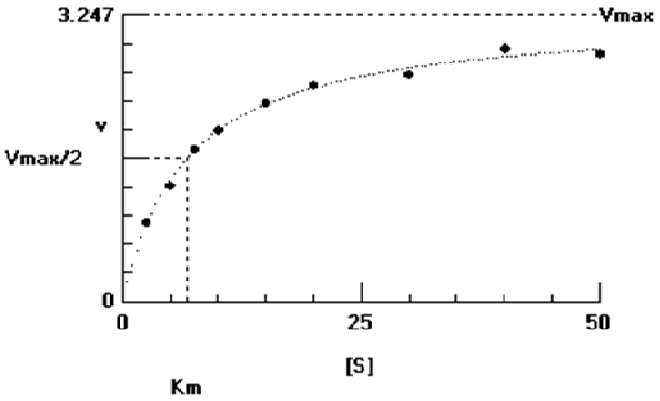
H-Pro-Phe-Arg-AMC



Appendix 2: Michaelis-Menten curves for C2

The plots generated by the Hyper® 32 software program appears to give an incorrect V_{max}, however the V_{max} value was verified by the GraphPad Prism® softeware program aswell. The Hyper® 32 software program does not allow for the addition of units to the axes. The units of v is $\mu M.s^{-1}$ and of [S] is µM

Z-Phe-Arg-AMC



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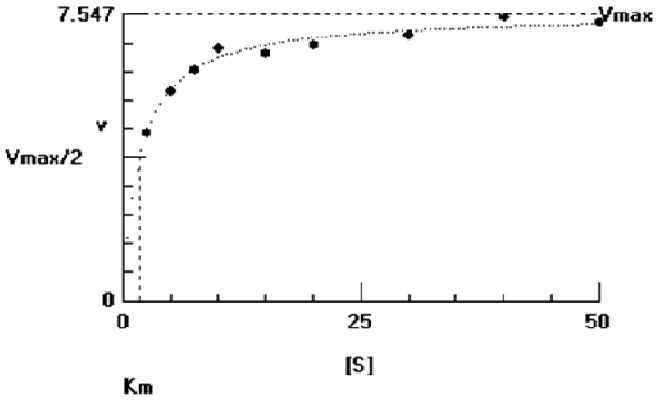

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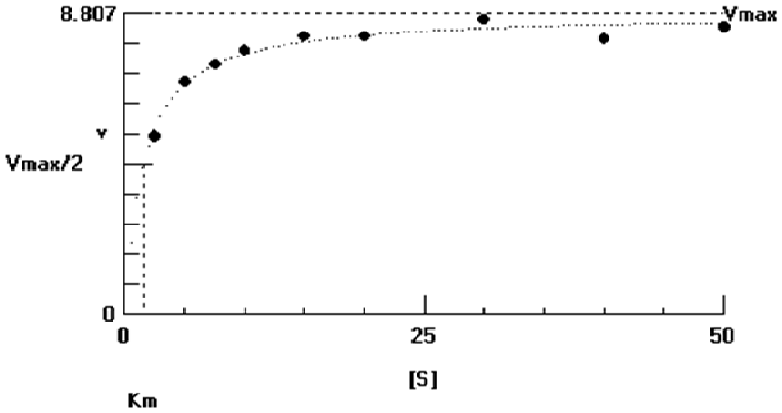
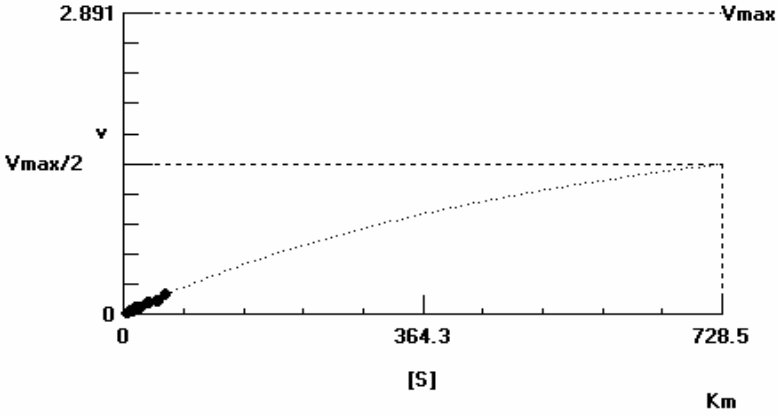
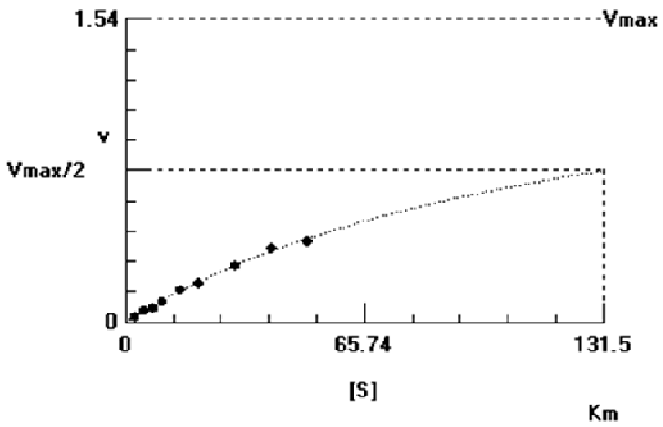


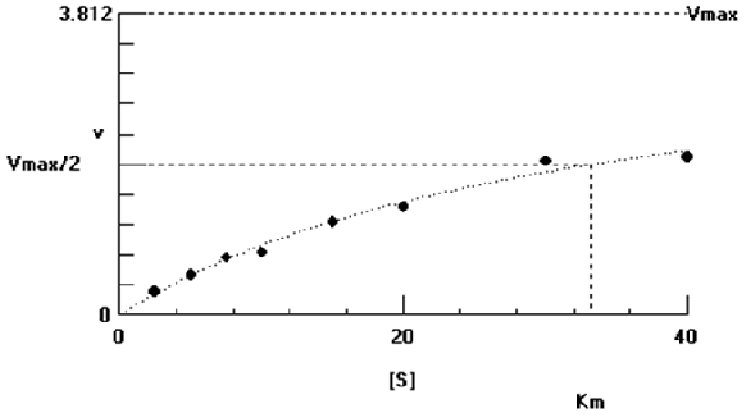
Suc-Leu-Tyr-AMC



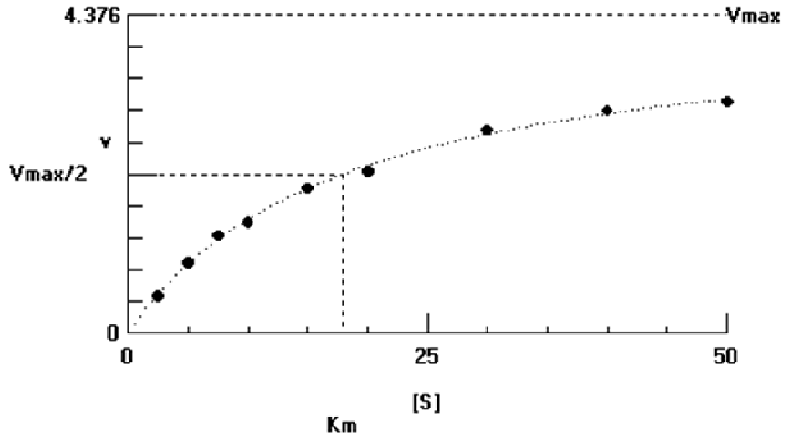
Suc-Ala-Phe-Lys-AMC



H-Ala-Phe-Lys-AMC



H-Pro-Phe-Arg-AMC



3. RECOMBINANT EXPRESSION OF THE FULL LENGTH INACTIVE MUTANT FORM OF VIVAPAIN (ΔFLVp)

P. Vather[1], A. F. V. Boulangé[1, 2] and T. H. T. Coetzer[1].

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

ABSTRACT

The use of many diagnostic tests based on clinical, parasitological, molecular and serological techniques has made it possible to diagnose trypanosomosis, which allows for the proper treatment of the disease. One of the key diagnostic tests is the serological enzyme-linked immunosorbent assay (ELISA). This test can be based on antibody detection or on antigen detection. In both cases, the use of a recombinant antigen is required for the tests. There is no antigen available to detect *T. vivax* infections like there are for *T. congolense* and *T. brucei* infections. In this study, a mutant full length inactive form of vivapain (ΔFLVp) was recombinantly expressed for potential use as an antigen for diagnostic tests. This antigen contains the Ala^{25}, His^{159} and Asn^{175} (papain numbering) in the active site instead of the conventional Cys^{25}, His^{159} and Asn^{175} (papain numbering) rendering it inactive. The ΔFLVp was cloned into pTZ57R/T vector and sub-cloned into the pPic9 yeast and pET-32a yeast expression vectors. The ΔFLVp was only able to be expressed in a bacterial system as a 70 kDa protein. Attempts were made to renature the protein. Antibodies against this antigen were produced in both chickens and mice. These antibodies were able to detect the recombinant ΔFLVp in western blots. The mice serum was able to detect native vivapain in a *T. vivax* lysate in a western blot. This finding suggests that this antigen could possibly be useful in field diagnosis of *T. vivax* infections especially in its use in ELISAs.

3.1 INTRODUCTION

Trypanosoma vivax is the major causative agent of trypanosomosis in African livestock (Lalmanach *et al.* 2002). Control and therapeutic methods for this disease are required due

to the negative impact it has on the economy of sub-Saharan Africa (Antoine-Moussiaux *et al.* 2009). The diagnosis of trypanosomosis is one of the areas that can provide sufficient and accurate information of the disease to allow for appropriate therapeutic measures to be taken (Mattioli *et al.* 2001; Eisler *et al.* 2004). A range of diagnostic tests that are being used, include, clinical, parasitological, molecular and serological tests. Diagnostic techniques used should be appropriate for use in the field and for the level of technology available in developing countries (Luckins 1992; Eisler *et al.* 2004).

The clinical diagnostic technique is dependent on the physical examination of cattle. The major problem with this technique is that the clinical signs are not specific for trypanosomosis (Nantulya 1990). The method involved in parasitological techniques are viewing of wet blood films and Giemsa-stained thick and thin fixed blood films with a light microscope. This technique varies in sensitivity due to the trypanosome species' similarities in morphology (Magona *et al.* 2003). For this reason, a haematocrit centrifuge can be used to increase the sensitivity by separating the buffy coat containing the trypanosomes from the red blood cells and plasma. The disadvantage of using this technique especially in the field is that fresh blood, a haematocrit centrifuge and microscopes are needed (Luckins 1992; Eisler *et al.* 2004). Polymerase chain reaction (PCR) is the molecular biology method used for the diagnosis of bovine trypanosomosis. PCR has shown to be a very sensitive method and it has the ability of species-specific detection of the trypanosomes (Solano *et al.* 1999; Desquesnes and Davila 2002). The problem of using this technique in the field is that it is very expensive since it involves the use of specialised equipment by an experienced technician.

The most promising techniques, especially for diagnosing *T. vivax* infections, are the serological techniques; more importantly, the enzyme-linked immunosorbent assay (ELISA) (Osório *et al.* 2008). The advantage of the ELISA technique is that it can be adapted to field use, requiring only a simple ELISA plate reader. This technique also allows for the processing of a large number of samples due to automation of the equipment. ELISA is also a very sensitive technique (Hopkins *et al.* 1998).

There are two types of ELISAs that can be used for the diagnosis; namely, the antibody-detection ELISA (Hopkins *et al.* 1998; Rebeski *et al.* 1999) and the antigen-detection ELISA (Nantulya and Lindqvist 1989). The absence of recombinant trypanosome antigens for the detection and production of anti-trypanosome antibodies is a major problem for both the antibody-detection ELISA and the antigen-detection ELISA. Thus far, ELISAs for both antibody and antigen detection have relied on crude preparation of whole parasite lysate and native antigens acquired from trypanosome culture; therefore, it is difficult to optimise and standardise the tests and a large volume of cultured trypanosomes is required (Greiner *et al.* 1997; Eisler *et al.* 2004; Tran *et al.* 2009). Due to this problem work increased on the identification and recombinant expression of antigens that showed promise for the detection of *T. brucei* (Tran *et al*. 2008), *T. congolense* (Davita 2009, MSc) and *T. evansi* (Verloo *et al.* 2001; Lejon *et al.* 2005; Tran *et al.* 2009) infections in the sera of infected animal species. There is no suitable antigen for the specific diagnosis of *T. vivax* infections (European Commission 6th Framework Research grant, Trypadvac2). For this reason, this study focused on producing a recombinant antigen that could potentially be used to detect *T. vivax* infections. This particular antigen was based on the *T. vivax* cysteine peptidase, vivapain. Due to high cross-reactivity observed between the trypanosome species in ELISA it is important to test infected sera using species-specific antigens; therefore, the production of this recombinant antigen specific to detecting *T. vivax* infections could potentially be used in conjunction with the existing recombinant antigens, such as the full length inactive mutant of congopain, to increase the sensitivity of the ELISA tests. Thus far the full length inactive mutant of congopain showed promise as a species-specific antigen for *T. congolense* detection (Dr Phillippe Büscher, Department of parasitology, Institute of Tropical Medicine, Antwerp, Belgium).

In the present study, the full length inactive mutant form of vivapain, ΔFLVp, was cloned into pTZ57R/T vector and sub-cloned into bacterial (pET-32a) and yeast (pPic9) expression vectors. This was followed by recombinant expression of ΔFLVp in both bacterial and yeast systems. Antibodies were produced against the bacterial expressed recombinant ΔFLVp, in both chickens and mice. The antibodies were tested in western blots to

determine if they would be able to detect the recombinant ΔFLVp used to produce the antibodies as well as native vivapain in *T. vivax* blood stream form lysates.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Molecular Biology: EcoRI, NotI, SalI, SacI [reference for nomenclature see, (Roberts *et al.* 2003)] shrimp alkaline phosphatase (SAP), T4 DNA ligase, 10 mM dNTPs mix, X-gal, IPTG, pTZ57R/T vector, DNA high and middle range molecular marker mix, Fermentas Spectra™ multicolour Broad range protein ladder, GeneJetTM Plasmid Miniprep Kit and TranformAid™ Bacterial Transformation Kit were obtained from Fermentas (Vilnius, Lithuania). BsgI and competent *Escherichia coli* JM 109 cells were obtained from New England Biolabs (Ipawitch, MA, USA). Taq polymerase, 10 x PCR reaction buffer and $MgCl_2$ were from Solis Biodyne (Tartu, Estonia). The DNA clean and concentrator kit was purchased from ZymoResearch (Orange, CA, USA). The E.Z.N.A® gel extraction kit was purchased from PreQLab (Erlangen, Germany). The *Pichia pastoris* yeast strain (GS 115) and the pPic9 vector were obtained from were purchased from Invitrogen (Carlsband, CA, USA). pET-32a expression vector and the BL21 (DE3) electrocompetent cells were purchased from Novagen (Madison, WI, USA).

***P. pastoris* expression:** *E. coli* glycerol stock of the inactive mutant catalytic domain of vivapain fragment (ΔCAVp) in pPic9 was obtained from Professor Theo Baltz (University of Victor-Segalen, Bordeaux, France). *E. coli* mutated C2 clone (H43W) in pPIC9 was obtained from Hlumani Ndlovu 2009, MSc (University of KwaZulu-Natal).

Purification: His-select nickel affinity resin were obtained from Sigma (Munich, Germany). DEAE resin was purchased from Whatman International (England).

Antibodies: IgY anti-congopain-N-terminus peptide antibody (affinity purified, 50 μg/mL) was raised against the 22 N-terminal residues of congopain (Mkhize, 2003)**.** Horse anti-mouse IgG Horse radish peroxidase (HRPO) conjugate was obtained from Southern Cross

Biotechnology, Vector Labs. Rabbit anti-IgY coupled to HRPO, Freund's complete and incomplete adjuvants were obtained from Sigma (Munich, Germany). Nunc-ImmunoTM 96-well plates were from Nunc Intermed (Denmark).

3.2.2 Isolation of *Trypanosoma vivax* from infected mouse blood

Two mice were infected with *T. vivax* Y486 stabilate (40 µl per mouse). The mice were bled on the third day post infection when parasitaemia peaked. Approval for procedures involving animals was obtained from the University of KwaZulu-Natal animal ethics committee, reference number 036/09/Animal. Half of the blood was used to make new stabilate and the other half was used to obtain the *T. vivax* parasites by purification according to Lanham and Godfrey (1970). Briefly, 5 g DEAE-50 resin was suspended in 20 mL phosphate buffered saline (PBS) [100 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl and 137 mM NaCl, pH 8]. The resin was allowed to settle for approximately 30 min and the PBS removed. This step was performed three times to equilibrate the resin. The resin slurry was poured into the column (30 x 120 mm) (usually for 1 mL of mouse blood, 4 mL of packed resin is required) and the resin was washed with one column volume of PBS. Thereafter, the resin was equilibrated with one column volume of phosphate saline glucose (PSG) [57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 42 mM NaCl, 50 mM glucose and 1 mM hypoxanthine]. The heparinised blood was added to the column. The column tap was opened, which allowed for the blood to settle, which took approximately 10 min. After a further 10 min, a drop of eluate was placed on a slide and analysed under a microscope. Once parasites were present in the eluate, collection of the *T. vivax* parasites occurred for approximately three column volumes or until no parasites were present when the elution drops were viewed under the microscope. Care had to be taken to ensure that the column did not run dry. This was done by continually adding PSG to the column (approximately 10 mL at a time). The parasites were collected from the eluates by centrifugation (3000 *g*, 10 min, RT). The pellets were resuspended in 70% (v/v) PSG and 30% (v/v) glycerol and stored at -80^{o}C until further use.

3.2.3 Extraction of *T. vivax* genomic DNA

The extraction of *T. vivax* genomic DNA was performed according to the protocol from Medina-Acosta and Cross (1993). The parasites isolated as described in Section 3.2.2, were resuspended in PBS (1 mL) and centrifuged (1000 *g*, 10 min, RT). The supernatant was removed and the pellet was dissolved in 150 µl TELT [50 mM Tris-HCl buffer, pH 8, 62.5 mM EDTA, pH 9, 2.5 M LiCl, 4% (v/v) TritonX-100]. This solution was incubated at RT for 5 min. Thereafter, 150 µl of phenol-chloroform [1:1 (v/v)] was added. The mixture was placed on a rocker for 5 min at RT. The two phases were separated by centrifugation (13000 *g*, 5 min, RT). The top phase was retained and to this 300 µl of absolute ethanol was added. This solution was centrifuged (13000 *g*, 5 min, RT) and the supernatant was poured out and 1 mL of absolute ethanol was added, followed by centrifugation (13000 *g*, 5 min, RT). Once again, the supernatant was removed and the pellet was incubated at 37^{o}C for 10-15 min. Thereafter, 100 µl TE buffer [100 mM Tris-HCl buffer, pH 7.5, 10 mM EDTA] was added as well as RNAse to a final of 2 mg/mL and incubated at 37^{o}C for 45 min. The genomic DNA was stored at -20^{o}C until further use. The extraction of the DNA was analysed by electrophoresis on a 1% (w/v) agarose gel in 1 x Tris-acetate-EDTA (TAE) buffer [40 mM Tris-HCl buffer pH 7.4, 20 mM glacial acetic acid and 0.1 mM EDTA] at 80 V for 30-40 minutes.

3.2.4 Amplification of the *FLVp* gene from *T. vivax* genomic DNA

The *T. vivax* genomic DNA (1 in 10 dilution) was used as the template for the amplification of the *FLVp* gene using specific full length vivapain primers (Table 3.1). The primers were designed to introduce EcoRI/NotI cloning sites at each extremity of the amplicon. EcoRI/NotI nomenclature according to Roberts *et al* (2003). The master mix for the PCR reaction contained FLVp Fw and Rv primers [0.25 µM for each], 1x PCR buffer, 2.5 mM $MgCl_2$, 0.25 U Taq and 0.5 mM dNTPs in a total reaction volume of 25 µl. The amplification method used is known as touch-down-PCR, which was carried out by denaturing the genomic DNA at 95^{o}C for 5 min, followed by 25 cycles of denaturing the DNA at 95^{o}C for 1 min, primers annealed at 55^{o}C for 1 min, decreasing by 1^{o}C after every cycle until the temperature reached 45^{o}C, elongation at 72^{o}C for 2 min and finally an

elongation at 72°C for 7 min. The PCR product was analysed by electrophoresis on a 1% (w/v) agarose gel in 1 x TAE buffer at 80 V for 30-40 minutes.

Table 3.1 Primers used for the amplification, cloning and screening of the *FLVp* and *ΔFLVp* genes. The full length primers were used to amplify the *FLVp* gene from *T. vivax* genomic DNA. The C-term primers were used to confirm the presence of the *FLVp* gene in the pTZ57R/T vector (T vector). The restriction sites for EcoRI and NotI are highlighted in bold.

Primer	Sequence	Tm (°C)
Full length (FL) Vp Fw	5'-AAA **GAA TTC** TAC ATG GCG GTG CTG CGC GCG GAG-3'	67
Full length (FL) Vp Rv	5'-AAA **GCG GCC GC**C CTA CCC TCG ATG TTG TGC ATC GCA GGG CGG CG-3'	78
C-terminal (C-term) extension Fw	5'-AAA GAA TTC TAC ATG ACA ACA ACA ACA ACA ACA AC-3'	59

3.2.5 Cloning of the full length vivapain (*FLVp)* gene into the pTZ57R/T vector

The PCR product (1.3 kb) from the amplification of the *FLVp* gene was cut out of the agarose gel and purified using the E.Z.N.A gel extraction kit (PreQLab). The DNA was eluted in elution buffer (30 µl). The purified DNA was ligated into the pTZ57R/T vector (T vector) (Fig 3.1) in a 3:0.25 ratio in the presence of 10 x ligation buffer and T4 DNA ligase overnight at 4°C. The ligation mix (2.5 µl) was transformed into competent *E. coli* JM 109 cells using the TransformAid™ bacteria transformation kit. The transformed cells (50 µl) were plated onto pre-warmed 2x YT plates [1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 15g/l bacteriological agar] containing ampicillin (100 µg/mL) and incubated at 37°C overnight. Prior to plating of the cells, X-gal (20 µg/mL) and IPTG (10 µg/mL) were spread on the 2x YT plate and incubated at 37°C for 30 min. This was performed to allow for blue and white colony selection.

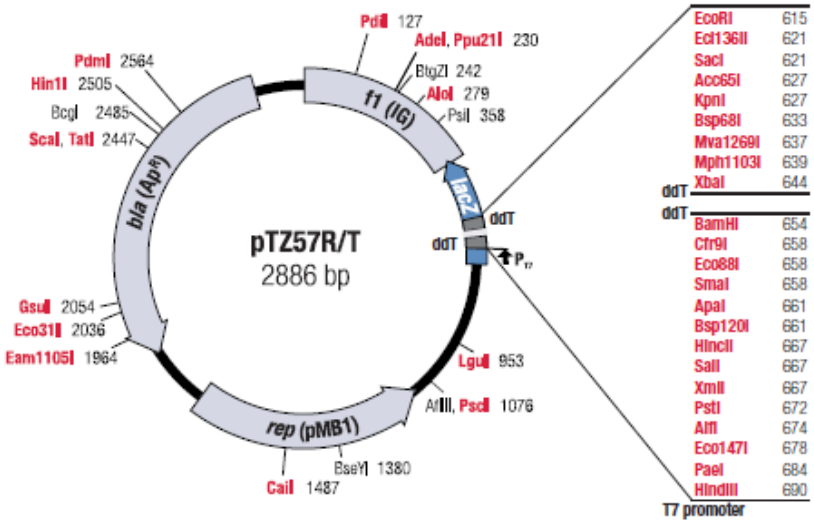


Figure 3.1 Map of the pTZ57R/T vector (Fermentas). This vector has a multiple cloning site, f1 origin of replication [*f1*(IG)], ampicillin resistance gene [*bla* (Ap^R)] used for the selection of recombinants and a *lacZ* gene which allows for blue and white colony screening.

Transformation revealed the presence of many more white colonies than blue colonies. The white colonies were screened by colony PCR using C-term Fw and FLVp Rv primers (Table 3.1). The colony PCR amplification method was carried out by denaturing the genomic DNA at 95^{o}C for 5 min, followed by 25 cycles of denaturing the DNA at 95^{o}C for 1 min, primer annealing at 55^{o}C for 1 min, elongation at 72^{o}C for 2 min and finally an elongation at 72^{o}C for 7 min. The amplification products were analysed by electrophoresis on a 1% (w/v) agarose gel in 1 x TAE buffer at 80 V for 30-40 minutes. The positive clones were grown in 2x YT liquid media (5 mL) [1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl] containing ampicillin (100 µg/mL) overnight at 37^{o}C, with shaking. The plasmid DNA was isolated from the overnight cultures, using the GeneJet™ plasmid miniprep kit. DNA was eluted in elution buffer (50 µl). The plasmid DNA (20 µl) was sent for sequencing to the Segoli Ilri laboratory unit, International livestock research unit, Nairobi, Kenya.

3.2.6 Subcloning of the full length inactive mutant vivapain (*ΔFLVp*) gene into the pTZ57R/T vector

The *FLVp* gene was cloned into the T vector to determine whether a natural mutation would occur in the catalytic domain of the gene, for the production of a full length inactive

mutant. This mutant is required for diagnostic tests for *T. vivax.* Sequencing of the *FLVp* gene cloned into the T vector revealed that only one out of the six clones matched up to the vivapain reference sequence on the database (Appendix 1). This clone contained the catalytic triad Cys^{25}, His^{159} and Asn^{175} (papain numbering) in the active site; thus, representing the full length active form of vivapain. Therefore, a different approach was taken to obtain the ΔFLVp fragment. In order to obtain the ΔFLVp fragment the full length active vivapain fragment (FLVp) cloned into the T vector and an inactive mutant catalytic domain of vivapain fragment (ΔCAVp) that was in pPic9 [obtained from Professor Theo Baltz (University of Victor-Segalen, Bordeaux, France) were used. The ΔCAVp fragment had an alanine substituted for cysteine in the catalytic triad. Therefore, the amino acids in the active site of ΔCAVp are Ala^{25}, His^{159} and Asn^{175}. Upon inspection of the gene sequence around that coding for the catalytic residues, Cys^{25}, His^{159} and Asn^{175} (called CHN), in the FLVp clone and the Ala^{25}, His^{159} and Asn^{175} (called AHN) in the ΔCAVp showed that these domains were flanked by two BsgI restriction sites. The double cutter restriction enzyme, BsgI (New England Biolabs), was used to excise the "CHN" coding fragment from the *FLVp* gene and the "AHN" coding fragment from the ΔCAVp inactive mutant catalytic domain of vivapain in the pPic9 vector. This "AHN" coding fragment was ligated with the cut *FLVp* gene (now without the CHN coding fragment), which rendered the full length inactive mutant vivapain (*ΔFLVp*) gene in T vector (Fig. 3.2).

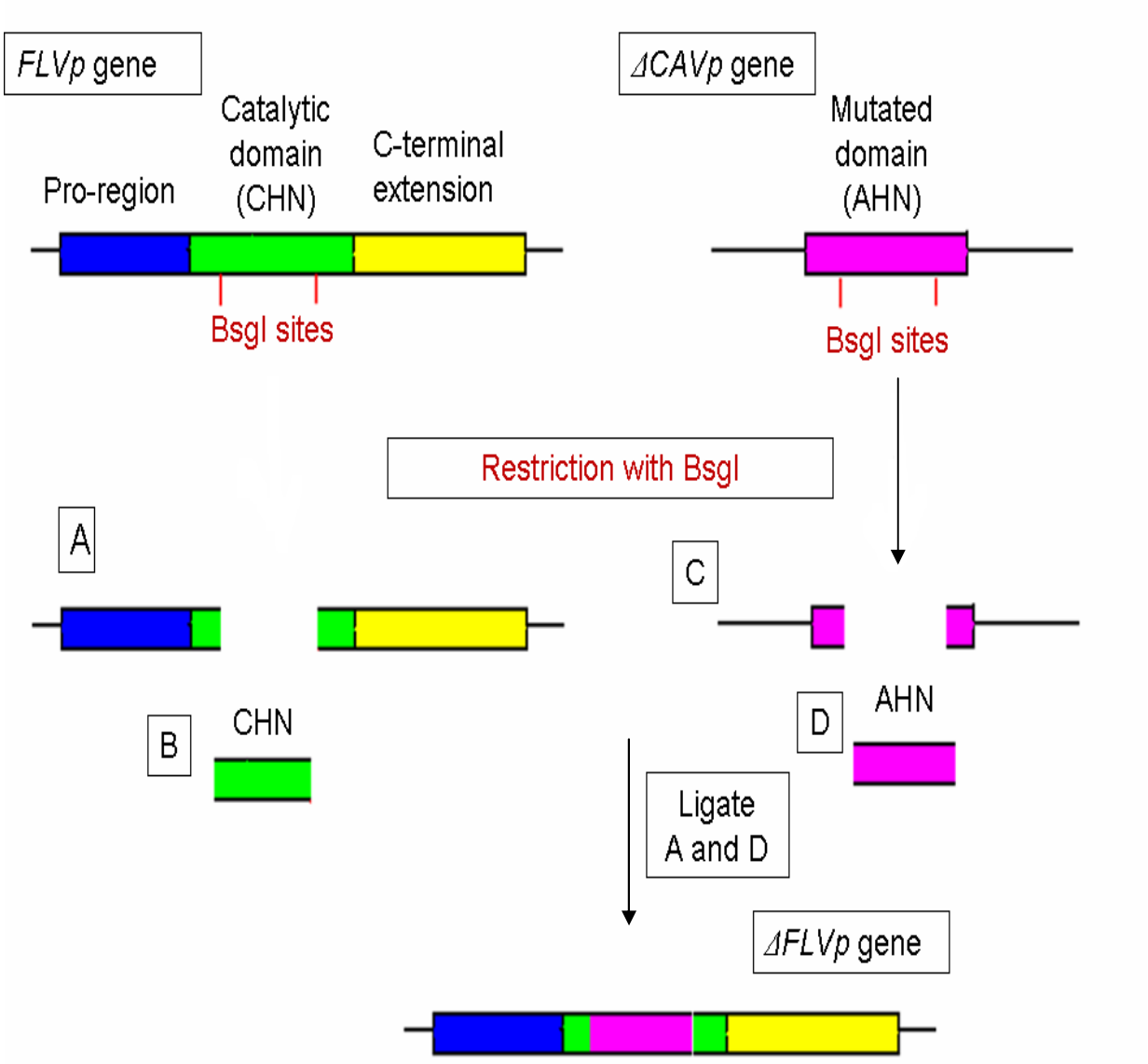


Figure 3.2 Schematic representation of the action of BsgI restriction enzyme on the FLVp gene in T vector and the ΔCAVp in pPic9. The restriction site for the BsgI restriction enzyme is 5'...GTGCAG(N)16↓...3' and 3'...CACGTC(N)14↓...5'.

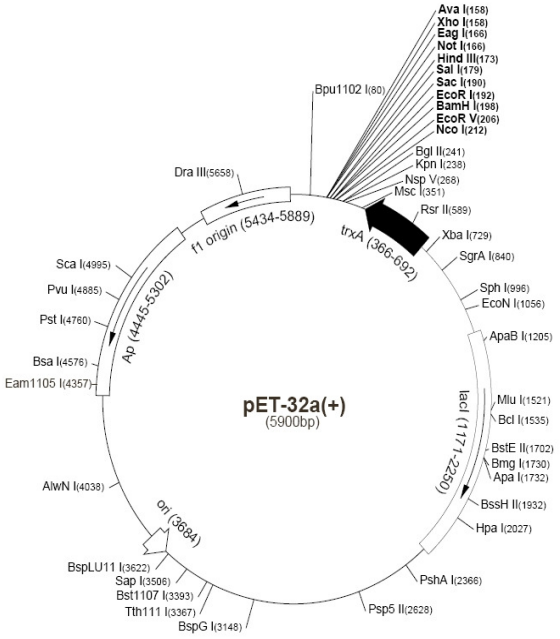
The plasmid DNA for the ΔCAVp in the pPic9 vector was obtained by streaking a glycerol stock of *E. coli* containing the pPic9-ΔCAVp on a 2x YT plate containing ampicillin (100 µg/mL). The plate was incubated overnight at 37°C. A single colony was grown in 2x YT liquid media, overnight at 37°C, with shaking. The plasmid DNA was isolated from the overnight cultures using the GeneJet™ plasmid miniprep kit. DNA was eluted in 50 µl elution buffer. The plasmid DNA for the ΔFLVp in the T vector was obtained from the remaining sample isolated for sequencing (Section 3.2.5). Both the plasmid DNA for FLVp in the T vector and pPic9-ΔCAVp were restricted with BsgI (50 U). The resulting restriction revealed that the "CHN" coding fragment from FLVp and the "ANH" coding fragment for ΔCAVp were cut out (Fig. 3.2). Thereafter, the T vector without the "CHN" coding fragment was ligated to the "AHN" fragment in a 0.25:3 ratio in the presence of 10 x ligation buffer and T4 DNA ligase overnight at 4°C. This ligation resulted in the ΔFLVp in the T vector. The ligation mix (2.5 µl) was transformed into competent *E. coli* JM 109 cells using the TransformAid™ bacteria transformation kit. The transformed cells were plated on pre-warmed 2x YT plates containing ampicillin (100 µg/mL), X-gal (20 µg/mL) and IPTG (10 µg/mL). The plate was incubated overnight at 37°C. Colonies were screened for recombinants by colony PCR with the C-term Fw and FLVp Rv primers (Table 3.1). The colony PCR amplification method was carried out by denaturing the genomic DNA at 95°C for 5 min, followed by 25 cycles of denaturing the DNA at 95°C for 1 min, primer annealing at 55°C for 1 min, elongation at 72°C for 2 min and finally an elongation at 72°C for 7 min. The results from the amplification were analysed by electrophoresis on a 1% (w/v) agarose gel in 1 x TAE at 80 V for 30-40 minutes. Positive clones were grown in 2x YT liquid media (5 mL) containing ampicillin (100 µg/mL) overnight at 37°C, with shaking. The plasmid DNA was isolated from the overnight cultures, using the GeneJet™ plasmid miniprep kit. DNA was eluted in elution buffer (50 µl). In order to verify the presence of the "AHN" fragment and the entire *ΔFLVp* gene in the T vector, duplicates of the plasmid DNA from a positive clone was restricted with either BsgI or with EcoRI and NotI. Thereafter, both clones were sequenced Segoli Ilri laboratory unit, International livestock research unit, Nairobi, Kenya.

3.2.7 Subcloning of the ΔFLVp construct into bacterial (pET-32a) and yeast (pPic9) expression vectors

The ΔFLVp clone containing the "AHN" fragment was cloned into expression vectors. The pET-32a and pPic9 expression vectors were prepared for transformation in the same manner. Briefly, the vector glycerol stock was streaked on a 2x YT plate containing ampicillin (100 µg/mL). The plate was incubated overnight at 37°C. A single colony was grown in 2x YT liquid media, overnight at 37°C, with shaking. The GeneJet™ plasmid miniprep kit was used to isolate the plasmid DNA. DNA was eluted in elution buffer (50 µl). The plasmid DNA was restricted with EcoRI and NotI, which generated a linear plasmid and treated with SAP (1 U) to dephosphorylate the exposed ends of the plasmid. The plasmid DNA was concentrated using the ZymoResearch® DNA clean and concentrator kit and eluted in 30 µl of elution buffer. The ΔFLVp-T vector plasmid DNA was restricted with EcoRI and NotI in order to remove the ΔFLVp insert. This restriction was analysed by electrophoresis on a 1% (w/v) agarose gel in 1 x TAE buffer at 80 V for 30-40 minutes. The ΔFLVp insert was cut out of the gel and purified using the E.Z.N.A gel extraction kit (PreQLab). The purified DNA was eluted in 30 µl of elution buffer. Both the purified pET-32a vector (EcoRI/NotI) (1 µl) and the ΔFLVp insert (EcoRI/NotI) (1 µl) were analysed on 1% (w/v) agarose gel in 1 x TAE buffer at 80 V for 30-40 minutes. This was done to determine the relative quantities of vector and insert. This was also performed with the purified pPic9 vector (EcoRI/NotI) (1 µl) and the ΔFLVp insert (EcoRI/NotI). The ligation of the ΔFLVp into pET-32a (Fig. 3.3 A) and pPic9 (Fig. 3.3 B) were performed in a 3:1 ratio into 10 x ligation buffer and T4 DNA ligase overnight at 4°C. The pET-32a-ΔFLVp and pPic9-ΔFLVp ligation mixes (2.5 µl) were transformed into competent *E. coli* JM 109 cells. This was performed using the TransformAid™ bacteria transformation kit. The transformed cells (50 µl) were plated onto pre-warmed 2x YT plates containing ampicillin (100 µg/mL) and incubated at 37°C overnight. The colonies for the pET-32a-ΔFLVp transformation were screened using the C-term Fw and pET Rv primers and the pET Fw and Rv primers for the control (Table 3.2). The template for the control was the pET-32a-ΔFLVp plasmid DNA. The colonies for the pPic9-ΔFLVp were

screened using the C-term Fw and FLVp Rv primers (Table 3.2). The amplification conditions for both colony PCR reactions are represented in Table 3.3.

A



B

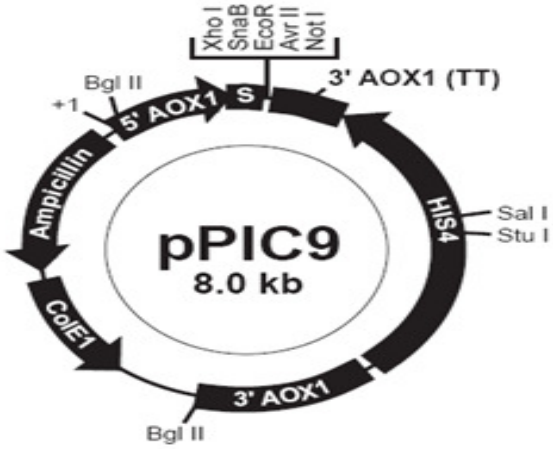


Figure 3.3 Expression vector maps of A) pET-32a (5.9kb) (Novagen) and B) pPic 9 (8kb) (Invitrogen). A) The vector contains a multiple cloning site, f1 origin of replication, ampicillin resistance gene (Ap), *lacI* coding sequence (lacI) and a T7 promoter and transcription start. B) The vector contains the following features which assist in the expression process: ColE1 origin of replication (pBR322), 3' AOX fragment (3' AOX), a *HIS4* gene, which is used for selection by complementation in the his4 yeast strain, *c-myc* epitope (TT), multiple cloning sites, α-factor secretion signal (S), 5' AOX fragment (5' AOX) and an ampicillin resistance gene.

Positive colonies from each transformation, ΔFLVp into pET-32a and ΔFLVp into pPic9, were grown in 2x YT liquid media, overnight at 37^{o}C, with shaking. The plasmid DNA of pET-32a-ΔFLVp and pPic9-ΔFLVp were obtained using the GeneJet™ plasmid miniprep kit. The DNA was eluted in 30 µl of elution buffer. Both the plasmid DNA for pET-32a-ΔFLVp (10 µl) and pPic9-ΔFLVp (10 µl) were restricted with EcoRI/NotI to verify the presence of the ΔFLVp insert. The result of this restriction was analysed on a 1% (w/v) agarose gel in 1 x TAE buffer at 80 V for 30-40 minutes.

Table 3.2 Primers used for the amplification, cloning and screening of the *ΔFLVp* gene. The C-term primers were used to confirm the presence of the *ΔFLVp* gene in pPic9 after transformation into the *E. coli* JM 109 cells. The C-term Fw and pET Rv primers were used to confirm the presence of the *ΔFLVp* gene in pET-32a after transformation into the *E. coli* JM 109 cells. The C-term Fw and AOX Rv primers were used to confirm the presence of the *ΔFLVp* gene in pPic9 after the transformation into the *Pichia pastoris* GS115 yeast cells. The AOX Fw and AOX Rv primers were used to confirm the presence of the *C2 (H43W)* gene in pPic9 after transformation into *P. pastoris* GS115 yeast cells.

Primer	Sequence	Tm (^{o}C)
Full length (FL) Vp Rv	5'-AAA GCG GCC GCC CTA CCC TCG ATG TTG TGC ATC GCA GGG CGG CG-3'	78
C-terminal (C-term) extension Fw	5'-AAA GAA TTC TAC ATG ACA ACA ACA ACA ACA ACA AC-3'	59
pET Fw	5'-TAATACGACTCACTATAGGG-3'	54
pET Rv	5'-GCTAGTTATTGCTCAGCGG-3'	57
AOX Fw	5'-ACTGGTTCCAATTGACAAGC-3'	50
AOX Rv	5'-GCAAATGGCATTCTGACATCC3'	52

Table 3.3 Colony PCR conditions for the screening of pET-32a and pPic9 colonies. The master mix for the PCR reaction contained Fw and Rv primers [0.25 µM for each], 1x PCR buffer, 2.5 mM $MgCl_2$, 0.25 U Taq and dNTPs [0.5 mM]. PCR reactions were subjected to 25 cycles.

	Colony PCR				
	C-term Fw/ pET Rv	pET Fw/ pET Rv	C-term Fw/ FL Vp Rv	AOX Fw/ AOX Rv	C-term Fw/ AOX Rv
Denaturation	95°C ; 1 min	95°C ; 1 min	95°C ; 1 min	95°C ; 1 min	95°C ; 1 min
Renaturation	55°C ; 1 min	55°C ; 1 min	55°C ; 1 min	55°C ; 1 min	55°C ; 1 min
Elongation	72°C ; 2 min	72°C ; 2 min	72°C ; 2 min	72°C ; 2 min	72°C ; 2 min
Reaction Volume (µl)	25	25	25	100	100

3.2.8 Expression of ΔFLVp in bacteria

The pET-32a-ΔFLVp plasmid DNA (1 µl) was combined with *E. coli* BL21 (DE3) electrocompetent cells. This mix was added to a gap vial and electroporated at 2.5 kV, 25 µF, 200 Ω (BioRad, Hercules, CA, USA). The cells were diluted in 2x YT liquid media, without ampicillin (100 µg/mL) and grown at 37°C for one hour, with shaking. The cells were pelleted by centrifugation (13000 *g*, 30-60 s). The supernatant was removed (approximately 800 µl) and the cells resuspended in the remainder of the supernatant and plated on pre-warmed 2x YT plates containing ampicillin (100 µg/mL) and incubated overnight at 37°C.

A single colony from the transformation of pET-32a-ΔFLVp in *E. coli* BL21 (DE3) cells was inoculated into terrific broth liquid media (800 µl) [1.2% (w/v) tryptone, 2.4 % (w/v) yeast extract, 0.4% (v/v) glycerol, 0.17 M KH_2PO_4, 0.72 M K_2HPO_4] containing ampicillin (100 µg/mL). A BL21 (DE3) non recombinant colony was also inoculated in terrific broth as a control. The glycerol in the media acts as a carbohydrate source. One of the advantages of using glycerol instead of glucose is that the glycerol does not ferment to acetic acid (Tartof and Hobbs 1987). The terrific broth cultures were incubated overnight at 37°C with shaking. Thereafter, the cells were harvested by centrifugation (5000 *g*, 10 min, 4°C). The

cell pellets were resuspended in TE buffer. The cells were pelleted once again by centrifugation (5000 *g*, 10 min, 4°C) and resuspended in lysis buffer [50 mM Tris-HCl buffer, pH 7.5, 100 mM NaCl, 1mM DTT and 5% (v/v) glycerol]. Peptidase inhibitors; leupeptin, E-64, AEBSF and EDTA were added with a final concentration 1 µM. Lastly, lysozyme at pH 8 was added to the lysate to lyse cells by degrading the bacterial cell wall. The lysate was stored overnight -20°C. The lysed cell pellet and supernatant were analysed on SDS-PAGE to determine if the ΔFLVp was expressed in the soluble fraction or as inclusion bodies.

3.2.9 Solubilisation of the bacterial-expressed ΔFLVp

The solubilisation of the recombinantly expressed ΔFLVp was performed according to Sijwali *et al.* (2001). The lysate was thawed and sonicated (8 x 15 s bursts) using a VirSonic 60 sonicator. This was followed by pelleting the lysed cells (inclusion bodies) by centrifugation (14000 *g*, 15 min, 4°C). The supernatant was discarded and the inclusion bodies were washed twice by a first wash buffer [2.0 M urea, 20 mM Tris-HCl buffer, pH 8, 2.5% (v/v) Triton X-100] and by a second wash buffer [20% sucrose and 20 mM Tris-HCl buffer, pH 8]. Following each wash, the inclusion bodies were pelleted by centrifugation (12000 *g*, 30 min, 4°C) and the supernatant was retained each time. The pelleted inclusion bodies were solubilised using solubilisation buffer [6 M guanidine-HCl, 20 mM Tris-HCl buffer, pH 8, 250 mM NaCl, 20 mM imidazole].

3.2.10 Purification of the bacterial-expressed ΔFLVp under denaturing conditions

Preparation of His-select nickel affinity resin

His-select nickel affinity resin (1 mL) was placed in a 15 mL conical centrifuge tube and centrifuged (800 *g*, 1 min, RT) to allow the resin to settle. The supernatant was removed and resin was resuspended by gentle tapping in 10 mL of sterile distilled water. The resin was allowed to settle by centrifugation (800 *g*, 1 min, RT). This step was performed twice and each time the supernatant was discarded. The resin was resuspended in 10 mL of the

solubilisation buffer (Section 3.2.9) and allowed to settle by centrifugation (800 *g*, 1 min, RT). This step was repeated with the removal of the supernatant after each step.

Purification

Two mL of the solubilised ΔFLVp (Section 3.2.9) was added to the prepared His-select nickel affinity column. The solubilised ΔFLVp was allowed to bind to the resin by end-over-end mixing for 1 h at RT. The resin was allowed to settle by centrifugation (800 *g*, 1 min, RT) and the supernatant was retained for analysis on an SDS-PAGE gel. Thereafter, the resin was washed with 10 mL of solubilisation buffer and settled by centrifugation (800 *g*, 1 min, RT). The resin was washed with a buffer containing 8 M urea, 20 mM Tris-HCl, pH 8, 0.5 M NaCl and allowed to settle by centrifugation (800 *g*, 1 min, RT). This was followed by washing the resin with a buffer containing 8 M urea, 20 mM Tris-HCl, pH 8, 0.03 M imidazole and the resin was allowed to settle by centrifugation (800 *g*, 1 min, RT). The resin was resuspended in 2 mL of elution buffer (8 M urea, 20 mM Tris-HCl, pH 8, 1 M imidazole) and transferred into a BioRad chromatography purification column. Fractions (100 µl) were collected and analysed on a reducing 12.5% SDS-PAGE gel. The supernatants from each wash step were also analysed on 12.5% SDS-PAGE gel. The resin was washed in 0.5 M NaOH (10 column volumes) and equilibrated in solubilisation buffer containing 20 mM imidazole and stored at 4^{o}C.

3.2.11 Renaturation of the bacterial-expressed ΔFLVp

The renaturation of the recombinantly expressed ΔFLVp was performed according to Sijwali *et al.* (2002). Any insoluble material from the solubilisation of ΔFLVp (Section 3.2.9) was removed by centrifugation (27000 *g*, 30 min, 4^{o}C). The solubilised ΔFLVp was diluted 100 fold with renaturation buffer [100 mM Tris-HCl buffer, pH8, 1 mM EDTA, 20% (v/v) glycerol, 1 M KCl, 1 mM GSH, 0.5 mM GSSG] added in small increments. The renaturation buffer and solubilised ΔFLVp were left for 20 h at 4^{o}C without stirring. Following renaturation the sample was concentrated down to 1/10th of the original volume using ultrafiltration (Amicon PM 30, 62 mm filter). Thereafter, the volume was brought back to the original volume with ice cold sterile distilled water. Once again, the sample was

concentrated but this time the volume was brought down to 1 mL. DTT (2.5 mM) was added to the concentrated sample before incubation at RT for 40 min with gentle stirring. The sample was stored at -20^{o}C until further use. The renatured samples were analysed by 12.5% SDS-PAGE gel as described in section 3.2.14. The samples were analysed by electrophoresis under reducing and non-reducing conditions.

3.2.12 Expression of ΔFLVp in yeast

Transformation of pPic9-ΔFLVp and pPic9-C2 (H43W) into Pichia pastoris GS115 cells.

An *E. coli* mutated C2 clone (H43W) in pPIC9 (Ndlovu 2009, MSc) was used as a positive control for transformation and expression of ΔFLVp in yeast. The transformation of pPic9-ΔFLVp and pPIC9-C2 (H43W) was based on the work done by Wu and Letchworth (2004). The recombinant vectors were transformed into methylotrophic *P. pastoris* GS 115 cells. A single GS 115 colony was grown in yeast extract peptone dextrose medium (YPD) (500 mL) [1% (w/v) yeast extract, 2% (w/v) peptone and 2% (w/v) dextrose (glucose)] at 30^{o}C overnight, with shaking until an OD_{600} of 2 was reached. The cells were pelleted by centrifugation (708 *g*, 20 min, 4^{o}C). The pelleted cells were resuspended in 400 mL of 100 mM lithium acetate, 10 mM DTT, 0.6 M sorbitol, 10 mM Tris-HCl buffer, pH 7.5 and incubated at RT for 30 min. This was followed by centrifugation (708 *g*, 20 min, 4^{o}C). The pelleted cells were resuspended in 45 mL of ice cold 1 M sorbitol and centrifuged as before. This step was repeated three times. The cells were finally resuspended in 1.5 mL of ice cold sorbitol, giving a final concentration of 10^{10} cells/mL. The pPic9-ΔFLVp and pPIC9-C2 (H43W) plasmid DNA (10 µl of each) was linearised with SalI and SacI, respectively. The linearised DNA (2 µl) was mixed with 200 µl of the GS 115 cells and transferred to gap vials. The linearised DNA was transformed into GS115 cells by electroporation at 1.5kV, 25µF, 200Ω using an electroporator (BioRad, Hercules, CA, USA). Immediately after electroporation, 1 mL of ice cold 1 M sorbitol was added to the cells. Cells were plated on minimal dextrose (MD) plate [1.34% (w/v) YNB, 0.00004% (w/v) biotin, 2% (w/v) dextrose (glucose), 15g/l bacteriological agar] and grown for four days at 30^{o}C. Colony PCR was performed using the AOX primers for the pPIC9-C2 (H43W) transformation and C-term Fw and AOX Rv primers for pPic9-ΔFLVp transformation

(Table 3.2). This was performed in order to confirm the presence of the foreign DNA in the *P. pastoris* genome. The AOX Fw and Rv primers could not be used for the pPic9-ΔFLVp transformation since linearisation with SalI directs insertion of the DNA into the *P. pastoris* genome at the *his4* gene; therefore, the insert will not be between the Fw and Rv AOX priming sites.

Small Scale Expression of ΔFLVp

The expression of both ΔFLVp and C2 (H43W) were performed using the same method described below. A single colony from the MD plate was inoculated into 10 mL YPD liquid media containing ampicillin (100 µg/mL) to prevent contamination of the culture. The YPD culture was placed in 250 mL baffled flasks and grown at 30°C in an orbital shaking incubator for two to three days. The culture was transferred into a 1 L baffled flask and the culture volume was made up to 100 mL with buffered complex glycerol media (BMGY) [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6.5, and 1.34% (w/v) yeast nitrogen base (YNB)]. The culture was grown for a further two days at 30°C in a shaking incubator (until an OD_{600} of 2-6 was reached). The BMGY media was used to increase the biomass of cells as it allows for better growth of the cells. The cells were harvested by centrifugation (2000 *g*, 10 min, 4°C) and resuspended in 25 mL of buffered minimal media (BMM) [100 mM potassium phosphate buffer, pH 6.5, 1.34 % (w/v) YNB, 0.00004% (w/v) biotin and 0.5% (v/v) methanol] The BMM culture was placed in 1 L baffled flasks and grown at 30°C in a shaking incubator for four to six days. Methanol (0.5%) was added daily to the cultures in order to maintain induction of expression of the protein of interest. The cells were harvested by centrifugation (5000 *g*, 10 min, 4°C) and the supernatant was retained. The supernatant was stored in plastic bottles at -20°C until further use.

3.2.13 Purification of ΔFLVp and C2 (H43W)

Three phase partitioning (TPP)

Three phase partitioning (Pike and Dennison 1989) was used to concentrate and purify the ΔFLVp and C2 (H43W) samples. The supernatants (25 mL) were thawed and filtered through Whatman No 1 filter paper. Thereafter, tertiary butanol [30% (v/v)] was added to the culture supernatant. The peptidases precipitated with ammonium sulfate [40% (w/v) of the total volume i.e. culture supernatant and tertiary butanol]. The TPP sample was centrifuged (6000 *g*, 10 min, 4^{o}C) in a swing-out rotor to separate the three layers: the tertiary butanol layer, the precipitated protein layer and an aqueous layer. The precipitated protein layer was retained and dissolved in dialysis buffer [phosphate buffered saline (PBS), pH 7.2]. The proteins were then dialysed overnight at 4^{o}C against PBS, pH 7.2 in a 10 kDa cut-off dialysis bag (Thermo Scientific Snakeskin® pleated dialysis tubing) in order to eliminate any residual tertiary butanol and $(NH_4)_2SO_4$. The dialysed protein samples were concentrated against solid polyethylene glycol (M_r 20 000) until the volume in the dialysis bag was brought down to 5 mL.

3.2.14 SDS-PAGE gel analysis of recombinant protein

Analysis of recombinant protein samples was performed by SDS-PAGE according to Laemmli (1970) using a discontinuous Tris-Glycine buffer and gel system. Samples that are being resolved on a gel are combined with a reducing treatment buffer [125 mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol] or non-reducing treatment buffer [125mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS and 20% (v/v) glycerol] in a 1:1 ratio. The proteins were first resolved through a stacking gel containing [500 mM Tris-HCl buffer, pH 6.8] and thereafter through a running gel containing [1.5 M Tris-HCl buffer, pH 8.8]. Electrophoresis was conducted at 18 mA per gel for approximately 1.5 - 2 h in a tank buffer [250 mM Tris-HCl buffer, pH 8.3, 192 mM glycine, 0.1% (m/v) SDS]. The relative migration distance of proteins on a gel has an inversely proportional relationship with the molecular weight of the protein. A standard curve of log M_w of proteins of known molecular weight against distance travelled (relative

mobility) was constructed (Fig. 3.4). The plot generated was used to determine the molecular weight of the protein of unknown size.

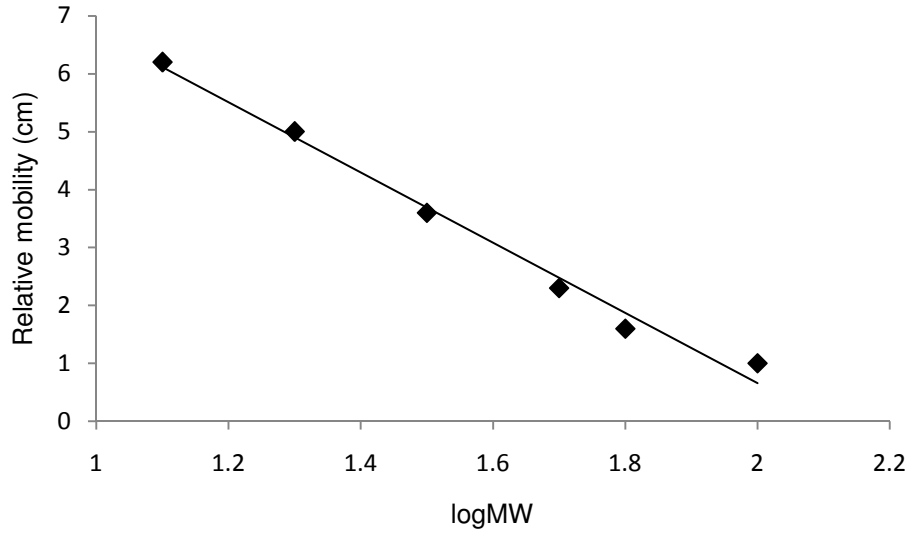


Figure 3.4 Standard curve of log Mw against relative mobility for the estimation of protein Mw by SDS-PAGE. The low range marker is made up of phosphorylase B (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (31 kDa), soybean trypsin inhibitor (21Da) and lysozyme (14 kDa). The equation of the trend line is y= -0.60602 + 12.778, with a correlation coefficient of 0.988. Microsoft excel 2007 was used to construct the plot.

The separated proteins were visualised by incubating the gel in a Coomassie blue R-250 stain [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid] for four hours followed by destaining in destain solution I [50% (v/v) methanol, 10% (v/v) acetic acid] overnight and then in destain solution II [5% (v/v) methanol, 7% (v/v) acetic acid] to allow for complete destaining. The detection limit of the Coomassie blue stain ranges from approximately 50-100 ng of protein (Laemmli 1970).

3.2.15 Production of anti-ΔFLVp antibodies in mice and chickens

The solubilised ΔFLVp protein band (from section 3.2.9) separated on SDS-PAGE was cut from the gel (Fig. 3.5) and liquid nitrogen added. The gel piece was crushed into a fine powder. The powder was washed five times with 1 mL distilled water and centrifuged

(5000 *g*, 2 min, RT) after each wash, followed by washing twice in 1 mL PBS and centrifuging (5000 *g*, 2 min, RT) after each wash. The pellet was resuspended in 1 mL of PBS.

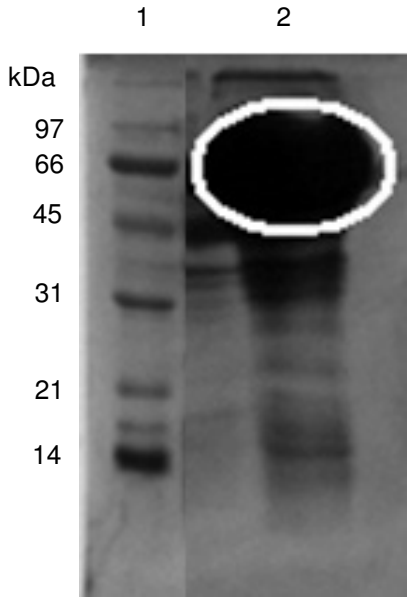


Figure 3.5 Analysis by a reducing 12.5% SDS-PAGE gel of recombinant solubilised ΔFLVp. Lane 1, Molecular weight markers; lane 2, pET-32a-ΔFLVp (15 µl). White oval indicates the section of ΔFLVp that was cut out the gel and used for immunisation. The sample wad mixed with an equal volume of reducing treatment buffer and boiled for 2 minutes prior to loading. Proteins were stained with Coomassie R-250.

For chicken immunisations the 1 mL ΔFLVp-PBS suspension was combined with 1 mL of Freund's complete adjuvant (Sigma) and the mixture was triturated to give a stable water-in-oil emulsion. Two chickens were immunised receiving 500 µl each (250 µl per breast muscle). Booster injections were administered at weeks 2, 4 and 6 using Freund's incomplete adjuvant (Sigma). Eggs were collected before immunisation and during the immunisation period.

For mice immunisation, 200 µl of the 1mL ΔFLVp-PBS suspension was combined with an equal volume of Freund's complete adjuvant (Sigma). The mixture was triturated to give a stable water-in-oil emulsion. The immunogen was injected into two mice (100 µl per

mouse). The mice received booster injections on weeks 3 and 6 using Freund's incomplete adjuvant (Sigma). Mice were bled prior to and during the immunisation period.

3.2.16 Isolation of IgY from egg yolk

Chicken IgY antibodies were isolated from egg yolk by a PEG precipitation technique described by Polson *et al.* (1985). The precipitation technique is made up of three steps. The first step involves removing the vitellin and fat fractions and the second and third steps involves the precipitation of the antibodies. The egg yolk was separated from the egg white and the yolk gently rinsed under running tap water to remove any remaining egg white. The yolk was collected by puncturing the yolk sac and the volume of the yolk was determined. Two volumes of 100 mM sodium phosphate buffer, pH 7.6, containing 0.02% (w/v) NaN_3 was mixed with the yolk. PEG 6000 was added to the total volume (yolk volume plus the two column volumes of buffer) to a final concentration of 3.5% (w/v) and dissolved with gently stirring. The mixture was centrifuged (4 420 *g*, 30 min, RT), and the supernatant filtered through absorbent cotton wool. The PEG 6000 concentration of the filtrate was increased to 12% (w/v) and dissolved once again by gentle stirring. The mixture was centrifuged (12 000 *g*, 10 min, RT). The pellet was retained and dissolved in a volume of sodium phosphate buffer equal to the yolk volume and 12% (w/v) PEG 6000 added and dissolved by gentle stirring. The solution was centrifuged (12 000 *g*, 10 min, RT). The pellet was retained and dissolved in a sixth of the yolk volume of 100 mM sodium phosphate buffer, pH 7.6, but this time containing 0.1% (w/v) NaN_3 and stored at 4°C.

3.2.17 Enzyme-linked immunosorbent assays (ELISAs)

The ELISA technique used in this study to evaluate the production of polyclonal antibodies in both mice and chickens was based on an original protocol (Huson *et al.* 2009). Recombinant ΔFLVp expressed in bacteria was used to coat the ELISA plate wells of 96 well Nunc-Immuno™ ELISA plates at a concentration of 1 µg/mL in PBS pH 7.2 (150 µl per well) overnight at 4°C. The coating antigen was removed and non-specific binding of the antibody was prevented by blocking the wells with 0.5% (w/v) BSA-PBS (200 µl per

well) for 1 h at 37°C. This was followed by washing the wells three times with 0.1% (v/v) PBS-Tween. Primary antibodies (chicken IgY or mice sera) were diluted appropriately with 0.5% (w/v) BSA-PBS and incubated in the wells (100 µl per well) for 2 h at 37°C. Wells were washed three times with 0.1% (v/v) PBS-Tween. The HRPO-conjugated anti-species secondary antibody (rabbit anti-chicken IgY or horse anti-mouse IgG), diluted in 0.5% (w/v) BSA-PBS, was added (120 µl per well) and incubated for 1 hour at 37°C. Wells were washed three times with 0.1% (v/v) PBS-Tween. This was followed by the addition of substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in 0.15 M citrate-phosphate buffer, pH 5.0] (150 µl per well) until colour developed in the dark. The A_{405} of each well was measured in a FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany).

3.2.18 Western blot analysis of recombinant proteins

Western blots were carried out to verify the expression of the recombinant proteins and to test the anti-ΔFLVp antibodies. Protein separated by SDS-PAGE (Section 3.2.14) were transferred onto a nitrocellulose membrane, using a wet blotter (BioRad Hercules, CA, USA) in the presence of blotting buffer [45 mM Tris-HCl buffer, pH 8.3, 173 mM glycine and 0.1% (w/v) SDS] for 16 hours at 200 mA (Towbin *et al.* 1979; Gershoni and Palade 1982). The nitrocellulose membrane was transiently stained with Ponceau S [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] and the molecular weight markers as well as the relevant protein bands marked with a pencil and the nitrocellulose membrane was destained with distilled water containing a drop of NaOH. The unreactive sites on the nitrocellulose membrane were blocked with low fat milk powder solution. [5% (w/v) low fat milk powder in Tris buffered saline (TBS) (20 mM Tris-HCl and 200 mM NaCl, pH 7.4)] for one hour at RT. The membrane was subsequently washed with TBS (3 x 5 minutes) and incubated in primary antibody diluted in 0.5% (w/v) BSA-TBS for 2 h at RT. This was followed by washing the membrane in TBS (3 x 5 minutes) and incubation in HRPO labelled secondary antibody diluted in 0.5% BSA TBS for one hour at RT. Lastly, the membrane was washed in TBS (3 x 5 minutes) and placed in substrate solution

[0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v) methanol, 0.0015 % (v/v) hydrogen peroxide H_2O_2 in TBS]. The blot was left in the dark until the protein bands developed.

3.3 RESULTS

3.3.1 Cloning of full length mutant of vivapain (ΔFLVp) into the pTZ57R/T vector (T vector)

The *T. vivax* parasites were purified from the blood of two mice infected with the Y486 *T. vivax* strain. The parasites were purified using a DEAE 50 cellulose column yielding 4.7 x 10^7 parasites/mL. Genomic DNA was isolated from the parasites using a phenol/chloroform method and revealed a single band with no degradation products (Fig. 3.6, lane 2).

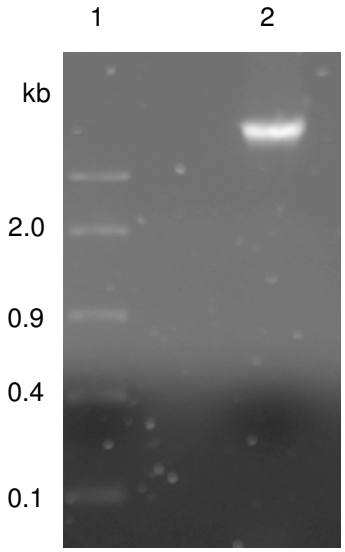


Figure 3.6 Agarose (1%) gel electrophoresis showing genomic DNA extracted from *T. vivax* parasites. Lane1, FastRuler™ DNA Ladder Middle Range; lane 2, *T. vivax* genomic DNA (1µl). Gel was stained with ethidium bromide.

Genomic DNA (Fig. 3.6) was used as the template for the PCR amplification of full length vivapain. A touch down PCR was performed since this was found to be the best method for the amplification of the *FLVp* gene. Agarose gel analysis of the PCR product revealed a prominent band at approximately 1.3 kb (Fig. 3.7) which corresponds to the expected size of the *FLVp* gene.

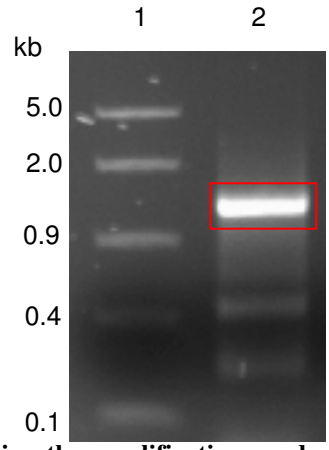


Figure 3.7 Agarose (1%) gel showing the amplification product of the full length vivapain gene from *T. vivax* genomic DNA using full length primers in the touch down PCR. Lane1, FastRuler™ DNA Ladder Middle Range; lane 2, PCR product of the *FLVp* gene amplification (1 µl). Red box showing the FLVp PCR product. Gel was stained with ethidium bromide.

The 1.3 kb fragment was cut out of the gel, purified and ligated into the pTZ57R/T vector (T vector). The ligation mix was transformed into *E.coli* JM109 cells. Blue-white colony selection was used to screen for transformants. Six white colonies were screened for recombinants by using colony PCR with specific C-term primers that were designed to amplify the C-terminal domain of the *FLVp* gene. All six colonies selected were recombinant due to the presence of a 350 bp product (Fig. 3.8, lanes 2-7) which corresponds to the size of the C-terminal domain of the *FLVp* gene.

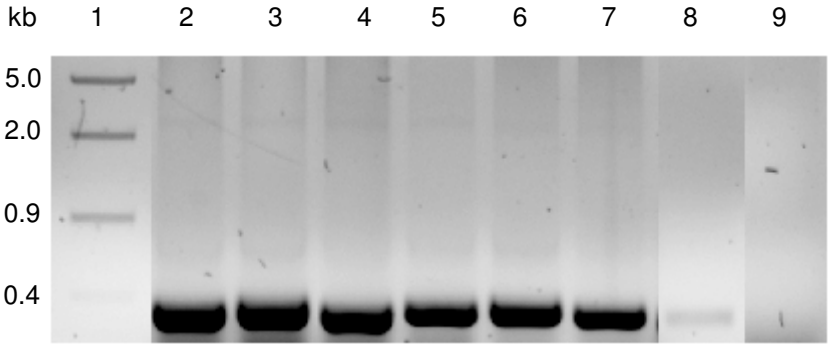


Figure 3.8 Agarose (1%) gel showing the screening of the six recombinant clones (T vector-FLVp) after transformation into *E. coli* JM 109 cells using C-term primers. Lane1, FastRuler™ DNA Ladder Middle Range; lanes 2-7, PCR product for the screening of the six colonies (20 µl); lane 8, positive PCR control (1 in 10 dilution of *T. vivax* genomic DNA as template) (20 µl); lane 9: negative control (no DNA template) (20 µl). Gel was stained with ethidium bromide.

The six recombinant T vector-FLVp plasmid DNA preparations were isolated and sequenced. The cloning of the *FLVp* gene into the T vector was performed to determine whether a natural mutation would occur in the catalytic domain coding region of the gene,

which would have led to the production of a full length inactive mutant that could have been used in diagnostic tests for *T. vivax.* The sequencing results revealed that only one out of the six clones matched up to the vivapain reference sequence in the database (Appendix 1). This sequence contained the catalytic triad Cys^{25}, His^{159} and Asn^{175} (papain numbering) in the active site. This meant that this particular clone's sequence constituted the full length active form of vivapain. Since the full length inactive form of vivapain (ΔFLVp) was required a different approach had to be taken.

The ΔFLVp fragment was obtained from the full length active vivapain (FLVp) fragment cloned into the T vector and an already existing mutant catalytic domain of vivapain fragment (ΔCAVp) cloned into a pPic9 vector. The ΔCAVp fragment had an alanine substituted for cysteine in the catalytic triad. Therefore, the amino acid residues in the active site of ΔCAVp are Ala^{25}, His^{159} and Asn^{175} (papain numbering). The ΔFLVp fragment was obtained by using a unique double cutter restriction enzyme, namely BsgI, which was unique to both the full length active vivapain fragment cloned in T vector and the mutant catalytic domain of vivapain fragment (ΔCAVp) in pPic9. The restriction sites for this enzyme were present on either side of the CHN domain of the FLVp clone and the AHN domain of the ΔCAVp clone. The result of this restriction is shown in Fig. 3.9.

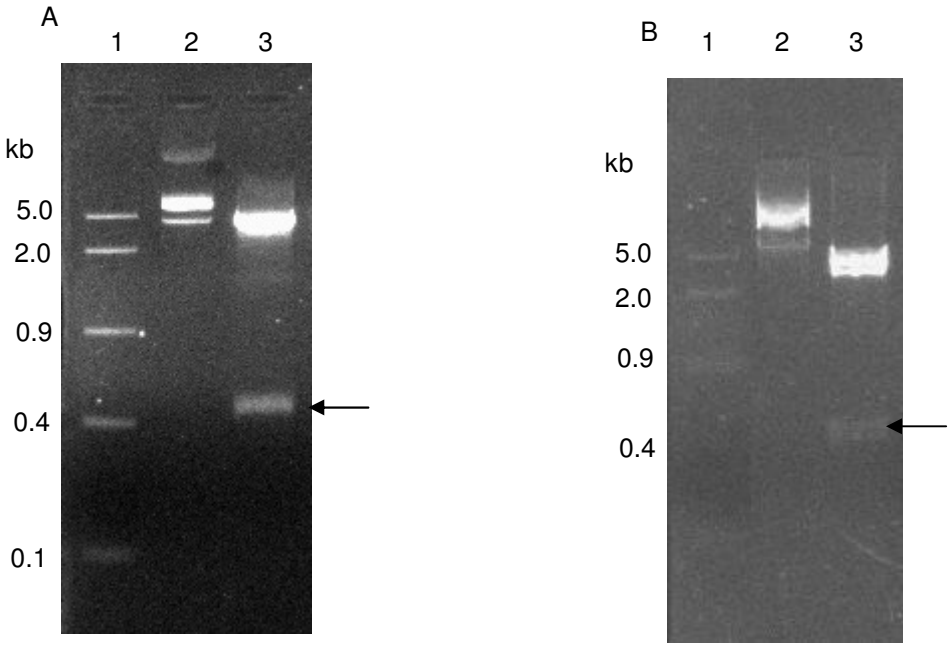


Figure 3.9 Agarose (1%) gel showing restriction with BsgI. A) full length active vivapain fragment cloned in T vector miniprep (CHN fragment) and B) mutant catalytic domain of vivapain fragment (ΔCAVp) in pPic9 miniprep (AHN fragment) using BsgI. Lane 1, FastRuler DNA Ladder Middle Range; lane 2, uncut miniprep (control) and lane 3, cut miniprep. The gel was stained with ethidium bromide. The arrow in (A) represents the CHN fragment and the arrow in (B) represents the AHN fragment

The AHN coding fragment was then ligated into the BsgI sites of the full length active vivapain coding fragment. The result of this ligation was DNA coding for a full length inactive mutant of vivapain. The ΔFLVp fragment in the T vector was transformed into *E.coli* JM 109 cells and colony PCR was performed to screen the white colonies with C-term primers (Table 3.2). This colony PCR (Fig. 3.10) revealed that all of the 17 cones selected were recombinant due to the presence of a 360 bp band in all the lanes of the agarose gel.

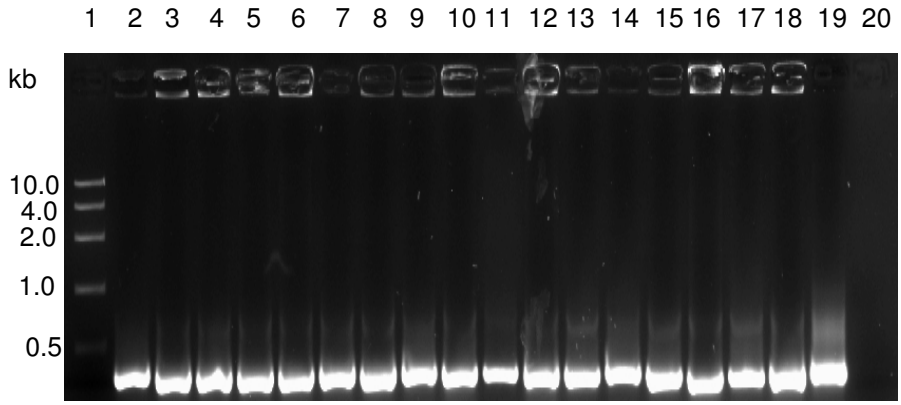


Figure 3.10 Screening of the recombinant (T vector-ΔFLVp) clones after transformation into *E. coli* JM 109 cells on 1% agarose gel. Lane1, FastRuler™ DNA Ladder High Range; lanes 2-18, PCR product for the screening of the 17 colonies (20 µl); lane 19, positive PCR control (*T. vivax* genomic DNA as template) (20 µl); lane 20, negative control (no DNA template) (20 µl). Gel was stained with ethidium bromide.

Since the C-term primers were used in the colony PCR, a further test to prove that the AHN coding fragment was ligated into the T vector was performed. A miniprep was performed on clones 1 and 10 (Fig. 3.10 lanes 2 and 11) in order to obtain the plasmid DNA. This plasmid DNA was then subjected to restriction by EcoRI and NotI (Fig. 3.11 A) to ensure that the ΔFLVp coding fragment was present in the T vector and by BsgI I to ensure that the AHN coding fragment was successfully ligated in the full length insert (Fig. 3.11 B).

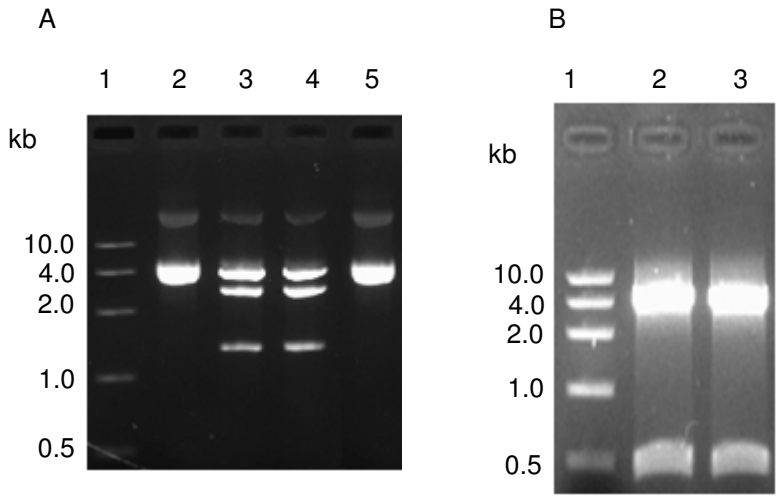


Figure 3.11 Analysis of restriction by EcoRI, NotI and BsgI using 1% Agarose gel. A) Restriction of clone one and ten by EcoRI and NotI. Lane 1, FastRuler™ DNA Ladder High Range; lane 2, clone one uncut plasmid DNA (1 µl); lane 3, clone one cut plasmid DNA (EcoRI/NotI) (10 µl); lane 4, clone 10 cut plasmid DNA (EcoRI/NotI) (10 µl); lane 5, clone ten uncut plasmid DNA (1µl). B) Restriction of clone one and ten by BsgI. Lane1, FastRuler™ DNA Ladder High Range; lane 2, clone one cut plasmid DNA (BsgI) (10 µl); lane 3, clone 10 cut plasmid DNA (BsgI) (10 µl). Gel was stained with ethidium bromide**.**

The sequencing of the clones revealed that the AHN coding fragment was successfully ligated into the full length vivapain fragment in the T vector (Fig. 3.12). This shows that the mutant AHN coding fragment was successfully ligated into to the BsgI sites of the full length vivapain coding fragment rendering the ΔFLVp. This clone was, therefore, used in the remainder of the study.

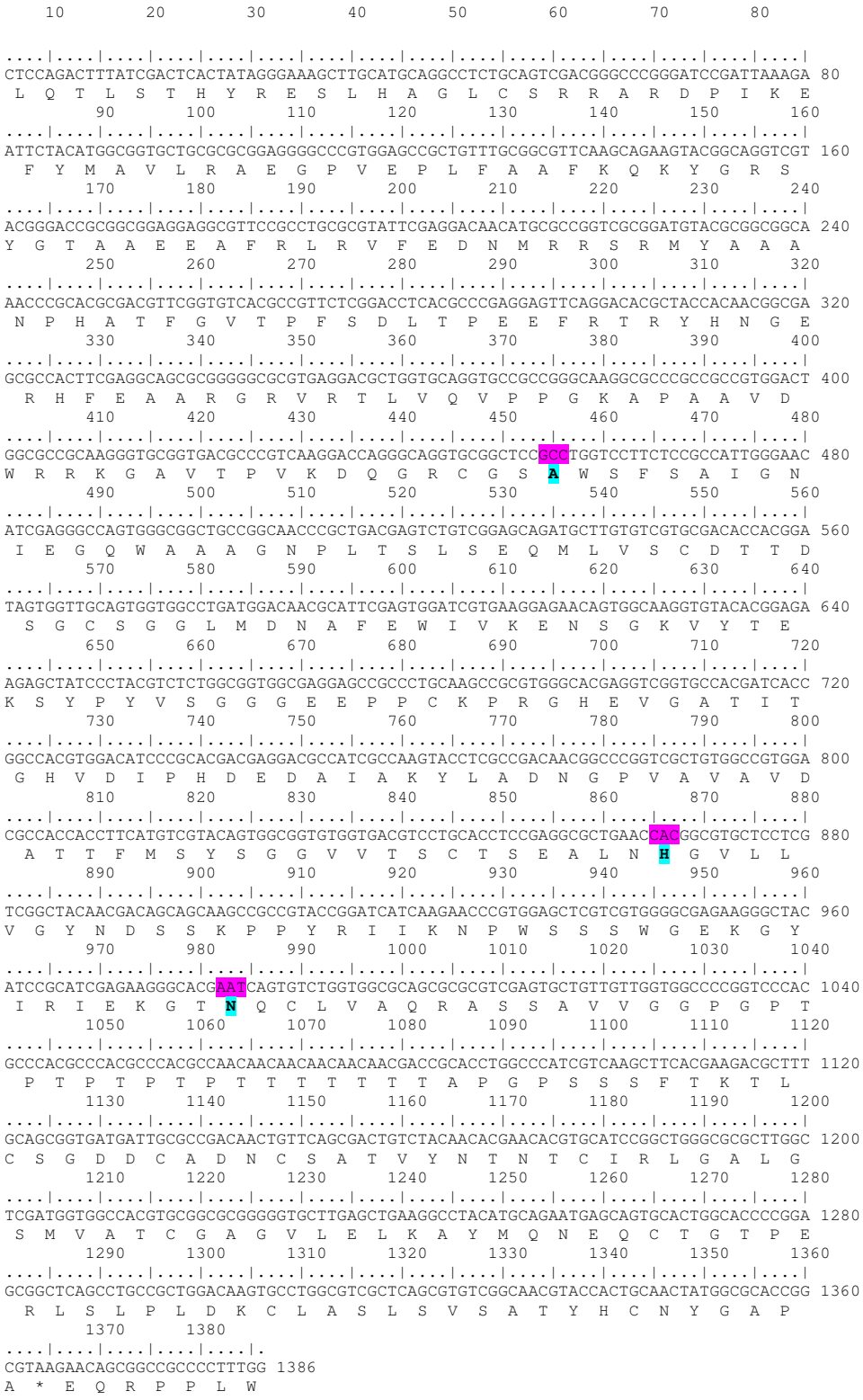


Figure 3.12 Sequence of the full length mutant vivapain (ΔFLVp), showing the amino acids in the active site, which are the "AHN" triad (blue) and the DNA sequence coding for each of these amino acids (pink).

3.3.2 Subcloning of full length inactive mutant of vivapain (ΔFLVp) into pET-32a and pPic9 expression vectors

Since the ΔFLVp fragment was successfully cloned into the T vector, the next step was to subclone the fragment into the bacterial expression vector, pET-32a, and the yeast expression vector expression vector, pPic9. The purified ΔFLVp fragment and the two expression vectors prepared and cut with EcoRI and NotI was analysed on an agarose gel to determine their relative quantities for use for ligation (Fig. 3.13 A and B). Figure 3.13 A shows the pET-32a expression vector at approximately 6 kb (lane 2) and the ΔFLVp fragment at approximately 1.3 kb (lane 4). Figure 3.13 B shows the pPic9 expression vector at approximately 8 kb (lane 2) and the ΔFLVp fragment at approximately 1.3 kb (lane 3).

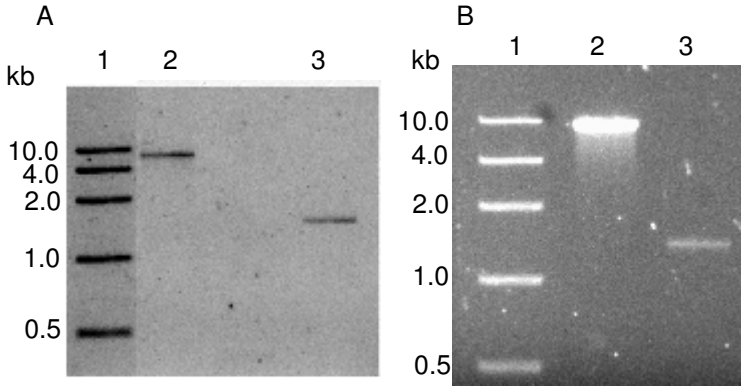


Figure 3.13 Agarose gel (1%) analysis of the purified ΔFLVp fragment and the expression vectors cut with EcoRI and NotI. A) the pET-32a expression vector and the ΔFLVp insert and B) the pPic9 expression vector and ΔFLVp insert in order to determine the relative quantities of DNA to use for ligation. A) Lane 1, FastRuler™ DNA Ladder High Range; lane 2, pET-32a expression vector (1 µl) and lane 3 ΔFLVp insert (1 µl). A) Lane 1, FastRuler™ DNA Ladder High Range; lane 2, pPic9 expression vector (1 µl) and lane 3 ΔFLVp insert (1 µl). Gel was stained with ethidium bromide.

The colonies obtained from ligating the ΔFLVp insert with the expression vectors and the transformation into *E. coli* JM 109 cells were screened for recombinants using colony PCR with C-term Fw and pET Rv primers (Fig. 3.14). All nine colonies that were chosen from the pET-32a-ΔFLVp transformation were positive (Fig. 3.14 A) due to the presence of a 0.456 kb band (made up from 0.360 kb from the C-terminal extension of ΔFLVp and 0.096 kb from the pET-32a plasmid). The recombinant pET-32a-ΔFLVp plasmid DNA was used as template DNA with the pET primers as a positive control for this PCR and,

consequently, a band at approximately 750 bp was obtained (Fig. 3.14 A, lane 12). Both colonies chosen from the pPic9-ΔFLVp transformation were positive since a PCR product of 0.360 kb was observed (Fig. 3.14 B), which is the expected size for the amplification of the C-terminal region of the *Vp* gene using the C-term primers.

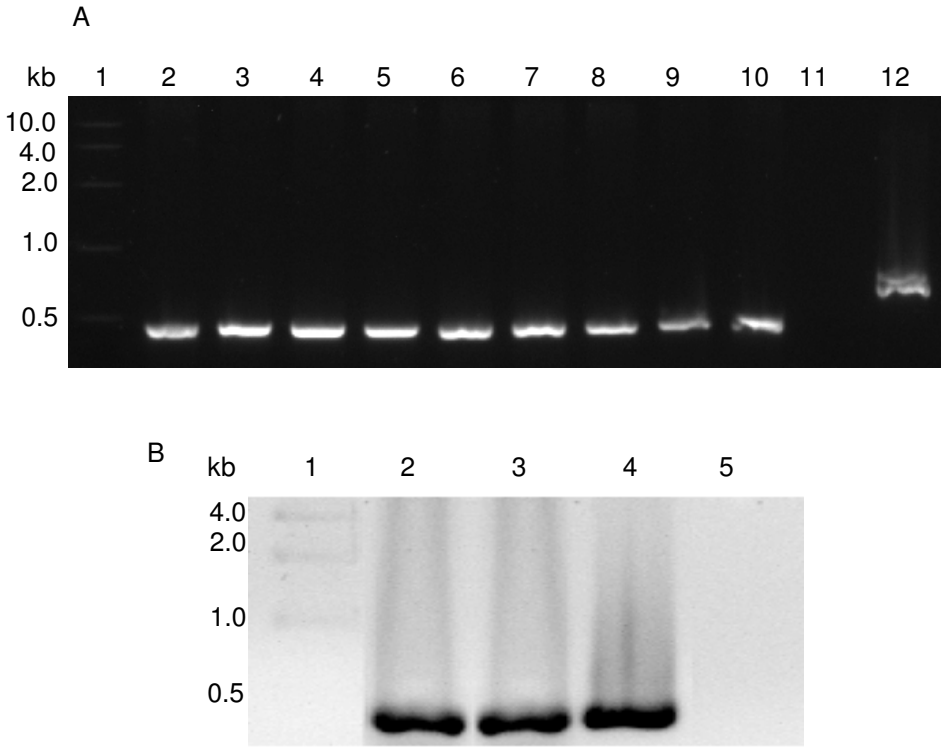


Figure 3.14 Agarose gel (1%) showing the screening of recombinant pET-32a-ΔFLVp and pPic9-ΔFLVp after transformation into *E. coli* JM 109 cells. A) recombinant pET-32a-ΔFLVp clones: lane 1, FastRuler™ DNA Ladder High Range; lanes 2-10, PCR product for the screening of the nine colonies (20 µl); lane 11, negative control (no DNA template) (20 µl); lane 12, positive PCR control (recombinant pET-32a vector plasmid DNA used as the template with pET primers) (20 µl). B) recombinant pPic9-ΔFLVp clones: lane 1, FastRuler™ DNA Ladder High Range; lanes 2-3, PCR product for the screening of the two colonies (20 µl); lane 4, positive PCR control (*T. vivax* genomic DNA as template) (20 µl); lane 5, negative control (no DNA template) (20 µl). Gel was stained with ethidium bromide.

The plasmid DNA of one clone from each of the transformations was isolated, and subjected to restriction digests using EcoRI and NotI. This restriction showed the presence of the ΔFLVp insert in pET-32a (Fig 3.15 A) and in pPic9 (Fig 3.15 B).

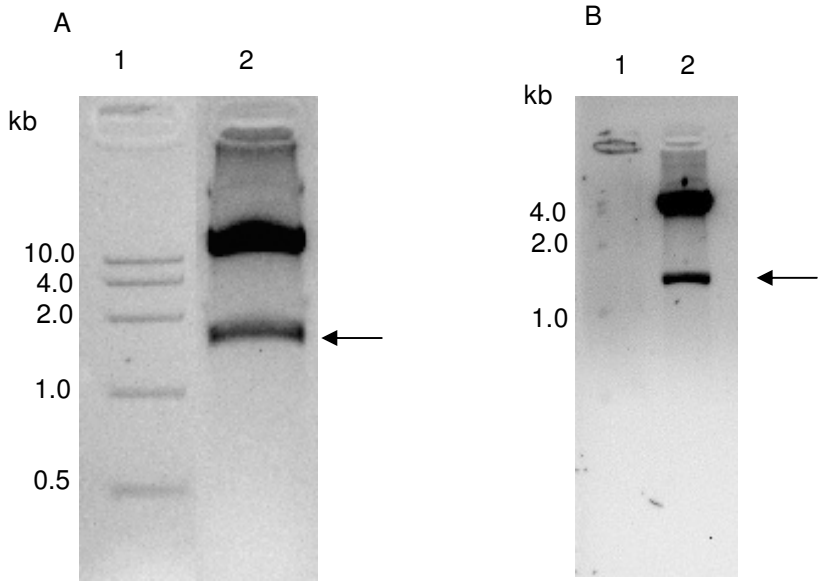


Figure 3.15 Agarose gel (1%) analysis of EcoRI and NotI restriction of pET-32a containing ΔFLVp and pPic9 containing ΔFLVp. A) Lane 1, FastRuler DNA Ladder High Range; lane 2, cut pET-32a-ΔFLVp miniprep DNA (10 µl). B) Lane 1, FastRuler DNA Ladder High Range; lane 2, cut pPic9-ΔFLVp miniprep DNA (10 µl). The arrow in (A) and in (B) represents the ΔFLVp insert in pET-32a and pPic9, respectively. The gel was stained with ethidium bromide.

3.3.3 Expression of the recombinant full length mutant of vivapain (ΔFLVp) in pET-32a

The recombinant plasmid DNA (pET-32a vector containing the ΔFLVp fragment) was transformed into BL21 (DE3) and expression was performed in terrific broth. Analysis of the lysed cell pellet and supernatant on SDS-PAGE (Fig. 3.16) revealed that majority of the expressed protein is found in the pellet as insoluble inclusion bodies at approximately 70 kDa (lane 2).

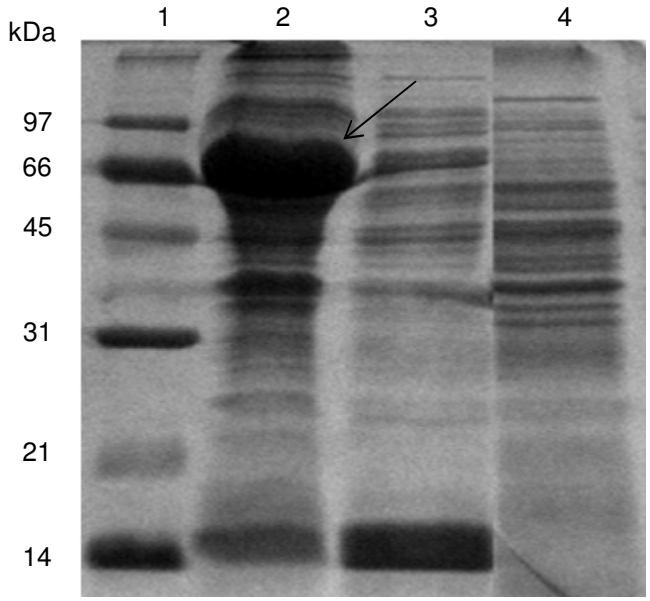


Figure 3.16 Analysis by a 12.5% reducing SDS-PAGE gel of recombinant ΔFLVp expression from bacterial lysate. Lane 1, Molecular weight markers; lane 2, pET-32a-ΔFLVp pellet (20 µl); lane 3, pET-32a-ΔFLVp supernatant (20 µl); lane 4, pET-32a-BL21 (DE3) (20 µl). Arrow indicates the size of ΔFLVp at 70 kDa in lane 2. Each of the samples were mixed with an equal volume of reducing treatment buffer and boiled for 2 minutes prior to loading. Proteins were stained with Coomassie R-250.

Since attempts to purify the recombinantly expressed ΔFLVp under denaturing conditions failed (results not shown), it was decided to renature the protein. Following renaturation, the protein sample was analysed on a SDS-PAGE gel (Fig. 3.17). Results of the renaturation are inconclusive because a clear shift in size between that of the renatured and non-renatured protein is not visible (Fig. 3.17). The renaturation process also led to a drastic loss in protein.

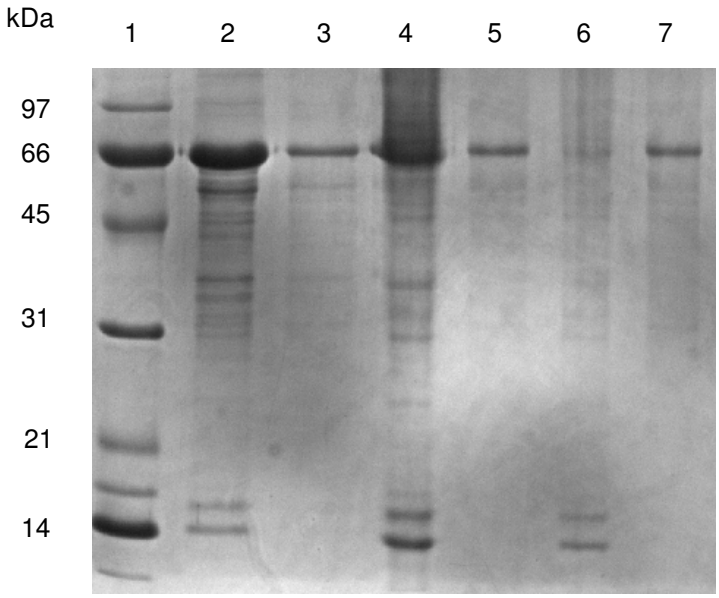


Figure 3.17 Analysis by 12.5% SDS-PAGE of the renaturation of full length mutant vivapain (ΔFLVp) expressed in pET-32a. Lane 1, molecular mass markers; lane 2, non-renatured ΔFLVp (5 µl reduced and boiled); lane 3, renatured ΔFLVp (5 µl reduced and boiled); lane 4, non-renatured ΔFLVp (5 µl non-reduced and boiled); lane 5, renatured ΔFLVp (5 µl non-reduced and boiled); lane 6, non-renatured ΔFLVp (5 µl non-reduced and non-boiled); lane 7, renatured ΔFLVp (5 µl non-reduced and non-boiled). Protein bands were stained with Coomassie R-250.

A western blot was carried out using anti-congopain N-terminal peptide antibody to confirm the recombinant expression of ΔFLVp. The positive control used in this blot was C2, which is detected at approximately 27 kDa (Fig. 3.18, lane 5). The anti-congopain N-terminal peptide antibody also detected a 70 kDa band in lane 2 (ΔFLVp solubilised) and lane 2 (ΔFLVp after renaturation), which is the expected size of ΔFLVp expressed in bacteria (Fig 3.16, lane 2).

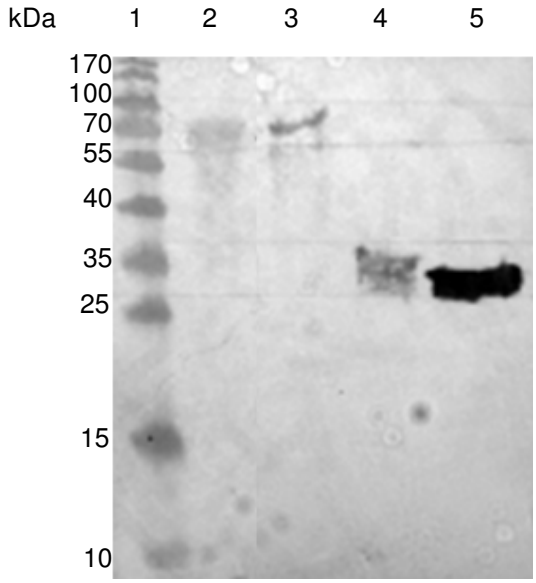


Figure 3.18 Western blot analysis of recombinant expression of ΔFLVp. Proteins were electrophoresed on a 12.5% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with anti-congopain N-terminal peptide antibody (1 µg/mL). Secondary antibody was rabbit anti-chicken HRPO-conjugate (1:10000). The reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, Fermentas Rainbow Marker ; lane 2, ΔFLVp after solubilisation; lane 3 ΔFLVp after renaturation; lane 4, the catalytic domain of vivapain (Vp); lane 5, catalytic domain of congopain (C2).

3.3.4 Expression of ΔFLVp in pPic9

3.3.4.1 Transformation into *P. pastoris* GS 115 cells

The recombinant plasmid DNA (pPic9 vector containing the ΔFLVp fragment) was linearised with SalI in preparation for transformation into *P. pastoris* GS 115 cells. SacI could not be used to linearise the plasmid DNA since sequencing revealed that a SacI site was present in the *ΔFLVp* gene. A positive control pPic9-C2 (H43W) transformed into JM 109 cells was also transformed and expressed in *P. pastoris* GS 115 cells. The pPic9-C2 (H43W) clones were screened using AOX primers (Fig. 3.19 A). In this colony PCR two prominent bands were obtained at approximately 0.96 and 2.2 kb, which correspond to the *C2* (*H43W*) insert gene and the native *AOX* gene of *P. pastoris* respectively. The pPic9-ΔFLVp clones were screened using the C-term forward primer and the AOX reverse primers (Fig. 3.19 B). This colony PCR revealed the presence of a 0.470 kb band in lanes 2-4 that correspond to the C-terminal extension portion of the *ΔFLVp* insert gene (0.36 kb)

and the portion of the pPic9 vector amplified (0.11 kb). The positive control used in this PCR was pPic9-ΔFLVp plasmid DNA, which also produced a band at 0.470 kb. The band present at 1 kb in Fig. 3.19 B is difficult to explain.

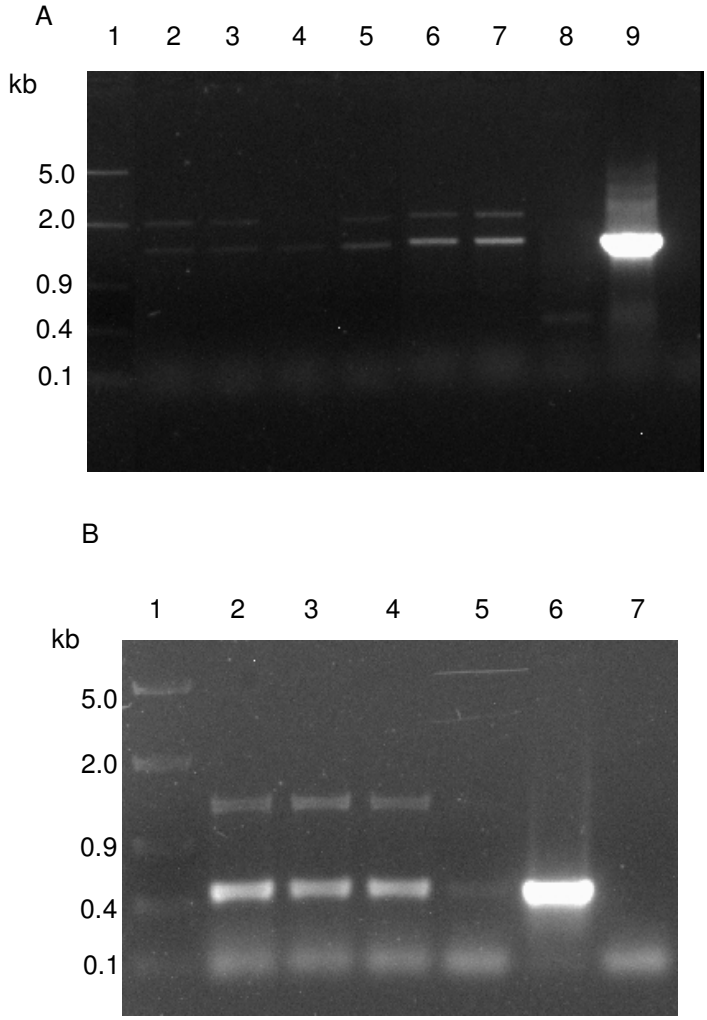


Figure 3.19 Agarose gel (1%) showing the screening of the pPic9-C2 (H43W and pPic9-ΔFLVp clones after transformation into GS115 *P. pastoris* yeast cells. A) Six [pPic9-C2 (H43W)] clones after transformation into GS115 *P. pastoris* yeast cells using AOX primers. Lane 1, FastRuler™ DNA Ladder Middle Range; lanes 2-7, PCR product for the screening of the six colonies (20 µl); lane 8, negative control (no DNA template) (20 µl); lane 9, positive PCR control (recombinant pPic9-C2 (H43W) vector plasmid DNA used as the template with AOX primers) (20 µl). B) Four (pPic9-ΔFLVp) clones after transformation into GS115 *P. pastoris* yeast cells using the C-term forward primer and the AOX reverse primers. Lane 1, FastRuler™ DNA Ladder Middle Range; lanes 2-5, PCR product for the screening of the four colonies (20 µl); lane 6, positive PCR control (recombinant pPic9-ΔFLVp vector plasmid DNA used as the template with C-term forward primer and the AOX reverse primers) (20 µl); lane 7, negative control (no DNA template) (20 µl).Gel was stained with ethidium bromide.

3.3.4.2 Expression in *P. pastoris* GS 115 cells

A small scale recombinant expression of ΔFLVp and C2 (H43W) was performed in *P. pastoris.* Three phase partitioning (TPP) was used to concentrate the recombinantly expressed proteins from the culture supernatant and expression was analysed by using chicken anti-N-terminal peptide antibodies (Fig. 3.20). The blot revealed that the ΔFLVp was not expressed since the antibody did not detect any band in lanes 2 and 3 (ΔFLVp before and after TPP) (Fig. 3.20). This is further verified by the observation that the primary antibody recognised bands at approximately 27 kDa in lanes 4 and 5 [C2 (H43W before and after TPP)] (Fig. 3.20), which was used as a positive control in this experiment.

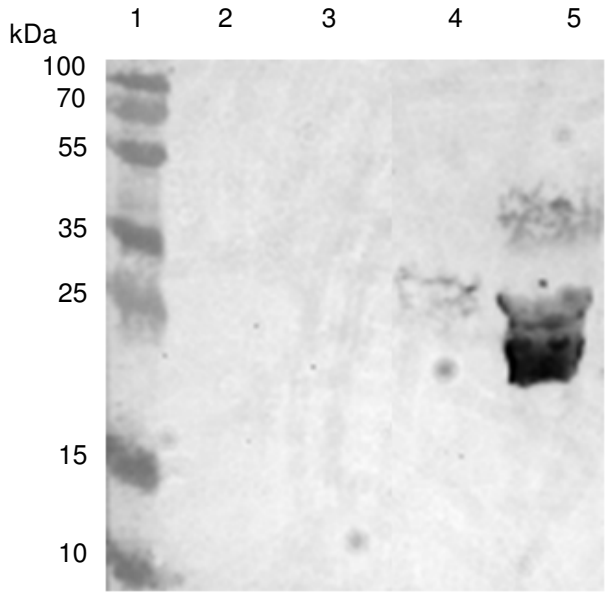


Figure 3.20 Western blot analysis of recombinant expression of ΔFLVp in *P. pastoris*. Proteins were electrophoresed on a 12.5 % SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with anti-congopain N-terminal peptide antibody (1 µg/mL). Secondary antibody was rabbit anti-chicken HRPO-conjugate (1:10000). Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, Fermentas Rainbow Marker ; lane 2, ΔFLVp supernatant culture from day 5 of expression (25 µl); lane 3, ΔFLVp after TPP (10 µl); lane 4, C2 (H43W) supernatant culture from day 5 of expression (25 µl); lane 5, ΔFLVp after TPP (10 µl).

3.3.5 Production of antibodies against the recombinantly expressed ΔFLVp (in *E. coli* JM 109 cells) in mice and chickens

Mice and chickens were injected with ΔFLVp separated on a gel and the production of antibodies was monitored by ELISA. The ability of the antibodies to recognise the recombinant ΔFLVp and native vivapain in a *T. vivax* lysate was evaluated by western blot.

3.3.5.1 Evaluation by ELISA of anti-ΔFLVp antibody production in chickens

The first ELISA was performed to analyse the progression of antibody production during the 12 week period (Fig. 3.21). The ELISA showed that for chicken 1 antibody production peaked at weeks five, nine and eleven. Antibody production for chicken 2 peaked at weeks four, six and nine. Chicken 2 seemed to produce slightly higher antibody levels (Fig. 3.21).

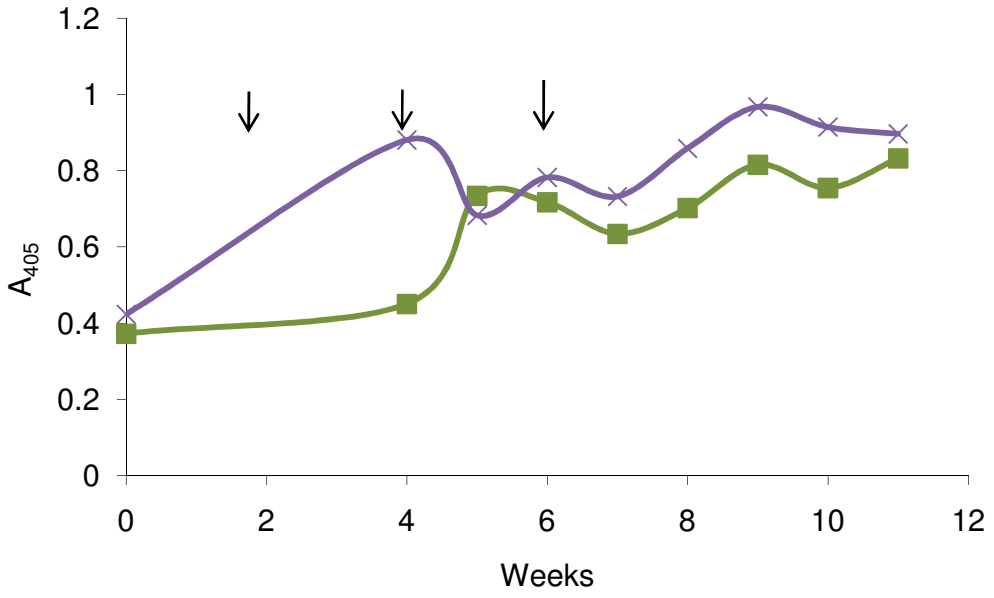


Figure 3.21 ELISA showing antibody production in chicken 1 (■) and chicken 2 (X) immunised with recombinant ΔFLVp. ELISA plates were coated with 1 µg/mL ΔFLVp. Primary antibody used was the IgY isolated from each chicken from weeks 1 to 12 at a concentration of 100 µg/mL. The interaction between ΔFLVp and the primary antibody were detected using rabbit anti-chicken IgY HRPO-conjugate (1:10000) and ABTS/H_2O_2. Absorbance of the reactions was read at $A_{405,}$ which represent the average of duplicate readings. Arrows indicate when the booster immunisations were administered. Microsoft excel 2007 was used to construct the plot.

The second ELISA was performed in order to determine antibody titre for each chicken using serial dilutions for the antibodies produced during each week (Fig. 3.22). The results from this ELISA revealed that chicken 2, once again, produced higher levels of antibodies (Fig. 3.22 B). The ELISA also shows that all the antibodies produced from week 6 in the case of chicken 1 and week 4 for chicken 2 were comparable and at significantly higher levels than the non-immune control. The titration curves suggest that a concentration of 10 µg/mL antibody should provide a good signal in the western blot.

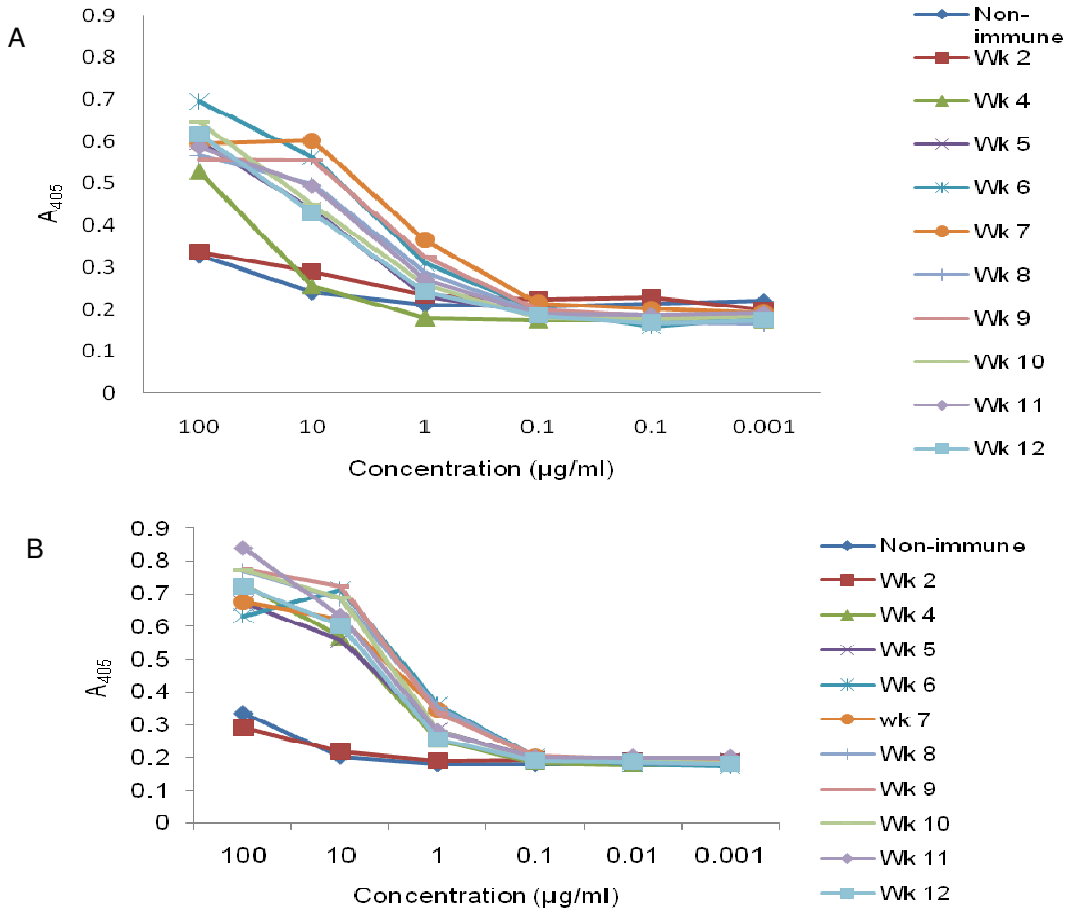


Figure 3.22 ELISA showing the antibody titre for chickens immunised with recombinant ΔFLVp. ELISA plates were coated with 1 µg/mL ΔFLVp. Primary antibody used was the IgY isolated for each chicken for chicken 1 (A) or chicken 2 (B) weeks 1 to 12 at a concentrations ranging from 100 µg/mL to 0.001 µg/mL. The interaction between ΔFLVp and the primary antibody were detected using rabbit anti-chicken IgY HRPO-conjugate (1:10000) and ABTS/H_2O_2. Absorbances of the reactions were read at A_{405}, which represent the average of duplicate readings. Microsoft excel 2007 was used to construct the plot.

3.3.5.2 Evaluation by ELISA of anti-ΔFLVp antibody production in mice

Two ELISAs were performed to analyse the antibody production in mice against the recombinant ΔFLVp over the eight week immunisation schedule. The first ELISA was conducted to monitor antibody production over the eight week immunisation period (Fig 3.23). This ELISA was performed using sera from samples collected three, four, five six, seven and eight weeks after the first immunisation. High antibody levels were observed four weeks after the first immunisation for both mice. Antibody production seemed to be constant from weeks four to six in mouse 1 and weeks four to five in mouse 2, thereafter, antibody production peaked at week 7 for mouse 1, and week six for mouse 2, which means that there was an increase in antibody production after the second booster (week six) for both mice (Fig. 3.23). It was decided to perform the big bleed on the mice at week eight. This serum was used for the subsequent ELISAs and western blots.

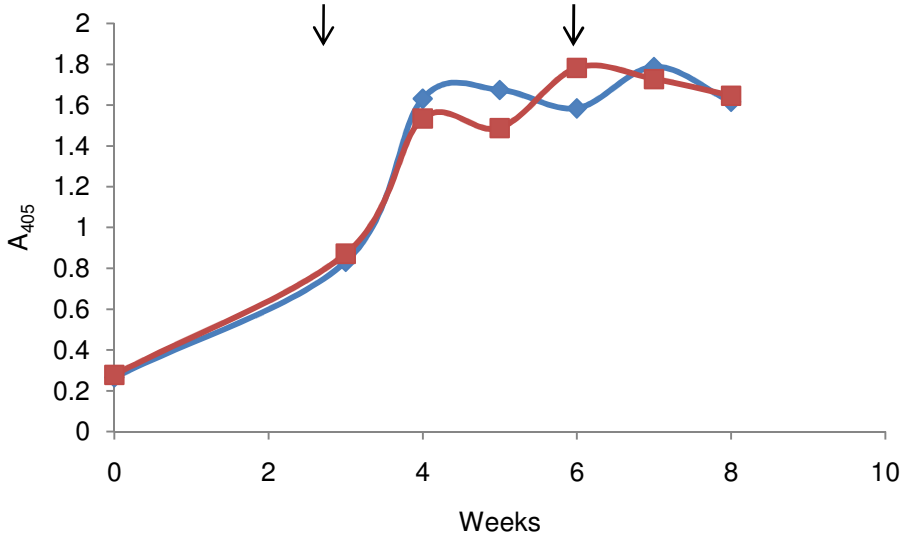


Figure 3.23 ELISA showing the antibody production in mice against ΔFLVp. Sera from mouse 1 (◆) and mouse 2 (■) were diluted 1:200 and incubated in ELISA plates coated with 1 µg/mL ΔFLVp. The interaction between ΔFLVp and the primary antibody were detected using horse anti-mouse IgG HRPO-conjugate and ABTS/H_2O_2. Absorbances of the reactions were read at A_{405}, which represent the average of duplicate readings. Arrows indicate when the booster immunisations were administered. Microsoft excel 2007 was used to construct the plot.

The second ELISA was carried out to determine which concentration would be the best to use in western blots and possibly diagnostic tests (Fig. 3.24). This was done by using serial dilutions of the mice sera collected after the eight week of the immunisation period. The ELISA revealed that serum from mouse 2 had slightly higher antibody levels than that of mouse 1 and the serum dilutions of 1:1000 or 1:2000 would be suitable for use in western blots (Fig. 3.24).

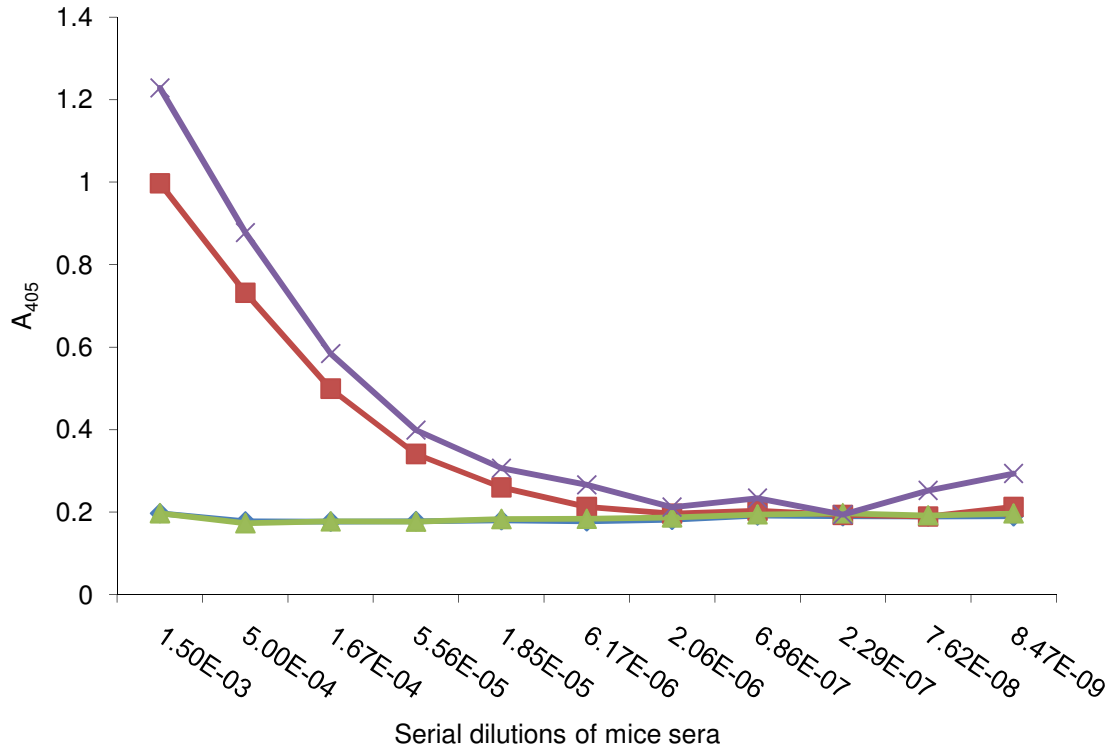


Figure 3.24 ELISA showing the antibody titre in mice. Sera from for mouse 1 (non-immune) (◆), mouse 1 (■), mouse 2 (non-immune) (▲), mouse 2 (X) were serially diluted in a range from 1:667 to 1:118 098 000 incubated in ELISA plates coated with 1 µg/mL ΔFLVp .The interaction between ΔFLVp and the primary antibody were detected using horse anti-mouse IgG HRPO-conjugate (1:10000) and ABTS/H_2O_2. Absorbance of the reactions were read at A_{405}, which represent the average of duplicate readings. Microsoft excel 2007 was used to construct the plot.

3.3.5.3 Detection of recombinant ΔFLVp and native vivapain in *T. vivax* lysate by antibodies in a western blot

The chicken anti-ΔFLVp antibodies and mouse anti-ΔFLVp sera were used in a western blot to detect recombinant ΔFLVp (Fig. 3.25) and native vivapain in a *T. vivax* lysate (Fig. 3.26). The antibodies collected at week eight from chicken 2 and serum from mouse 2 were used in the western blots. The chicken anti-ΔFLVp antibodies and mouse anti-ΔFLVp sera were able to detect the recombinant at approximately 70 kDa (Fig. 3.25 A and B). The chicken anti-ΔFLVp antibodies did not detect native vivapain in parasite lysate (results not shown). The mouse serum detected a band at approximately 55 kDa (Fig. 3.26, lanes 3 and 4), which corresponds to the native vivapain in the *T. vivax* lysate.

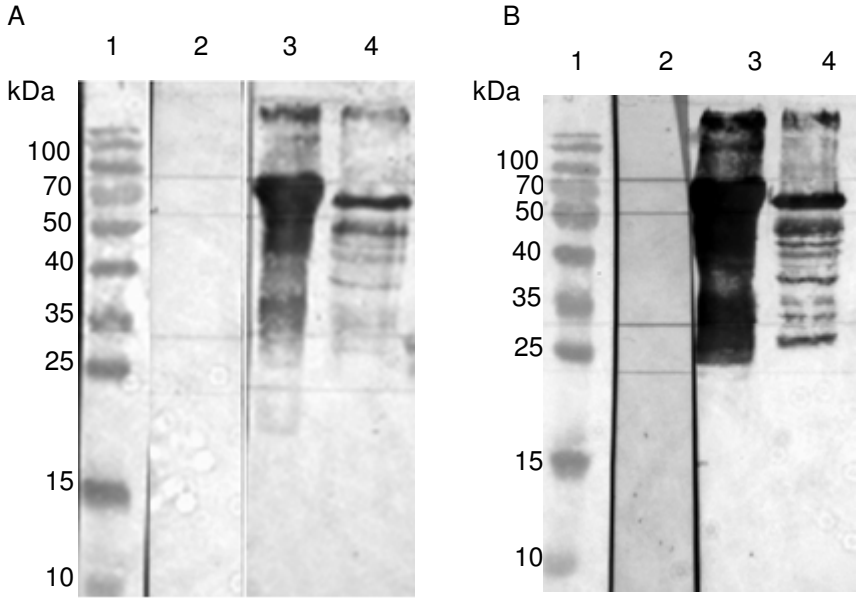


Figure 3.25 Western blot detection of recombinant ΔFLVp by chicken anti-ΔFLVp antibodies and mouse anti-ΔFLVp sera. Proteins were electrophoresed on a 12.5% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred protein was incubated with A) chicken anti-ΔFLVp antibodies (2.5 µg/mL) and non-immune chicken antibody in lane 2 (2.5 µg/mL) and with B) mice sera (1:5000) and a non-immune serum in lane 2 (1:5000). Secondary antibody in A) rabbit anti-chicken HRPO-conjugate (1:10000) and B) horse anti-mouse HRPO-conjugate (1:10000). Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, Fermentas Spectra™ multicolour Broad range protein ladder ; lanes 2-3, recombinant ΔFLVp; lane 4, recombinant ΔFLVp renatured. The pencil lines were drawn in to indicate the expected positions of ΔFLVp.

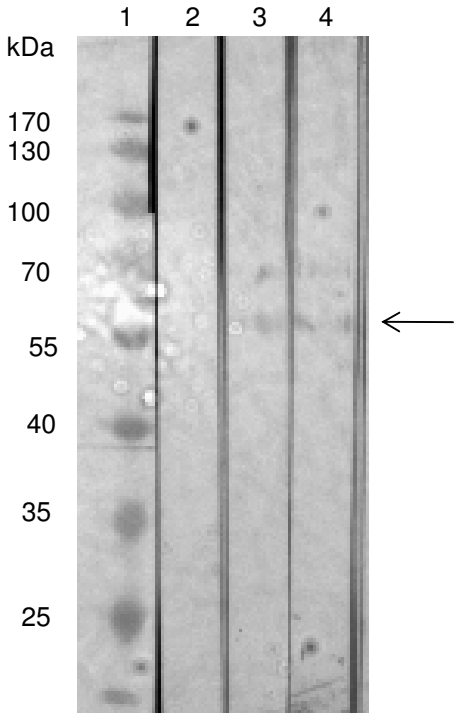


Figure 3.26 Western blot detection of native vivapain in *T. vivax* lysate. *T.* vivax lysate (1 x 10^5 parasites per well) proteins were electrophoresed on a 12.5% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane and membrane was cut in strips for incubation with different mouse serum samples. The antibody-vivapain interaction was detected using horse anti-mouse HRPO-conjugate (1:10000). Reaction was developed with 4-chloro-1-naphthol/H_2O_2. Lane 1, Fermentas Rainbow Marker; lane 2, non-immune serum; lane 3, mouse two serum (1:1000); lane 4, mouse two serum (1:2000). Arrow indicates the detection band of the native vivapain at 55 kDa.

3.4 DISCUSSION

The diagnosis of trypanosomosis is a very important aspect since it gives insight into the disease and allows for the correct use of chemotherapeutics. This study was performed to produce a recombinant antigen that will be useful in the field diagnosis of *T. vivax* in the sera of the infected animals. The most promising technique is the serological ELISA technique. There are two types of ELISA techniques that can be used; the antigen-detection ELISA and the antibody-detection ELISA. For the first method, the recombinant antigen will be used to produce antibodies that will be used to detect the antigen in infected sera and the second would use the antigen directly to detect antibodies in infected animals.

The cloning and expression of the full length inactive mutant of vivapain (ΔFLVp) was undertaken for possible use in diagnostic tests for *T. vivax.* It is not possible to express a full length (FL) active form of vivapain that would be useful for the detection of antibodies in *T. vivax* infected animals due to prior investigations with the full length active form of congopain from *Trypanosoma congolense* (Boulangé A, University of KwaZulu-Natal, personal communication). The expression levels of the full length active form of congopain were very poor and the expressed protein was very unstable. Therefore, for this study attempts were made to clone and recombinantly express ΔFLVp in bacterial and yeast expression systems.

The 1.3 kb ΔFLVp fragment was obtained from a full length active vivapain fragment cloned into the pTZ57R/T vector (T vector) and mutant catalytic domain of vivapain fragment (ΔCAVp) in a pPic9 vector. The full length active vivapain fragment that was cloned in the T vector was obtained by using the touch down PCR method. The full length vivapain gene was amplified from *T. vivax* genomic DNA extracted from *T. vivax* parasites. The full length vivapain gene was ligated into a T vector and successfully transformed into *E. coli* JM 109 cells. The transformation was verified by performing a colony PCR with primers that amplify the C-terminal extension coding sequence. Bands corresponding to the amplified C-terminal portion coding region of the full length vivapain fragment were present, confirming the presence of the full length vivapain fragment in the T vector. The mutant catalytic domain of vivapain fragment (ΔCAVp) in a pPic9 vector was obtained from Professor Theo Baltz (University of Victor-Segalen, Bordeaux, France). The ΔCAVp fragment has an alanine coding sequence substituted for a cysteine coding sequence in the catalytic triad, thus coding for an active site Ala^{25}, His^{159} and Asn^{175} (papain numbering) as compared to active vivapain, which has Cys^{25}, His^{159} and Asn^{175} (papain numbering) in the active site. The ΔFLVp fragment was obtained by using a unique double cutter restriction enzyme, BsgI, which was unique to both the full length active vivapain coding fragment cloned into the T vector and mutant vivapain catalytic domain coding fragment (ΔCAVp) in pPic9. Therefore, this enzyme was able to remove both the active site (CHN) coding sequence from the full length active vivapain fragment and the inactive AHN coding

sequence from the ΔCAVp fragment. Thereafter, the AHN coding fragment was ligated into the BsgI restriction sites of the full length active vivapain fragment which was in the T vector, leading to the production of an inactive form of FLVp (ΔFLVp). The ΔFLVp in the T vector was transformed into *E. coli* JM 109 cells. Once again, the transformation efficiency was examined by colony PCR using C-terminal extension primers. The presence of the ΔFLVp fragment in the T vector was verified by the presence of the band corresponding to the C-terminal extension coding portion of the full length inactive vivapain fragment. The presence of the ΔFLVp fragment in the T vector was further verified by sequencing.

For preparation of expression in the methylotrophic *Pichia pastoris* yeast expression system, the ΔFLVp fragment was sub-cloned into the pPic9 expression vectors via the EcoRI and NotI restriction sites. The recombinant pPic9 colony plasmid DNA linearised with SalI containing the open reading frame (ORF) coding for the full length mutant form of vivapain was transformed into *P. pastoris* GS115 cells. A positive control, pPic9-C2 (H43W) in JM 109 cells was also transformed into *P. pastoris* GS115 cells. The pPic9-C2 (H43W) plasmid DNA was linearised with SacI. A colony PCR was performed to detect the presence of either the pPic9-ΔFLVp or the pPic9-C2 (H43W) DNA in the yeast genome. For the pPic9-ΔFLVp the C-terminal extension forward primer and the AOX reverse primer were used. These primers amplified a band that correlated to the positive control in the PCR. The AOX primers were used to detect the presence of the pPic9-C2 (H43W) DNA. This PCR revealed the presence of a band that correlated to the insert DNA and to the wild-type *AOX* gene. Both colony PCR assays confirmed that the transformed yeast colonies were recombinant.

The reason for choosing the methylotrophic *P. pastoris* yeast expression system was due to the simplicity of the technique and its ability to express high levels of foreign proteins, but most of all for its ability to produce multiple eukaryotic post translational modifications. These include disulfide bond formation, glycosylation, protein folding and proteolytic processing, which eliminates the need for renaturation of the protein after expression (Cereghino and Cregg 2000; Macauley-Patrick *et al.* 2005; Aloulou *et al.* 2006). However,

attempts made to express the recombinant ΔFLVp in the yeast expression system were unsuccessful. In contrast the C2 (H43W) positive control was successfully expressed as shown by the recognition by anti-congopain N-terminal peptide antibody before and after the three phase partitioning concentration step. Therefore, it can be concluded that it was not possible to express the ΔFLVp in the *P. pastoris* yeast expression system.

Due to the inability to express the ΔFLVp in the *P. pastoris* yeast expression system, it was decided to attempt expression in a bacterial system. There is evidence of successful expression of cysteine peptidases in bacteria; such as, full length cruzipain, the major cysteine peptidase of *T. cruzi,* in *E. coli* (Eakin *et al.* 1993). The ΔFLVp fragment was sub-cloned into pET-32a via the EcoI and NotI restriction sites. The pET-32a-ΔFLVp plasmid DNA was transformed into *E. coli* JM 109 cells. The recombinant pET-32a-ΔFLVp colony plasmid DNA was further transformed into the electrocompetent BL21 (DE3) cells before expression. The BL21 (DE3) strain was chosen due to the fact that it contains the T7 RNA polymerase and one of the key features of the DE3 lysogen is that is peptidase deficient (pET Expression System Manual).

Very high levels of ΔFLVp were expressed in the pET-32a BL21 (DE3) bacterial expression system and detected by anti-congopain N-terminal peptide antibody. This antibody had a good chance of detecting the ΔFLVp due to the high sequence identity between congopain and vivapain (Fig 1.8, Section 1.8.5) Although the expected size of the ΔFLVp is approximately 50 kDa, the protein was expressed as a 70 kDa protein as a result of 20 kDa sequence that is contributed by the pET-32a vector. The results of a solubility test revealed that the bacterial cells expressed the ΔFLVp as insoluble inclusion bodies. Attempts to purify the ΔFLVp under denaturing conditions failed. Therefore, it was decided to solubilise and renature the protein before further attempts were made to purify the expressed protein. The renaturation protocol was adapted from that used by Sijwali *et al* (2001) for the refolding of falcipain-2, a cysteine peptidase from *Plasmodium falciparum*. The renaturation process usually involves the use of a buffer which contains reduced and oxidised thiol agents such as glutathione, cysteine or cystamine for the formation of disulfide bonds (Singh and Panda 2005). In the present study, the renaturation buffer used

was based on the buffer optimised for the refolding of falcipain-2, which contained reduced and oxidised glutathione in a 1:0.5 ratio (Sijwali *et al.* 2001). The refolding buffer also contains EDTA, which prevents the metal-catalysed oxidation of the cysteine residues (Singh and Panda 2005). The buffer further contains the stabilising agent glycerol, which allows for the correct folding of the protein and prevents the aggregation of partially folded intermediates of the protein formed during the renaturation process (Jaspard 2000; Vallejo and Rinas 2004).

The renaturation process involves the gradual dilution of the ΔFLVp protein after it had been solubilised. This was followed by incubation in the renaturation buffer for approximately 20 hours; thereafter, the solution is concentrated by ultrafiltration. Analysis of the renaturation of ΔFLVp using SDS-PAGE revealed that there was no shift in the size evident between the non-renatured and renatured ΔFLVp samples, and there was a drastic loss in amount of protein present after the renaturation process. Therefore, results were inconclusive for the renaturation of ΔFLVp. Since there was such a major loss in the amount of protein present after the renaturation process, it was not feasible to perform further purification steps.

Since the ultimate goal of the recombinant expression of ΔFLVp was its use in diagnostic tests, it was decided to make antibodies against this recombinant preparation in both chickens and mice to use as positive controls in antigen detection ELISAs and to determine whether the antibodies could detect the peptidase in *T. vivax* lysates. Such antibodies would also be useful to follow vivapain gene silencing in RNAi and gene knockout studies. Strong antibody responses against the recombinant ΔFLVp were observed in both mice and chickens. An ELISA to determine the progression of antibody production in chickens over the 12 week period revealed that antibody production peaked from week five for chicken one and weeks four for chicken 2 with slight fluctuations in antibody levels over the subsequent weeks to week 11 for which analyses were performed. The titres of the antibodies produced after 5 weeks did not differ significantly.

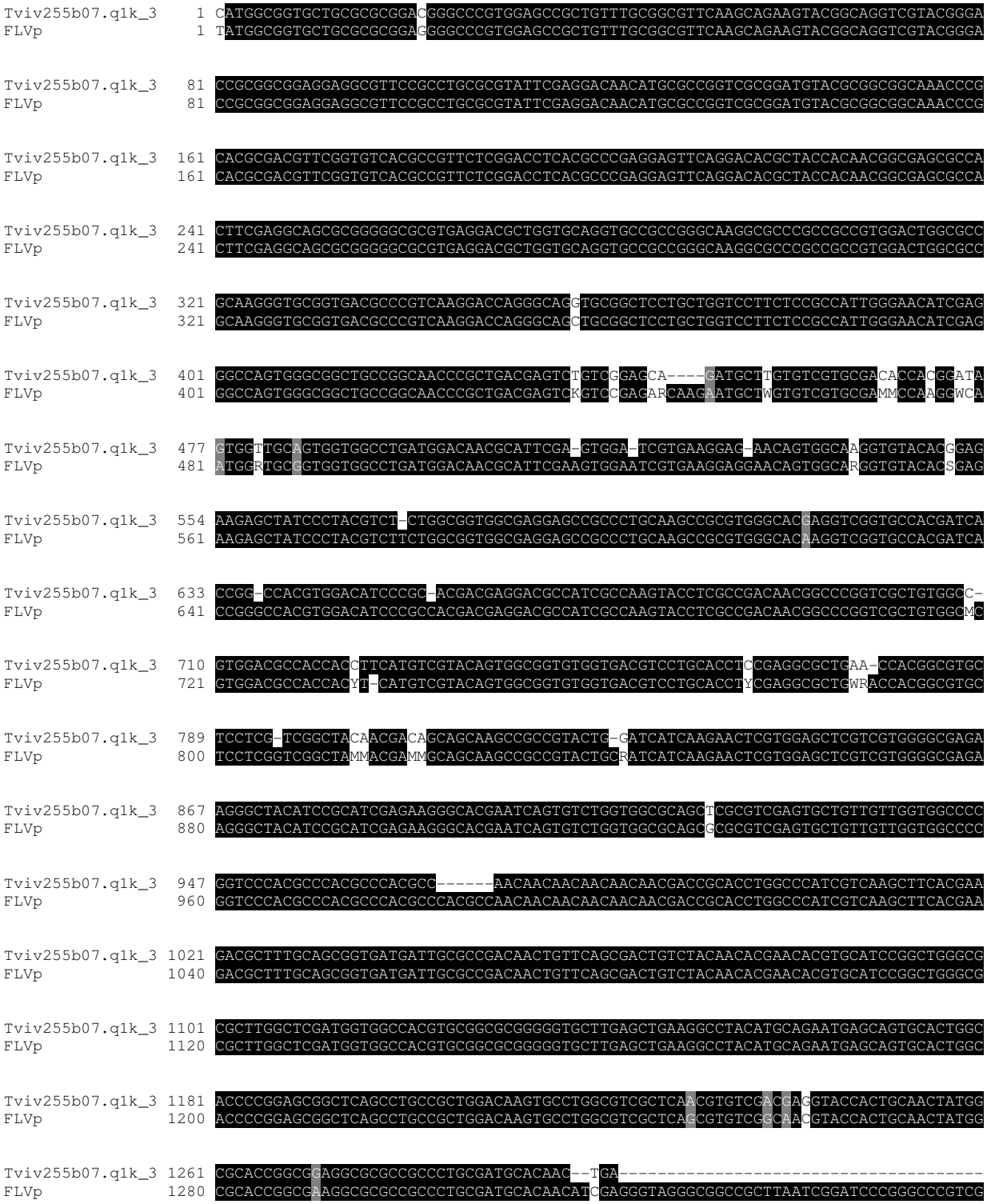
The general trend for both mice serum samples showed that antibody production peaked four weeks after the first immunisation. Thereafter, antibody production was relatively constant. The antibody titre revealed that mouse 2 produced higher levels of antibodies.

The antibodies produced in chickens and mice were able to detect the recombinant ΔFLVp used to produce these antibodies. The antibodies were tested on a *T. vivax* lysate in a western blot. Antibodies produced in chicken 2 and mouse 1 was used for the blot since the ELISA revealed that they produced higher levels of antibody. In contrast to the chicken antibodies, the mice serum was able to detect native vivapain in a *T. vivax* lysate.

In this study, the *ΔFLVp* gene was successfully cloned into the pTZ57R/T vector (T vector) and sub-cloned into the pPic9 and pET-32a expression vectors. The ΔFLVp was only able to be successfully expressed in the bacterial expression system. Antibodies were made against the recombinantly expressed ΔFLVp in both mice and chickens. The mice serum was able to detect the native vivapain in a *T. vivax* lysate. Therefore, the ability of the ΔFLVp to detect the native vivapain is a good indication that this antigen will be useful in detecting the *T. vivax* infections in the sera of infected animal for field diagnosis, using techniques such as the ELISA.

Appendix 1: The sequence alignment of FLVp with the vivapain sequence obtained from the database

The sequence alignment was performed using Clustal W2 program (http://www.ebi.ac.uk/Tools/clustalw2/index.html). The areas shaded in black shows where the sequences align



4. GENERAL DISCUSSION

Animal trypanosomosis is a major disease affecting livestock in sub-Saharan Africa. The trypanosome parasite causing the disease is transmitted by the *Glossina spp.* tsetse fly (Hursey 2001; Krafsur 2003; Antoine-Moussiaux *et al.* 2009). This disease is responsible for economic losses of US $4.5 billion annually in sub-Saharan Africa (Antoine-Moussiaux *et al.* 2009). Therefore, there is a great need for quick and effective diagnosis as well as methods for control of this devastating disease.

Current control measures that are being used for African animal trypanosomosis are tsetse fly eradication, the use of trypanocidal drugs and trypanotolerant cattle (McDermott and Coleman 2001; Naessens *et al.* 2002; Aksoy 2003; Holmes *et al.* 2004). Due to the negative impact that the trypanocidal sprays used for the eradication of tsetse flies have on the environment, the emergence of drug resistant parasites and the decreased used of trypanotolerant cattle because of their limited productivity, there is a great demand for improved control measures. The ability of the trypanosome to continually change its variable surface glycoprotein (VSG) coat, by a process known as antigenic variation, has made it extremely difficult to produce vaccines, especially one based on the VSGs (Vickerman 1978). For this reason, chemotherapy seems to be one of the key approaches for the control of trypanosomosis (McDermott and Coleman 2001; Antoine-Moussiaux *et al.* 2009). An interesting point to make is that diagnostic tools may also be used to monitor tsetse fly control and eradication methods, and provide information on the efficiency of chemotherapy and the development of trypanocidal drug resistance (Eisler *et al.* 2004).

The chemotherapeutic approach is based on targeting molecules that are essential for parasite survival and hence kill the parasites. The parasite cysteine peptidases are promising targets for chemotherapeutic drug design since they play an important role in the biology of trypanosomes (Sajid and McKerrow 2002; Antoine-Moussiaux *et al.* 2009). The cysteine peptidases assist the parasite in differentiation at the different stages of the life cycle, invading the host by parasite migration through tissue barriers and altering the host immune defence (Chagas *et al.* 1997; McKerrow *et al.* 2006).

Chemotherapy based on targeting the parasite cysteine peptidases has been introduced for many other parasitic diseases such as Chagas disease (McKerrow *et al.* 1995), malaria (Rosenthal 1998; Rosenthal 2004), schistosomiasis (Abdulla *et al.* 2007) and leishmaniasis (Croft and Coombs 2003). The backbone of this approach is to produce synthetic inhibitors, targeting the cysteine peptidase, which could potentially be used in the production of new drugs. This approach has been very promising in the last decade since the synthetic peptide inhibitors against the cysteine peptidases have been able to reduce and in some cases even cure *Plasmodium, Leishmania* and *Trypanosoma* infections (McKerrow *et al.* 1999). Synthetic inhibitors that proved to be promising as trypanocidal agents were the irreversible vinyl sulfones, the peptidyl ketones; namely, chloromethylketones, diazomethylketones and fluromethylketones (Anene *et al.* 2001). These inhibitors were trypanocidal against *T. brucei* (Scory *et al.* 1999; Troeberg *et al.* 1999), *T. congolense* (Mbawa et al. 1992) and *T. cruzi* infections (Ashall *et al.* 1990; Bonaldo *et al.* 1991; Harth *et al.* 1993; Engel *et al.* 1998). These findings brought about the need for this part of the study, which involved recombinantly expressing and enzymatically characterising a cysteine peptidase from *T. vivax*, known as vivapain. To date, there has been no published data on the enzymatic characterisation of this enzyme as compared to the cysteine peptidases of *T. brucei*, *T. congolense, T. cruzi.* By enzymatically characterising these cysteine peptidases, and more importantly, acquiring information on the amino acid residues preferred in the P_1 and P_2 positions of substrates, this would give the necessary information for the production of synthetic irreversible inhibitors. These inhibitors would be comprised of the preferred amino acid residues linked to vinyl sulfones or peptidyl ketones.

The trypanosomal cathepsin L-like cysteine peptidases belong to the C1 family of clan CA papain superfamily (Barrett and Rawlings 2004; Caffrey and Steverding 2009). This family of peptidases is characterised by the presence of a Cys^{25}, His^{159} and Asn^{175} (papain numbering) catalytic triad in the active site. The primary structure of these cysteine peptidase possess a pre-region, pro-region and a catalytic domain. The peptidases also contain a C-terminal extension, which is unique to parasite cysteine peptidases (Authié *et al.* 2001). The C-terminal extension of congopain and trypanopain are proline rich, while

that of vivapain is threonine rich. The exact function of this region is unknown; however, since it has proven to be very immunogenic it could potentially play a role in altering the host's immune defences. The presence or absence of this region does not affect the activity of the peptidase in any way (Robertson *et al.* 1996).

L-trans-Epoxysuccinyl-leucylamido(4-guanidino)butane (E-64) is the irreversible inhibitor for all clan CA cysteine peptidases. This inhibitor was able to irreversibly inhibit vivapain in the present study and was used as the active site titrant. The substrate specificity of cysteine peptidases is determined by the occupation of the S2 pocket on the peptidase (Sajid and McKerrow 2002; Rosenthal 2004; Caffrey and Steverding 2009). Cysteine peptidases belonging to this family prefer bulky hydrophobic amino acid residues such as phenylalanine in P_2 (Alves *et al.* 2001; Lecaille *et al.* 2001) and a wide range of amino acids are accepted in P_1; however, there is a preference for basic amino acids such as arginine in this position (Alves *et al.* 2001). Most of the peptidases belonging to the papain family possess endopeptidase activity (Mbawa *et al.* 1992), while Vp was also shown to have exopeptidase activity.

Since there has been no published data on vivapain, it was decided to use congopain as a positive control in this study. Both the catalytic domains of vivapain (Vp) and congopain (C2) were successfully expressed in the *P. pastoris* yeast expression systems and purified. Purification of Vp was conducted using the TPP method and molecular exclusion chromatography. Evidence of their expression was verified by detection with suitable antibodies in western blots. Interesting to note is that the mature vivapain has a glycosylation site at position Asn^{288} [determined using a prediction program; NetNGlyc 1.0 Server (www.cbs.dtu.dk/services/NetNGlyc)], since the double band characteristic of the vivapain sample was present before and after activation at pH 4.2. This is not a characteristic of cruzipain and rhodensain, for example, since these cysteine peptidases do not contain a glycosylation site in the mature region of the protein (Eakin *et al.* 1993; Caffrey *et al.* 2001). Both recombinantly expressed proteins, Vp and C2, were catalytically active and the exact amount of active enzyme was determined by active site titration using E64.

The enzymatic studies performed revealed that Vp and C2 were able to hydrolyse synthetic substrates with a phenylalanine residue (hydrophobic) in P_2 and an arginine residue (basic) in P_1, which was expected. Literature revealed that other cysteine peptidase were also able to hydrolyse the same substrates such as native congopain from *T. congolense* (Authié *et al.* 1992; Mbawa *et al.* 1992; Chagas *et al.* 1997), trypanopain from *T. b. brucei* (Troeberg *et al.* 1996), native and recombinant falcipain-2 from *Plasmodium falciparum* (Shenai *et al.* 2000), native and recombinant cruzipain from *T. cruzi* (Cazzulo *et al.* 1990; Serveau *et al.* 1996), mammalian cathepsin L (Brömme *et al.* 1989), cathepsin L-like cysteine peptidase from *Fasciola hepatica* (Wijffels *et al.* 1994), and a cysteine peptidase from *L. infantum* (Salvati *et al.* 2001). Both Vp and C2 were also able to hydrolyse substrates with a hydrophobic residue (leucine) in P_2 and a basic residue (lysine) in P_1. Trypanopain from *T. brucei* was also able to hydrolyse these substrates (Troeberg *et al.* 1996). Interesting to note is that Vp and C2 were only able to hydrolyse synthetic peptides if position P_2 was occupied by a hydrophobic amino acid residue such as Phe and Leu. These peptidases were also unable to hydrolyse peptides with a hydrophobic residue in P_1.

The hydrolysis of Z-Phe-Arg-AMC or D-Val-Leu-Lys-AMC by Vp and C2 was reversibly inhibited by the low molecular weight leupeptin, antipain and chymostatin. Native trypanopain from *T. b. brucei,* native and recombinant cruzipain from *T. cruzi*, native congopain from *T. congolense*, native and recombinant falcipain-2 from *P. falciparum* and a cathepsin L-like cysteine peptidase from *F. hepatica* (Cazzulo *et al.* 1990; Eakin *et al.* 1992; Mbawa *et al.* 1992; Wijffels *et al.* 1994; Troeberg *et al.* 1999; Shenai *et al.* 2000) were also inhibited by these reversible inhibitors. The hydrolysis of Z-Phe-Arg-AMC and D-Val-Leu-Lys-AMC by Vp and C2 was irreversibly inhibited by iodoacetic acid (IAA) and iodoacetamide (IAN). By analysing the interaction of an enzyme with its potential inhibitor, the reaction kinetic constants can be determined. These constants may be used to compare different inhibitors in order to find the best inhibitor for a particular peptidase. The kinetic constant values can give an indication of how much inhibitor to use and how much time should be allocated for the inhibition to take place (Salvesen and Nagase 1989).

The pH optimum obtained for Vp was pH 7.0 and for C2 was pH 6.5. This is surprising since most of the cysteine peptidases are located in the lysosome and the pH in this environment is usually acidic (Rosenthal 1999) and it would thus be expected that the pH optimum should be lower. Cysteine peptidases such as congopain are believed to be involved in intracellular proteolytic processing (Mbawa *et al.* 1991a). Due to the pH optimum of C2 being very close to neutral, it can be deduced that this peptidase may have roles outside the lysosome, and the same could, therefore, be said for Vp. Most other parasite cysteine peptidases also have a pH optimum in the same range of between 6 and 7.5 (Wijffels *et al.* 1994; Serveau *et al.* 1996; Troeberg *et al.* 1996; Shenai *et al.* 2000), which contributes to their role in the pathogenesis of the diseases.

The results obtained for the enzymatic studies performed gives insight and vital information such as the amino acid residues that Vp prefers in P_1, P_2 etc which could be used for potential future design of synthetic inhibitors for the development of chemotherapeutic drugs that could be potentially trypanocidal to *T. vivax.* The work would involve producing synthetic peptide inhibitors that would have the chemical groups such as the vinyl sulfones and the peptidyl ketones attached to the preferred amino acid residues in P_1, P_2 etc and testing for their inhibitory action on Vp. Since trypanosomal cysteine peptidases are similar to the host peptidases, care must be taken to allow for the least amount of toxicity to the host (Steverding *et al*. 2006; Pink *et al.* 2005).

The diagnosis of African animal trypanosomosis can be performed by a number of techniques, such as clinical, parasitological, molecular and serological. In most cases, the animals affected by trypanosomosis are found in areas that have poor physical and economic conditions; therefore, the diagnostic methods used should be suitable for use in the field. Other requirements for a diagnostic test are that the diagnosis needs to be specific and sensitive. The results obtained from the diagnostic tests should also be easy to interpret (Luckins 1992). One of the main reasons for diagnosis is to provide information to determine the most suitable method of therapeutic application (Eisler *et al.* 2004).

The most suitable serological diagnostic techniques to be used in conjunction with parasitological techniques for the diagnosis of *T. congolense, T. vivax* and *T. brucei* infections is the ELISA (Luckins 1992). This is due to the sensitivity, simplicity, accuracy and reliability of the technique as well as the fact that it does not require extremely specialised equipment and many samples can be tested at one time due to the automation of the equipment used (Nantulya 1990; Rebeski *et al.* 1999; Eisler *et al.* 2004). The antibody-detection ELISA and the antigen-detection ELISA are the two forms of ELISA that can be used for diagnosis. The most important requirement for these ELISAs is a species-specific antigen. These antigens can be used in the antibody-detection ELISA and they can also be used to produce antibodies that can be used in the antigen-detection ELISAs. There is, however, a lack of available recombinant trypanosome antigens required for the detection and production of antibodies for both ELISA techniques. Until recently, the antigens used in these ELISAs were based on crude preparations of whole parasite lysate. In one such study, the antigen used for the antibody-detection ELISA was obtained from tissue culture preparations of the procyclic and blood stream forms of *T. brucei* trypanosomes (Greiner *et al.* 1997). The use of native antigen preparation from whole parasite lysate for the ELISAs made it difficult to optimise and standardise the diagnostic tests (Greiner *et al.* 1997; Eisler *et al.* 2004; Tran *et al.* 2009).

The invariant surface glycoprotein ISG75 of *T. brucei*, full length mutant of congopain, FL28 and RoTat 1.2 variable surface glycoprotein recombinant antigens have been produced and used to detect *T. brucei* (Tran *et al*. 2008), *T. congolense* (Davita, 2009)(MSc) and *T. evansi* (Verloo *et al.* 2001; Lejon *et al.* 2005), respectively. Recombinant invariant surface glycoprotein ISG75 of *T. evansi* has also been expressed and was also able to detect *T. evansi* infections (Tran *et al.* 2009). However, none of these antigens were specific for the diagnosis of *T. vivax* infections (European Commission 6th Framework Research grant, Trypadvac2). Due to the high amount of cross-reaction that occurs in the ELISA methods when testing sera of animals infected with trypanosomosis, it is important for diagnostic sensitivity that sera is tested against an array of trypanosome species-specific antigens. The main pathogens causing disease in the African livestock are

T. congolense, T. vivax and to a lesser extent *T. brucei.* There are recombinant antigens; namely, the invariant surface glycoprotein ISG75 of *T. brucei* and full length mutant of congopain, FL28 specific for *T. brucei* and *T. congolense* infections, respectively. Therefore, there is a need to produce an antigen that would be specific in detecting *T. vivax* infections, since these three parasites are the main causative agents of bovine trypanosomosis.

For this reason, this study also focussed on the production of a recombinant antigen derived from the *T. vivax* that could potentially be used for specific diagnosis of *T. vivax* infections. The antigen that was produced was the full length (FL) inactive mutant form of vivapain (ΔFLVp). The decision to express the ΔFLVp instead of full length active form of vivapain was based on prior studies which showed that the expression of the full length active form of congopain resulted in a very low yield and that the expressed protein was very unstable (Boulangé A, University of KwaZulu-Natal, personal communication).

The ΔFLVp clone was obtained by firstly cloning a FL active form of vivapain, obtained by PCR using specific FL primers and *T. vivax* genomic DNA as the template, into a pTZ57R/T vector. Thereafter, the Cys^{25} residue from the catalytic domain of the FL active form was replaced with an Ala^{25} residue from an already existing mutated catalytic domain (C25A) clone which was present in the pPic9 vector. This mutation changes the conventional Cys^{25}, His^{159}, Asn^{175} (papain numbering) catalytic domain of the FL active form of vivapain to Ala^{25}, His^{159}, Asn^{175}. This mutation was made possible by using a specific restriction enzyme, i.e. BsgI that was specific to the full length active clone and the mutated catalytic clone. The restriction sites for this enzyme were present on either side of CHN coding domain in the full length active clone and the ANH coding domain on the mutated catalytic clone. Therefore, the use of this restriction enzyme allowed for the replacement of the entire section of the gene that codes for the Cys, His and Asn residues

from the full length active clone as well as the surrounding residues with the Ala, His and Asn and surrounding residues from the mutated catalytic domain clone.

This FL mutant was cloned into a yeast expression vector, pPic9, and a bacterial expression vector, pET32, but expression of the ΔFLVp was only successful in the bacterial expression system. Although this expression system resulted in very high yields of the recombinant ΔFLVp, the expressed protein was insoluble. This made purification difficult; hence attempts were made to renature the protein first. The method used for renaturation was based on that used for the refolding of falcipain-2 (Sijwali *et al.* 2001). The recombinant ΔFLVp solubilised in and treated with renaturation buffer that contained 100 mM Tris-Cl, 1 mM EDTA, 20% Glycerol, 1 M KCl, 1 mM GSH, 0.5 mM GSSG. The results from the renaturation were inconclusive since there was no clear shift in size between the renatured and non-renatured protein as was seen for falcipain-2 (Sijwali *et al.* 2001). This process also led to a drastic loss of the recombinant ΔFLVp. If this work were to be repeated then expression of the mutant should be performed under different conditions such as lowering the temperature at which the culture is grown. Studies have shown that lowering the incubation temperature leads to a decreased amount of inclusion bodies and an increase in the production of soluble protein (Shirano and Shibata 1990). By obtaining more soluble protein, this would alleviate the need to renature the protein and hopefully prevent such a drastic loss of protein in the purification steps.

Antibodies were produced against the recombinant solubilised ΔFLVp in both mice and chickens as indicated by ELISA. The antibodies from both chickens (IgY) and mice (sera) detected the recombinant ΔFLVp in western blots. The blots showed significant levels of non-specific antibody binding. This could have been due to the fact that the chicken and mouse antibodies were not affinity purified. It could also be due to the possibility that too much protein was loaded on the SDS-PAGE gel. A very positive and interesting point to note from a diagnostic point of view is that the serum from one of the mice was able to detect native vivapain in a *T. vivax* lysate. This suggests that the antibodies produced against the ΔFLVp antigen could potentially be used to detect *T. vivax* in sera of infected animals.

In this study, the catalytic domain of vivapain (Vp) was expressed in a yeast expression system and enzymatically characterised. This information could provide insight for future work to synthesise synthetic inhibitors which targets vivapain and could potentially be trypanocidal to the *T. vivax* parasite. This study also involved the expression of ΔFLVp in the bacterial expression system. The ΔFLVp was expressed for use as an antigen or it could be used to produce antibodies, which ultimately could be useful in the ELISA diagnostic tests. Since the ΔFLVp was expressed as an insoluble protein, future work will be based on attempting to express the mutant cultures at lower incubation temperatures, which could potentially lead to the production of more soluble protein. Antibodies against the ΔFLVp recombinant protein were produced in both mice and chickens. The mouse serum was able to detect the native vivapain from a *T. vivax* lysate.

The findings from enzymatic studies of Vp gives information that is vital for the future production of new chemotherapeutic drugs, which could potentially aid in the control of this disease. The ΔFLVp antigen could finally be used to specifically diagnose *T. vivax* infections as well as be used in conjunction with other antigens specific for *T. congolense* and *T. brucei* infections to validate diagnosis of trypanosomosis in cattle.

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