

**BIOCHEMICAL STUDIES ON TRYPANOSOMAL  
PROLYL OLIGOPEPTIDASE FAMILY PATHOGENIC  
FACTORS**

**SIJABULISIWE FAITH NDLOVU**

BSc (Hons) Biochemistry

Submitted in fulfillment of the academic requirements for the degree of Master of  
Science in the Department of Biochemistry, School of Life Sciences  
University of KwaZulu-Natal

Pietermaritzburg

2014

## PREFACE

The experimental work described in this dissertation was carried out in Biochemistry, School of Life Sciences, University of KwaZulu-Natal (Pietermaritzburg campus), from January 2013 to December 2014, 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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Sijabulisiwe Faith Ndlovu (candidate)


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Professor Theresa H. T. Coetzer (supervisor)

## DECLARATION - PLAGIARISM

I, Sijabulisiwe Faith Ndlovu declare that

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## ABSTRACT

African Animal trypanosomosis, also known as Nagana, is a parasitic disease which affects many mammalian species, mainly livestock such as cattle, sheep and goats. The disease also affects humans (Human African Trypanosomosis) and in this case is referred to as sleeping sickness. Nagana is caused by the *Trypanosoma* parasite, which is transmitted to the host by a bite from the tsetse fly (*Glossina spp*). The *Trypanosoma* causing trypanosomosis in animals are *Trypanosoma congolense*, *T. vivax* and *T. brucei brucei*.

Vaccine development has been unsuccessful, due to the presence of the variant surface glycoprotein on the surface of parasites which undergoes antigenic variation therefore enabling the parasite to avoid detection by vaccines. A chemotherapeutic drug such as isometamidium chloride combined with diminazene and suramin have also had little success due to the increase in drug resistance. During infection of the host, trypanosomal parasites utilise various proteolytic enzymes such as the oligopeptidases, which hydrolyse important host factors such as peptide hormones. These proteolytic enzymes are thus considered to be pathogenic factors which contribute to the manifestation of various trypanosomosis symptoms such as anaemia, fever, paralysis and disturbances in sleep cycle patterns. It is these pathogenic factors which are now being considered as drug targets in the hope to eradicate the spread or continuous advancement of trypanosomosis. Three trypanosomal pathogenic factors, which are serine oligopeptidases which belong to the prolyl oligopeptidase family of serine proteases (Clan SC in subfamily S9) were the focus of this study, namely, prolyl oligopeptidase (POP) from *T. b. brucei* (*TbPOP*) and *T. congolense* (*TcoPOP*) as well as oligopeptidase B (OPB) from *T. congolense* (*TcoOPB*). The full length *TbPOP* gene was cloned into pTZ57R/T cloning vector and successfully sub-cloned into pET32a expression vector and recombinantly expressed in its insoluble form at a size of approximately 100 kDa using the *Escherichia coli* BL21 DE3 expression system. *TbPOP* expression was confirmed by western blot probed with anti-His tag antibodies. Expression of *TbPOP* was optimised under varying temperatures and IPTG concentrations in an attempt to solubilise the inclusion bodies. However, the protein was expressed as part of inclusion bodies. Therefore, urea denaturation was used for its solubilisation. Following solubilisation, recombinant *TbPOP* was partially purified on a Ni<sup>2+</sup> affinity resin. Further attempts to purify *TbPOP* by molecular exclusion chromatography (MEC) were unsuccessful, this could be due to aggregation of the protein during the refolding step. Therefore refolding by a Sephadex G-25 desalting

column was attempted as it removes some impurities. However, further purification by MEC and ion exchange chromatography (IEC) were unsuccessful. The full-length *TcoPOP* gene was successfully cloned into pGEM-T® cloning vector and subsequently sub-cloned into pET32a expression vector. However, upon sequencing of the plasmid DNA, it was discovered that a mutation had occurred in the recombinant *TcoPOP* DNA sequence forming the stop codon “TAG” which resulted in the termination of protein expression, therefore, further work on *TcoPOP* was not pursued.

*TcoOPB* was successfully recombinantly expressed in pET28a using the *E. coli* BL21 DE3 expression system. The protein had a size of approximately 80 kDa. The protein was affinity purified using a Ni<sup>2+</sup> affinity resin. Expression of *TcoOPB* was confirmed by western blot using chicken raised anti-*TcoOPB* antibodies. Cross-reactivity of chicken anti-*TcoOPB* antibodies with *TbPOP* was also assessed and no cross-reactivity was found which was expected as POP and OPB only share 25% sequence identity. In order to determine the biochemical characteristics of *TbPOP* and *TcoOPB*, various activity assays and kinetics studies were conducted. It was found that *TbPOP* was able to hydrolyse type I collagen from rat tail. In contrast however, *TbPOP* was unable to digest gelatin which is a denatured form of collagen. Upon further analysis of *TbPOP* with the synthetic peptide substrate Z-Gly-Pro-AMC, it was found not to have activity as it was unable to hydrolyse the substrate, this is thought to be due to the misfolding of the protein during the refolding step. *TcoOPB* on the other hand was unable to hydrolyse either collagen or gelatin. Further biochemical analysis of *TcoOPB* was conducted using synthetic peptide substrates, the kinetic parameters of *TcoOPB*  $K_m$ ,  $k_{cat}/K_m$  were determined and it was found that OPB had a high affinity for the substrates Z-Arg-Arg-AMC, Z-Gly-Gly-Arg-AMC, H-Ala-Phe-Lys-AMC and Z-Pro-Arg-AMC and lower affinity for the substrates H-Pro-Phe-Arg-AMC, H-D-Val-Leu-Lys-AMC, Z-Gly-Pro-AMC, Suc-Ala-Phe-Lys-AMC, Boc-Leu-Gly-Arg-AMC. OPB was also found to have an optimal pH of 8 – 9 and retained 79% of its optimal activity at the physiological pH of 7.4. *TcoOPB* was found not to have good diagnostic potential as an indirect ELISA revealed that the antigen was unable to detect antibodies in *T. congolense* infected cattle sera. This study laid the foundation to conduct further studies on *TbPOP*, *TcoPOP* and *TcoOPB* as chemotherapeutic and diagnostic targets for Nagana.

## **ACKNOWLEDGEMENTS**

I would like to express my gratitude and appreciation to the following people and institutions:

My supervisor, Professor Theresa H. T. Coetzer for all her assistance, support and expert guidance throughout this study and extremely helpful analysis of my dissertation.

Charmaine Ahrens and Pat Joubert for all their assistance in financial and administrative matters.

My fellow post-graduate students, Phindile Ximba, Omolara Baiyegunhi, Lauren Eyssen, Sanele Mnkandla, Kayleen Brien, Alex Delpport, Jacky Viljoen, Kelvin Addicott, Faiaz Shaik and Robert Krause for their advice and happy times in the lab.

The National Research Foundation for the financial support.

To mom and dad and my siblings for all their support, love and words of encouragement during the dark days.

To Siboniso Latha, for love, patience, support and the happy memories.

And lastly to the Lord Jesus Christ for all he has done for me in my life.

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## ABBREVIATIONS

2x YT	2 x yeast extract, tryptone
A <sub>280</sub>	absorbance at 280 nm
A <sub>405</sub>	absorbance at 405 nm
A <sub>595</sub>	absorbance at 595 nm
ABTS	2,2-azino-di-[3-ethylbenzthiazoline sulfonate]
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
AMC	7-amino-4-methylcoumarin
AMT	acetate-MES-Tris
ANF	atrial natriuretic factor
ATP	adenosine triphosphate
BCA	bicinchoninic acid
Bis	N,N'-methylenebisacrylamide
Bis-Tris	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol
Boc	butyloxycarbonyl
bp	base pair
BSA	bovine serum albumin
Bz	benzoyl
C-terminal	carboxy-terminal
dH <sub>2</sub> O	distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay

<i>g</i>	relative centrifugal force
h	hour (s)
HRPO	horseradish peroxidase
[I]	inhibitor concentration
IAA	iodoacetic acid
IgG	immunoglobulin G
IgY	immunoglobulin Y
IPTG	isopropyl- $\beta$ -D-thiogalactopyranoside
$k_{ass}$	rate of complex association
$k_{cat}$	turnover number
kDa	kilo-Dalton
$K_i$	inhibition constant
$K_{iapp}$	apparent inhibition constant
$K_m$	Michaelis-Menten constant
$k_{obs}$	pseudo first-order inhibition rate constant
MEC	molecular exclusion chromatography
MeoSuc	methoxy-succinyl
MES	acetate-2(N-morpholino)ethanesulfonic acid
min	minute (s)
N-Cbz	benzyl chloroformate
N-terminal	amino-terminal
OPB	oligopeptidase B
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline, pH 7.2
PCR	polymerase chain reaction
pI	isoelectric point
POP	prolyl oligopeptidase

RNAi	RNA interference
RT	room temperature
s	second (s)
SAP	Shrimp alkaline phosphatase
[S]	substrate concentration
SD	standard deviation
SDS	sodium dodecyl sulfate
Suc	succinyl
$t_{1/2}$	half-life
TAE	tris-acetate-EDTA
TBS	tris buffered saline
TEMED	N,N,N',N'-tetramethyl ethylene diamine
TLCK	N-tosyl-L-lysyl chloromethylketone
TPCK	N-tosyl-L-phenylalanyl chloromethylketone
TPP	three phase partitioning
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
$V_{max}$	maximum velocity
$v_0$	initial velocity
$V_t$	total column volume
X-gal	isopropyl- $\beta$ -D-thiogalactopyranoside
Z	benzyloxycarbonyl



# CHAPTER 1

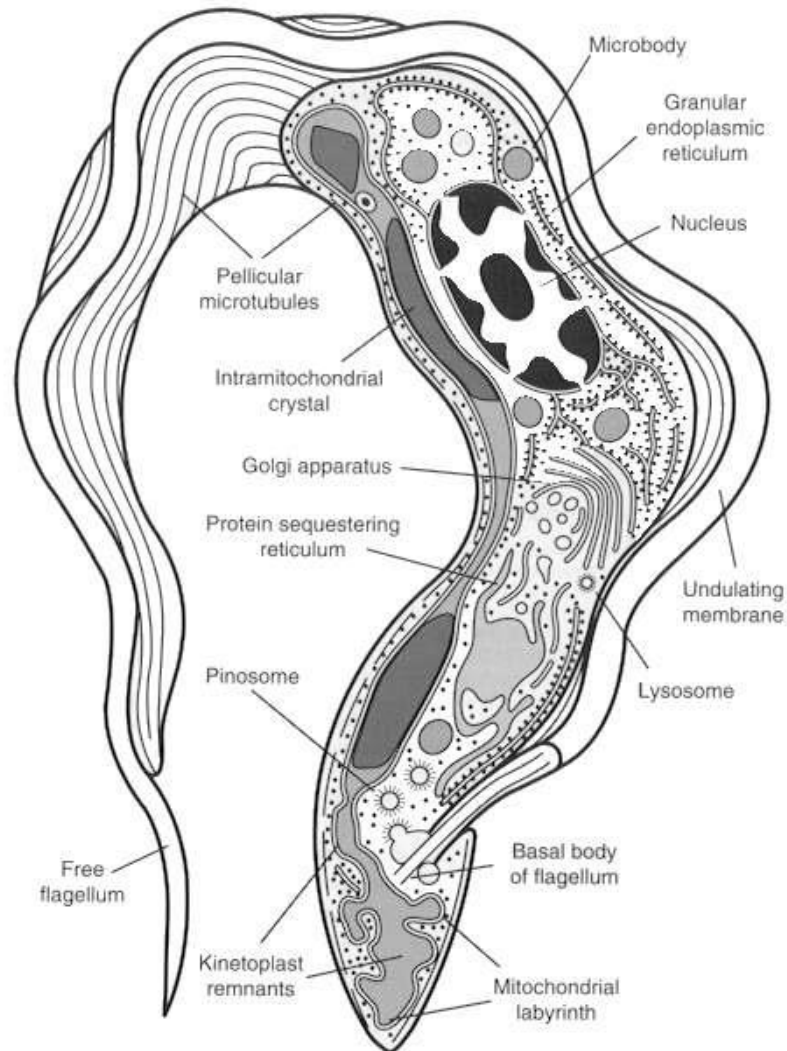
## Literature Review

### 1.1. Trypanosomosis

Animal African trypanosomosis (AAT), also known as Nagana, is a parasitic disease which affects mainly livestock such as cattle, sheep and goats (Nantulya, 1990). The disease also affects humans (Human African trypanosomosis, HAT) and in this case is referred to as sleeping sickness (Chappuis *et al*, 2005). Nagana is caused by the *Trypanosoma* parasite which is transmitted to the host by a bite from the tsetse fly (*Glossina spp.*) which acts as the disease vector (Steverding, 2008). There are various protozoan species from the *Trypanosoma* genus which cause Nagana, namely, *Trypanosoma congolense*, *T. vivax* and *T. b. brucei* (Nantulya, 1990). *Trypanosoma brucei* can be sub-divided further into three subspecies, *T. b. rhodesiense* and *T. b. gambiense* which both cause HAT (Barrett *et al*, 2003) whilst the third subspecies, *T. b. brucei* only infects animals (Vickerman, 1985). Symptoms of trypanosomosis include inflammation due to parasites proliferating at the point of infection (Barrett *et al*, 2003), fever, paralysis (Steverding, 2008), weight loss, severe anaemia and even infertility (Nantulya, 1990) to name just a few. Since the disease affects mostly livestock, it leads to devastating economic losses of approximately US \$ 1.3 billion per annum (Shaw, 2004) mainly in the African agriculture industry (Chamond *et al*, 2010).

#### 1.1.1 Trypanosomes and their classification

The Genus *Trypanosoma*, belongs to the Family Trypanosomatidae and the Order Kinetoplastida (Haag *et al*, 1998). These unicellular flagellated protozoan parasites (Figure 1.1) (Daniels *et al*, 2010) have a single flagellum which extends from the posterior end (Chappuis *et al*, 2005) and is attached to the cell via a microtubule system known as the flagellum attachment zone (Gull, 1999), the parasites also have a microtubule cytoskeleton with an underlying plasma membrane which gives them their elongated shape. They also contain various single copies of cell organelles such as the kinetoplast, basal body, nucleus and mitochondrion (Christensen & Kabat, 1991).

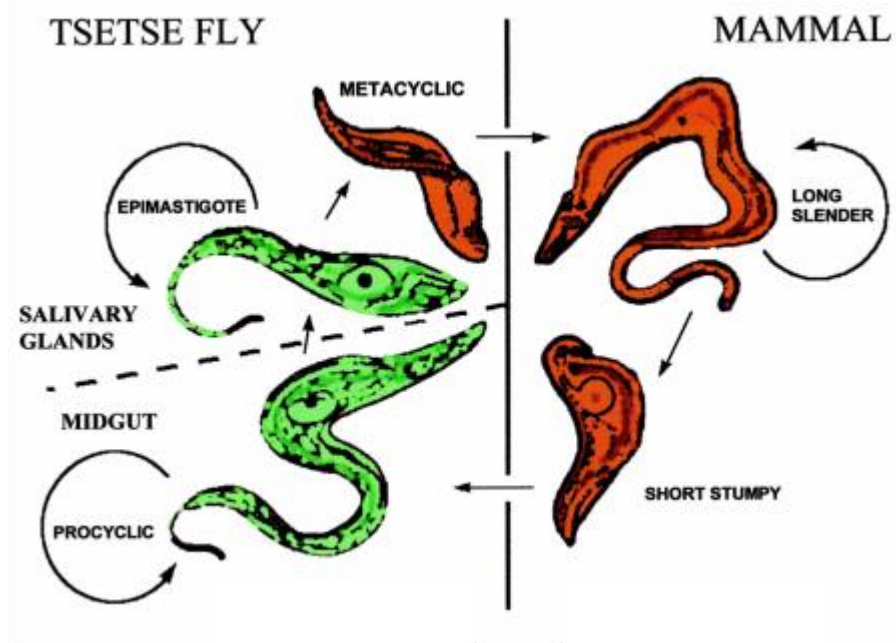


**Figure 1.1: The anatomy of the *Trypanosoma evansi* parasite.** The parasite has a single free flagellum emerging from the flagella pocket, the kinetoplast, a microtubule cytoskeleton and large nucleus. <http://www.parasitemuseum.com/trypanosome/> (accessed: 17 December 2013).

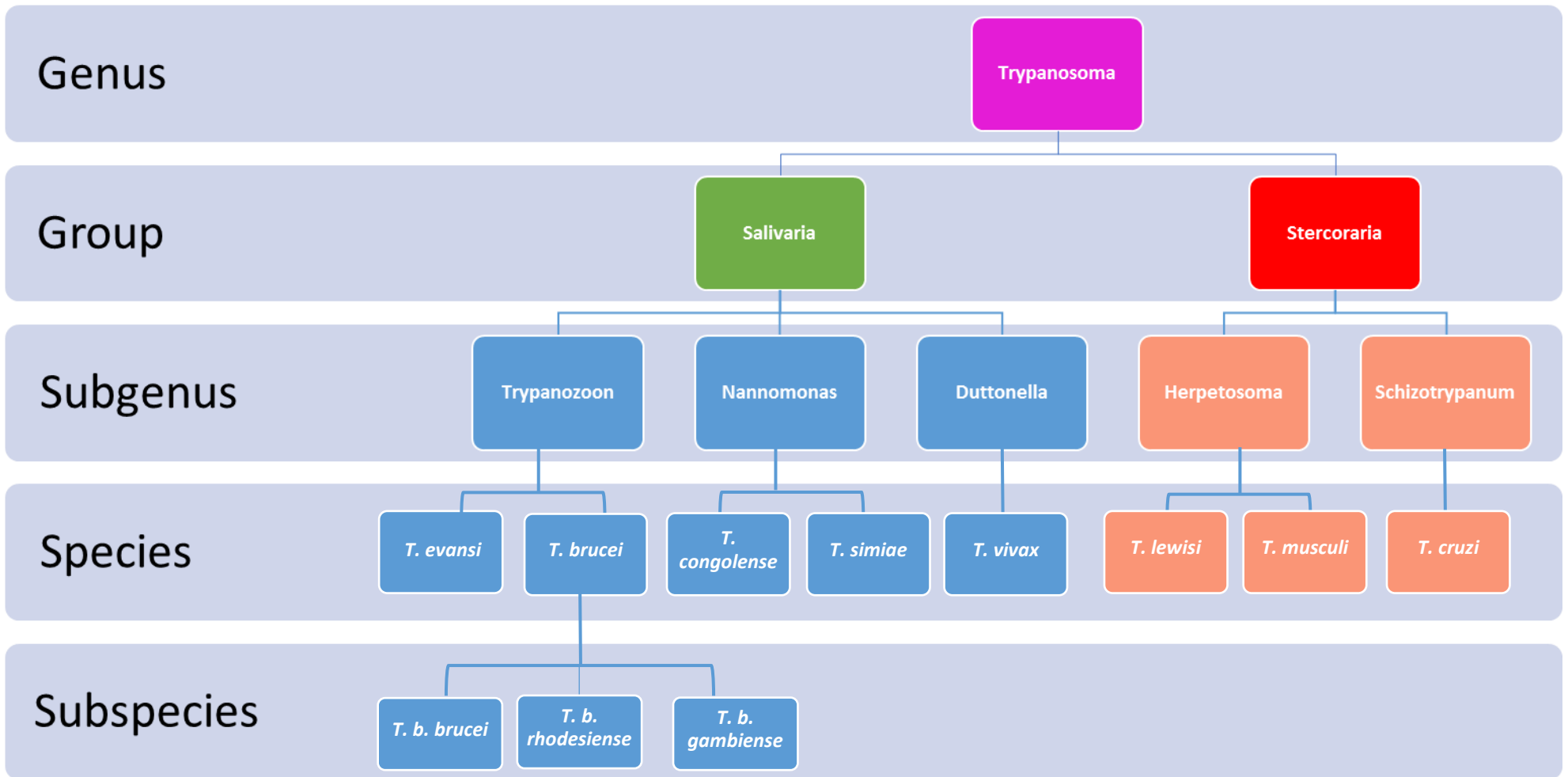
As trypanosomes were discovered, they were distinguished or classified into species and subspecies according to their morphology, capacity to cause disease, the range of hosts they infect and geographical distribution (Gibson, 2007). Mammalian trypanosomes are divided into two groups, the Salivaria and Stercoraria (Fig. 1.3), depending on the method by which they are transmitted to a host (Haag *et al*, 1998). Trypanosomes such as *T. brucei*, which belong to the salivarian group, are transmitted to the host through the saliva of the vector and trypanosomes such as *T. cruzi*, which belong to the stercoraria group, are transmitted to the host through the faeces of vectors such as leeches and infect the host through open wounds (Stevens & Gibson, 1999).

### 1.1.2 Trypanosome life cycle and mechanism of infection

In order for the trypanosome parasite to infect the host and survive in the host bloodstream, following transmission by the tsetse fly vector, it goes through a life cycle of two stages and undergoes several morphological changes (Vickerman, 1969). Figure 1.2 shows the different forms of the parasite in various stages of development in both the tsetse fly and the mammalian host (El-Sayed *et al*, 2000). The blood stages of the parasite proliferate in infected mammals after which they are ingested by the tsetse fly. The parasites then migrate to the tsetse midgut where they undergo differentiation into the procyclic form (Vickerman, 1985) and they subsequently migrate to the salivary glands (Donelson & Rice-Ficht, 1985) and attach as epimastigote forms. Both the procyclic and epimastigote forms of the parasite are non-infective and undergo proliferation. The parasites then finally differentiate into the metacyclic forms which are highly infective and do not undergo proliferation (Vickerman, 1969). They are then transmitted as long and slender forms, to a new host during the next feeding of the tsetse fly (Vickerman, 1985).



**Figure 1.2:** The life cycle of the trypanosome parasite *T. brucei*. The various stages of the development of the parasite are depicted in both the disease vector, the tsetse fly, and the mammalian host. Adapted from El-Sayed *et al* (2000).



**Figure 1.3: Classification of the *Trypanosoma* genus.** All the species and subspecies from the *Salivaria* and *Stercoraria* groups which are infectious to mammals. Adapted from Haag *et al* (1998).

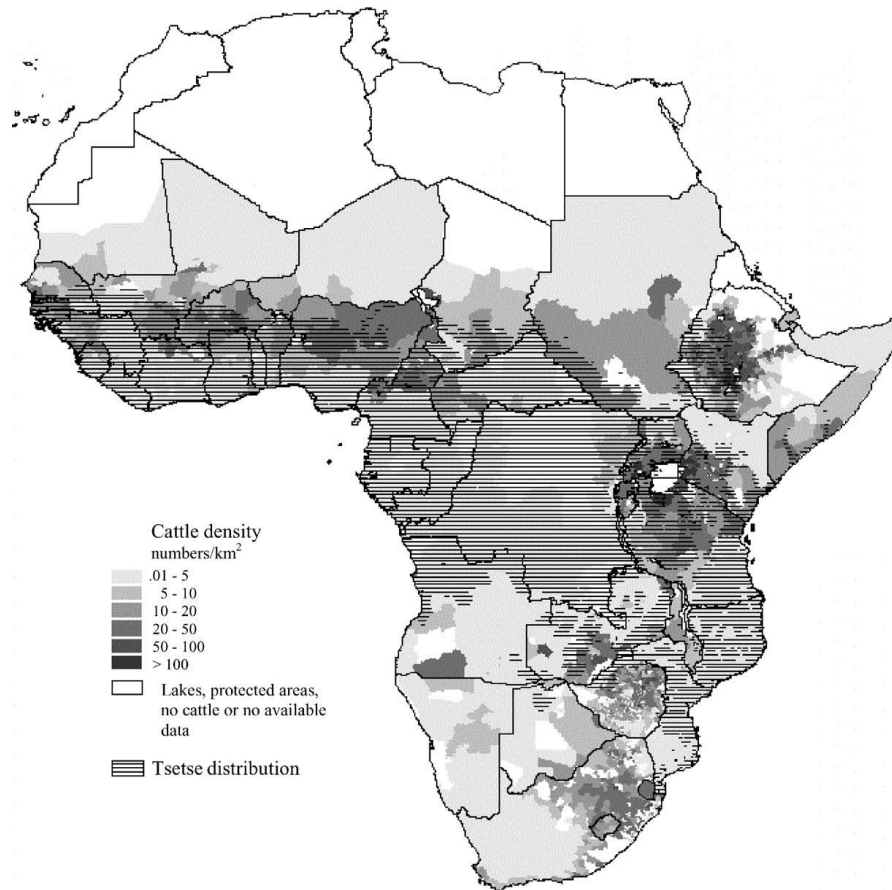
When the trypanosome parasites are transmitted to the mammalian host, they enter the dermal tissue in their metacyclic form (Vickerman, 1985). The parasites then travel to the host's bloodstream and lymphatic system where they are able to traverse along the blood and lymph vessels (Vickerman, 1985) until they reach vital tissues and organs such as the central nervous system (CNS) (Chappuis *et al*, 2005) through the blood brain barrier (Masocha & Kristensson, 2012). This is what leads to most of the disease symptoms such as anaemia, fever and paralysis (Steverding, 2008).

### **1.1.3 Antigenic variation**

Trypanosomes have a surface coat which contains variable surface glycoproteins (VSGs). The VSGs enable the parasite to evade detection by the host's immune system during infection (Berriman *et al*, 2005). Each parasite has approximately  $7 \times 10^6$  VSG molecules on its surface (Turner, 1982) and has hundreds of genes which code for each VSG, however, only one can be expressed at a single time (Pays *et al*, 2004). Each VSG is composed of 500 amino acids and contains an N-terminal signal peptide and a hypervariable region which is on the surface of the parasite and thus exposed to the immune system. The VSGs also contain a conserved C-terminal hydrophobic tail which is anchored on the surface of the parasite (Donelson & Rice-Ficht, 1985; Berriman *et al*, 2005). During the process of antigenic variation, transcriptional activation of a new VSG gene occurs, causing a switch from one VSG coat to another. The cause of this automatic switch however, is currently unknown (Hall *et al*, 2013). It is this process which allows the parasite to evade vaccine detection thus making it difficult to produce a vaccine against the disease.

### **1.1.4 Trypanosome distribution and vector control**

Trypanosome parasites are distributed in regions where tsetse flies occur. Tsetse flies are found in a large region in central Africa which is south of the Sahara and north of the Kalahari deserts (Barrett *et al*, 2003). Figure 1.4 shows the distribution of tsetse and overlap between the areas of cattle farming, placing cattle at high risk of infection from the trypanosome parasite.



**Figure 1.4: Geographic distribution of the trypanosomosis disease vector, the tsetse fly and cattle throughout Africa.** Tsetse distribution in the African belt south of the Sahara and north of the Kalahari deserts. Cattle in this region are at high risk of trypanosome infection (Naessens *et al*, 2002).

Trypanosomosis is a major health risk throughout Africa and countless attempts have been made to eradicate or control the spread of the disease. Attempts include controlling the spread of the disease vectors, the tsetse fly, by ground and aerial spraying with insecticides such as dichlorodiphenyltrichloroethane DDT (Allsopp, 2001) as well as a process called ‘pour-ons’, which is spraying insecticides on animals which tsetse feed on (Schofield & Maudlin, 2001; Hargrove *et al*, 2012). Other methods to control the disease vector are to remove bushes where tsetse flies rest (Schofield & Kabayo, 2008), or to build targets and traps which will not completely eradicate tsetse flies, but will significantly reduce their numbers and therefore reduce the risk of infection in animals. The traps which are pre-treated with insecticides, also have visual stimuli (e.g. blue colour; Harrison trap) to trap the tsetse (Grant, 2001).

### 1.1.5 Trypanotolerance and chemotherapy

Due to the limited success of current methods to control trypanosomosis and the lack of a vaccine for the disease, breeding of trypanotolerant cattle in tsetse-overrun areas is encouraged (Murray *et al*, 1990). Trypanotolerance is the ability of animals to remain healthy without manifestation of disease symptoms during infection. Trypanotolerance is largely witnessed in west African taurine breeds such as the west African Short Horn and the N'Dama (d'Ieteren & Kimani, 2001) and this is thought to be due to their long standing co-habitation with tsetse flies (Naessens *et al*, 2002). Although there has been a concern about the use of trypanotolerant cattle in areas away from western Africa due to the theory that they would only be resistant to local strains of *T. congolense* trypanosomes and succumb to other strains, it was discovered, however, that trypanotolerance was not limited to local trypanosome strains, but other *T. congolense* strains as well, as evidenced by the successful relocation and breeding of cattle from west Africa in distant tsetse areas such as the Central African Republic (Murray *et al*, 1990). Trypanotolerant cattle acquire their tolerance from their ability to control or prevent parasite proliferation in their circulation, maintain their body weight, quick immune response and control of anaemia (Hanotte *et al*, 2003; Naessens, 2006).

Another method used to control trypanosomosis is chemotherapy, which is the use of drugs to treat early as well as advanced stages of the disease. For treatment of early stages of HAT, pentamidine, an aromatic diamidine, is used (Baker *et al*, 2013) and although it is known to starve trypanosomes of glucose because of its hypoglycaemic effect, it is also thought to kill the parasites through kinetoplast disruption (Shapiro & Englund, 1990). To treat advanced stages of HAT, the highly toxic melaminophenyl arsenical, melarsoprol is used. A new drug known as eflornithine was made available at no cost in 2001, however, the large doses required for treatment resulted in melarsoprol being the drug of choice (Simarro *et al*, 2012). The drug isometamidium chloride in combination with diminazene is used to treat AAT in cattle kept in ranch areas in Tanzania (Geerts *et al*, 2001). This treatment, however, is not doing well and could be due to the development of drug resistance (Chitanga *et al*, 2011) of infected hosts. Some of the trypanocides such as pentamidine and suramin have been shown to exert their effect in part by inhibiting a trypanosome proteolytic enzyme, oligopeptidase B (Morty *et al*, 1998). Dying trypanosomes release proteolytic enzymes such as cysteine and serine proteases

into the bloodstream of infected hosts which hydrolyse host proteins and peptide hormones (Bastos *et al*, 2013) which leads to the manifestation of disease symptoms. Proteases are therefore considered as chemotherapeutic as well as diagnostic targets because of their presence in the infected host bloodstream.

## **1.2. Proteolytic peptidases in trypanosomes**

During trypanosome infection, parasitic proteolytic enzymes may be responsible for symptoms such as disturbances in sleep cycle patterns (Chappuis *et al*, 2005), fever, anaemia and paralysis (Steverding, 2008) when they are released into the bloodstream of infected animals (Bastos *et al*, 2010) either by secretion or upon the death of parasites (Antoine-Moussiaux *et al*, 2009). These parasitic proteolytic enzymes include cysteine proteases such as cathepsins (Pupkis & Coombs, 1984), or serine proteases such as prolyl oligopeptidase (POP) (Grellier *et al*, 2001). These enzymes contribute to pathogenesis by breaking down specific host factors resulting in the manifestation of the disease symptoms (Bastos *et al*, 2010). There are various classes of peptidases responsible for the hydrolysis of protein substrates. These peptidases are grouped into several classes depending on their catalytic mechanism as well as the amino acid residue(s) which acts as the nucleophile within the active site during substrate hydrolysis. The four peptidase classes which were initially identified by Rawlings and Barrett (1993) were the serine, cysteine, metallo and aspartic peptidases. During a study of the catalytic mechanism of the 20S proteasome from *Archaeobacterium Thermoplasma acidophilum* by Seemuller *et al* (1995), they discovered that a threonine residue at the N-terminus of the proteasome was responsible for nucleophilic attacks on substrates, hence recognition of a fifth class of peptidases known as the threonine peptidases. Similarly, a sixth class, the glutamate peptidases was observed in scytalidocarboxyl peptidase B (SCP-B) hydrolysis of angiotensin II (Fujinaga *et al*, 2004). More recently, the seventh class of peptidases was discovered to be the asparagine peptidases as observed in the self-cleaving Tsh protein from *E. coli* which cleaves itself at its carbonyl carbon using asparagine as a nucleophile (Rawlings *et al*, 2011).

The most common mechanism for the hydrolysis of peptide bonds is polarisation of the peptide bond by nucleophilic attack on the carbonyl carbon, either directly or assisted by a nitrogen in the peptide bond (Neurath, 1984). In the alternative mechanism, it is the amino acid residues within the peptidases which are responsible for the nucleophilic attack on



the peptide bond; they also act as the proton donors without the assistance of a water molecule. It is these amino acid residues responsible for the nucleophilic attack on the peptide bond which determine the class of peptidase as seen in Table 1.1. The serine class of peptidases will be discussed further since prolyl oligopeptidase and oligopeptidase B, the central topic of this study, belong to this class of peptidase.

**Table 1.1: The seven classes of peptidases. The classes are distinguished by the amino acids or metal ions present within their active sites.**

Class	Active Group	Example	Inhibitors
<b>Serine</b>	-OH group of Ser	<sup>a</sup> Prolyl Oligopeptidase	<sup>a</sup> Benzyloxycarbonyl-Pro-prolinal
<b>Cysteine</b>	-SH group of Cys	<sup>b</sup> Congopain ( <i>Tc</i> CATL)	<sup>b</sup> E-64
<b>Aspartic</b>	$\beta$ -COO <sup>-</sup> group of Asp	<sup>c</sup> Cathepsin E	<sup>b</sup> Pepstatin
<b>Metallo</b>	Zn/Co/Ni	<sup>c</sup> Metalloendopeptidase	<sup>c</sup> EDTA
<b>Threonine</b>	-OH group of Thr	<sup>d</sup> 20S Proteasome	<sup>g</sup> Bortezomib
<b>Glutamate</b>	$\gamma$ -COO <sup>-</sup> group of Glu	<sup>e</sup> Scytilidocarboxyl Peptidase B (SCP-B)	<sup>e</sup> 1,2-epoxy-3-(p-nitrophenoxy)propane
<b>Asparagine</b>	$\beta$ -COO <sup>-</sup> group of Asn	<sup>f</sup> Asparagine Peptide Lyases	<sup>h</sup> 5-azacytidine

<sup>a</sup>Polgar (2002), <sup>b</sup>Vermelho *et al* (2012), <sup>c</sup>Rawlings & Barrett (1993), <sup>d</sup>Seemuller *et al* (1995), <sup>e</sup>Fujinaga *et al* (2004), <sup>f</sup>Rawlings *et al* (2011), <sup>g</sup>Fierabracci (2012), <sup>h</sup>Lyko & Brown (2005)

### 1.2.1 Serine peptidases

Serine peptidases are grouped into six clans which are distinguished according to the arrangement of catalytic residues within their active sites (Barrett & Rawlings, 1995). The catalytic triad in serine peptidases comprises of the serine, aspartic acid and histidine amino acid residues. The six clans include Clan A, which are the serine peptidases that share a common ancestry with chymotrypsin and have a  $\beta/\beta$  hydrolase fold. The

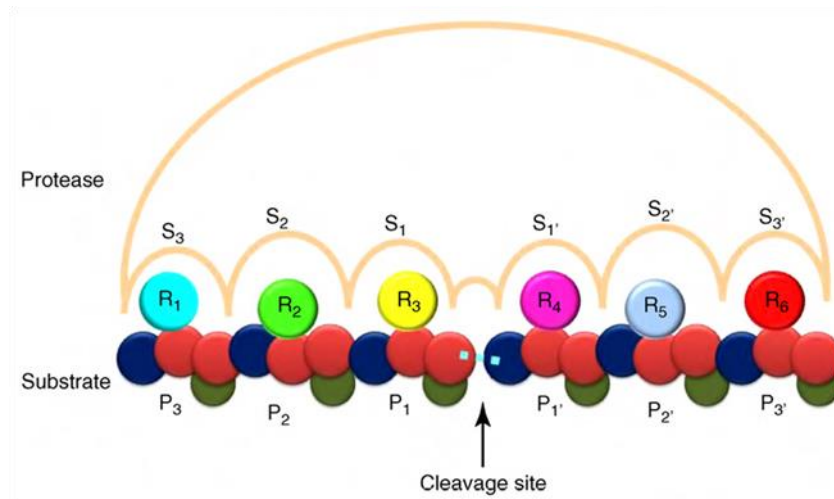
arrangement of the catalytic residues within the polypeptide chain in this clan of peptidases is His/Asp/Ser. Clan B peptidases share a common ancestry with subtilisin and have an  $\alpha/\beta$  hydrolase fold, their order of catalytic residues is Asp/His/Ser. The Clan C (carboxypeptidase C) peptidases also have  $\alpha,\beta$ -hydrolase folds in their tertiary structures, however, their catalytic residues have the order Ser/Asp/His. Serine peptidases which belong to Clan E (D-Ala-D-Ala-Peptidase A) do not have a catalytic triad, instead, they have Ser and Lys catalytic residues in a Ser-Xaa-Xaa-Lys motif. The Clan F peptidases have a catalytic Ser/Lys dyad within their active sites and finally, Clan G consists of the ATP-dependent peptidases which hydrolyse substrates in the presence of ATP (Barrett & Rawlings, 1995). The two largest clans of serine peptidases are the chymotrypsin-like ( $\beta/\beta$  hydrolase fold) and subtilisin-like ( $\alpha/\beta$  hydrolase fold) clans which share similar arrangements of the His, Asp and Ser residues in their catalytic triad (Siezen & Leunissen, 1997). Table 1.2 summarises the arrangement of the amino acid residues in the catalytic sites of the various serine peptidase clans.

**Table 1.2: Summary of the arrangement of catalytic residues that distinguish the serine peptidases into the six clans**

Clan	Order of catalytic residues	Example
A	His/Asp/Ser	<sup>a</sup> Chymotrypsin
B	Asp/His/Ser	<sup>b</sup> Subtilisin
C	Ser/Asp/His	<sup>b</sup> Prolyl oligopeptidase
E	Ser-Xaa-Xaa-Lys	<sup>a</sup> <i>Escherichia</i> D-Ala-D-Ala peptidase A
F	Ser/Lys	<sup>a</sup> Repressor LexA
G	ATP dependent catalysis	<sup>a</sup> Endopeptidase La

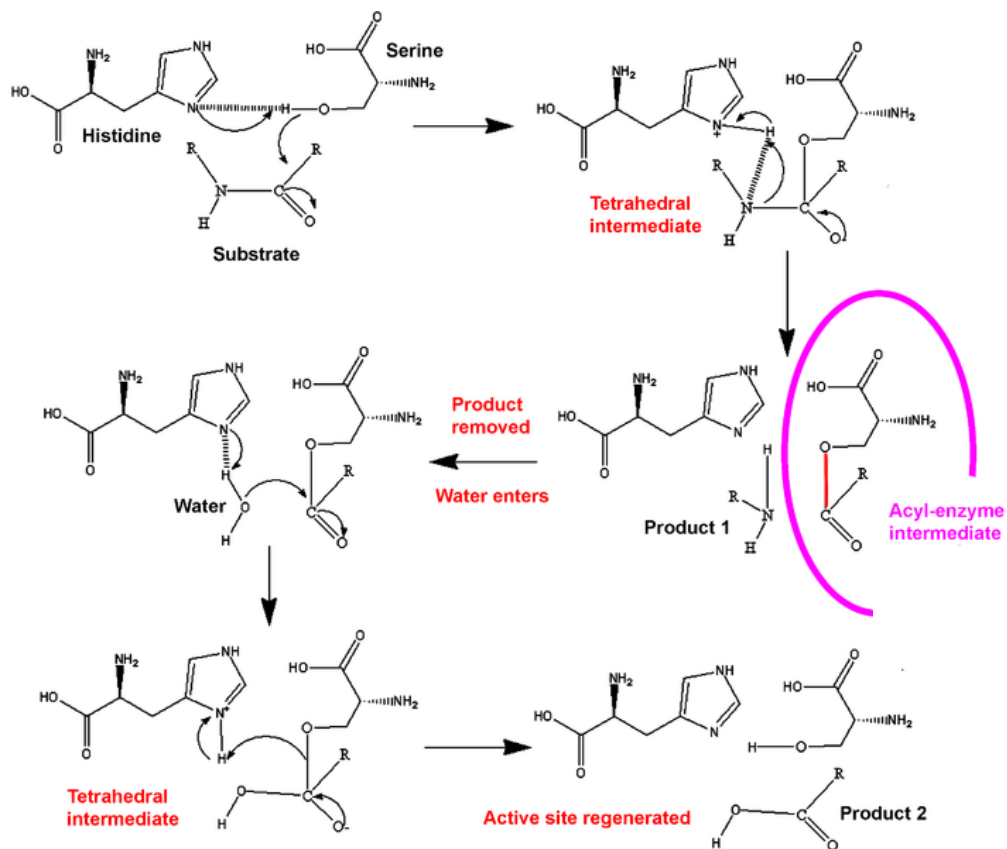
<sup>a</sup>Barrett & Rawlings (1995); <sup>b</sup>Page & Di Cera (2008)

During substrate hydrolysis, peptidases cleave substrates at specific cleavage sites i.e. scissile bond. The amino acids of the substrate bind to specific binding sites in the catalytic domain of the peptidase. The peptidase binding sites are numbered S1, S2....Sn from the cleavage site towards the N-terminus and S1', S2'.....Sn' towards the C-terminus (Fig. 1.5). The substrate amino acids within these binding sites are numbered P1, P2....Pn from the cleavage site towards the N-terminus and P1', P2'....Pn' towards the C-terminus (Berger & Schechter, 1970).



**Figure 1.5: Schematic diagram of peptidase active site represented by Schechter and Berger notation** (Smooker *et al*, 2010).

Serine peptidases are largely specific for substrates with hydrophobic residues at the P1 position (Perona & Craik, 1995). The catalytic mechanism of these peptidases is a process which involves the addition and removal of specific products within the reaction which results in the formation of tetrahedral intermediates (Polgar, 2005). Figure 1.6 depicts the catalytic mechanism of the serine peptidases and how the tetrahedral intermediates are formed. The hydrolysis of a substrate peptide bond involves nucleophilic attack by the hydroxyl group of the serine residue on the carbonyl carbon of the peptide bond in the substrate polypeptide chain. This nucleophilic attack is stabilised by the imidazole group of the histidine in the catalytic site which results in the formation of the tetrahedral intermediate and an imidazolium ion. The intermediate subsequently breaks down into an acyl-enzyme, imidazole base and an amine product (product 1) through acid catalysis (Polgar, 2005). Following acid catalysis, a deacylation reaction occurs which breaks down the acyl-enzyme intermediate. The ester bond in the acyl-enzyme undergoes nucleophilic attack from a water molecule to generate a second tetrahedral intermediate complex which subsequently breaks down to form the regenerated active site and a carboxylic acid (product 2) (Perona & Craik, 1995).



**Figure 1.6: Schematic representation of the serine peptidase catalytic mechanism.** The mechanism shows the Ser and His residues in the active site as well as the generation of the tetrahedral intermediates and the release of two bi-products. <http://en.wikipedia.org/wiki/Serpin> (accessed: 20 December 2013).

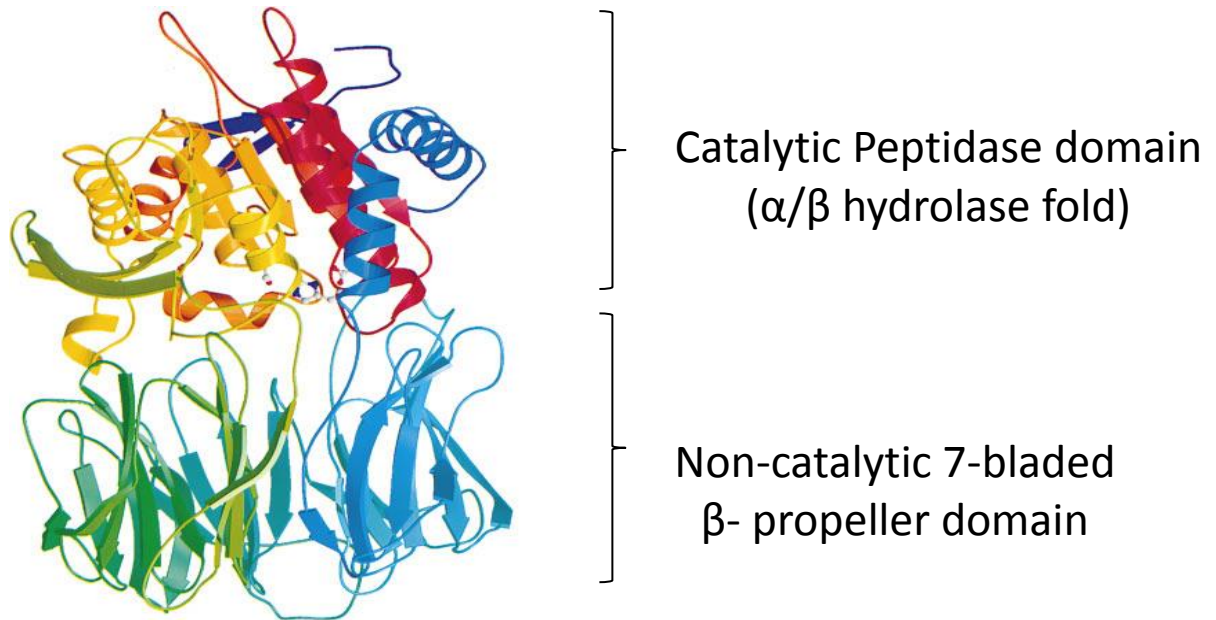
### 1.2.2 Prolyl Oligopeptidase

Prolyl oligopeptidase is a naturally occurring enzyme in living organisms such as plants and mammals where it degrades neuropeptides such as oxytocin (Goossens *et al*, 1995; Rosenblum & Kozarich, 2003). The natural levels of POP, however, can become elevated, leading to diseases such as schizophrenia and alzheimers' disease in human subjects. The drug valproate is used to treat patients and bring POP levels back to normal (Polgar, 2002). During trypanosome infection, POP (Bastos *et al*, 2005) is highly distributed in the brain of the host (Rosenblum & Kozarich, 2003) where it degrades peptide hormones (Garcia-Horsman *et al*, 2007) and neurotensin (Venäläinen *et al*, 2006) resulting in reduced cognitive function and neurological dysfunction (Bastos *et al*, 2010). Oligopeptidase B (OPB), (Morty *et al*, 1999; Coetzer *et al*, 2008) a serine protease not

present in mammals is released by the dying trypanosomes into the bloodstream of the host, where it hydrolyses regulatory peptide hormones such as atrial natriuretic factor (Rea *et al*, 2006). *Trypanosoma cruzi* OPB is also involved in cell invasion through a  $\text{Ca}^{2+}$  signaling pathway (Caler *et al*, 1998).

Prolyl oligopeptidase, along with OPB, acyl aminoacyl peptidase as well as dipeptidyl peptidase IV (Szeltner *et al*, 2004) are serine peptidases which belong to the prolyl oligopeptidase family of serine proteases (Clan SC in subfamily S9) (Venäläinen *et al*, 2004). Although they have similar 3D structures, their amino acid sequences differ (Polgar, 2002). They also share similarities in the substrates they hydrolyse as they have a high affinity for peptides containing ~30 amino acid residues or less containing Arg/Lys and Pro/Ala residues (Coetzer *et al*, 2008). They are referred to as oligopeptidases as they hydrolyse peptides which contain a small number of amino acids of approximately 10 - 50 residues. POP and OPB will be discussed further in ensuing paragraphs.

Prolyl oligopeptidase (POP), previously referred to as post-proline cleaving enzyme (Männistö *et al*, 2007) is a large serine peptidase of ~80 kDa found in the cytosol of the cell (Tarragó *et al*, 2005). It cleaves proline-containing peptides at the carboxy terminus of the proline residue (Rea & Fülöp, 2006). POP is much larger than the classic serine peptidases such as trypsin and subtilisin whose sizes range from 25 – 30 kDa (Polgar, 2002). This serine peptidase has an  $\alpha/\beta$  hydrolase fold 3D structure, it consists of two domains, the catalytic peptidase domain and the non-catalytic seven-bladed  $\beta$ -propeller domain (Szeltner *et al*, 2013) as shown in Figure 1.7.



**Figure 1.7: Tertiary structure of Porcine prolyl oligopeptidase.** The N to C-terminus is colored from blue to red respectively. The Ser, Asp and His catalytic residues are represented in ball and stick formation and the catalytic peptidase and non-catalytic  $\beta$ -propeller domains are shown. Adapted from Fülöp *et al* (1998).

The peptidase domain has  $\alpha/\beta$  hydrolase fold characteristics, it consists of an eight-stranded  $\beta$ -sheet in the center where all the strands are parallel except the second  $\beta$ -strand. The  $\beta$ -sheet is also adjacent to two  $\alpha$ -helices on one side and six  $\alpha$ -helices on the other (Polgar, 2002). The  $\beta$ -propeller domain consists of a 7-fold repeat of 4-stranded antiparallel  $\beta$ -sheets (Racys *et al*, 2010). Figure 1.8 shows how this 7-fold repeat forms a “propeller-like” conformation, hence the name  $\beta$ -propeller.

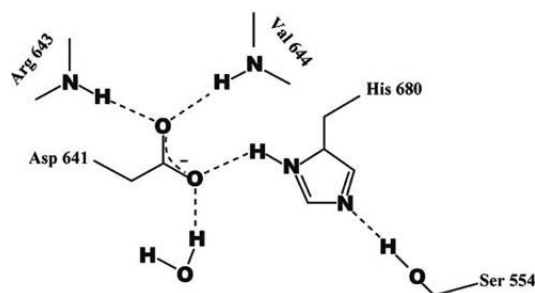


**Figure 1.8: Non-catalytic  $\beta$ -propeller domain of prolyl oligopeptidase.** The  $\beta$  sheets are twisted and have spiral arrangement around a central tunnel stabilized by hydrophobic amino acid residues (Fülöp *et al*, 1998).

The hydrophobic interactions within this  $\beta$ -propeller domain provide the structural stability of the  $\beta$ -sheets (Fülöp *et al*, 1998). In order for substrates to enter the catalytic site via POP's central tunnel, the opening or "Velcro" of the  $\beta$ -propeller is not closed between the first and last blades (Fülöp *et al*, 2000). The peptidase and  $\beta$ -propeller domains of POP are held together by two peptide chains with a combination of hydrogen bonds, salt bridges and hydrophobic interactions (Polgar, 2002).

### 1.2.3 POP active site

The active site of Porcine POP contains the catalytic triad Ser<sup>554</sup>, Asp<sup>641</sup> and His<sup>680</sup> (Szeltner *et al*, 2003) which is located at the interface of the catalytic peptidase and non-catalytic  $\beta$ -propeller domains where it is hidden by the central tunnel of the  $\beta$ -propeller (Szeltner *et al*, 2000). The propeller is perfectly positioned to act as a filter and control substrate entry into the active site (Morty *et al*, 2002). Small peptides of ~30 amino acid residues are easily accessible to the active site, whereas larger peptides are unable to enter through the small Velcro and therefore avoid catalysis (Rea & Fülöp, 2006). The catalytic residues are located in ideal positions to allow for maximum substrate binding as well as catalysis. Ser<sup>554</sup> is located at a nucleophile elbow (sharp turn within the structure of the molecule). This is a typical characteristic of the  $\alpha/\beta$  hydrolases (Polgar, 2002). The OH group of Ser<sup>554</sup> is highly exposed and easily accessible to the catalytic imidazole group of His<sup>680</sup> on one side and the carbonyl carbon of the substrate on the other side (Polgar, 2002). The His<sup>680</sup> is located in the middle of a loop. One of the oxygen atoms from the carboxylate group of Asp<sup>641</sup> is simultaneously hydrogen bonded to the imidazole ring of His<sup>680</sup> as well as a water molecule. The other oxygen atom from Asp<sup>641</sup> is bonded to two NH groups from Arg<sup>643</sup> and Val<sup>644</sup> (Polgar & Szeltner, 2008). Figure 1.9 shows the orientation of the catalytic residues within the active site of POP.



**Figure 1.9: Prolyl oligopeptidase catalytic triad showing Ser554, Asp641 and His680.** Hydrogen bonds with surrounding residues are shown (Polgar & Szeltner, 2008).

#### 1.2.4 POP catalytic mechanism

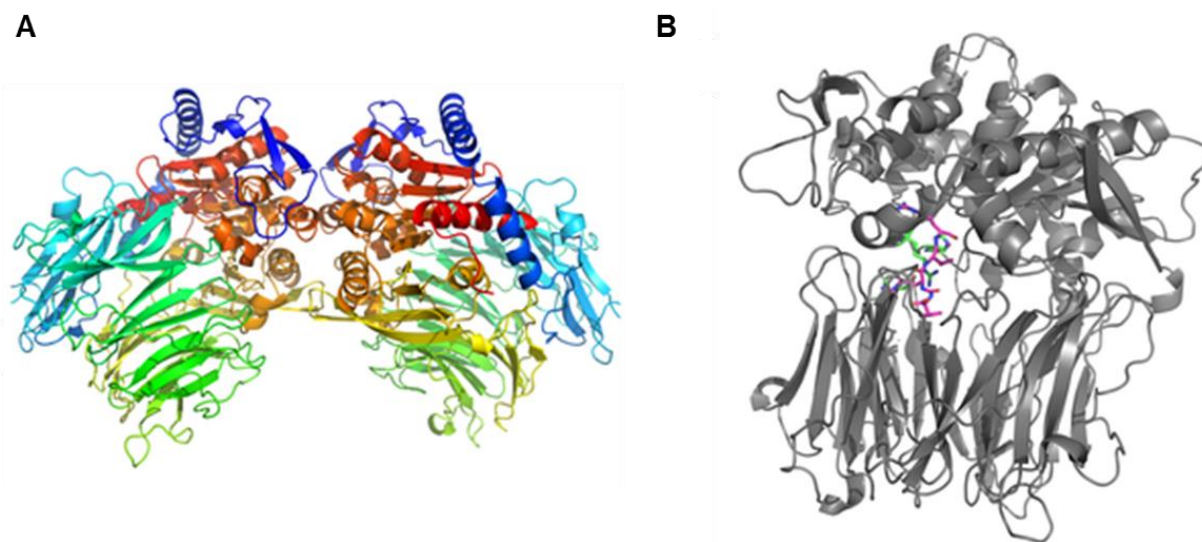
The binding mechanism of POP to substrates is similar to that of classic serine peptidases such as chymotrypsin. The major difference occurs during the stabilisation of the negatively charged tetrahedral intermediate (Szeltner *et al*, 2002). As in the classic serine peptidase mechanism, the catalytic Ser<sup>554</sup>, attacks the carbonyl carbon atom from the substrate peptide bond. During this substrate hydrolysis, the stacking of the Trp<sup>595</sup> indole ring against the substrate proline ring enhances the specificity of the enzyme-substrate complex (Gass & Khosla, 2007). Figure 1.6 shows that the catalytic mechanism of classic serine peptidases involves the formation and disintegration of a negatively charged tetrahedral intermediate which is stabilised by hydrogen bonds between peptide bond amides within the enzyme active site and the oxyanion binding site of the tetrahedral intermediate (Hedstrom, 2002). The hydrogen bonds between the peptide bond amides form a pocket known as an oxyanion hole which is positively charged, it is this oxyanion hole which stabilises the tetrahedral intermediate complex during substrate hydrolysis (Bryan *et al*, 1986). In the chymotrypsin-like peptidases, the oxyanion hole is formed by hydrogen bond interactions between the NHs of Ser<sup>195</sup> and Gly<sup>193</sup>, the positive charge of the oxyanion hole subsequently stabilises the oxyanion of the tetrahedral intermediate complex which has a negative charge. In contrast during POP substrate hydrolysis, the hydrogen bond interactions which form the oxyanion hole, are between the main-chain NH group of Asn<sup>555</sup> and the OH group of Tyr<sup>473</sup> (Szeltner *et al*, 2000; Hedstrom, 2002). Further hydrolysis of the substrate continues as that of the classic serine peptidases.

#### 1.2.5 Oligopeptidase B (OPB)

Oligopeptidase B is a large serine peptidase of approximately 80 kDa. It belongs to the prolyl oligopeptidase family (Clan SC, family S9) (Canning *et al*, 2013) and shares approximately 25% amino acid sequence identity with porcine POP (Rea & Fülöp, 2006), but unlike POP, OPB is responsible for the hydrolysis of oligopeptides at the carboxyl side of the basic amino acid residues arginine and lysine with a higher preference for arginine (Rea & Fülöp, 2006). Although OPB and POP share very low sequence identity (primary structure), they do, however, have highly similar tertiary structures. Similar to POP, OPB also consists of two domains, a catalytic domain as well as a  $\beta$ -propeller domain which



are connected by a hinge region (Fülöp *et al*, 2000). The catalytic domain is an  $\alpha/\beta$  hydrolase fold which consists of a central eight-stranded  $\beta$ -sheet, eight  $\alpha$ -helices and seven short sections of helices. The  $\beta$ -propeller domain consists of seven 4-stranded antiparallel  $\beta$ -sheets which are connected by general loops (Figure 1.10). The 'Velcro' is absent in OPB and is instead replaced by hydrophobic interactions and a hydrogen bond between Gly<sup>106</sup> and Gln<sup>412</sup> (McLuskey *et al*, 2010). Crystallisation studies conducted by Canning *et al* (2013) on *Tb*OPB revealed that *Tb*OPB exists as a dimer in solution. The  $\alpha/\beta$  hydrolase and  $\beta$ -propeller domains are linked together by disulfide bridge between Cys<sup>169</sup>, this forms the dimer which opens and closes at the interface during substrate hydrolysis.

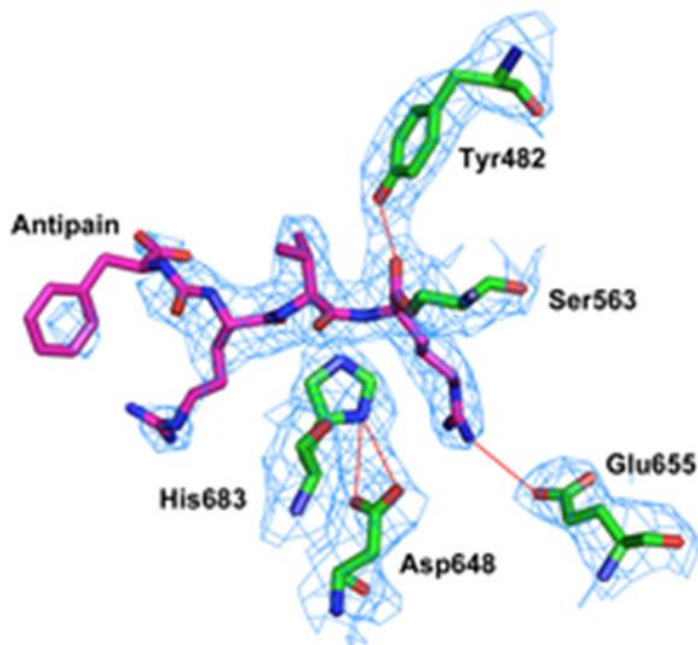


**Figure 1.10: Tertiary structure of oligopeptidase B from *T. brucei*.** **A:** The  $\beta$ -propeller domain is shown in green and the general loops in blue. The hinge region which joins the two domains is shown in yellow. In the catalytic domain, the eight-stranded  $\beta$ -sheets are coloured in a light shade of red and orange and the eight  $\alpha$ -helices are dark red and blue. N to C-terminus coloured blue to red. **B:** *Tb*OPB and antipain complex, antipain coloured magenta (Canning *et al*, 2013).

### 1.2.6 OPB active site and catalytic mechanism

Oligopeptidase B isolated from *T. brucei* was previously co-crystallised with its inhibitor antipain in order to determine the mechanism of OPB in substrate recognition and catalysis (Canning *et al*, 2013). The OPB active site contains the catalytic triad Ser<sup>563</sup>, Asp<sup>648</sup> and His<sup>683</sup> within a large cavity between the catalytic and  $\beta$ -propeller domains.

Ser<sup>563</sup> binds to antipain via antipain's aldehyde group, the resulting tetrahedral intermediate complex is stabilised by the NH of Ala<sup>564</sup> and the OH of Tyr<sup>482</sup> (Figure 1.11) (Canning *et al*, 2013).



**Figure 1.11: The catalytic residues Ser563, His683 and Asp648 of TbOPB are shown in ball and stick formation.** TbOPB carbons are green, oxygens are red and nitrogens are blue. Antipain is magenta. The catalytic residues are bound to Arg at the P1 position (Canning *et al*, 2013).

Like its homologue POP, the OPB catalytic mechanism is similar to the classic serine peptidases. The difference occurs during the stabilisation of the negatively charged tetrahedral intermediate (oxyanion) during substrate hydrolysis (Coetzer *et al*, 2008). The tetrahedral intermediate is stabilised by the formation of hydrogen bonds between its oxyanion binding site and NHs from amino acids in the main chain. The OPB tetrahedral intermediate is stabilised by hydrogen bonds from the amide group of Ala<sup>564</sup> and the OH of Tyr<sup>482</sup> (Canning *et al*, 2013), a similar mechanism was observed by McLuskey *et al* (2010) where the amide group of Ala<sup>578</sup> in the main chain as well as the OH from Tyr<sup>496</sup> of OPB from *Leishmania* stabilised the tetrahedral intermediate complex.

### 1.3. Role of POP and OPB in trypanosomosis pathogenesis

As previously stated, during trypanosome infection, various pathogenic factors are released into the bloodstream of the host by infective parasites or upon the death of the parasites (Antoine-Moussiaux *et al*, 2009). During intracellular *T. cruzi* infection, in order for the parasites to spread and penetrate the hosts' extracellular matrix (ECM) which is mainly composed of collagen, *TcrPOP* (Caffrey & Steverding, 2009), which also acts as a collagenase, is released by the parasite and hydrolyses human type I and IV collagens (Bastos *et al*, 2013). This enables easy passage of the parasites through the hosts ECM, which can generate host inflammatory responses that can lead to further tissue damage (Bastos *et al*, 2013). OPB has also been shown to play a role in trypanosomosis pathogenesis. This was first discovered in a study of the trypanosomal parasite, *T. cruzi*, where *TcrOPB* was shown to control parasite invasion of non-phagocytic mammalian cells by signal transduction (Burleigh *et al*, 1997). Figure 1.12 depicts how *T. cruzi* trypomastigotes release *TcrOPB* which cleaves an inactive precursor triggering an active  $\text{Ca}^{2+}$  agonist which binds to the host cell receptor. This activates phospholipase C which generates inositol triphosphate ( $\text{IP}_3$ ), this subsequently leads to the release of intracellular  $\text{Ca}^{2+}$  which recruits host cell lysosomes by signal transduction, to fuse with the parasite site of attachment where the parasites invade the host lysosomes (Caler *et al*, 1998). The acidic environment of the lysosome promotes differentiation of metacyclic infectious trypomastigotes to replicative amastigotes (Tomlinson *et al*, 1995) which spread within the host. It was also shown that *TbOPB* and OPB from *T. evansi* remain active in the bloodstream of infected animals and retain the ability to hydrolyse peptide hormones such as atrial natriuretic factor (Morty *et al*, 2001; Morty *et al*, 2005b).

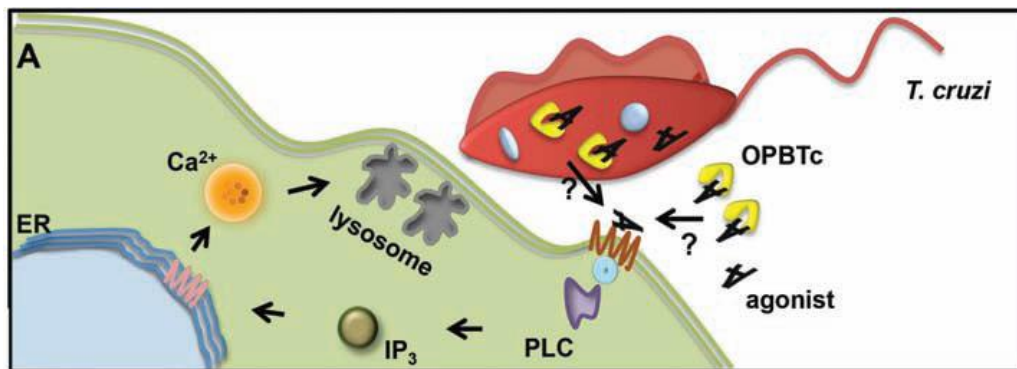


Figure 1.12: Representation of the role of *TcrOPB* in host cell invasion (Bastos *et al*, 2013).

In an attempt to fully understand the role of OPB in trypanosomosis pathogenesis, gene knock-out studies using RNA interference (RNAi) gene regulation were carried out. It was found that when the OPB gene was knocked-out in the kinetoplastida *Leishmania* there was an up-regulation in enzymatically inactive enolase which acts as a virulence factor for *Leishmania* parasites by binding to plasminogen in host cell membranes. Furthermore, it was also found that macrophages showed low levels of infection in OPB knocked-out parasites (Swenerton *et al*, 2011). In another study carried out by Kangethe *et al* (2012), it was found that in OPB knocked-out *T. b. brucei* parasites, there was a significant increase in *TbPOP*-like activity. This shows that other oligopeptidases, mainly *TbPOP* or a *TbPOP*-like peptidase are affected by the absence of the *TbOPB* gene which suggests that these enzymes have interconnected functions or roles during trypanosome infection since one compensates for the absence of the other by the up-regulation of other pathogenic factors with similar functions. Kangethe *et al* (2012) also pointed out that both *TbOPB* and *TbPOP* hydrolysed the same peptide hormones such as atrial natriuretic factor (ANF) and neurotensin. Each of these peptides contain either Arg/Lys or Pro/Ala residues, further reinforcing that these oligopeptidases do share the same substrates. This suggests that OPB along with other similar pathogenic factors such as POP would be good simultaneous drug targets. Additionally, POP and OPB may induce the production of antibodies in the infected host and could therefore be diagnostic targets.

#### **1.4. Objectives of the present study**

The first aim of this study was to determine the biochemical characteristics of the two oligopeptidases, prolyl oligopeptidase and oligopeptidase B which have been identified as potential drug targets in trypanosome infections as well as compare their distinct characteristics and similarities.

The specific objectives were to:

- Clone the *T. b. brucei* prolyl oligopeptidase (*TbPOP*) and the *T. congolense* prolyl oligopeptidase (*TcoPOP*) genes into pTZ57R/T and pGEM-T® easy T-vectors respectively and sub-clone the genes into pGEX-4-T-1 and pET32a expression vectors.

- Recombinantly express *TbPOP* and *TcoPOP* using the *E. coli* (BL21-DE3) expression system.
- Recombinantly express *TcoOPB* in pET28a expression vector using the *E. coli* BL21 DE3 expression system.
- Purify the His-tagged proteases on a Ni<sup>2+</sup> affinity resin.
- Further purify the proteases by molecular exclusion chromatography (MEC) and ion exchange chromatography (IEC).
- Conduct enzymatic assays on *TbPOP*, *TcoPOP* (Chapter 2) and *TcoOPB* (Chapter 3) to determine substrate specificity, optimal pH conditions and inhibitor susceptibility.

The second aim of the study was to assess if *TcoOPB* has diagnostic potential by screening for antibodies in *T. congolense* infected cattle sera (Chapter 3).

The specific objectives were to:

- Conduct an indirect ELISA using *TcoOPB* as the antigen to screen for antibodies in *T. congolense* infected cattle sera

## CHAPTER 2

### Cloning, recombinant expression and purification of *Trypanosoma brucei brucei* and *T. congolense* Prolyl Oligopeptidase

#### 2.1. Introduction

Trypanosomes are parasites which release several proteases, which act as pathogenic factors, into the bloodstream of infected hosts (Troberg *et al*, 1996; Morty *et al*, 2001). There are several pathogenic factors utilised by trypanosome parasites, these include cysteine proteases such as congopain (*TcoCATL*) from *T. congolense* (Lalmanach *et al*, 2002), cruzipain (*TcrCATL*) from *T. cruzi* (Engel *et al*, 1998) and trypanopain (*TbbCATL*) from *Trypanosoma brucei brucei* which are lysosomal proteases involved in the hydrolysis of host proteins. Trypanosome parasites also utilise serine proteases such as oligopeptidase B from *T. congolense* (*TcoOPB*) which hydrolyses the peptide hormone atrial natriuretic factor (Morty *et al*, 2005b) and prolyl oligopeptidase from *T. b. brucei* (*TbPOP*) which has been found to hydrolyse type I and type IV collagen that allows the traversal of parasites through the hosts' extracellular matrix (Grellier *et al*, 2001; Bastos *et al*, 2013). POP belongs to the prolyl oligopeptidase S9 family of serine proteases and hydrolyses peptides at the carboxy-terminus of Pro and Ala residues (Bastos *et al*, 2010).

Previous methods to eradicate trypanosomosis have not been highly successful; these methods include vector control using insecticides destruction of tsetse habitats, as well as removal of animal hosts from tsetse over-run regions (Taylor, 1998). Another attempt to eliminate the disease is by treatment of cattle with diminazene and suramin, however, the parasites have since developed drug resistance (Chitanga *et al*, 2011). Therefore, there have been ventures to develop a vaccine against trypanosomosis, however, this pursuit has been futile as well due to the surface coat of the trypanosome parasites which contains over 10 million copies of a surface glycoprotein which undergoes antigenic variation (La Greca & Magez, 2011). More recently, other methods have been explored to treat trypanosomosis, such as identifying pathogenic factors used by trypanosomes during infection and targeting them for the development of new drugs (Bastos *et al*, 2013).

The peptidase POP from *T. b. brucei* (*TbPOP*) and *T. congolense* (*TcoPOP*) will be the main focus of the work reported in this chapter. Kangethe *et al* (2012) found that when the *TbOPB* gene was knocked-out in *T. b. brucei* parasites, there was an increase in *TbPOP*-

like activity, indicating that these proteases may have similar functions during trypanosomiasis infection due to the fact that when one is knocked-down, the other compensates for the absence of the other. Therefore the objective of this part of the study was to clone, recombinantly express and purify the peptidases *TbPOP* and *TcoPOP* in order to conduct enzymatic assays on the proteases to determine their biochemical characteristics which will assist in identifying how they can be utilised as drug targets.

## 2.2. Materials and methods

### 2.2.1 Materials

**Molecular biology:** GeneJET<sup>®</sup> plasmid miniprep kit, Transform Aid<sup>®</sup> bacterial transformation kit, shrimp alkaline phosphatase (SAP), T4 DNA ligase, dATP, dGTP, dTTP, dCTP, X-gal, IPTG, MassRuler<sup>®</sup> DNA ladder mix (O' GeneRuler 1 kb) were from Fermentas. The DNA clean and concentrator kit were purchased from ZymoResearch (Orange, CA, USA). The pTZ57R/T<sup>®</sup> and pGEM-T<sup>®</sup> Easy vectors as well as restriction enzymes Sall<sup>a</sup>, XhoI and 2 x Tango Buffer were purchased from Thermo Scientific. Gel extraction kit was from PEQlab (Erlangen, Germany). FIREpol<sup>®</sup> Taq polymerase, 25 mM MgCl<sub>2</sub> and 10 x PCR reaction buffer were purchased from Solis Biodyne (Tartu Estonia). Molecular biology grade agarose was purchased from Conda laboratories. Tryptone, yeast extract and bacteriological agar were purchased from Merck. Ampicillin sodium salt was from USB Corporation and kanamycin sulfate was from Fluka. *Escherichia coli* JM 109 and BL21(DE3) cells were obtained from New England Biolabs.

**Recombinant *TbPOP* purification:** His-select<sup>®</sup> nickel affinity column as well as imidazole were purchased from Sigma Aldrich (St. Louis, MO, USA). HiPrep<sup>™</sup> 16/60 Sephacryl<sup>™</sup> S200 HR column (1.6 cm x 60 cm), HiPrep QFF 16/10 resin (20ml), HiPrep SP FF 16/10 resin (20ml) and ÄKTAdesign<sup>™</sup> system were from GE Healthcare Life Sciences (Buckinghamshire, UK). The BCA<sup>™</sup> Protein Assay Kit was purchased from Pierce (Rockford, IL, USA).

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<sup>a</sup>Nomenclature for restriction enzymes (Roberts *et al*, 2003) where genes are italics but restriction enzymes are not.

**Western blot analysis:** Horse radish peroxidase (HRPO) conjugated mouse anti-His tag antibody was purchased from Roche, rabbit anti-chicken IgY (whole molecule)-peroxidase conjugate was purchased from Sigma Aldrich (St. Louis, MO, USA). PageRuler™ Prestained Protein Ladder was purchased from ThermoScientific and PageRuler™ Unstained Broad Range Protein Ladder was purchased from Fermentas.

**Enzymatic characterisation:** Purified collagen type I from rat tail was purchased from Sigma Aldrich (St. Louis, MO, USA).

## 2.2.2 Methods

### 2.2.2.1 Cloning of full length *TbPOP* and *TcoPOP* gene

#### *Isolation of T. b. brucei genomic DNA*

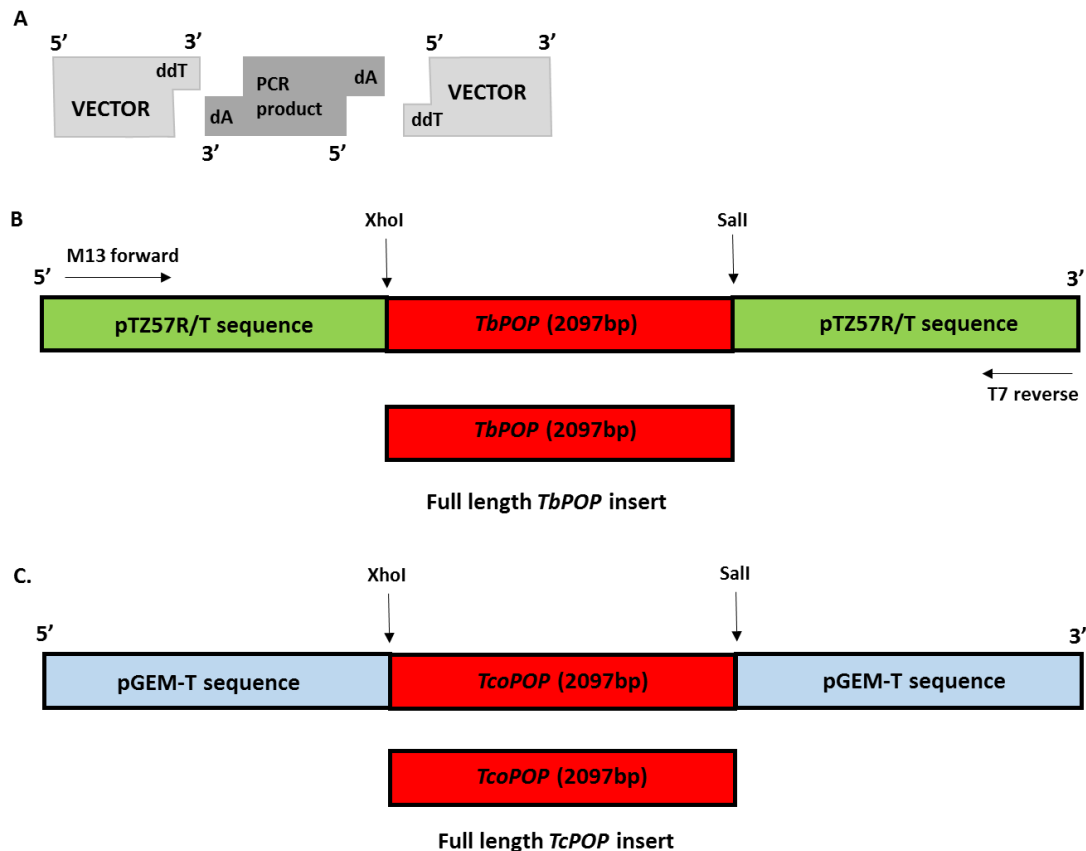
A pellet from *T. b. brucei* parasites (strain Lister 427; lab stocks) was used to extract genomic DNA according to Fish *et al* (1989). Briefly, the pelleted parasites were resuspended in 1 ml PBS [136 mM NaCl, 2.68 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O, 1.5 mM KH<sub>2</sub>PO<sub>4</sub>, pH 7.4], centrifuged (3000 *g*, 15 min, RT) and the supernatant removed. The pellet was resuspended in 300 µl TELT buffer [50 mM Tris-HCl, pH 8, 80 mM EDTA, 2.5 mM LiCl] and incubated for 5 min at RT. Phenol-chloroform [300 µl; 1:1 (v/v)] was added and agitated for 5 min at RT. The solution was centrifuged (17 000 *g*, 5 min, RT) and the supernatant added to 600 µl absolute ethanol and centrifuged (17 000 *g*, 10 min, RT) to precipitate the genomic DNA. The supernatant was removed and the DNA washed with 1 ml absolute ethanol and centrifuged once again (17 000 *g*, 5 min, RT). The ethanol was subsequently removed and the pellet of genomic DNA incubated at 37°C for 10 min to evaporate excess ethanol. The dry DNA was dissolved in 100 µl of TE buffer [10 mM Tris-HCl, pH 8, 1 mM EDTA] containing 1 mg/ml RNase and incubated at 37°C for 45 min after which, the DNA was quantified using the NanoDrop™ 2000/2000c Spectrophotometer (Thermo Fisher Scientific, Roskilde, Denmark) and analysed on a 1% (w/v) agarose gel.



### *Cloning into pTZ57R/T and pGEM-T<sup>®</sup> Easy vectors*

The DNA sequences coding for prolyl oligopeptidase from *T. b. brucei* [EMBL accession no. AJ496456 (Bastos et al, 2010)] and *T. congolense* [EMBL accession no. HE575323.1] were selected to design primers for the amplification of the full length 2097 bp *POP* fragment in both *T. b. brucei* and *T. congolense*. The primers were designed to introduce Xho I and Sal I restriction sites at each end of the PCR product for cloning and subcloning into pTZ57R/T (Appendix 2) and pGEM-T<sup>®</sup> Easy (Appendix 3) cloning vectors and the pET32a (Appendix 5) expression vector. The primers for *TbPOP* were: forward, 5'-**AAGTCGAC**ATGCGCCTCGCTTACC-3' with a SalI restriction site (underlined) and a start codon (bold); reverse, 5'-CACTCGAGCACCGTCAGTCTGTCCACTG-3' with a XhoI restriction site (underlined) and a stop codon (bold). The primers for *TcoPOP* were: forward, 5'-GTGTCGAC**ATGCGTCTCGTGTACCCCA**-3' with a SalI restriction site (underlined) and a start codon (bold); reverse, 5'-GACTCGAGCCCAATT**AGTCCGTCCACTG**-3' with a XhoI restriction site (underlined) and a stop codon (bold). Genomic DNA was used as a template for PCR. Briefly, the master mix for the PCR reaction contained the primers designed for *TbPOP* (0.2 μM each) along with *T. b. brucei* DNA (50 ng), 1 X PCR buffer, 2.5 mM MgCl<sub>2</sub>, 1.25 U Taq, 0.25 mM dNTPs and the total reaction volume was made to 20 μl with sterile dH<sub>2</sub>O. The master mix for *TcoPOP* contained the same reagents with the exception of primers designed for *TcoPOP* (0.2 μM each) along with 50 ng *T. congolense* DNA (lab stocks).

The PCR amplification was done under the following conditions, Taq polymerase activation step at 94°C for 2 min, followed by 40 cycles of a DNA denaturing step at 94°C for 15 s, a primer annealing step at 55°C for 15 s and an elongation step at 72°C for 1 min. A final elongation step was added at the end of 40 cycles at 72°C for 7 min. The generated PCR products (5 μl) were analysed on a 1% (w/v) agarose gel in 1 × Tris-acetate-EDTA (TAE) buffer [10 mM EDTA, 18 mM glacial acetic acid, 40 mM Tris] at 80 V. The remaining 15 μl was gel purified using the PEQLab Gel extraction kit and quantified using the NanoDrop™ 2000/2000c Spectrophotometer prior to ligation into pTZ57R/T and pGEM-T<sup>®</sup> Easy cloning vectors (Fig. 2.1) using a 3:1 ratio in 1 × ligation buffer and 1 U T4 DNA ligase in a 10 μl reaction mix incubated at 4°C overnight.



**Figure 2.1: Orientation of *TbPOP* and *TcoPOP* PCR products within the pTZ57R/T and pGEM-T® Easy vectors.** The Taq polymerase adds 5'-A overhangs to each end of the PCR product which allow for easy binding of the insert to the 3'-ddT overhangs of the vector. **A:** Orientation of the PCR product in the cloning vector. **B:** Representation of the Sall and XhoI restriction sites and release of the *TbPOP* insert from pTZ57R/T after restriction digest. **C:** Representation of the Sall and XhoI restriction sites and release of the *TcoPOP* insert from pGEM-T® Easy after restriction digest.

The ligation mix was then transformed into competent *E. coli* JM109 cells using the TransformAid™ bacterial transformation kit as per manufacturer's instructions. The transformed *E. coli* JM109 cells (40 µl) were plated onto pre-warmed 2x YT plates [1.6% (w/v) tryptone, 0.5% (w/v) NaCl, 1% (w/v) yeast extract, 15 g/l bacteriological agar] containing ampicillin (50 µg/ml), X-gal (20 µg/ml) and IPTG (10 µg/ml) and incubated overnight at 37°C. Recombinant colonies were determined using blue/white screening. White colonies which contained possible recombinant T-vector were selected for colony PCR using *TbPOP* and *TcoPOP* primers and analysed on a 1% (w/v) agarose gel in 1 × TAE buffer at 80 V. The positive clones were used to inoculate 2 ml 2x YT medium

containing 50 µg/ml ampicillin and the plasmid DNA extracted using the GeneJET™ plasmid miniprep kit as per manufacturer's instructions. Sequencing of recombinant plasmid DNA was done at Stellenbosch University (Central Analytical Facility).

#### *Sub-cloning into pET32a and pGEX-4-T expression vectors*

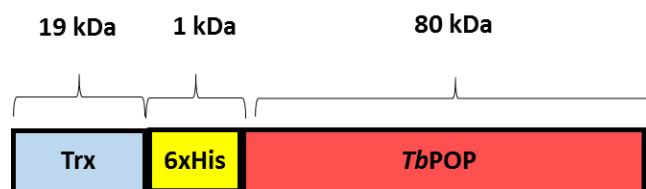
Recombinant *TbPOP*/pTZ57R/T and *TcoPOP*/pGEM-T® Easy plasmid DNA was restricted with 1 U Sall and 1 U XhoI in 2 × Tango buffer™ and incubated at 37°C overnight (Fig. 2.1). The restriction digest was analysed on a 1% (w/v) agarose gel in 1 × TAE buffer. The “dropped-out” insert was excised from the agarose gel and purified using the peQLab Gel extraction kit. The pET32a and pGEX-4-T (Appendix 4) expression vectors were also restricted with Sall and XhoI as described above. The linearised vectors were subsequently dephosphorylated with 1 U Shrimp alkaline phosphatase (SAP) and concentrated using the ZymoResearch™ DNA Clean and Concentrator kit. The insert was ligated into pET32a and pGEX-4-T and the ligation mix was once again transformed into competent *E. coli* JM109 cells and recombinant colonies were selected for colony PCR using *TbPOP* and *TcoPOP* primers. Recombinant colonies were used to inoculate 2x YT medium and the plasmid DNA isolated. The *TbPOP*/pET32a, *TbPOP*/pGEX-4-T and *TcoPOP*/pET32a plasmid DNA was subsequently transformed into competent *E. coli* BL21(DE3) cells. Recombinant colonies were screened by colony PCR using *TbPOP* and *TcoPOP* primers. Sequencing of recombinant plasmid DNA was done at Stellenbosch University (Central Analytical Facility).

#### **2.2.2.2 Recombinant expression of full-length *TbPOP* and *TcoPOP***

##### *Expression of *TbPOP* and *TcoPOP* in *E. coli* BL21(DE3) cells*

A single colony of recombinant *E. coli* BL21(DE3) cells containing the full length *TbPOP* and *TcoPOP* gene in the pET32a expression vector was used to inoculate terrific broth [24 g/l yeast extract, 0.4% (v/v) glycerol, 12 g/l tryptone, 0.17 M KH<sub>2</sub>PO<sub>4</sub> and 0.72 M K<sub>2</sub>HPO<sub>4</sub>; 500 ml] containing 50 µg/ml ampicillin and grown overnight at 37°C. Cells were harvested by centrifugation (2000 g, 10 min, 4°C), the supernatant was discarded, the pellet resuspended in PBS-T lysis buffer [PBS, Triton X-100, 1% (v/v), 1 mg/ml lysozyme, pH 7.4] and incubated at 37°C for 30 min after which the suspension was frozen at -20°C for 30 min. Frozen samples were then thawed and sonicated 4 times for 10 s for each sonication. The expression lysate containing recombinant *TbPOP* with the N-terminal

thioredoxin-Histidine (Trx-His) dual affinity tag (Fig. 2.2) was separated on a 12.5% SDS-PAGE gel (Section 2.2.2.8) alongside untransformed BL21 DE3 cells and the non-recombinant pET32a expression vector to confirm expression.



**Figure 2.2: Composition of recombinantly expressed *TbPOP*.** The enzyme now contains the N-terminal Trx-His dual affinity tag which can be used for affinity purification.

In order to verify *TbPOP* solubility, a solubility test was done. After expression, the cells were lysed and separated by centrifugation (10 000 *g*, 10 min, 4°C). The soluble fraction (supernatant) and insoluble fraction (pellet) were separated on 12.5% SDS-PAGE (Section 2.2.2.8) gel to verify solubility.

#### *Expression optimisation of TbPOP*

After confirming that *TbPOP* was expressed in an insoluble state, expression was optimised at 16°C and 27°C in an attempt to obtain soluble protein. Briefly, seven colonies were used to inoculate 2x YT (10 ml) containing ampicillin (50 µg/ml), the cultures were then incubated at 37°C overnight. Following overnight incubation, each culture was diluted into 40 ml 2x YT containing ampicillin (50 µg/ml) and incubated at 37°C until an OD<sub>600</sub> of ~0.7 nm was reached. Each culture was then induced with 1 mM IPTG. Following induction, three cultures were incubated at 16°C and expression stopped at 3, 4 and 5 h, the rest of the cultures were incubated at 27°C and expression stopped at 2, 3, 4 and 5 h. Cells were harvested by centrifugation (2000 *g*, 10 min, 4°C), the supernatant was discarded and the pellet resuspended in PBS-T lysis buffer and incubated at 37°C for 30 min after which the suspension was frozen at -20°C for 30 min. Frozen samples were thawed and sonicated 4 times for 10 s for each sonication. The sonicated samples were once again separated by centrifugation (10 000 *g*, 10 min, 4°C); both the pellets and supernatants were analysed on a 12.5% SDS-PAGE gel (Section 2.2.2.8). Expression of *TbPOP* was also optimised at various IPTG concentrations ranging from 0 mM to 1 mM in an attempt to solubilise the enzyme.

### 2.2.2.3 Solubilisation and refolding of full-length *TbPOP*

*TbPOP* was expressed at high levels in the form of inclusion bodies (Raina *et al*, 2004) which required solubilisation for purification purposes. In order to solubilise the *TbPOP* inclusion bodies, urea, which acts as a denaturant (Tsumoto *et al*, 2003) was used. The manner in which urea solubilises proteins is by the breakdown of inter- and intra-molecular interactions (Rabilloud, 2009) resulting in complete unfolding of the protein structure. After the process of solubilisation, the protein is refolded to its native state by dialysis against decreasing concentrations of the denaturant (Tsumoto *et al*, 2003). Briefly, the insoluble *TbPOP* fraction (pellet) was washed 3 times with pellet wash buffer [2 M urea, 0.02 M Tris-HCl, 2.5% (v/v) Triton X-100, pH 8.0] and the soluble *TbPOP* collected by centrifugation (12 000 *g*, 30 min, 4°C) from the supernatant. Unfolded *TbPOP* was refolded by dialysis using 10 kDa cut-off dialysis tubing cellulose membrane flat width 33 mm (Sigma Aldrich) against two changes of dialysis buffer 1 [4 M urea, 0.02 M Tris-HCl, 5% (v/v) glycerol, pH 8.0], 6 h, RT, followed by dialysis against dialysis buffer 2 [2 M urea, 0.02 M Tris-HCl, 10% (v/v) glycerol, pH 8.0], 18 h, RT. It was then dialysed against two changes of dialysis buffer 3 [1 M urea, 0.02 M Tris-HCl, 15% (v/v) glycerol, pH 8.0], 6 h, RT, followed by dialysis against dialysis buffer 4 [0.5 M urea, 0.02 M Tris-HCl, 20% (v/v) glycerol, pH 8.0], 18 h, 4°C and finally against three changes of equilibration buffer [0.02 M Tris-HCl, 0.25 M L-arginine, 0.5 mM PMSF, 0.1% (w/v) NaN<sub>3</sub>, 20% (v/v) glycerol, pH 8.0], 6 h, 4°C. Refolded *TbPOP* was analysed by 12.5% SDS-PAGE (Section 2.2.2.8).

**Table 2.1: Summary of refolding steps for solubilised *TbPOP***

Buffer	Vol (ml)	Time (h)	Temp. (°C)	Number of changes	[Urea] (M)
Dialysis buffer 1	250	6	RT	2	4.0
Dialysis buffer 2	1000	18	RT	1	2.0
Dialysis buffer 3	500	6	RT	2	1.0
Dialysis buffer 4	1000	6	4	2	0.5
Equilibration buffer	1000	6	4	3	0

#### 2.2.2.4 Affinity purification of *TbPOP*

His Select<sup>®</sup> nickel affinity resin (1 ml) was placed in a 10 ml chromatography column washed with 5 column volumes of distilled water and equilibrated with 10 column volumes equilibration buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M NaCl, 10 mM imidazole, pH 6.8]. Soluble refolded *TbPOP* lysate (10 ml) was added to the resin and incubated overnight at 4°C while mixing using an end-over-end rotor. The unbound fraction was collected, after which the resin washed with equilibration buffer until an A<sub>280</sub> of ~0.02 was obtained. The bound fraction was eluted using 5 ml elution buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.5 M, pH 6.8] containing increasing concentrations of imidazole 50 mM – 250 mM with 1 ml fractions collected. The nickel resin was regenerated with 2 column volumes distilled water, 5 columns of 6 M guanidine-HCl, another wash with 3 column volumes distilled water and finally, another 5 column volumes wash with equilibration buffer before storage in 30% (v/v) ethanol at 4°C. All fractions were analysed by 12.5% SDS-PAGE (Section 2.2.2.8) for efficiency of the purification. Eluted *TbPOP* fractions were dialysed against two changes of MEC buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0] overnight at 4°C to remove imidazole.

#### 2.2.2.5 Molecular Exclusion Chromatography (MEC)

Since *TbPOP* was only partially purified by affinity chromatography, further purification was required and molecular exclusion chromatography was conducted using a HiPrep<sup>™</sup> 16/60 Sephacryl<sup>™</sup> S200 HR column (1.6 cm x 60 cm) on the ÄKTAdesign<sup>™</sup> system (GE Healthcare Life Sciences, Buckinghamshire, UK). The resin separates proteins of molecular weight within the range 10 kDa – 150 kDa. The resin was washed with 60 ml ultrapure dH<sub>2</sub>O (manual run, flow rate 0.5 ml/min, 0.2 MPa, RT) and thereafter, equilibrated with 2 column volumes of MEC buffer [50 mM NaH<sub>2</sub>PO<sub>4</sub>, 300 mM NaCl, pH 8.0] (manual run, flow rate 1 ml/min, 0.2 MPa, RT). Semi pure, polyethylene glycol, 20 000 (PEG) concentrated *TbPOP* (2.4 ml) was filtered through a 0.45 µM filter and subsequently injected into the system and separated with 2 column volumes MEC buffer (manual run, flow rate 0.5 ml/min, 0.2 MPa, RT). Separation was monitored by collecting 2 ml fractions and measuring the A<sub>280</sub>. Following elution, the resin was washed with 60 ml 0.2 M NaOH, followed by 480 ml ultrapure dH<sub>2</sub>O and finally 60 ml 20% ethanol (manual

run, 0.5 ml/min, RT) and stored. All reagents were prepared with ultra-pure dH<sub>2</sub>O, filtered and degassed using a vacuum degasser before use.

#### **2.2.2.6 Refolding of *TbPOP* using HiTrap Desalting resin**

Since the removal of urea by step-wise dialysis was highly time consuming and did not remove much of the contaminants, a new approach to the removal of urea was attempted. After solubilisation of *TbPOP* by urea, the denaturant was removed using the HiTrap desalting Sephadex G-25 resin (5 ml) on the ÄKTAdesign™ system. Removal of urea on the G-25 resin is also a form of purification as the resin not only removes the urea, but impurities as well. The resin was washed with 2 column volumes of ultrapure dH<sub>2</sub>O and equilibrated with 5 column volumes desalting buffer [20 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.15 mM NaCl, pH 7.0] (manual run, 5 ml/min, 0.2 MPa, RT), solubilised *TbPOP* (1.5 ml) was filtered through a 0.45 µm filter and injected into the system. The desalting procedure was conducted using the ÄKTAdesign™ system template method “Desalting HiTrap Desalting”. After the run, the resin was washed with 2 column volumes 0.2 M NaOH, followed by 5 column volumes ultrapure dH<sub>2</sub>O and finally, 5 column volumes 20% ethanol. All reagents were prepared with ultra-pure dH<sub>2</sub>O, filtered and degassed using a vacuum degasser before use.

#### **2.2.2.7 Ion exchange chromatography (IEC)**

##### *Anion exchange chromatography*

Following desalting of solubilised *TbPOP*, further purification was conducted using anion exchange chromatography. In anion exchange chromatography, the resin is positively charged therefore the protein of interest would have to be negatively charged in order to bind to the resin. A gradient increase in salt concentration is used to elute the resin. *TbPOP* has a pI (isoelectric point) of 5.80 (calculated using ExPaSy programme). The buffer used in the purification is required to have a pH at least 1 unit above the pI of *TbPOP*, this will give the protein a negative charge and therefore bind easily to the positively charged resin. The resin used was the HiPrep QFF 16/10 resin (20 ml) which a strong anion exchanger on the ÄKTAdesign™ system. The resin was equilibrated with

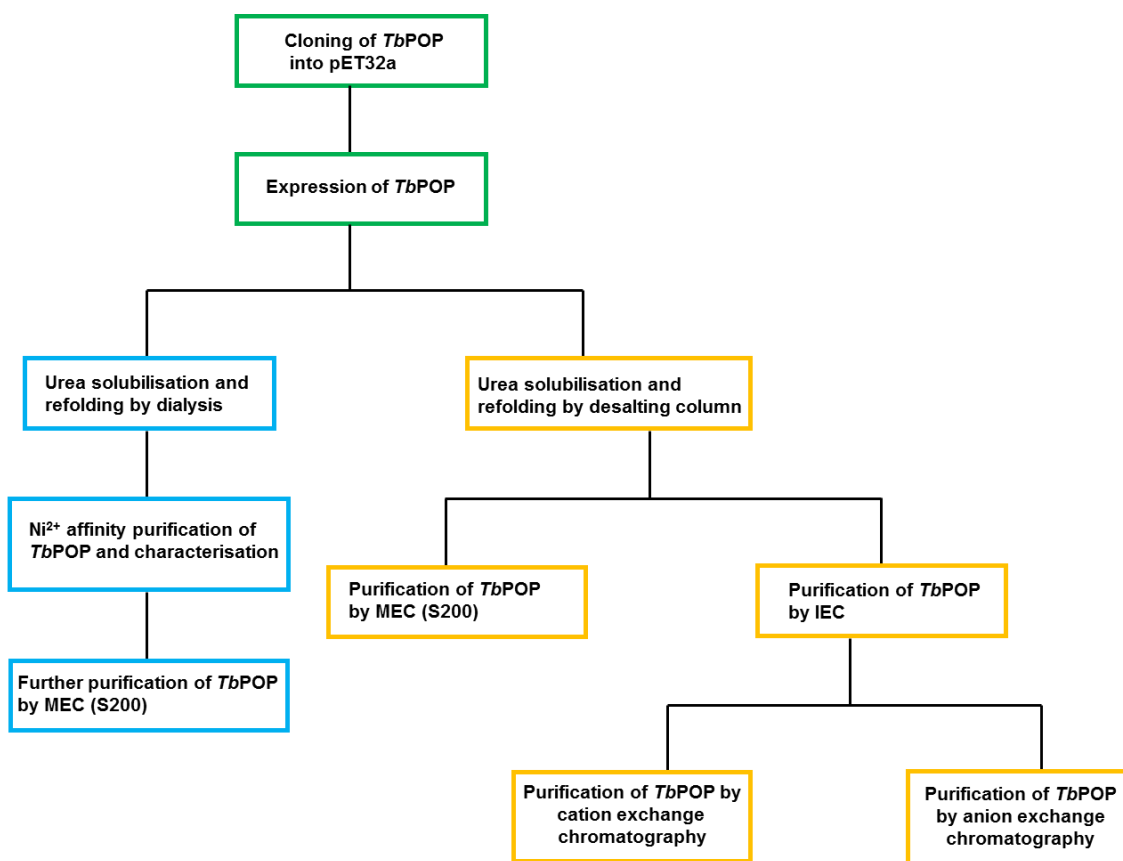
100 ml of start buffer [20 mM bis-Tris, pH 6.8]. Desalted *TbPOP* (4 ml) was diluted in start buffer (1 ml), filtered through a 0.45  $\mu$ M filter and subsequently injected into the system. The resin was washed with 40 ml start buffer, after elution of the unbound material, a gradient of 0 – 1 M NaCl in start buffer was applied over 5 column volumes followed by 40 ml of start buffer containing 2 M NaCl to elute any remaining proteins with collection of 1 ml fractions which were monitored by measuring the  $A_{280}$ . Following elution, the resin was washed with 100 ml ultra-pure dH<sub>2</sub>O followed by 100 ml 20% ethanol. The flow rate was maintained at 5 ml/min and the pressure at 0.2 MPa throughout the purification process. All reagents were prepared with ultra-pure dH<sub>2</sub>O, filtered and degassed using a vacuum degasser before use.

#### *Cation exchange chromatography*

Desalted *TbPOP* was also purified using cation exchange chromatography. In cation exchange chromatography, the resin is negatively charged therefore the protein of interest would have to be positively charged in order to bind to the resin. A gradient increase in salt concentration is also used to elute the resin. The buffer used in the purification is required to have a pH at least 1 unit below the pI of *TbPOP*, this will give the protein a positive charge and therefore bind easily to the negatively charged resin. The resin used was the HiPrep SP FF 16/10 resin (20 ml) which is a strong cation exchanger on the ÄKTAdesign™ system. The resin was equilibrated with 100 ml of start buffer [20 mM sodium citrate, pH 4.8]. Desalted *TbPOP* (4 ml) was diluted in start buffer (1 ml), filtered through a 0.45  $\mu$ M filter and subsequently injected into the system. The resin was washed with 40 ml start buffer and after elution of the unbound material, a gradient of 0 – 1 M NaCl in start buffer was applied over 5 column volumes followed by 40 ml of start buffer containing 2 M NaCl to elute any remaining proteins with collection of 1 ml fractions which were monitored by measuring the  $A_{280}$ . Following elution, the resin was washed with 100 ml ultra-pure dH<sub>2</sub>O followed by 100 ml 20% ethanol. The flow rate was maintained at 5 ml/min and the pressure at 0.2 MPa throughout the purification process. All reagents were prepared with ultra-pure dH<sub>2</sub>O, filtered and degassed using a vacuum degasser before use.



The flow diagram below is a summary of all purification steps of *TbPOP* for clarification on the process.



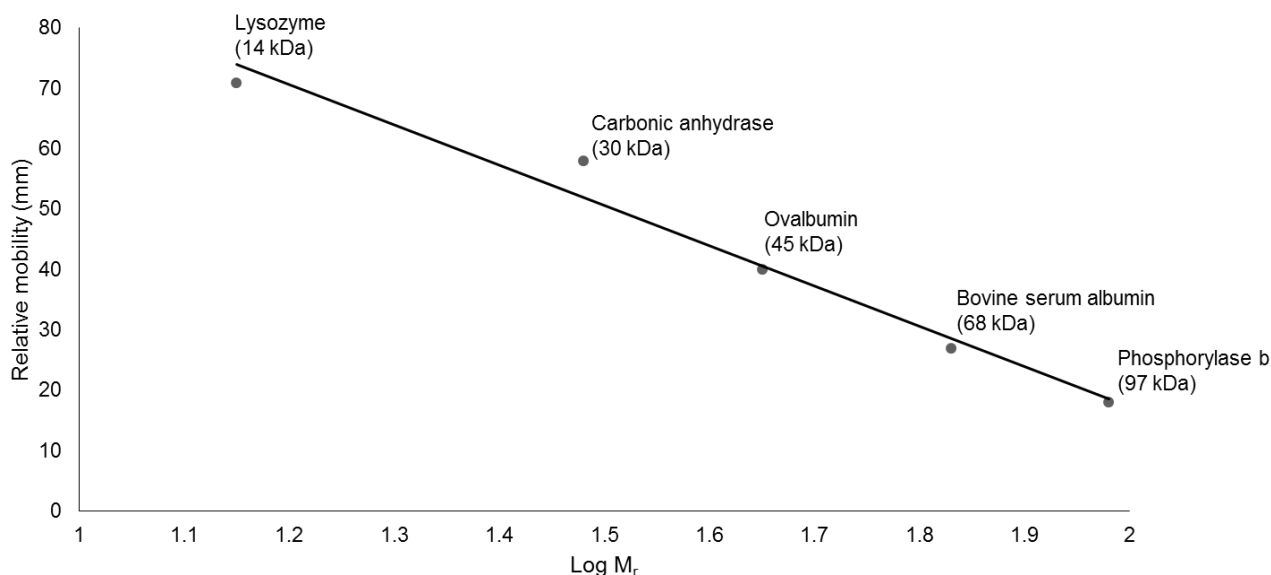
**Figure 2.3: Summary of the *TbPOP* purification steps.** Following expression in insoluble inclusion bodies, *TbPOP* was solubilised, refolded and purified by molecular exclusion and ion exchange chromatography.

### 2.2.2.8 Visual analysis of proteins

#### *Laemmli* SDS-PAGE

All SDS-PAGE analysis was carried out according to (Laemmli, 1970). The proteins were resolved through a stacking gel buffer (pH 6.8) and a running gel buffer (pH 8.8). Prior to loading onto the gel, samples were treated with reducing treatment buffer [125 mM Tris-HCl buffer, 4% (w/v) SDS, 20% (v/v) glycerol and 10% (v/v) 2-mercaptoethanol] at a 1:1 ratio (Laemmli, 1970). The gel was run at 20 mA per gel for 2 h in tank buffer [250 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS]. In order to determine the molecular weight ( $M_r$ ) of a protein analysed by SDS-PAGE, a standard curve (Fig. 2.4) was constructed using proteins of known  $M_r$ . Proteins were visualised by staining the gel with

Coomassie blue R-250 [0.125% Coomassie brilliant blue R-250, 50% (v/v) methanol and 10% (v/v) acetic acid]. Destaining of the gel in order to view proteins, was done using destaining solution [50% (v/v) methanol, 10% (v/v) acetic acid].



**Figure 2.4: Standard curve for determination of  $M_r$  of unknown proteins analysed by SDS-PAGE.** The marker containing proteins of known molecular mass were separated alongside proteins of unknown molecular weight in order to determine their sizes. The equation of the trend line is  $y = -66.763 + 150.82x$ , with a correlation coefficient of 0.9744.

#### *Silver staining of proteins*

For more sensitive protein staining, a silver stain (Blum *et al*, 1987) was used. Briefly, the proteins were resolved by reducing SDS-PAGE, after which the running gel containing proteins was incubated in fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) 37% formaldehyde] for 1h. The gel was then washed with washing solution 1 [50% (v/v) ethanol] (3 × 20 min) followed by soaking in pretreatment solution [4 mg/ml  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ ] for 1 min. The gel was then rinsed in  $\text{dH}_2\text{O}$  (3 × 20 sec) and soaked in impregnation solution [0.2% (m/v)  $\text{AgNO}_3$ , 0.75% (v/v) 37% formaldehyde] for 20 min. The gel was once again rinsed in  $\text{dH}_2\text{O}$  (2 × 20 sec). The gel was subsequently incubated in developing solution [60 g/l  $\text{Na}_2\text{CO}_3$ , 50% (v/v) 37% formaldehyde,  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5 \text{H}_2\text{O}$ ] until the first protein bands became visible after which the gel was immersed in  $\text{dH}_2\text{O}$  until the protein bands had fully developed. Once all bands were visible, the gel was incubated in stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] for 10 min to stop over

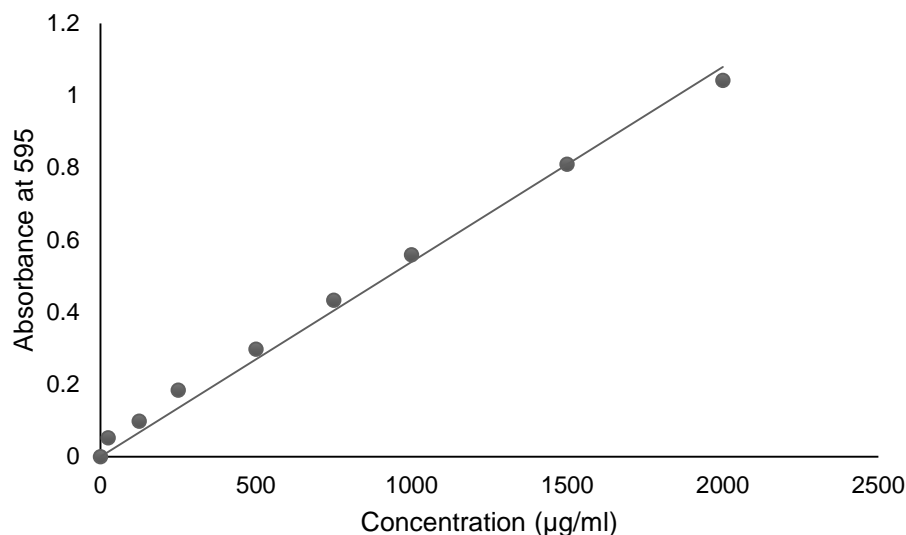
development of protein bands and finally, washed with washing solution 2 [50% (v/v) methanol].

#### *Western blot analysis of TbPOP*

Proteins resolved by SDS-PAGE were transferred onto nitrocellulose membranes using a semi-dry blotter (Sigma, St. Louis, MO, USA) , 20 V, 50 min in blotting buffer [45 mM Tris, 173 mM glycine, 18% (v/v) methanol and 0.1% SDS]. Following blotting, the nitrocellulose membrane was stained with Ponceau S [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] to confirm if protein bands were successfully transferred. The membrane was destained with distilled water containing 3 drops of 500 mM NaOH. The membrane was then rinsed with TBS [20 mM Tris-HCl buffer, pH 7.4, 200 mM NaCl] then blocked with low fat milk [5% (w/v) low fat milk in TBS] for 1 h. The membrane was washed with TBS (3 x 5 min) and incubated in horse radish peroxidase (HRPO) conjugated mouse anti-His-tag antibody overnight at 4°C. The membranes were washed once again with TBS (3 x 5 min) and incubated with Pierce™ ECL western blotting substrate solution as per manufacturer's instructions, the fluorescent bands were visualised using the Syngene G:Box Chemi XR5 system (Vacutec, California, USA).

#### **2.2.2.9 Quantification of proteins using BCA Assay**

In order to determine protein concentration, the BCA™ Protein Assay Kit (Pierce, Rockford, IL, USA) was used. Bovine serum albumin (BSA) standards were prepared in triplicates at concentrations ranging from 0 – 2000 µg/ml. Working reagent solution was prepared by combining reagent B with reagent A at a 1:20 ratio. Duplicate standards (25 µl) were mixed with the working reagent (200 µl) in a Nunc® 96 microtiter plate and incubated at 37°C, 30 min. The purple coloured product was measured at 595 nm and a standard curve was constructed (Fig. 2.5) to determine protein concentration.



**Figure 2.5: BSA standard curve for determination of protein concentration.** BSA standards of known concentration were mixed with the working reagent and the  $A_{595}$  measured. The trend line equation is  $y = 0.0005x$ , with a correlation coefficient of 0.9921.

### 2.2.2.10 Enzymatic characterisation of *TbPOP*

#### *Gelatin SDS-PAGE analysis*

Enzymatic activity was analysed by Laemmli (1970) SDS-PAGE modified according to Heussen and Dowdle (1980). Gelatin 1% (w/v) was prepared by dissolving 0.1 g gelatin in 10 ml running gel buffer with heating. Gelatin solution (1.5 ml) was added to running gel buffer (2.25 ml) and the SDS-PAGE gel was further prepared as described in Section 2.2.2.9. Following electrophoresis, the gel was soaked in 2 changes of 2.5% (v/v) Triton X-100 for 1 h at RT. The gel was subsequently incubated in assay buffer [100 mM Na-acetate, 1 mM  $\text{Na}_2\text{EDTA}$ , 0.02% (m/v)  $\text{NaN}_3$ , 40 mM cysteine, pH 5.0] overnight at 37°C. The gel was stained with amido black [0.1% (w/v) amido black, 30% (v/v) methanol, 10% (v/v) acetic acid] for 1 h and destained with destaining solution [30% (v/v) methanol, 10% (v/v) acetic acid].

#### *Hydrolysis of collagen type I*

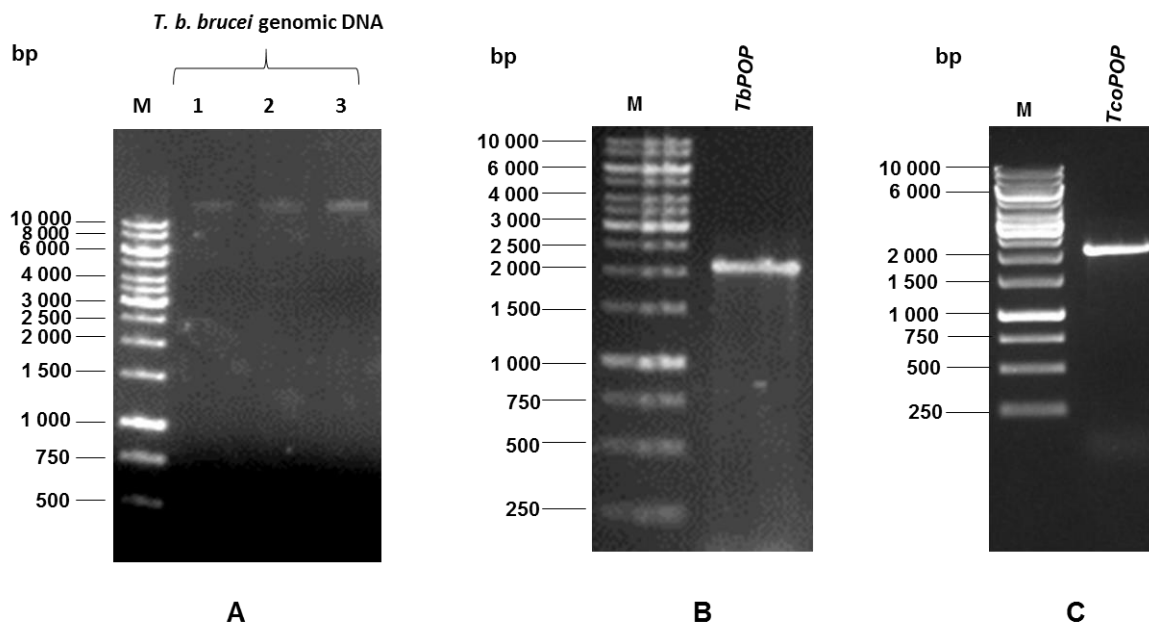
Proteolytic activity of *TbPOP* was tested against the protein substrate type I collagen from rat tail as previously described by Bastos *et al* (2010). Collagen hydrolysis was conducted by incubating 100 µg type I collagen with 5 µg *TbPOP* in 50 µl assay buffer [50 mM Tris, 50 mM NaCl, 5 mM DTT, pH 8.0] for 16 h at 37°C. Controls consisted of type I collagen

incubated in assay buffer in the absence of *TbPOP*. Following incubation, the reaction was stopped by the addition of equal volume of reducing treatment buffer and boiling for 2 min. Hydrolysis was analysed by SDS-PAGE and visualised by silver staining (Sections 2.2.2.8 and 2.2.2.9).

## 2.3. Results

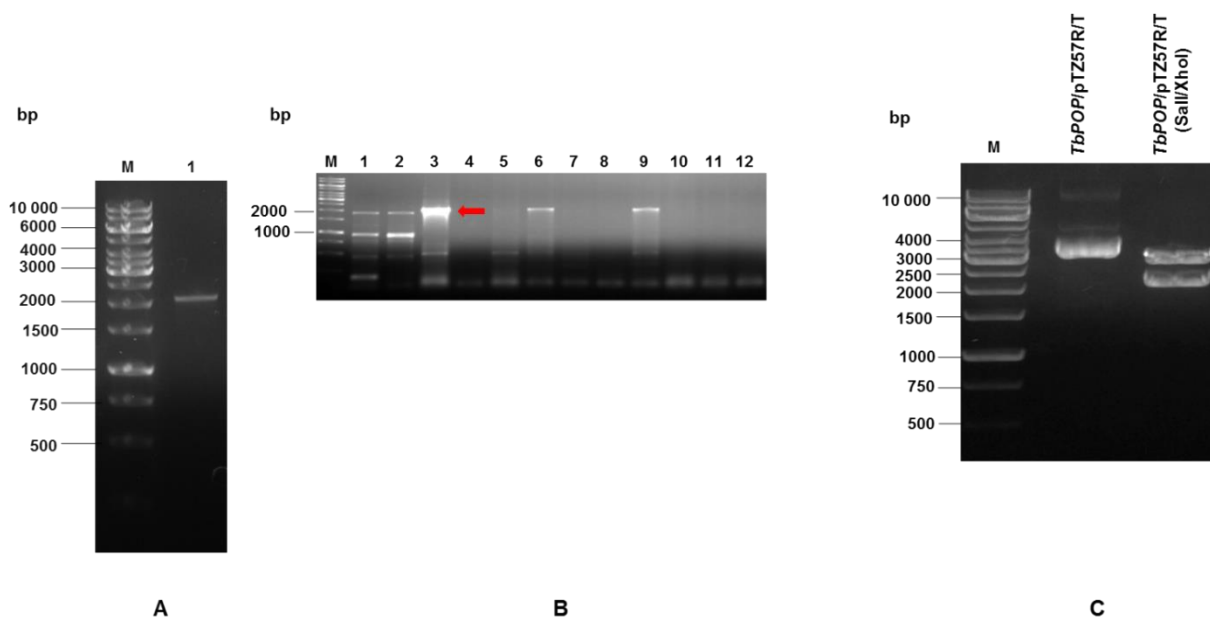
### 2.3.1 Cloning of full-length *TbPOP* and *TcoPOP* into pTZ57R/T and pGEM-T® Easy cloning vectors

Genomic DNA from *T. b. brucei* 427 Lister strain was isolated (Fig. 2.6, panel A lanes 1, 2 and 3) and used as a template for PCR amplification using primers designed specifically to amplify the *TbPOP* gene. The PCR amplification was successful as seen by the presence of an ~2097 bp PCR product in Fig. 2.6, panel B. *TcoPOP* gene was also successfully amplified using the gene specific primers (Fig 2.6, panel C) as seen by the presence of an ~2097 bp product.



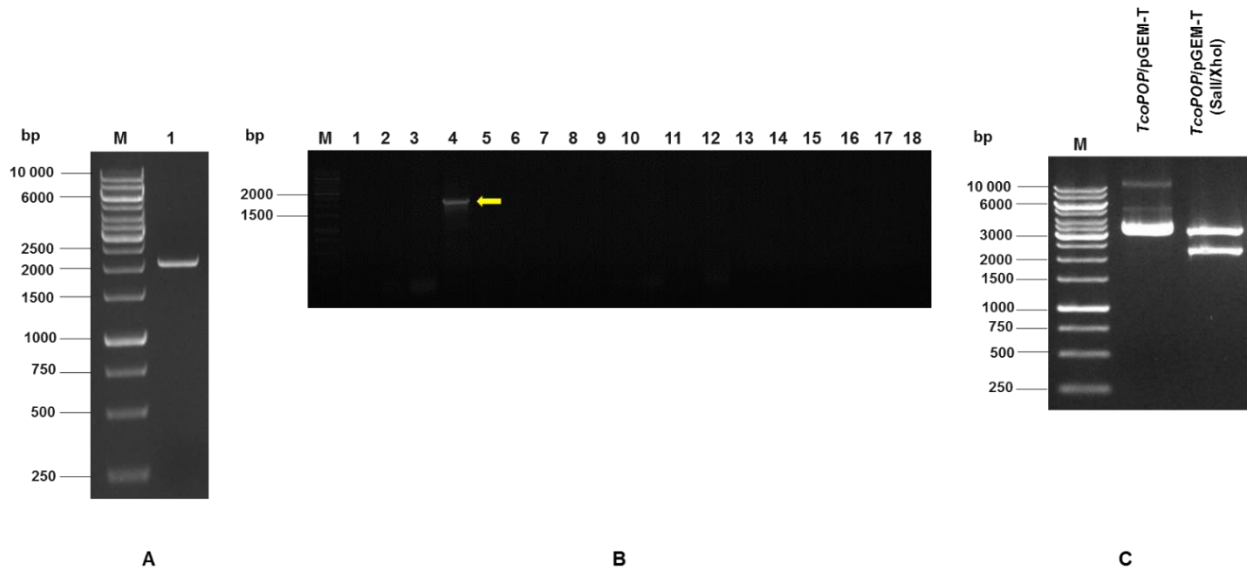
**Figure 2.6:** *T. b. brucei* genomic DNA isolation and amplification of *TbPOP* and *TcoPOP* genes. M, MassRuler® DNA ladder mix. A: 1, 2 and 3, Genomic DNA isolated from *T. b. brucei* 427 Lister strain; B: Amplified 2097 bp *TbPOP* PCR product. C: Amplified 2097 bp *TcoPOP* PCR product. Samples were analysed on a 1% (w/v) agarose gel stained with ethidium bromide (3 µg/ml).

The amplified *TbPOP* PCR product was gel purified (Fig. 2.7, panel A) and ligated to pTZ57R/T and transformed into competent *E. coli* JM109 cells. Recombinant colonies were screened using blue/white screening and colony PCR. From the positive clone, number 3 (indicated by the red arrow) was selected (Fig. 2.7, panel B). The plasmid DNA from the recombinant colony was isolated and subjected to a double restriction digest with *Sall* and *XhoI*. The *TbPOP* insert was successfully excised from the T-vector as the inert was dropped at ~2097 bp after the double digest (Fig. 2.7, panel C).



**Figure 2.7: Colony PCR screening of recombinant colonies after ligation of *TbPOP* PCR product into pTZ57R/T vector and double digest of *TbPOP/pTZ57R/T* recombinants.** M, MassRuler® DNA ladder mix. **A:** Gel purified *TbPOP* PCR product (13.3 ng/μl). **B: Lanes 1-12,** Colonies selected for colony PCR, amplification of the conserved region was done using *TbPOP* primers. *TbPOP/pTZ57R/T* recombinant colony indicated by red arrow. **C:** Isolation of plasmid DNA from recombinant colony and double digest of the *TbPOP/pTZ57R/T* recombinants using *Sall* and *XhoI* restriction enzymes. Samples were analysed on a 1% (w/v) agarose gel stained with ethidium bromide (3μg/ml).

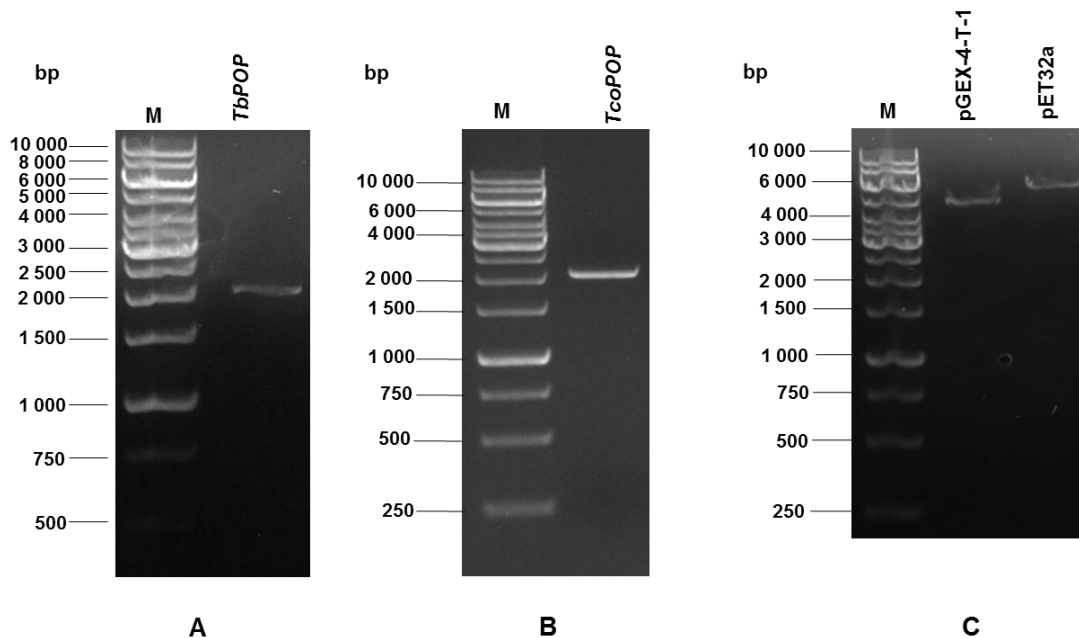
The gel purified *TcoPOP* PCR product (Fig. 2.8, panel A) was ligated into pGEM-T® Easy cloning vector, after analysis with colony PCR, one colony (indicated by the yellow arrow) was positive for the *TcoPOP/pGEM-T*® Easy recombinant plasmid (Fig. 2.8, panel B). The plasmid DNA from the recombinant colony was isolated and subjected to a double restriction digest with *Sall* and *XhoI*. The *TcoPOP* insert was successfully excised from the T-vector as the inert was dropped at ~2097 bp (Fig. 2.8, panel C).



**Figure 2.8: Colony PCR screening of recombinant colonies after ligation of *TcoPOP* PCR product to pGEM-T® Easy T-vector and double digest of *TbPOP*/pGEM-T® Easy recombinants. M, MassRuler® DNA ladder mix. **A:** Gel purified *TcoPOP* PCR product (28.5 ng/μl). **B:** Lanes 1-18, Colonies selected for colony PCR, amplification of the conserved region was done using *TcoPOP* primers. *TcoPOP*/pGEM-T® Easy recombinant plasmid indicated by yellow arrow. **C:** Isolation of plasmid DNA from recombinant colony and double digest of the *TcoPOP*/pGEM-T® Easy recombinants using Sall and XhoI restriction enzymes. Samples were analysed on a 1% (w/v) agarose gel stained with ethidium bromide (3 μg/ml).**

**2.3.2 Sub-cloning of *TbPOP* and *TcoPOP* into pGEX-4-T-1 and pET32a expression vectors**

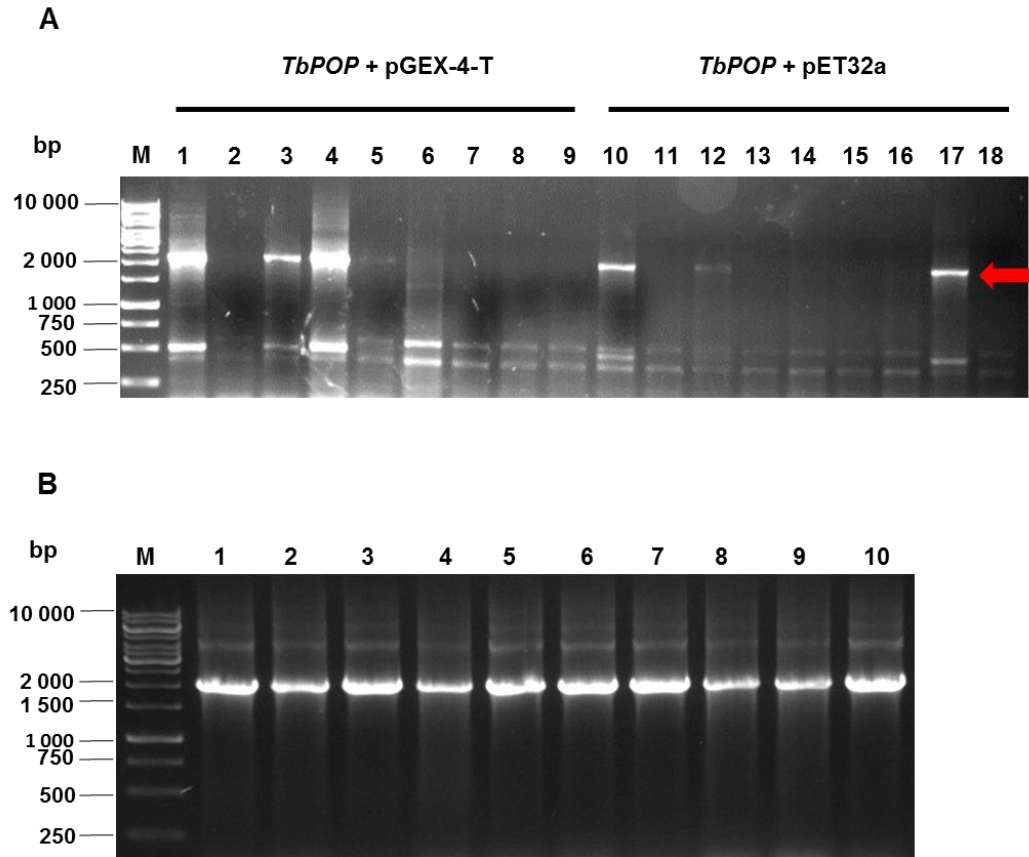
Following excision of the *TbPOP* and *TcoPOP* inserts from the T-vectors, both inserts were successfully gel purified as seen in Fig. 2.9, panels A and B. The expression vectors were prepared by double digestion with Sall and XhoI restriction enzymes and de-phosphorylation to prevent re-circularisation. They were subsequently concentrated and analysed on a 1% (w/v) agarose gel (Fig 2.9, panel C).



**Figure 2.9: Agarose gel evaluations of gel purified *TbPOP* and *TcoPOP* inserts and concentrated pGEX 4-T-1 and pET32a expression vectors. M, MassRuler® DNA ladder mix A: Gel purified *TbPOP* insert (15.8 ng/μl). B: Gel purified *TcoPOP* insert (13.6 ng/μl). C: Purified and concentrated expression vectors, pGEX-4-T-1 (24 ng/μl) and pET32a (8.1 ng/μl). Samples were analysed on a 1% (w/v) agarose gel stained with ethidium bromide (3 μg/ml).**

The gel purified inserts were sub-cloned into pGEX-4T-1 and pET32a expression vectors and transformed into BL21(DE3) competent cells. For *TbPOP*, sub-cloning into pGEX-4T-1 produced three positive clones (lanes 1, 3 and 4) and sub-cloning into pET32a also produced two positive clones (lanes 10 and 17), however, only one clone, as indicated by the red arrow (Fig. 2.10, panel A) was selected for recombinant and expression. Colonies shown in lanes 1, 3, 5 and 9 were used to inoculate terrific broth to test for expression of *TcoPOP* (Fig. 2.10, panel B).

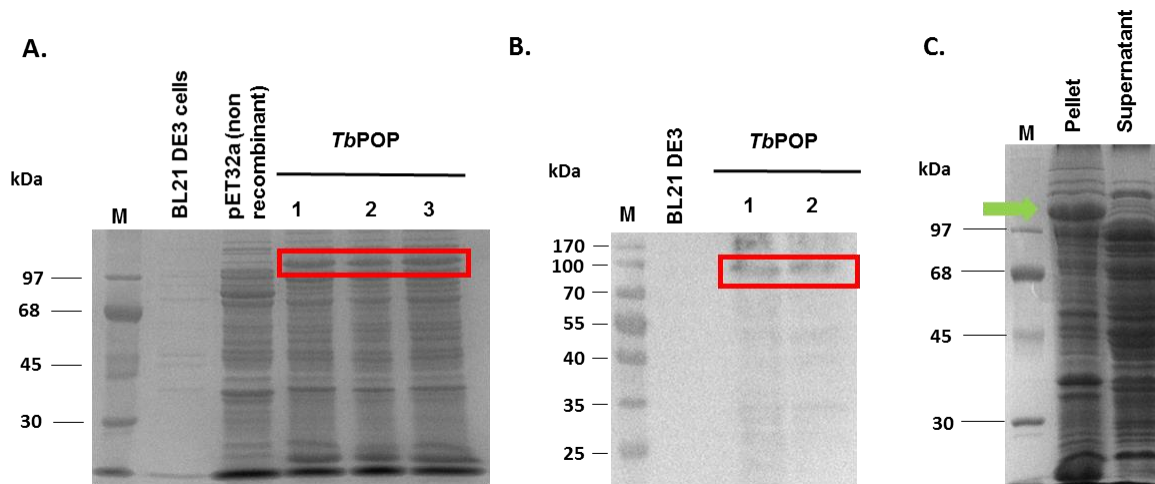




**Figure 2.10: Colony PCR screening for recombinants after *TbPOP* and *TcoPOP* ligation to pGEX-4-T-1 and pET32a expression vectors; and evaluation of amplification of conserved region using insert primers. M, MassRuler® DNA ladder mix. **A:** Lanes 1-9, Colonies from *TbPOP*/pGEX 4-T-1 ligation selected for colony PCR, Lanes 10-18, colonies from *TbPOP*/pET32a ligation selected from colony PCR. Recombinant *TbPOP*/pET32a colony indicated by red arrow. **B:** Colonies from *TcoPOP*/pET32a ligation selected for colony PCR. Amplification of the conserved regions was done using insert primers. Samples were analysed on a 1% (w/v) agarose gel stained with ethidium bromide (3 µg/ml).**

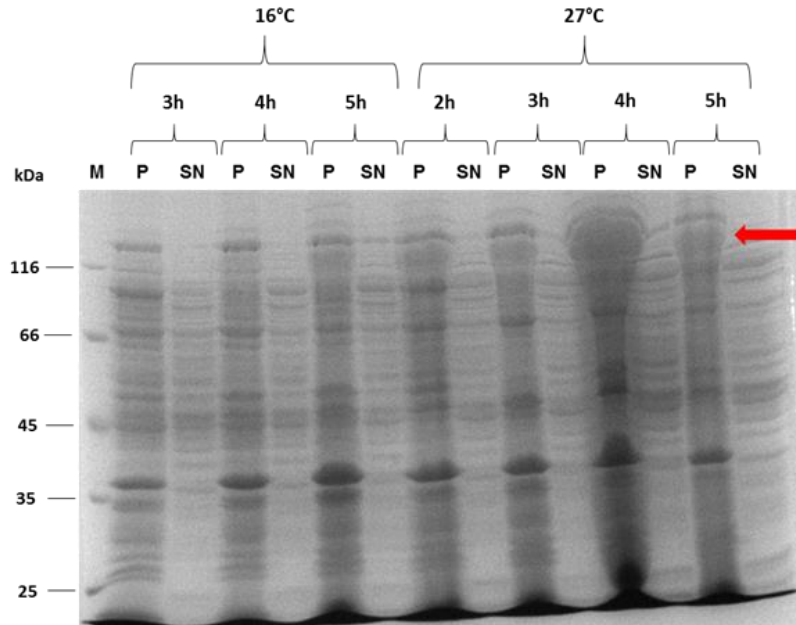
### 2.3.3 Recombinant expression, solubilisation and purification of *TbPOP*

Recombinant expression of *TbPOP* was successful as seen by the presence of a band at ~ 100 kDa (Fig. 2.11, panel A). Expression was also confirmed by western blot analysis using mouse  $\alpha$ -His tag antibodies (Fig. 2.11, panel B). After confirmation of expression, a solubility test was done in order to determine if *TbPOP* was expressed in its soluble state for purification purposes. Fig. 2.11, panel C shows that *TbPOP* was expressed in an insoluble state in the lysate pellet and therefore required solubilisation in order to purify the protein.



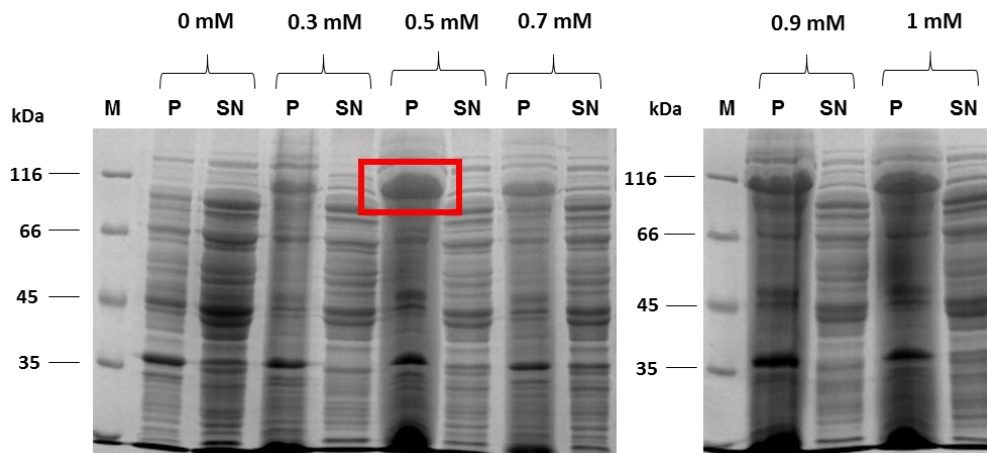
**Figure 2.11: Reducing SDS-PAGE (12.5%) and western blot analysis of *TbPOP* expression.** **M**, Molecular weight marker **A**: Expression of recombinant *TbPOP*. Expression lysate run alongside untransformed cells and non-recombinant vector confirmed expression. **Lanes 1, 2 and 3**: Recombinantly expressed *TbPOP*. **B**: **M**, PageRuler™ Prestained protein ladder. Western blot confirming *TbPOP* expression; blot was probed with mouse anti-His-tag antibodies (1:5000 dilution) and visualised with ECL substrate. **Lanes 1 and 2**: Recombinantly expressed *TbPOP*. **C**: Solubility test confirmed *TbPOP* was expressed in the pellet in its insoluble state. The green arrow shows insoluble recombinant *TbPOP*. Gels were stained with Coomassie blue R-250.

After the solubility test confirmed that *TbPOP* was expressed in the pellet in its insoluble state, expression was optimised at 16°C and 27°C (Fig. 2.12), in an attempt to result in expression in the soluble form of the enzyme. Expression at lower temperatures sometimes assists in the solubilisation of proteins (Baneyx, 1999), however, expression of *TbPOP* at these temperatures was unsuccessful at producing soluble protein as indicated by the red arrow in Fig. 2.12.



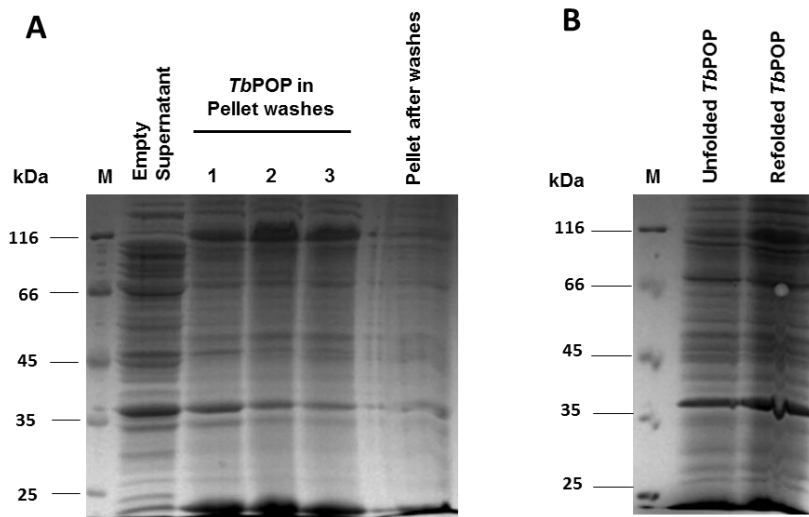
**Figure 2.12: Reducing SDS-PAGE (12.5%) analysis of *TbPOP* expression under various temperatures.** M, PageRuler™ unstained protein ladder. P: Pellet. SN: Supernatant. Expression at lower temperatures still produced insoluble *TbPOP*. The gel was stained with Coomassie blue R-250.

The IPTG concentration was also optimised to determine the optimal concentration to use during expression. The optimal concentration was found to be 0.5 mM as seen by the presence of a sharp band at 0.5 mM in the pellet indicated by the red box in Fig 2.13.



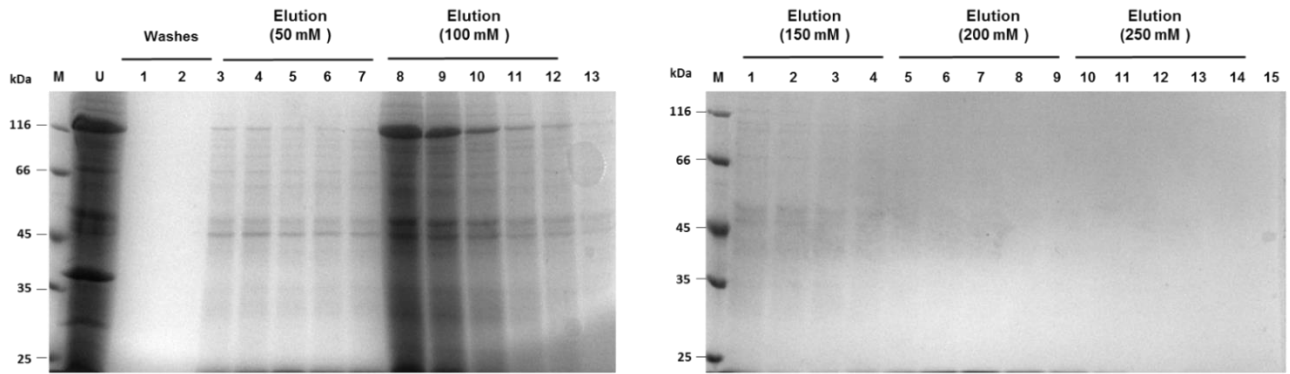
**Figure 2.13: Reducing SDS-PAGE (12.5%) analysis of *TbPOP* expression at various IPTG concentrations.** M, PageRuler™ unstained protein ladder. P: Pellet. SN: Supernatant. Optimal concentration was found to be 0.5 mM. Gels were stained with Coomassie blue R-250.

Since attempts at expression of *TbPOP* at various temperatures was unsuccessful at producing soluble protein, it was then solubilised using urea and refolded by dialysis. The solubilisation of *TbPOP* was successful after only 3 washes of the pellet with pellet wash buffer containing urea, (Fig. 2.14, panel A) showing the now soluble *TbPOP* present in the supernatant after the washes. Solubilised *TbPOP* was also refolded as seen in Fig. 2.14, panel B due to a slight shift in size back to approximately 100 kDa. A definite



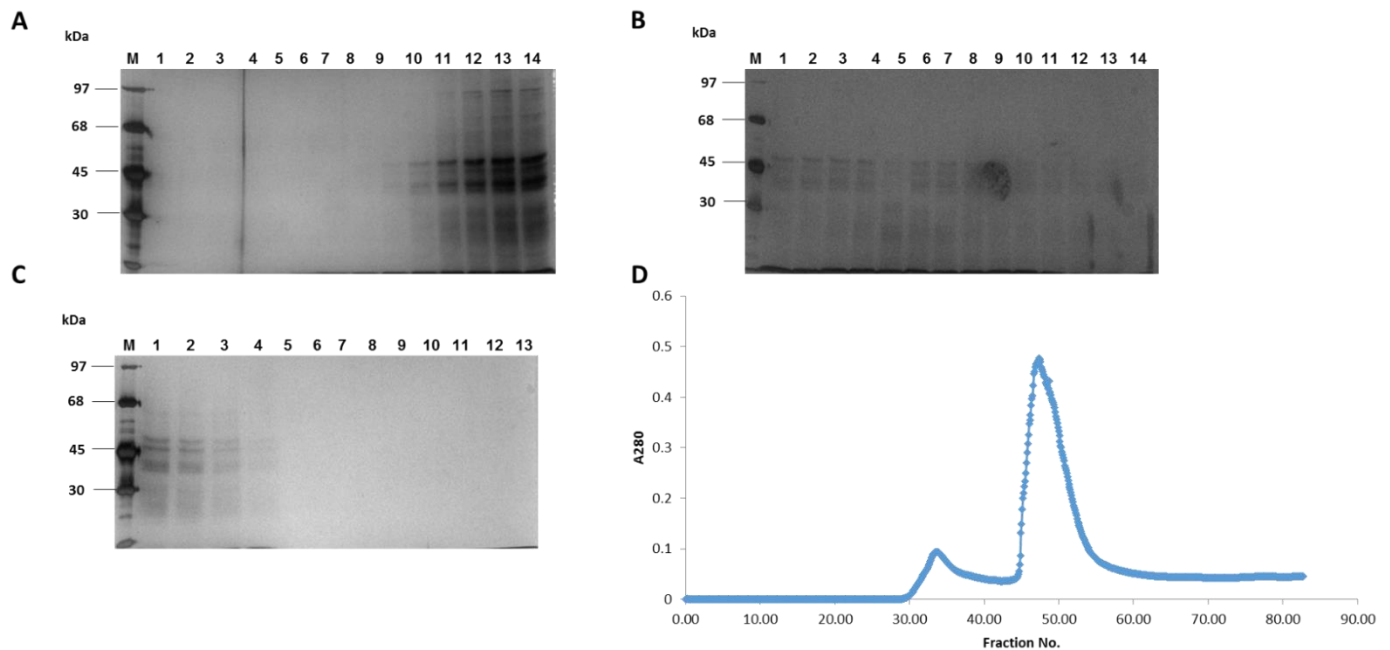
**Figure 2.14: Reducing SDS-PAGE (12.5%) analysis of solubilisation of and refolding of *TbPOP*.** M, PageRuler™ unstained protein ladder. **A:** Initial supernatant had no soluble *TbPOP* present. Soluble *TbPOP* present in the washes and no longer in the pellet. The remaining pellet no longer contains insoluble protein. **B:** *TbPOP* successfully refolded by dialysis. Gels were stained with Coomassie blue R-250.

After refolding, soluble *TbPOP* could now be purified. Since the recombinant enzyme had the Trx-his dual affinity tag, it was purified by affinity purification on a Ni<sup>2+</sup> chelate resin and eluted with an increasing gradient of imidazole from 50 mM to 250 mM. *TbPOP* was eluted at 100 mM imidazole. Unfortunately the protein was only partially purified (Fig. 2.15) as seen by the presence of some contaminants in the eluted fraction.



**Figure 2.15: Reducing SDS-PAGE (12.5%) analysis of affinity purification of *TbPOP* on Ni affinity resin.** M, PageRuler™ unstained protein ladder. U: Unbound fraction. In eluted fractions, *TbPOP* eluted with some contaminants. Gels were stained with Coomassie blue R-250.

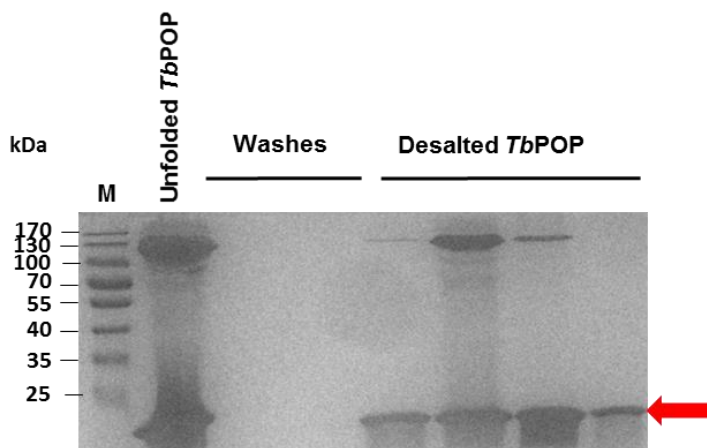
Following affinity purification, the semi purified fractions eluted at 100 mM imidazole Lanes 8 -11 (Fig. 2.15) were concentrated and loaded on a gel filtration column (Fig. 2.16)



**Figure 2.16: Purification of *TbPOP* on a HiPrep™ 16/60 Sephacryl™ S200 HR molecular exclusion resin.** M, molecular weight marker **A:** Reducing SDS-PAGE (12.5%) analysis of *TbPOP* peak fractions 25– 38. **B:** Reducing SDS-PAGE (12.5%) analysis of *TbPOP* peak fractions 39-52. **C:** Reducing SDS-PAGE (12.5%) analysis of *TbPOP* peak fractions 53-65. proteins visualised by silver staining. **D:** Elution profile of *TbPOP* purification on a HiPrep™ 16/60 Sephacryl™ S200 HR resin (1.6 cm x 60 cm, flow rate 0.5 ml/min, 0.2 MPa).

Although the elution profile showed some separation of the proteins, further analysis by SDS-PAGE showed that separation of the impurities from *TbPOP* by gel filtration was unsuccessful as seen in fractions 34–38 (Fig. 2.16, panel A lanes 10–14). The *TbPOP* higher molecular weight bands are eluted first along with some impurities, but also become diffuse and the lower molecular weight bands become more prominent. Fractions 39–60 (Fig. 2.16, panels B and C) represent the lower molecular weight proteins which are further eluted in the second peak.

It was considered that these impurities may be difficult to remove due to the refolding process of solubilised *TbPOP* which was by stepwise dialysis (Section 2.2.2.3) which leads to proteins further aggregating during long periods of dialysis (De Bernardez Clark, 1998). So another method to remove urea using a desalting column was considered as desalting columns may remove some impurities along with the denaturing agent. The G-25 desalting column seemed to remove most of impurities during the desalting process as seen in Fig. 2.17. However, there now seemed to be the development of a lower molecular weight band at approximately 20 kDa (indicated by red arrow) in both unfolded and desalted fractions.

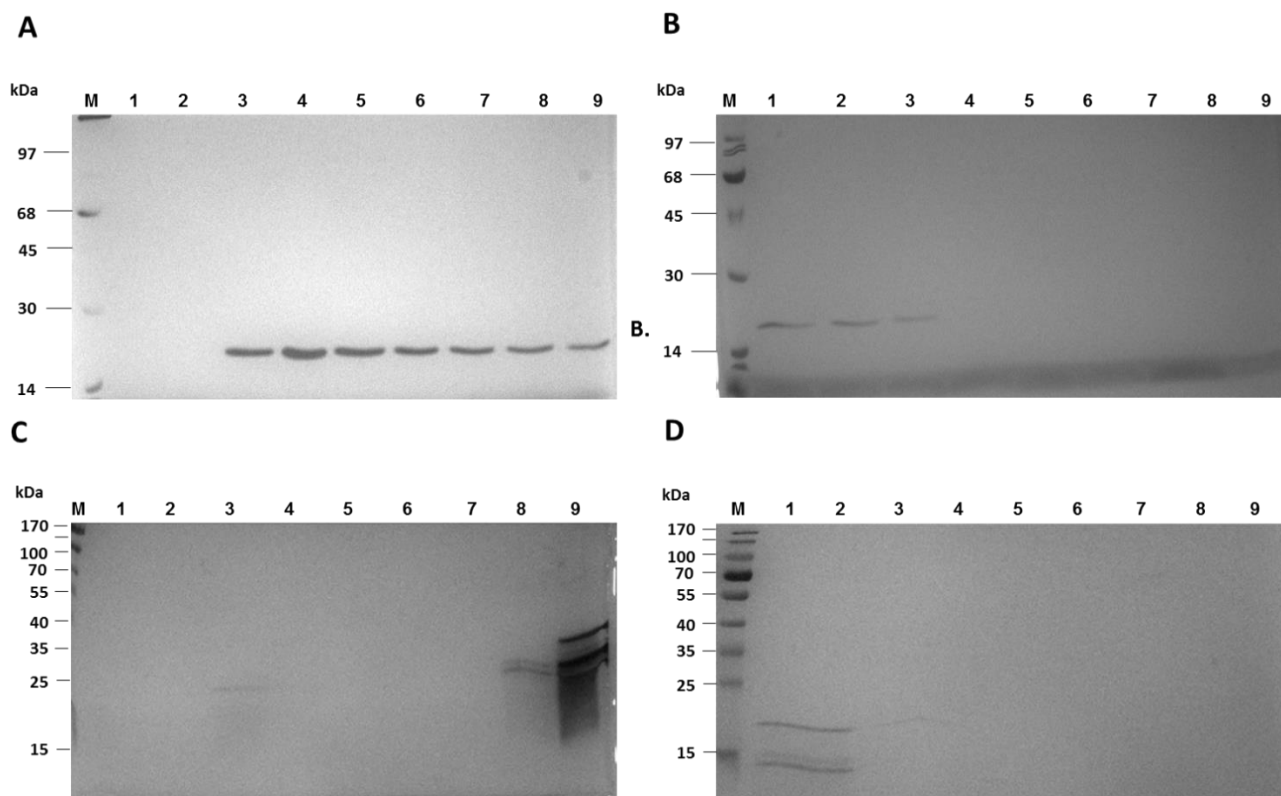


**Figure 2.17: Reducing SDS-PAGE (12.5%) analysis of desalted *TbPOP* by HiTrap desalting G-25 resin.** M, PageRuler™ Prestained protein ladder. Unfolded or solubilised *TbPOP* was run alongside desalted *TbPOP*. Red arrow indicates lower molecular weight band. Proteins visualised by silver staining.

The lower molecular weight band at approximately 20 kDa which developed after desalting, could be separated from 100 kDa *TbPOP* using gel filtration as the difference in

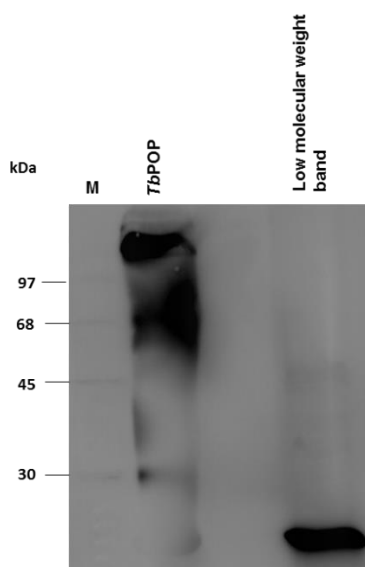
size between the two proteins was sufficient to separate them (Section 2.2.2.5). Unfortunately, the procedure was unsuccessful, as the elution profile showed no separation of the proteins (result not shown). When assessing the fractions constituting the single peak by SDS-PAGE, no clear protein bands could be distinguished even after silver staining (result not shown).

Since gel filtration was unsuccessful, ion exchange chromatography was attempted on desalted *TbPOP*. Purification of *TbPOP* by anion exchange chromatography was also unsuccessful as it resulted in the complete disappearance of *TbPOP* at ~100 kDa and only the lower molecular weight band remained following elution (Fig. 2.18, panels A and B). Cation exchange chromatography also resulted in the disappearance of *TbPOP* and only residual proteins were eluted (Fig. 2.18, panels C and D).



**Figure 2.18: Purification of desalted *TbPOP* on a HiPrep QFF 16/10 anion exchange and HiPrep SP FF 16/10 cation exchange resin. M, molecular weight marker. A and B: Reducing SDS-PAGE (12.5%) analysis of *TbPOP* peak fractions from anion exchange resin. C and D: Reducing SDS-PAGE (12.5%) analysis of *TbPOP* peak fractions from cation exchange resin. Proteins visualised by silver staining.**

A western blot using anti-His-tag antibodies was conducted on *TbPOP* as well as the lower molecular weight band which developed after *TbPOP* desalting and ion exchange chromatography. It was found that the band was recognised by anti-his-tag antibodies (Fig. 2.19), so it can be speculated that the band could be the *trx*-his tag because similar to the tag, it has a size of ~20 kDa or it could be part of a degraded form of *TbPOP*. However, the only way to confirm these speculations would be to have the band sequenced in future work.



**Figure 2.19: Western blot analysis of *TbPOP* and low molecular weight band.** Blot was probed with mouse anti-His-tag antibodies (1:5000 dilution) and visualised with ECL substrate.

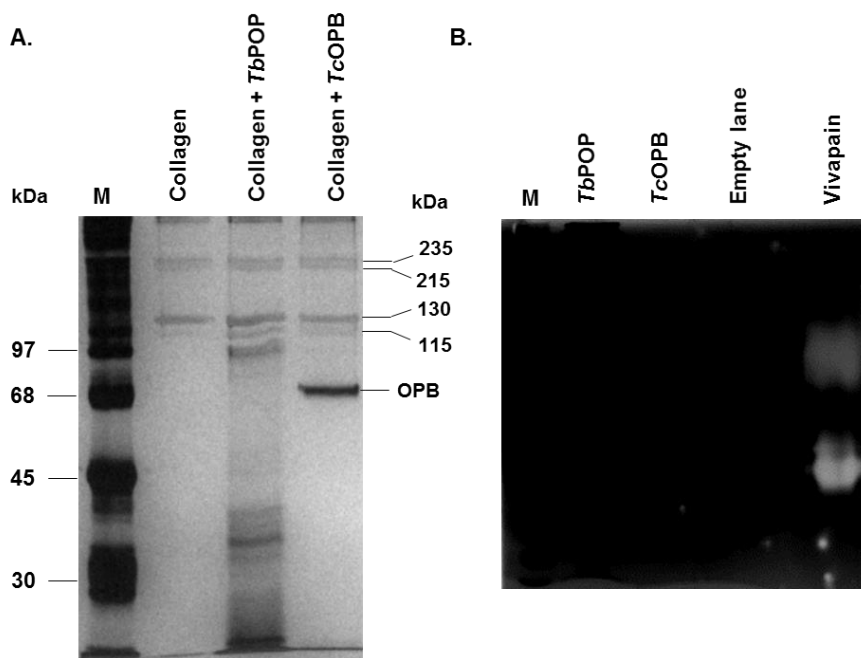
Purification of *TbPOP* proved to be quite a challenge as affinity chromatography failed to separate the protein from contaminants and any further attempts to remove the contaminants led to possible degradation of *TbPOP* and the development of another lower molecular weight band at ~20 kDa.

### 2.3.4 Enzymatic characterisation of *TbPOP*

Following partial Ni<sup>2+</sup> affinity purification of *TbPOP*, two enzymatic assays were conducted to determine if the enzyme had activity. A collagen type I hydrolysis assay was conducted (Fig. 2.20, panel A) as well as a gelatin hydrolysis assay (Fig. 2.20, panel B). The assays were conducted with *TbPOP* as well as another oligopeptidase (oligopeptidase B, OPB)



which will be discussed further in chapter 3. Panel A revealed that *Tb*POP does hydrolyse type I collagen, as shown by the presence of degradative collagen bands at the 115 kDa and a 130 kDa doublet when incubated with *Tb*POP. The degradation products were seen at ~97 kDa and ~35 kDa. As expected, OPB did not hydrolyse collagen type I, instead of the presence of degradative collagen bands being seen, only the type I collagen doublet at 115 kDa and 130 kDa and the doublet at 215 kDa and 235 kDa as well OPB at ~80 kDa are visible. Panel B revealed that neither of the oligopeptidases hydrolyse gelatin, only the vivapain control showed activity. These results show that although *Tb*POP was only partially purified, it still retained hydrolytic activity. However, further analysis of its biochemical characteristics to determine kinetic parameters such as  $K_m$  and  $K_{cat}$  was not possible as that would require the enzyme to have high purity.

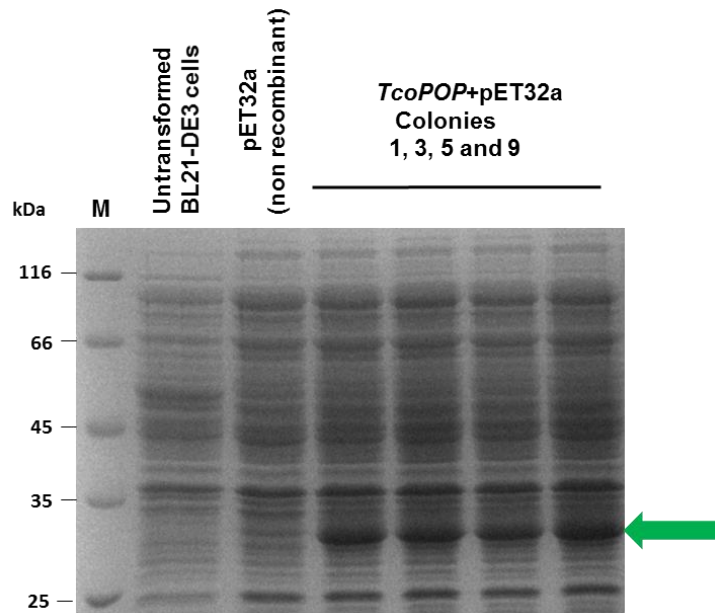


**Figure 2.20: Enzymatic characterisation of *Tb*POP using type I collagen and gelatin substrates. M, molecular weight marker. A:** Reducing SDS-PAGE (10%) of type I collagen hydrolysis by *Tb*POP. Proteins visualised by silver staining. **B:** Non-reducing SDS-PAGE (12.5%) incorporated with 1% gelatin. Gel stained with amido black.

### 2.3.5 Recombinant expression of *Tco*POP

Recombinant expression of *Tco*POP was unsuccessful as expression seemed to end prematurely at a size of approximately 30 kDa (Fig. 2.21) indicated by the green arrow.

When comparing the *TcoPOP* expression lysates to the untransformed BL21 DE3 cells and the non-recombinant pET32a (Fig. 2.21), it is clear that the band is not present in these samples, it is only present after auto induction of expression in terrific broth, so one can conclude that expression had occurred, however, it seems that a mutation was introduced in the *TcoPOP* gene during the cloning stages which resulted in termination of protein translation.



**Figure 2.21: Reducing SDS-PAGE (12.5%) of *TcoPOP* expression. M, PageRuler™ unstained protein ladder.** Expression lysate run alongside untransformed BL21-DE3 cells and non-recombinant pET32a vector confirmed that expression had occurred and produced an incomplete protein at ~30 kDa indicated by the green arrow. Gel stained with Coomassie blue R-250.

After cloning the *TcoPOP* gene into the pGEM-T® T-vector and pET32a expression vector, the recombinant DNA was sent for sequencing and the cloned sequences aligned with that of non-recombinant *TcoPOP* (Fig. 2.22 and Fig. 2.23 respectively) in order to determine if the sequence of the recombinant DNA was still viable. The alignments showed that the recombinant *TcoPOP* DNA had undergone a mutation where a guanine in the *TcoPOP* sequence (highlighted in yellow) was replaced by an adenine in the recombinant sequence (highlighted in red) which led to the development of the stop codon “TAG” which subsequently led to a stop in translation and hence termination of protein expression.



TcPOP	ATGCGTCTCGTGTACCCCACTGTCAGGCGTTCGGCGGCGACATACACCCTTCACAATGTC	60
TcPOP+_pET32a	-----AARCWMTTTCAMMGTTYGGSCWGCRACTACACSCTKCAYAATGTC * *       *** **               ***** ** * *****	45
TcPOP	ACGGTATCTGAGCCGTACGACTACCTGGAGGACCCTGAAGACCCACAACAAGTCATTT	120
TcPOP+_pET32a	ACGGTATCTGAGCCGTACGACTACCTGGAGGACCCTGAARACCCACAACRAAGTCATTT *****	105
TcPOP	GTA CTGCGCAAACAATTTATTCACGAGTATATGACTTCCACAGCAGAGCTTCGTGAG	180
TcPOP+_pET32a	GTA CTGCGCAAACAATTTATTCACGAGTATATGACTTCCACAGMAGARCTTCGKGAG *****	165
TcPOP	AAAGTCTTTAATCGCATCACTCAGGTGATGAACTTCCCCGCACATCCAGTCCAAGCTTC	240
TcPOP+_pET32a	AAAGTCTTTAATCGCATCACTCAGGTGATGAACTTCCCCGACRTCCAGTCCAAGCTTC *****	225
TcPOP	CGCAACGGGCACTATTACTACTACTTTAACACCGGCTACAGAATCAAGACGTTTTAATG	300
TcPOP+_pET32a	CGCAACGGGCACTATTACTACTACTTTAACACCGGCTACAGAATCAAGACGTTTTAATG *****	285
TcPOP	CGCGCCACGTCCCTTACCGACAAGAACCCACCACATTCCTGGATCCCAACATTCTGAAC	360
TcPOP+_pET32a	CGCGCCACGTCCCTTACCGACAAGAACCCACCACATTCCTGGATCCCAACATTCTGAAC *****	345
TcPOP	AGCGATGGAACGAGCGCACTGAGGTCCACAGCGTGGAGCAAAGACGAATCGTTGTTTGCG	420
TcPOP+_pET32a	AGCGATGGAACGAGCGCACTGAGGKCCACAGCGTGGAGCAAAGACGAATCGTTGTTTGCG *****	405
TcPOP	TACAGCGTGAGCGATAAGGGAAGTGACTGGCAACACGTTACGTCGCGCGTCTGACACC	480
TcPOP+_pET32a	TACAGCGTGAGCGATAAGGGAAGTGACTGGCAACACGTTACGTCGCGCGTCTGACACC *****	465
TcPOP	TCCCAGGACCTACCGGATGTTGTTGAATGGGCGAAGTTCACGGGCATCGCATGGTGGCAC	540
TcPOP+_pET32a	TCCCARGACCTACCGGATGTTGTTGAATGGGCGAAGTTCACGGGCATCGCATGGTAGCAC *****	525
TcPOP	AGTGATGGATTTTTTTATACCCGCTACCCGACCTTGAAGGGGACGTGGATAAAGGGCGCT	600
TcPOP+_pET32a	AGTGATGGATTTTTTTATACCCGCTACCCGACCTTGAAGGGGACGTGGATAAARGGCGCT *****	585
TcPOP	GAGACGGACTGCAAAGGACCATTATATGTTTTTCATCGTCTTGGAACGAAGCAGGAG	660
TcPOP+_pET32a	GAGACGGACTGCAAAGGAYCWTTTTTRTATGTTTTTCATCGTCTTGGACGAAGCAGGAG *****	645
TcPOP	GAAGATGTTGTTGTGTTTTCCATGCCTGAGAACCATATTGGAGCCTCTCGGCTGAGGTG	720
TcPOP+_pET32a	GAAGATGTTGTTGTGTTTTCCATGCCTGAGAACCATATTGGAGCCTCTCGGCTGAGGTG *****	705

**Figure 2.23: Sequence alignment of recombinant *TcoPOP* in pET32a vector with non-recombinant *TcoPOP*.** Sequencing was performed using gene specific primers. Sequence highlighted in yellow shows the non-mutated form of *TcoPOP* gene. Sequence highlighted in red shows the guanine replaced with an adenine forming the stop codon “TAG” within the recombinant *TcoPOP* sequence. Sequences were aligned using Clustal Omega <http://www.ebi.ac.uk/Tools/msa/clustalo/> (accessed date: 20 October 2014).

It is clear from the sequencing results that the mutation occurred prior to ligation into the T-vector, probably during the gel purification steps as the DNA needs to be exposed to UV light during viewing and extraction from the agarose gel and UV light is known to damage DNA. Furthermore, the DNA polymerase used does not have 3'-5' exonuclease activity therefore errors or mutations occurring within the DNA sequence could not be corrected.

Sequencing of the *TcoPOP*/pGEM-T® Easy recombinant DNA was done while proceeding with sub-cloning into expression vector, but following analysis of the sequencing results, further work on *TcoPOP* was not pursued.

## 2.4. Discussion

Trypanosomiasis causes devastating losses of up to \$ 1.3 billion per annum for cattle farmers (Shaw, 2004) and many attempts to treat the disease have been futile. Attempts such as eliminating of the disease vector, the tsetse fly (*Glossina ssp.*) with insecticides (Bauer *et al*, 2011) or treatment of infected animals with chemotherapeutic drugs to treat the disease have not been very successful (Baker *et al*, 2013). This has led to the prospect of developing drugs which target certain pathogenic factors released by the parasite during infection. One of these pathogenic factors is prolyl oligopeptidase (POP) which is responsible for the hydrolysis of host factors such as hormones which leads to the manifestation of disease symptoms in animals (Bastos *et al*, 2010). Therefore further research needs to be conducted to understand the biochemical characteristics of this pathogenic factor in order to develop therapeutic drugs against it.

In this study, it was shown that *TbPOP* was successfully cloned into pTZ57R/T and pET32a vectors and successfully expressed, however, the protein was over expressed in the form of inclusion bodies. During protein over-expression in *E. coli*, the over-expressed protein accumulates to form insoluble aggregates known as inclusion bodies (Singh & Panda, 2005). Although formation of inclusion bodies can be advantageous due to high levels of expressed protein as well as low susceptibility of proteins to degradation and easy recovery of proteins (Raina *et al*, 2004; Singh & Panda, 2005), it can also be a disadvantage due to the fact that there can be poor recovery of active protein after treatment with denaturing agents during solubilisation and refolding (Singh & Panda, 2005). Furthermore, proteins expressed from inclusion bodies can be highly unstable and difficult to purify (Thapa *et al*, 2008). In this study, several experiments to solubilise *TbPOP* were conducted. As Bastos *et al* (2005) and Bastos *et al* (2010) demonstrated that expression at 16°C produced soluble POP from *T. cruzi* (*TcrPOP*) and *TbPOP* respectively, experiments to express soluble *TbPOP* in this study were conducted at lower temperatures of 16°C and 27°C in an attempt to produce soluble protein. However, the

attempts were futile as soluble protein was not obtained at any of these temperatures, even when cultures were incubated for extended periods of time.

There are several other methods to solubilise inclusion bodies, these include treatment with chaotropic agents such as guanidine hydrochloride and urea as well as detergents such as SDS (Lilie *et al*, 1998; Singh & Panda, 2005). In this study, as in the studies by Misawa and Kumagai (1999) and Natarajan *et al* (2005), the chaotropic agent, urea was used to solubilise *TbPOP* inclusion bodies. However, solubilisation with these denaturing agents results in the complete unfolding of the protein structure due to the break in inter and intra-chain interactions within the protein (Tsumoto *et al*, 2003), therefore, in order to return the protein to its native conformation, a process of refolding is required. There are several refolding methods, such as step-wise dialysis against decreasing concentrations of the denaturant, buffer exchange with desalting columns and by dilution of unfolded samples into refolding buffer (Tsumoto *et al*, 2003) to name a few. In this study, *TbPOP* was refolded using two methods, the first was by step-wise dialysis against decreasing concentrations of urea (Table 2.1.), the second by buffer exchange with a desalting column. After successful solubilisation and refolding by dialysis, affinity purification by Ni<sup>2+</sup> resin was conducted. The purification was partially successful as low molecular weight contaminants were still present after gradient elution with imidazole. Even after adjusting conditions such as lowering the pH of the elution buffer (Robichon *et al*, 2011) and increasing incubation periods of the protein on the resin were unsuccessful at decreasing contaminating proteins. The contaminants are thought to be certain *E. coli* proteins which also contain histidine residues on the surface of their tertiary structure rendering them with the ability to bind to the Ni<sup>2+</sup> resin as well (Robichon *et al*, 2011). Further purification of affinity purified *TbPOP* by gel filtration resulted in no separation between *TbPOP* and contaminants.

Since refolding by dialysis occurs for long periods of time and can lead to further aggregation of proteins (De Bernardez Clark, 1998), the refolding process was therefore switched to the use of a desalting column which is quicker and more efficient (De Bernardez Clark, 1998). This process led to the development a lower molecular weight protein at ~20 kDa. Since the size difference between *TbPOP* and the 20 kDa protein was so significant, it was speculated that purification by gel filtration would be successful at separating the two proteins, however, after the purification process, there was no separation, but rather the complete diffusion or disappearance of *TbPOP* and the 20 kDa

protein. Following yet another unsuccessful purification attempt with gel filtration, anion and cation exchange chromatography was conducted. Anion exchange chromatography led to the elution of only the 20 kDa protein and following cation exchange chromatography purification, only residual proteins were eluted without a trace of *TbPOP* or the 20 kDa protein. Western blot analysis done on the 20 kDa protein revealed that the protein was recognised by mouse anti-His tag antibodies. From these results, it was speculated that the protein could be a possible *TbPOP* degradation product or the Trx-His tag itself as its size is quite similar to that of the 20 kDa protein. Further studies can be conducted in the form of sequencing the protein in order to confirm its identity.

Other purification techniques were attempted, such as Three phase partitioning (TPP) as conducted by Raghava *et al* (2008) which resulted in all proteins precipitating at 10% ammonium sulfate (see appendix 7). Another method used was electro elution which was conducted according to (Weldingh *et al*, 2000). Following SDS-PAGE analysis of electro-elution, *TbPOP* produced what seemed to be degradation products as several other bands emerged (see appendix 8), this could be due to peptide chain cleavage during the electro elution process (Dunn, 1996). It is clear from the unsuccessful purification attempts of *TbPOP* that the purification of protein from inclusion bodies can be a significant challenge (Lilie *et al*, 1998), even the presence of the thioredoxin (Trx) affinity tag which is known to enhance the solubility of a fusion protein (Thapa *et al*, 2008) was unsuccessful at producing soluble protein (Owen *et al*, 2013).

Although the purification of *TbPOP* proved difficult, characterisation of partially Ni<sup>2+</sup> affinity purified *TbPOP* revealed some interesting information, it showed that *TbPOP* is able to hydrolyse collagen, which is surprising as enzymes which belong to the POP family of serine peptidases are known to only hydrolyse peptides of 30 amino acids or less (Bastos *et al*, 2013). In an experiment conducted by Bastos *et al* (2010), they obtained similar results where *TbPOP* showed collagenolytic activity. These results led to the conclusion that another of *TbPOP*'s biological functions during trypanosomosis infection is the hydrolysis of collagen (as it has an abundance of Gly-Pro residues) in order to allow easy traversal of trypanosome parasites through host tissue (Bastos *et al*, 2010). Since *TbPOP* is able to hydrolyse collagen, it was thought it would also be effective in hydrolysing gelatin, which is a denatured form of collagen (Tabata & Ikada, 1998), however, this was not the case, *TbPOP* was unable to hydrolyse gelatin. It was thought that the low pH of the assay buffer affected the lack of hydrolysis of gelatin by the oligopeptidases, however,

in a study conducted by Kangethe *et al* (2012), they also found that the oligopeptidases *TbOPB* and *TbPOP* did not hydrolyse gelatin.

*TcoPOP* was successfully cloned into the pGEM-T® cloning vector as well as the pET32a expression vector, however, upon expression of the protein, SDS-PAGE analysis showed that there was a premature termination of expression, resulting in the expression of an incomplete protein. Sequencing results of both *TcoPOP*/pGEM-T® and *TcoPOP*/pET32a clones revealed that a point mutation had occurred in a single codon within the *TcoPOP* DNA sequence. This mutation is referred to as a “non-sense” mutation, where a single nucleotide base is substituted with another, resulting in the formation of a premature stop codon within a DNA sequence (Brognia, 1999; Gatfield *et al*, 2003). This mutation leads to the expression of an incomplete protein (Urlaub *et al*, 1989). The example in Fig. 2.24 represents what occurred within the *TcoPOP* sequence.

<i>TcoPOP</i> DNA:	5' ATC GCA TGG <b>TGG</b> CAC 3'
Protein:	Ile Ala Trp Trp His
<i>TcoPOP</i> /pET32a DNA:	5' ATC GCA TGG <b>TAG</b> CAC 3'
Protein:	Ile Ala Trp Stop

**Figure 2.24: Representation of point mutation in *TcoPOP*/pET32a and *TcoPOP*/pGEM-T® recombinant DNA.** The guanine is replaced by an adenine which leads to a premature stop codon in the *TcoPOP* sequence. Decoding of codons into amino acids done using DNA codon table from [http://en.wikipedia.org/w/index.php?title=DNA\\_codon\\_table&oldid=576071692](http://en.wikipedia.org/w/index.php?title=DNA_codon_table&oldid=576071692). (Access date: 12 October 2014)

Since sequencing results revealed that further work with *TcoPOP* could not be pursued, future work would be to repeat the cloning and sub-cloning of the *TcoPOP* gene into a T-vector and an expression vector. Furthermore, more attempts to further purify *TbPOP* need to be pursued. POP has been shown to perform similar functions to another oligopeptidase, oligopeptidase B (OPB) during trypanosomosis pathogenesis. A study conducted by Kangethe *et al* (2012) showed through gene knock-out studies that when OPB was knocked-down, POP-like activity increased, indicating that the one compensates



for the other. This led to the conclusion that these oligopeptidases possibly work in conjunction during pathogenesis. This led to the study described in chapter 3 on OPB, which aims to characterise OPB and determine its biochemical characteristics and compare them to POP.

# CHAPTER 3

## Recombinant expression, purification and enzymatic characterisation of Oligopeptidase B from *Trypanosoma congolense*

### 3.1. Introduction

During trypanosome infection, there are various pathogenic factors which are released into the bloodstream of infected animals when the trypanosomes lyse. These pathogenic factors are trypanosomal peptidases such as the cysteine peptidase *TbCATL* (trypanopain) which is released by *Trypanosoma b. brucei* parasites (Troeborg *et al*, 1996), *TcrCATL* (cruzipain) released by *Trypanosoma cruzi* parasites (Mottram *et al*, 2003; Caffrey *et al*, 2011), tropolysin, a metallopeptidase released by *T. b. brucei* parasites (Morty *et al*, 2005c) and oligopeptidase B which is released by *T. congolense* and *T. b. brucei* parasites (Troeborg *et al*, 1996; Morty *et al*, 1999a; Coetzer *et al*, 2008). The focus of this chapter will be on the pathogenic factor oligopeptidase B (OPB), which belongs to the prolyl oligopeptidase family (Clan SC) of serine proteases (Morty *et al*, 2005b). OPB displays trypsin-like activity by hydrolysing substrates at the carboxy-terminal side of the basic amino acids arginine and lysine (Morty *et al*, 2001). During infection, it hydrolyses peptide hormones such as atrial natriuretic factor (Morty *et al*, 1999b; Morty *et al*, 2005b) and this renders blood vessels weak and leads to uncontrolled bleeding during infection (Bastos *et al*, 2013).

Treatment of trypanosomiasis by chemotherapeutic drugs such as diminazene and suramin, which are thought to block essential processes vital for parasite survival or block important enzyme metabolic pathways (Al-Mohammed, 2008) have only been partially successful due to animal drug resistance (Chitanga *et al*, 2011). The targets of the drugs are largely unclear, but in some instances have been identified, such as OPB from *T. b. brucei* by Morty *et al* (1998) who found that the enzyme was inhibited by the drugs pentamidine as well as suramin. These findings strongly suggest that trypanosomal peptidases can be possible drug targets (Morty *et al*, 1998; de Matos Guedes *et al*, 2007). Serine protease inhibitors can be further researched in order to undergo further development into chemotherapeutic drugs which can specifically target trypanosomal peptidases and hopefully stop the manifestation of disease symptoms. Effective chemotherapy is critically dependent on accurate diagnosis of trypanosomiasis. Several

immunodiagnostic methods have been developed with variable sensitivity and specificity. The card agglutination test for trypanosomosis (CATT) detects antibodies against the variant surface coat antigens on the surface of trypanosome parasites (Nantulya, 1990). The antibody detection test by indirect ELISA is also a good method for diagnosis of trypanosomosis, it is conducted by coating the ELISA plate with the antigen, after which the infected sera is added and finally, an enzyme-labelled species-specific antibody is added and the reaction developed with a chromogenic substrate. This method is preferred over microscopy because it is highly specific, sensitive and saves time as it is able to test a large number of samples (Pillay *et al*, 2013). Davison *et al* (1999) used the indirect ELISA method for the detection of antibodies in *T. evansi* infected buffalo sera, which they compared to the CATT test and found that the ELISA showed high sensitivity and the CATT method, high specificity.

It has recently been shown that the use of recombinantly expressed and purified antigens to screen for antibodies increases the sensitivity of the ELISA, this was shown by Pillay *et al* (2013) who recombinantly expressed and purified the *T. vivax* GM6 antigen which was successfully used for the diagnosis of *T. vivax* and *T. congolense* in cattle. An alternative to the antibody detection test, is the antigen detection test where species-specific monoclonal antibodies against an antigen are used to screen for a specific antigen in infected sera by sandwich ELISA.

OPB from *Trypanosoma congolense* (*TcoOPB*) will be the main focus of the research reported in this chapter. The aim is to determine the enzyme's biochemical characteristics and properties and compare them to those of *TbPOP* and *TcoPOP* in order to understand the possible role it plays in the bloodstream of infected animals. Therefore the objective of this part of the study was to recombinantly express and purify *TcoOPB*, enzymatically characterise *TcoOPB* and use the peptidase as an antigen in an indirect ELISA to test for antibodies in sera from *T. congolense* infected cattle.

## 3.2. Materials and methods

### 3.2.1 Materials

**TcoOPB purification and quantification:** His-select<sup>®</sup> nickel affinity column and imidazole were purchased from Sigma Aldrich (St. Louis, MO, USA). The BCA<sup>™</sup> Protein Assay Kit was from Pierce (Rockford, IL, USA) and Nunc-Immuno<sup>™</sup> Maxisorp ELISA plates were purchased from Thermo Fisher Scientific (Roskilde, Denmark).

**Western blot analysis:** Chicken anti-TcoOPB antibodies were previously produced in our laboratory. PageRuler<sup>™</sup> Prestained Protein Ladder purchased from Thermo Scientific. Secondary antibody rabbit anti-chicken-horseradish peroxidase conjugate purchased from Sigma (St. Louis, MO, USA). Pierce<sup>™</sup> Enhanced Chemiluminescence (ECL) western blotting substrate purchased from Thermo Fisher Scientific (Roskilde, Denmark).

**Enzyme assays:** The peptide substrates (Z-benzyloxycarbonyl)-Arg-Arg-7-amino-4-methylcoumarin, Z-Gly-Gly-amino-4-methylcoumarin (AMC), H-Pro-Phe-Arg-AMC, Z-Pro-Arg-AMC, MeoSuc-Asp-Tyr-Met-AMC, H-Gly-AMC, Suc-Ala-Phe-Lys-AMC, H-Ala-Phe-Lys-AMC, H-D-Val-Leu-Lys-AMC and (N-benzyl chloroformate)-glycyl-L-proline-AMC were all purchased from Bachem (Bubendorf, Switzerland). Boc-Leu-Gly-Arg-AMC was purchased from Novabiochem (Nottingham UK). The inhibitors antipain, leupeptin, chymostatin, aminopeptidase, bestatin, 1-10 phenanthroline, TLCK, TPCK, AEBSF, E-64 and Iodoacetic acid were purchased from Sigma (St. Louis, MO, USA). Nunc Black 96-well plates were purchased from Thermo Fisher Scientific (Roskilde, Denmark).

**Indirect ELISA:** Sera from infected and non-infected cattle obtained from ClinVet International (PTY) LTD (Bloemfontein, South Africa). Rabbit anti-bovine IgG-HRPO (A8917) conjugate purchased from Sigma Aldrich (St. Louis, MO, USA). Bovine serum albumin (BSA) purchased from Sigma Aldrich (St. Louis, MO, USA).

### 3.2.2 Methods

#### 3.2.2.1 Recombinant expression of TcoOPB

A construct of TcoOPB which was previously cloned (Kangethe *et al*, 2012) was used to express TcoOPB. Briefly, a single colony of recombinant *E. coli* BL21 DE3 cells containing the full length TcoOPB gene in the pET28a expression vector was used to inoculate

terrific broth [24 g/l yeast extract, 0.4% (v/v) glycerol, 12 g/l tryptone, 0.17 M  $\text{KH}_2\text{PO}_4$  and 0.72 M  $\text{K}_2\text{HPO}_4$ ; 500 ml] containing 34  $\mu\text{g/ml}$  kanamycin and grown overnight at 37°C. Cells were harvested by centrifugation (2000 *g*, 10 min, 4°C), the supernatant was discarded and the pellet re-suspended in PBS-T lysis buffer and incubated at 37°C for 30 min, after which the suspension was frozen at -20°C for 30 min. Frozen samples were then thawed and sonicated 4 times for 10 s for each sonication. The samples were once again centrifuged (10 000 *g*, 10 min, 4°C). The supernatant and pellets were analysed by 10% SDS-PAGE (see section 2.2.2.9).

### **3.2.2.2 Affinity purification of *TcoOPB***

His Select<sup>®</sup> nickel affinity resin (1 ml) was placed in a 10 ml chromatography column, washed with 5 column volumes of distilled water and equilibrated with 10 column volumes equilibration buffer [50 mM  $\text{NaH}_2\text{PO}_4$ , 0.5 M NaCl, 10 mM imidazole, pH 6.8]. Soluble *TcoOPB* lysate (10 ml) was added to the resin and incubated overnight at 4°C while mixing using an end-over-end mixer. The unbound fraction was collected, after which the resin was washed with equilibration buffer until an  $A_{280}$  of ~0.02 was obtained. The bound fraction was eluted using 5 ml elution buffer [50 mM  $\text{NaH}_2\text{PO}_4$ , 0.5 M, pH 6.8] containing increasing concentrations of imidazole 50 mM – 150 mM in a step-gradient and 1 ml fractions collected. The nickel resin was regenerated with 2 column volumes distilled water, 5 column volumes of 6 M guanidine-HCl, another wash with 3 column volumes distilled water and finally, another 5 column volumes wash with equilibration buffer before storage in 30% (v/v) ethanol at 4°C. All fractions were analysed on 12.5% SDS-PAGE for efficiency of the purification.

### **3.2.2.3 Western blot analysis of *TcoOPB***

Proteins resolved by SDS-PAGE were transferred onto nitrocellulose membranes using a semi-dry blotter, 20 V, 50 min in blotting buffer [45 mM Tris, 173 mM glycine, 18% (v/v) methanol and 0.1% (w/v) SDS]. Following blotting, the nitrocellulose was stained with Ponceau S [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] to confirm if protein bands were successfully transferred. The membrane was destained with distilled water containing 3 drops of NaOH. The membrane was then rinsed with TBS [20 mM Tris-HCl buffer, pH 7.4, 200mM NaCl] then blocked with low fat milk [5% (w/v) low fat milk in TBS] for 1 h. The membrane was then washed with TBS (3 x 5 min) and incubated in anti-*TcoOPB* primary antibody. Following overnight incubation, the nitrocellulose was

incubated in rabbit anti-chicken IgY-horseradish peroxidase conjugate secondary antibody for 1 h at 4°C. The membrane was then washed once again with TBS (3 x 5 min) and incubated with Pierce™ ECL western blotting substrate solution as per manufacturer's instructions, the chemi-luminescent bands were visualised using the Syngene G:Box Chemi XR5 system from Vacutec.

#### **3.2.2.4 Protein quantification**

As per section 2.2.2.10

#### **3.2.2.5 Enzymatic characterisation of *TcoOPB***

##### *Peptidolytic activity of *TcoOPB**

Purified *TcoOPB* was tested for activity by incubation in *TcoOPB* assay buffer [200 mM Tris-HCl, 10 mM DTT, 0.02% (w/v) NaN<sub>3</sub>, pH 8.0] in increasing concentrations 0.0005 – 0.5 µg/ml [diluted in 0.1% (w/v) Brij-35] for 10 min at 37°C. The substrate Z-Arg-Arg-AMC (20 µM) was then added and the hydrolysis measured at excitation 360 nm; emission 460 nm (Ex<sub>360 nm</sub>; Em<sub>460 nm</sub>) using the FLUOStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany).

##### *Inhibition of *TcoOPB* using class-specific protease inhibitors*

The determination of *TcoOPB* class was conducted using various protease inhibitors TPCK (10 µM), E-64 (10 µM), chymostatin (10 x 10<sup>3</sup> µM), EDTA (1 x 10<sup>3</sup> µM), cystatin (1 µM), 1-10 phenanthroline (10 µM), bestatin (10 µM), leupeptin (10 µM) and antipain (10 µM), the inhibitor concentrations were prepared according to (Beynon & Bond, 1989). Briefly, *TcoOPB* [0.5 µg diluted in 0.1% (w/v) Brij-35] was incubated with the inhibitors [diluted in 0.1% (w/v) Brij-35] and *TcoOPB* assay buffer for 15 min at 37°C. Hydrolysis of Z-Arg-Arg-AMC (20 µM) was then measured at excitation 360 nm; emission 460 nm (Ex<sub>360 nm</sub>; Em<sub>460 nm</sub>) using the FLUOStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany). The residual activity was then calculated as the % relative activity.

##### *Active site titration with tight-binding inhibitor antipain*

The active concentration of *TcoOPB* was determined using the tight-binding reversible inhibitor which has been used for co-crystallisation, antipain. Briefly, *TcoOPB* [1 µM

diluted in 0.1% (w/v) Brij-35] was incubated with antipain [0 – 1  $\mu$ M diluted in 0.1% (w/v) Brij-35] in *TcoOPB* assay buffer for 30 min at 37°C. Hydrolysis of Z-Arg-Arg-AMC (20  $\mu$ M) was measured (EX<sub>360 nm</sub>; Em<sub>460 nm</sub>). The active concentration of *TcoOPB* was determined by plotting fluorescence against antipain concentrations.

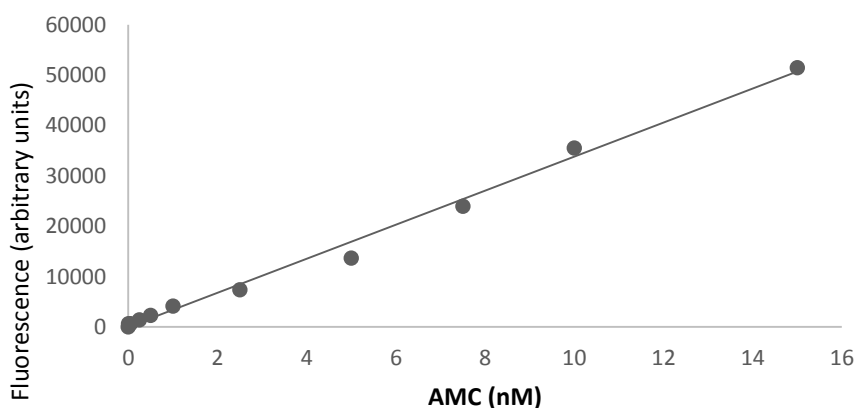
#### *Effect of pH on TcoOPB activity*

The pH optimum of *TcoOPB* was determined using the constant ionic strength AMT buffer [100 mM acetic acid, 100 mM MES, 200 mM Tris-HCl], titrated from pH 4.0 to pH 11 in 1.0 pH unit intervals with NaOH or HCl (Ellis & Morrison, 1981). *TcoOPB* [0.5  $\mu$ g diluted in 0.1% (w/v) Brij-35] was incubated with each buffer for 10 min at 37°C. The substrate Z-Arg-Arg-AMC (20  $\mu$ M) was added to each of the solutions and the fluorescence measured (EX<sub>360 nm</sub>; Em<sub>460 nm</sub>).

### 3.2.2.6 Determination of kinetic constants

#### *Construction of AMC standard curve*

During hydrolysis of synthetic peptides, the fluorescence released was quantified using the AMC standard curve (Fig. 3.1). AMC dilutions (0.5 – 15 nM) were prepared in *TcoOPB* assay buffer and incubated at 37°C for 5 min. The fluorescence was then measured (EX<sub>360 nm</sub>; Em<sub>460 nm</sub>).



**Figure 3.1: AMC standard curve showing an increase in AMC concentration results in an increase in fluorescence (Ex<sub>360 nm</sub>; Em<sub>460 nm</sub>).** The equation of the trendline is  $y = 3378x + 29.59$ , with a correlation coefficient of 0.9938.

### *Substrate specificity*

Substrate specificity of *TcoOPB* was determined by incubation of the enzyme [1.5 ng diluted in 0.1% (w/v) Brij-35] (as determined by antipain active site titration) with assay buffer for 10 min at 37°C, after which, various substrates (Z-Gly-Gly-Arg-AMC, Z-Arg-Arg-AMC, H-Ala-Phe-Lys-AMC, Z-Pro-Arg-AMC, H-D-Val-Leu-Lys-AMC, Suc-Ala-Phe-Lys-AMC, Boc-Leu-Gly-Arg-AMC, H-Gly-AMC, Z-Gly-Pro-AMC, MeoSuc-Asp-Tyr-Met-AMC and Cbz-glycyl-L-proline-AMC) each at concentrations ranging from 7.5 – 50  $\mu$ M were added. Fluorescence was measured ( $Ex_{360\text{ nm}}$ ;  $Em_{460\text{ nm}}$ ) continuously in order to determine the initial steady-state velocity ( $v_0$ ) of *TcoOPB*. The kinetic constants  $K_m$  and  $V_{max}$  were determined using the Hyper32<sup>®</sup> software. The  $k_{cat}$  was determined using the formula  $k_{cat} = V_{max}/[E]_0$ , where  $[E]_0$  represents the active enzyme concentration.

### *Reversible inhibition of TcoOPB*

For the determination of the inhibition kinetic constant ( $K_i$ ) according to Salvesen and Nagase (1989), the uninhibited rate of Z-Arg-Arg-AMC substrate hydrolysis ( $v_0$ ) was first determined by incubating *TcoOPB* [1.5 ng diluted in 0.1% (w/v) Brij-35] in *TcoOPB* assay buffer for 15 min at 37°C. After which the substrate Z-Arg-Arg-AMC (20  $\mu$ M) was added and the fluorescence measured ( $Ex_{360\text{ nm}}$ ;  $Em_{460\text{ nm}}$ ) for 5 min. The reversible inhibitors (antipain, leupeptin, chymostatin, bestatin and 1-10 phenanthroline) were then added (2.5 – 40 nM) and a new steady-state velocity determined in the presence of inhibitor ( $v_i$ ). Following the addition of the inhibitor, the apparent inhibition constant in the presence of substrate [ $K_{i(app)}$ ] was determined from the formula  $v_0/v_i = 1 + [I]/K_{i(app)}$ . The graph of  $v_0/v_i$  against  $[I]$  is derived from the equation, where  $[I]/K_{i(app)}$  is the slope. When calculating the true inhibition constant ( $K_i$ ) for competitive reversible inhibitors, the presence of substrate is accounted for and is derived from the formula,  $K_i = K_{i(app)}/1 + [S]/K_m$ .

### *Irreversible inhibition of TcoOPB*

The effect of irreversible inhibitors (TPCK, TLCK, AEBSF, E-64 and iodoacetic acid) on *TcoOPB* was determined as described by Salvesen and Nagase (1989). *TcoOPB* [1.5 ng diluted in 0.1% (w/v) Brij-35] was incubated in assay buffer at 37°C for 1 h along with inhibitor (28 nM). Aliquots (75  $\mu$ l) were removed at 5 min intervals and residual activity ( $v_t$ ) measured against Z-Arg-Arg-AMC (20  $\mu$ M). The experiment was conducted under



pseudo-first order conditions where the inhibitor is combined in large excess of the enzyme (Salvesen & Nagase, 1989). The pseudo-first order rate constant ( $-k_{obs}$ ) is the slope from the plot  $\ln v_t/v_0$  against time where  $v_0$  is the enzyme activity prior to the addition of inhibitor. Under second-order conditions, the apparent second-order rate constant ( $k_a$ ) is determined from the relationship  $k_a = k_{obs}/[I]$ , where  $[I]$  is the inhibitor concentration. In the presence of an irreversible inhibitor, the time required for the free enzyme concentration to decrease by 50% (half-life,  $t_{1/2}$ ) is given by  $t_{1/2} = 0.693/k_a[I]$ .

### **3.2.2.7 Indirect ELISA for the detection of antibodies in bovine sera infected with *Trypanosoma congolense* parasites**

An indirect enzyme-linked immunosorbent assay (ELISA) was conducted on various infected and non-infected cattle sera in order to detect the presence of antibodies. The sera used were 201 (+7), 201 (+19). Sera, 186 (-7) and 53 (-28) served as the negative controls and 48 (+98) served as the positive control. In order to optimise the antigen coating concentration, TcoOPB at concentrations of 5  $\mu\text{g/ml}$ , 2.5  $\mu\text{g/ml}$  and 1  $\mu\text{g/ml}$  (100  $\mu\text{l/well}$ ), diluted in PBS [NaCl (8 g/l), KCl (0.2 g/l),  $\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$  (1.15 g/l),  $\text{KH}_2\text{PO}_4$  (0.2 g/l), pH 7.4] was used to coat the wells of 96 well Nunc-Immuno™ Maxisorp ELISA plates for 16 h at 4°C. Non-coated wells which were left empty were included and served as the no coat controls. The coating solution was removed and the plate patted dry on tissue to remove any residual coating solution. Blocking solution BSA-PBS/T [0.5% (w/v) BSA in PBS, 0.1% Tween-20] (300  $\mu\text{l/well}$ ) was added to each well and incubated at 37°C for 1 h to prevent non-specific binding. Sera (1 in 100 dilution in PBS, 100  $\mu\text{l/well}$ ) were added to each well and incubated for 2 h at 37°C. The plate was then washed 3 times with PBS-Tween 20 [0.1% (v/v) Tween-20 in PBS] using the BIOTEK® ELx50™ Microplate washer from BioTek Instruments Inc. (USA) after which the rabbit anti-bovine IgG HRPO conjugate (1 in 10 000 dilution in blocking solution; 100  $\mu\text{l/well}$ ) was added to each well and incubated for 1 h at 37°C.

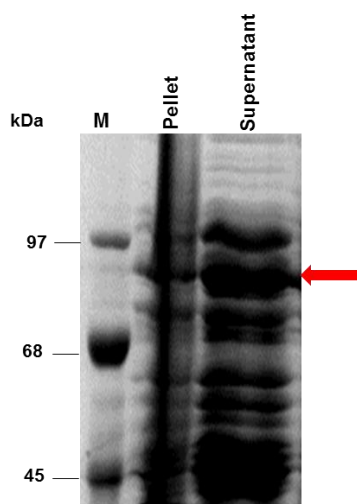
The plate was washed once again and the ABTS- $\text{H}_2\text{O}_2$  chromogen substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v)  $\text{H}_2\text{O}_2$  in citrate buffer, pH 5.0] (100  $\mu\text{l/well}$ ) added to each well. The plate was incubated in the dark for 10 min prior to measuring the absorbance at 405 nm in 10 min intervals for 50 min using the FLUOStar Optima

spectrophotometer (BMG Labtech, Offenburg, Germany). After obtaining the optimum antigen coating concentration, further experiments using indirect ELISAs were conducted on other infected and non-infected sera to test for the presence of antibodies. This time a no serum control was included.

### 3.3. Results

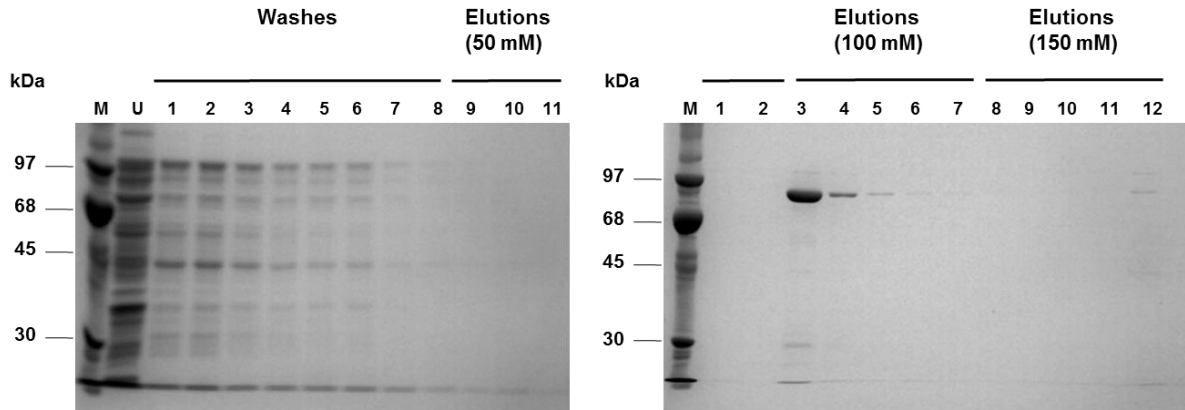
#### 3.3.1 Recombinant expression and purification of *TcoOPB*

Expression of *TcoOPB* in BL21 DE3 *E. coli* expression system was successful as seen by the presence of the recombinant protein band at approximately 80 kDa (Fig. 3.2). The solubility test showed that *TcoOPB* was expressed in its soluble state as the protein was found in the lysate supernatant.



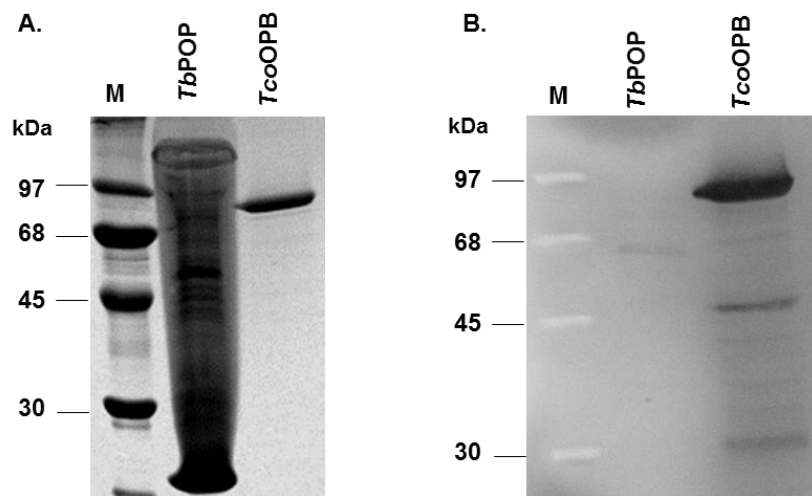
**Figure 3.2: Reducing SDS-PAGE (10%) of *TcoOPB* expression.** M, Molecular weight marker. *TcoOPB* was successfully expressed in its soluble state in the supernatant. Gel stained with Coomassie blue.

Following expression, soluble *TcoOPB* was purified by affinity purification on a Ni<sup>2+</sup> affinity resin. The protein was eluted using a step-wise gradient of increasing concentration of imidazole and was eluted at 100 mM. The purification was successful as shown by the presence of a single band with no contaminants at approximately 80 kDa (Fig. 3.3, lane 3).



**Figure 3.3: Reducing SDS-PAGE (12.5%) of *TcoOPB* following  $\text{Ni}^{2+}$  affinity purification. M, Molecular weight marker. U: Unbound fraction. *TcoOPB* was successfully eluted at 100 mM Imidazole. Gel stained with Coomassie blue.**

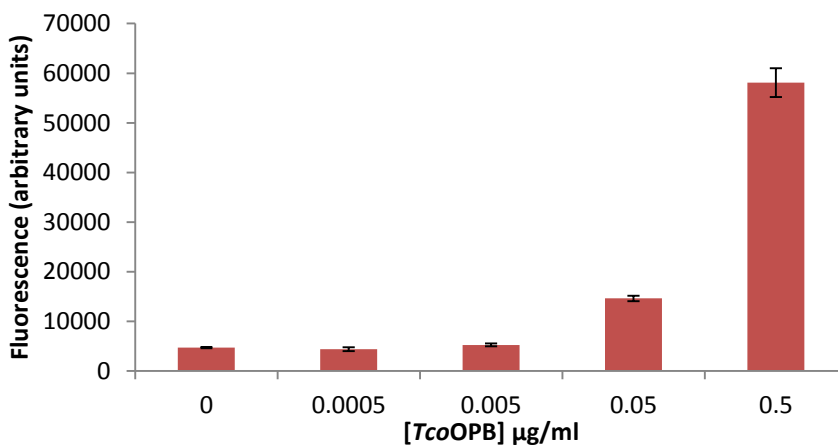
Western blot analysis of *TcoOPB* was conducted and chicken the anti-*TcoOPB* antibodies were also to tested for cross reactivity with *TbPOP* as they are both oligopeptidases and share approximately 25% amino acid sequence identity with each other (Rea & Fülöp, 2006). As shown in Fig. 3.4, panel B, there was no cross reactivity as *TbPOP* was not recognised by the *TcoOPB* antibodies, this can be due to the low identity of the enzymes to each other. The antibodies did however, successfully recognise *TcoOPB* as expected.



**Figure 3.4: Reducing SDS-PAGE (12.5%) and western blot analysis of *TcoOPB* and *TbPOP*. M, Molecular weight marker. A: Reference gel showing *TbPOP* and *TcoOPB*. Gel stained with Coomassie blue. B: Western blot of *TbPOP* and *TcoOPB*, the blot was probed with 10  $\mu\text{g}/\text{ml}$  chicken anti-*TcoOPB* followed by rabbit anti-chicken-HRPO conjugate (1:5000). Blot visualised with ECL chemiluminescence substrate.**

### 3.3.2 Enzymatic characterisation of *TcoOPB*

Following successful purification of *TcoOPB*, an assay was conducted to test for activity against the peptide substrate Z-Arg-Arg-AMC, the preferred substrate (Morty *et al*, 2002). Fig. 3.5 shows that there was a direct relationship between enzyme concentration and substrate hydrolysis. At low enzyme concentration, there is little substrate hydrolysis, however, at higher enzyme concentration, there is an increase in hydrolysis, this shows that *TcoOPB* was active.



**Figure 3.5: Hydrolysis of Z-Arg-Arg-AMC by increasing concentrations of *TcoOPB* (0.5 ng to 0.5 µg).** *TcoOPB* was assayed against 20 µM Z-Arg-Arg-AMC. Fluorescence measured at  $E_{x360\text{ nm}}$ ;  $E_{m460\text{ nm}}$ . Each data point represents the mean activity  $\pm$  SD ( $n = 3$ ).

The effect of class specific protease inhibitors on Z-Arg-Arg-AMC hydrolysing activity of *TcoOPB* was determined and expressed as % relative activity. Table 1 shows that antipain and leupeptin were the most effective inhibitors of *TcoOPB* activity resulting in only 6.44% and 7.11% relative activity respectively. This reinforces the fact that *TcoOPB* is a serine protease as it was effectively inhibited by serine protease inhibitors. In contrast however, *TcoOPB* activity was weakly inhibited by TPCK and chymostatin which are also serine protease inhibitors, this result was not expected. Furthermore, the inhibitor E-64, which is a cysteine protease inhibitor, did not inhibit *TcoOPB* activity, however the enzyme was sensitive to cystatin which is also a cysteine protease inhibitor, suggesting the presence of a cysteine residue near the active site which assists in substrate hydrolysis (Morty *et al*, 2005a). Unexpectedly, *TcoOPB* activity was weakly inhibited by EDTA and 1-

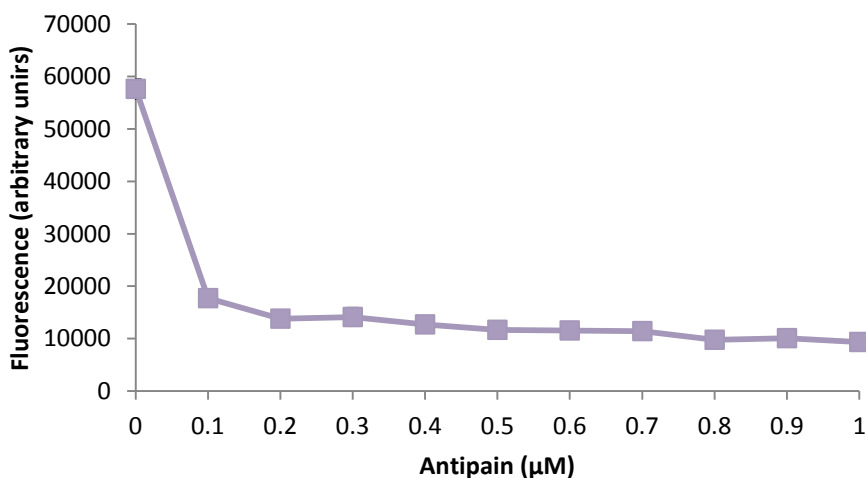
10 phenanthroline which are metalloprotease inhibitors, bestatin, an aminopeptidase inhibitor and cystatin.

**Table 3.1: Inhibition of *TcoOPB* activity using class specific protease inhibitors**

Inhibitor	Concentration ( $\mu\text{M}$ )	% Relative Activity
No inhibitor		100
TPCK	10	99.7
E-64	10	91.2
Chymostatin	$1 \times 10^4$	83.3
EDTA	$1 \times 10^3$	36.7
Cystatin	1	38.1
1-10 Phenanthroline	10	33.2
Bestatin	10	25.6
Leupeptin	10	7.11
Antipain	10	6.44

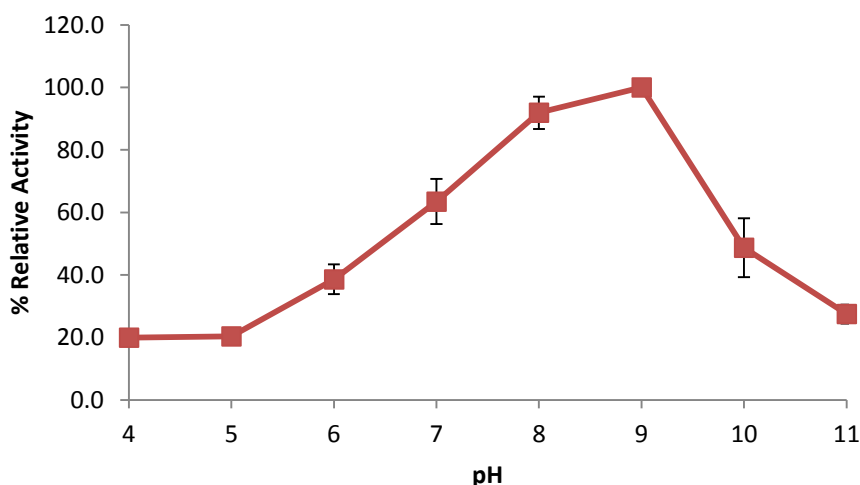
Assays performed by incubation of  $0.5 \mu\text{g}$  *TcoOPB* active enzyme with inhibitor for 15 min before the addition of  $20 \mu\text{M}$  Z-Arg-Arg-AMC.

Since antipain proved to be the most effective inhibitor of *TcoOPB*, an active site titration to determine the active concentration of *TcoOPB* was conducted. The enzyme was incubated with increasing concentrations of antipain and the residual activity measured using the preferred substrate Z-Arg-Arg-AMC. Fig. 3.6 shows that *TcoOPB* was only 10% active.



**Figure 3.6: Active site titration of *TcoOPB*.** A constant concentration ( $1 \mu\text{M}$ ) of *TcoOPB* was incubated with increasing concentrations of antipain ( $0 - 1 \mu\text{M}$ ) for 15 min at  $37^\circ\text{C}$ . Hydrolysis of Z-Arg-Arg-AMC was measured. Each data point represents the mean activity  $\pm$  SD ( $n = 3$ ).

The effect of pH on *TcoOPB* was determined using the constant ionic strength AMT buffer with pH ranging from 4 – 11. The hydrolysis of Z-Arg-Arg-AMC was measured and Fig. 3.7 shows that *TcoOPB* has high rate of hydrolysis at pH ranging from pH 8 – 9 which are alkaline conditions and retained 79% of maximal activity at physiological pH, 7.4.

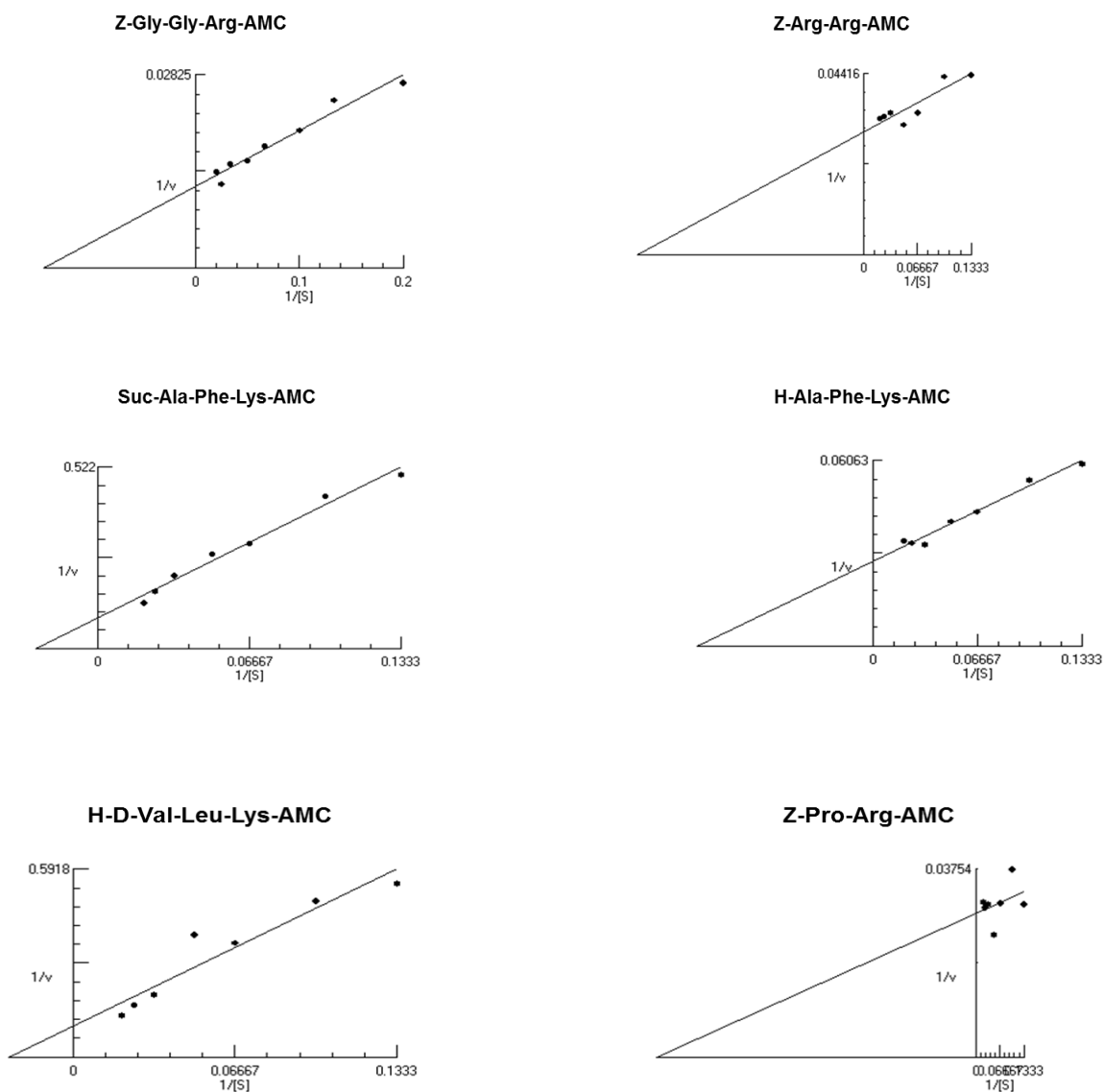


**Figure 3.7:** *TcoOPB* pH profile constructed using the constant ionic strength AMT buffer over a pH range of 4 - 11. Hydrolysis of Z-Arg-Arg-AMC was measured. Each data point represents the mean activity  $\pm$  SD ( $n = 3$ ).

### 3.3.3 Determination of kinetic parameters

The hydrolysis of various fluorogenic substrates by *TcoOPB* were represented in the form of Lineweaver-Burk plots (Fig. 3.8) and the kinetic parameters ( $K_m$  and  $k_{cat}$ ) tabulated in Table 3.2. *TcoOPB* had high  $k_{cat}/K_m$  values for Z-Gly-Gly-Arg-AMC, Z-Arg-Arg-AMC and Z-Pro-Arg-AMC. These results are consistent with the fact that *TcoOPB* has a preference for substrates with the basic residue Arg at  $P_1$ . *TcoOPB* showed the highest  $k_{cat}/K_m$  value for Z-Pro-Arg-AMC indicating that the enzyme has a higher preference for Pro over Gly and Arg at  $P_2$ . It also shows that the enzyme has a preference for hydrophobic residues such as Pro at  $P_2$ . The lack of hydrolysis of H-Pro-Phe-Arg-AMC, however, shows that although the enzyme accepts hydrophobic residues at  $P_2$ , it does show less hydrolysis when there are hydrophobic residues at both  $P_2$  and  $P_3$  positions. *TcoOPB* also has a preference for Lys at  $P_1$ , however, it did not hydrolyse the substrates H-Ala-Phe-Lys-AMC,

H-D-Val-Leu-Lys-AMC and Suc-Ala-Phe-Lys-AMC efficiently, this confirms that *TcoOPB* has a higher preference for Arg at P<sub>1</sub> than Lys as previously stated by Morty *et al* (1999a) and Coetzer *et al* (2008). As expected, there was no hydrolysis of H-Gly-AMC which is an aminopeptidase substrate and likewise for MeoSuc-Asp-Tyr-Met-AMC, a metalloprotease substrate as well as Z-Gly-Pro-AMC and N-Cbz-glycyl-L-proline-AMC which are both prolyl oligopeptidase substrates.



**Figure 3.8: Lineweaver-Burk plots of various substrates hydrolysed by *TcoOPB*.** Enzyme was incubated with substrates and the fluorescence measured. Y-axis ( $s^{-1}$ ), x-axis ( $\mu M^{-1}$ ). The plots were derived from the Hyper32<sup>®</sup> software.

**Table 3.2: Kinetic parameters for the hydrolysis of fluorogenic substrates by recombinant *TcoOPB*<sup>a</sup>**

Substrate	$K_m$ ( $\mu\text{M}$ )	$k_{\text{cat}}$ ( $\text{s}^{-1}$ )	$k_{\text{cat}}/K_m$ ( $\text{s}^{-1}\mu\text{M}^{-1}$ )
Z-Gly-Gly-Arg-AMC	7.74	99.8	12.8
Z-Arg-Arg-AMC	3.16	37.6	11.8
H-Ala-Phe-Lys-AMC	8.78	41.3	4.70
Z-Pro-Arg-AMC	1.0	40.0	40.0
H-D-Val-Leu-Lys-AMC	224	47.2	0.21
Suc-Ala-Phe-Lys-AMC	74.2	20.7	0.28

<sup>a</sup>*TcoOPB* was incubated in assay buffer at 37°C.  $K_m$  values and Lineweaver-Burk plots were calculated using the Hyper32<sup>®</sup> software. No activity was detected against Boc-Leu-Gly-Arg-AMC, H-Gly-AMC, H-Pro-Phe-Arg-AMC, MeoSuc-Asp-Tyr-Met-AMC, Z-Gly-Pro-AMC and Cbz-Cly-L-Pro-AMC

The inhibition of *TcoOPB* by competitive, reversible inhibitors is shown in Table 3.3. *TcoOPB* was strongly inhibited by antipain followed by leupeptin which are both serine protease inhibitors, however, antipain proved to be the competitively stronger inhibitor as it showed the lower  $K_i$  compared to leupeptin. Chymostatin, which is both a serine and cysteine protease inhibitor proved to be the weakest inhibitor of *TcoOPB*. No inhibition was seen with bestatin as expected as it is an aminopeptidase inhibitor, the same was observed with 1-10 phenantroline, a metalloprotease inhibitor. These results once again prove that *TcoOPB* is a serine protease as it is strongly inhibited by the serine protease inhibitors. The data also shows that it is slightly inhibited by cysteine protease inhibitors that suggests that it is dependent on a cysteine residue at, or near the active site during substrate hydrolysis.

**Table 3.3: Inhibition of *TcoOPB*<sup>a</sup> by competitive reversible inhibitors**

Inhibitor	$K_i$ (mM) ( $\times 10^3$ )
Antipain	7.6
Leupeptin	9.23
Chymostatin	20

<sup>a</sup>*TcoOPB* was incubated with inhibitors in assay buffer at 37°C. No inhibition was observed with Bestatin and 1-10 Phenanthroline



The inhibition of *TcoOPB* by irreversible inhibitors is shown in Table 3.4. AEBSF and TPCK which are serine protease inhibitors were the most effective inhibitors of *TcoOPB* as they showed similar high  $k_a$  values of  $71.43 \text{ mM}^{-1}\text{s}^{-1}$  and  $71.42 \text{ mM}^{-1}\text{s}^{-1}$  respectively. TLCK on the other hand, which is also a serine protease inhibitor showed the weakest inhibition of *TcoOPB*. The cysteine protease inhibitors, E-64 and Iodoacetic acid did not inhibit the enzyme.

**Table 3.4: Inhibition of *TcoOPB*<sup>a</sup> by irreversible inhibitors**

Inhibitors <sup>b</sup>	$k_a$ ( $\text{mM}^{-1}\text{s}^{-1}$ )	$t_{1/2}$ (s)
TLCK	$32.14 \pm 0.192$	773
TPCK	$71.42 \pm 1.20$	348
AEBSF	$71.43 \pm 4.67$	348

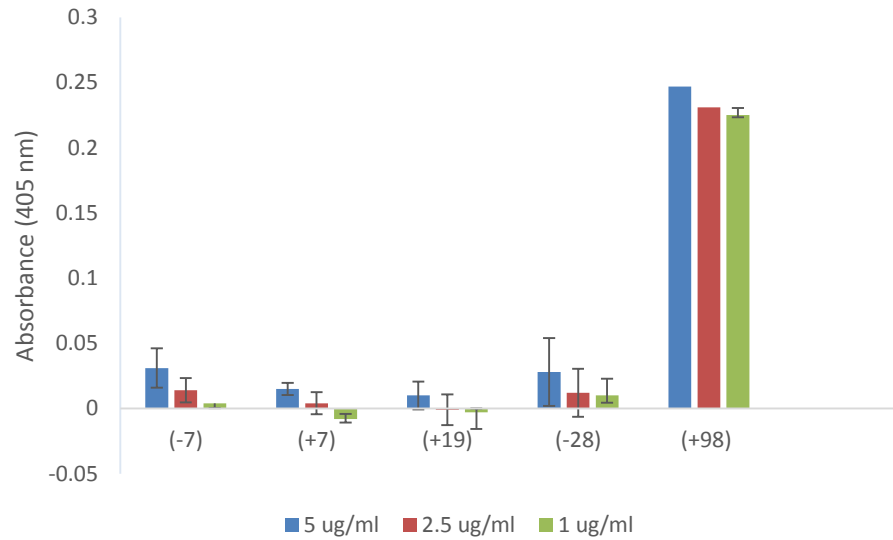
<sup>a</sup> *TcoOPB* was incubated with inhibitors in assay buffer at 37°C. No inhibition was observed with E-64 and Iodoacetic acid.

<sup>b</sup> 28 nM inhibitor used

Data reflect the mean  $k_a \pm \text{SD}$  (n = 3)

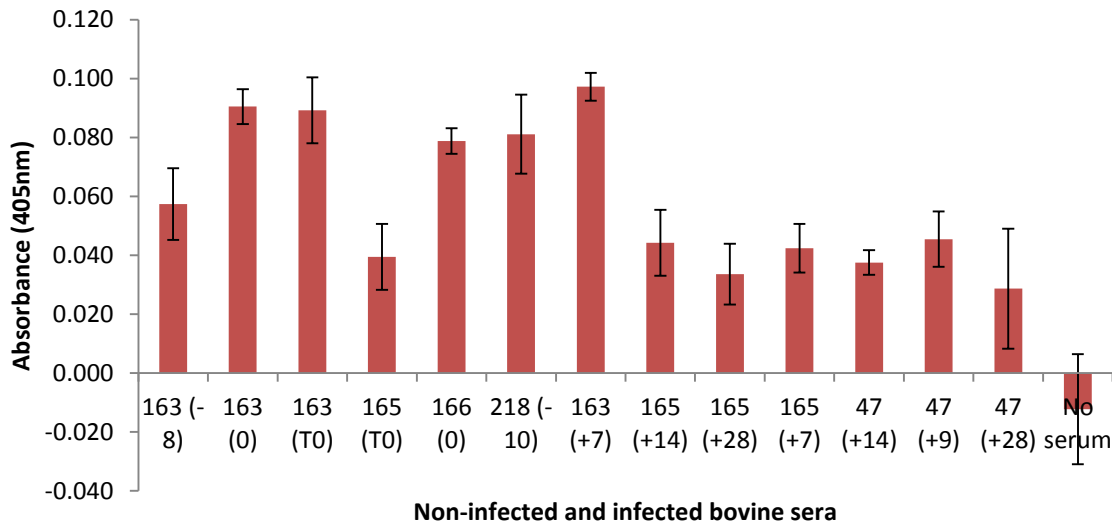
### 3.3.4 Antibody detection in infected bovine sera by indirect ELISA

Since *TcoOPB* is one of the antigens released by trypanosomal parasites during infection, a screening process for antibody production in sera of cattle infected with *T. congolense* parasites was conducted using an indirect ELISA with the *TcoOPB* antigen. In order to optimise the antigen coating concentration, *TcoOPB* was diluted in PBS to concentrations of 5  $\mu\text{g/ml}$ , 2.5  $\mu\text{g/ml}$  and 1  $\mu\text{g/ml}$  and tested against CVB 201 (+7) (CV12/885) and CVB 201 (+19) (CV12/885) infected sera as well as CVB 186 (-7) (CV12/885) and 53 (-28) (CV12/884) non-infected sera and 48 (+98) (CV12/928) infected sera which served as the positive control and an indirect checkerboard ELISA was conducted. Fig. 3.9 shows that only the positive control serum, 48 (+98), showed the presence of antibodies, as seen by the relatively high  $A_{405 \text{ nm}}$  values compared to the other infected and non-infected sera which is arbitrary as 201 (+7) and 201 (+19) infected sera should have shown positive results as well. The 5  $\mu\text{g/ml}$  coating concentration seemed to be optimal as it had the highest  $A_{405 \text{ nm}}$  values compared to the lower coating concentrations.



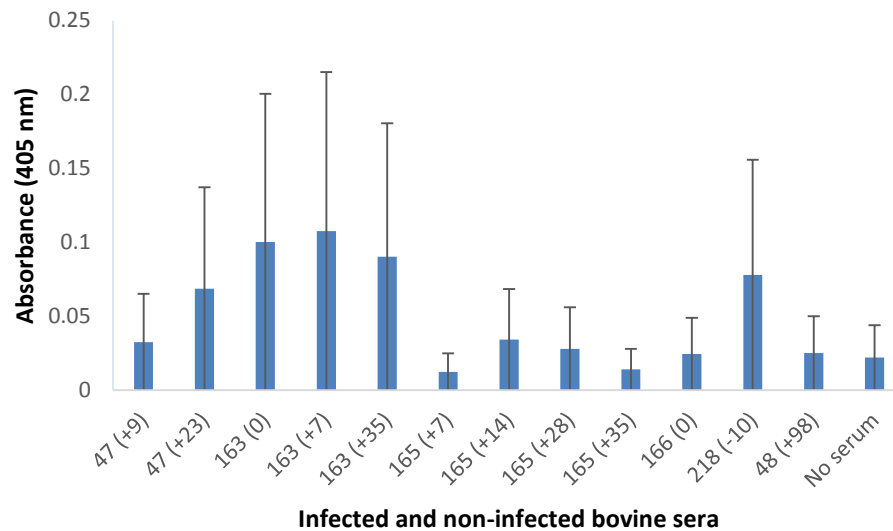
**Figure 3.9: Indirect checkerboard ELISA using various *TcoOPB* coating concentrations against infected and non-infected bovine sera.** ELISA plates coated with 5, 2.5 and 1  $\mu\text{g/ml}$  *TcoOPB*. Sera was used at a 1:100 dilution and rabbit anti-bovine IgG was used at 1:10 000 dilution and developed for 60 min using ABTS.H<sub>2</sub>O<sub>2</sub> substrate. Each data point represents the mean no coat control corrected absorbance  $\pm$  SD (n = 3).

Since the optimal antigen coating concentration was established to be 5  $\mu\text{g/ml}$ , the next batch of infected and non-infected sera could be tested and this time as a 1:10 dilution in PBS-BSA/T and a no serum negative control was included. The tested sera included CVB163 (-8), CVB163 (0), CVB163 (+7), CVB163 (T0), CVB165 (+14), CVB165 (+28), CVB165 (+7), CVB165 (T0), CVB166 (0), CVB218 (-10), 47 Group (+14), 47 Group (+9) and 47 Group (+28). In Fig. 3.11, CVB163 (+7) infected serum shows the highest  $A_{405 \text{ nm}}$  value surpassing that of the positive control serum 47 Group (+28), this indicates that the animal had produced antibodies against the *T. congolense* parasites which were able to recognise the *TcoOPB* antigen, other sera from infected cattle however, showed lower absorbance readings indicating that although the parasites triggered an immune response in the animal, the immune system did not produce a large amount of antibodies. The sera from non-infected cattle which showed high absorbance readings are probably the results of non-specific binding occurring during the indirect ELISA screening process. As expected, the control where serum was omitted showed negative  $A_{405 \text{ nm}}$  values.



**Figure 3.10: Indirect ELISA screening for antibody production in sera from non-infected and infected cattle.** ELISA plates coated with 5 µg/ml *TcoOPB*. Sera were used at a 1:10 dilution and rabbit anti-bovine IgG was used at 1:10 000 dilution and developed for 60 min using ABTS.H<sub>2</sub>O<sub>2</sub> substrate. Non-infected bovine sera samples were 163 (-8), 163 (0), 163 (T0), 165 (T0), 166 (0) and 218 (-10). Infected bovine sera samples were 163 (+7), 165 (+14), 165 (+7), 47 (+14), 47 (+9) and 47 (+28). Each data point represents the mean no coat control corrected absorbance ± SD (n = 3).

In the previous screening, the absorbance values were rather low, therefore when the following ELISA was conducted, a few conditions were altered, a higher coating concentration of *TcoOPB* (10 µg/ml) was attempted as well as a 1:5000 dilution of rabbit anti-bovine IgG to try and obtain higher more distinctive absorbance values. The sera tested under these conditions were 47 Group (+9) (CV12/928), 47 Group (+23) (CV12/928), 163 (0), CVB163 (+7) (CV12/928), CVB163 (+35) (CV12/928), CVB165 (+7) (CV12/928), CVB165 (+14) (CV12/928), CVB165 (+28) (CV12/928), CVB165 (+35) (CV12/928), CVB166 (0) (CV12/928), CVB218 (-10) (CV12/885) and CVB48 (+98) (CV12/928) at a 1:10 dilution in PBS-BSA/T. The results for the sera tested shown in Fig. 3.11 were similar to those obtained in Fig. 3.10 where the highest absorbance value for the infected sera 163 (+7) was 0.1, therefore increasing the concentration of coating antigen or antibody, did not have any effect. The absorbance of 163 (+7) was also higher than that of the positive control, 48 (+98). Other sera from infected cattle used in this test also showed low absorbances indicating low antibody production. The no serum control, however, showed a higher absorbance than previously obtained which was unexpected.



**Figure 3.11: Indirect ELISA screening for antibody levels in infected and non-infected cattle.** ELISA plates coated with 10 µg/ml *TcoOPB*. Sera were used at a 1:10 dilution and rabbit anti-bovine IgG was used at 1:5000 dilution and developed for 60 min using ABTS.H<sub>2</sub>O<sub>2</sub> substrate. Non-infected bovine sera samples were 163 (0), 166 (0), and 218 (-10). Infected bovine sera samples were 47 (+9), 47 (+23), 163 (+7), 163 (+35), 165 (+7), 165 (+14), 165 (+28), 165 (+35) and 48 (+98). Each data point represents the mean no coat control corrected absorbance ± SD (n = 3).

### 3.4. Discussion

The results presented in the present study show that *TcoOPB* was successfully expressed in its soluble state at a size of approximately 80 kDa. It was also successfully purified by affinity chromatography. Western blot analysis with anti-OPB specific antibodies also recognised the antigen.

Studies were conducted on the enzyme to determine its biochemical properties. The optimal pH of *TcoOPB* was determined using constant ionic strength AMT buffers and was found to have maximal activity in a pH range of 8 – 9, these results are consistent with those obtained by Morty *et al* (1999b) where the optimal pH of OPB from *T. brucei* (*TbOPB*) was found to be pH 9. These results show that OPB is highly active under alkaline conditions. Morty *et al* (1999b) found that *TbOPB* retained 75% of maximal activity at the physiological pH of 7.4 and *TcoOPB* retained 73% of maximal activity (Morty

*et al*, 1999a). In the present study, *TcoOPB* retained 79% of maximal activity at physiological pH (7.4). This is indicative of the fact that OPB would be active in the bloodstream of infected hosts as shown by Morty *et al* (2005b) who found that in the blood plasma of *T. evansi* infected rats, as blood parasitemia levels increased, the atrial natriuretic factor (the OPB substrate) levels decreased indicating that OPB was released into the bloodstream of the infected rats where it hydrolysed the peptide hormone, decreasing its levels. Burleigh *et al* (1997) also showed that OPB from *T. cruzi* (*TcrOPB*) is a cytosolic peptidase which may be involved in cell invasion using a  $\text{Ca}^{2+}$  signaling pathway in host cells. They generated *TcrOPB* specific antibodies and found that the antibodies not only inhibited *TcrOPB* proteolytic activity, but also inhibited  $\text{Ca}^{2+}$  signaling, therefore preventing host cell invasion by *T. cruzi* parasites.

Substrate specificity studies done on *TcoOPB* using fluorogenic substrates revealed that *TcoOPB* hydrolysed substrates with the basic amino acids Arg or Lys at the  $\text{P}_1$  position with a high preference for Arg over Lys which is a property of enzymes which display trypsin-like activity (Morty *et al*, 2002). OPB's preference for basic residues at  $\text{P}_1$  is due to the presence of two catalytic dyads in the  $\text{S}_1$  and  $\text{S}_2$  pockets of OPB which consist of acidic residues that bind electrostatically to the substrate Arg or Lys in  $\text{P}_1$  (Coetzer *et al*, 2008). In OPB homologs, the  $\text{S}_1$  binding pocket consists of two Glu residues with the exception of *TcoOPB* and OPB from *Leishmania major* (*L. major* OPB) where one of the Glu residues is replaced by a Gln. The  $\text{S}_2$  binding pocket consists of two Asp residues (Coetzer *et al*, 2008). *TcoOPB* showed higher  $k_{\text{cat}}/K_m$  values for Z-Arg-Arg-AMC, Z-Gly-Gly-Arg-AMC and Z-Pro-Arg-AMC, these results are consistent with those obtained by (Morty *et al*, 1999a) for OPB from *T. congolense* and results obtained by (Morty *et al*, 2005b) for OPB from *T. evansi*. The results also indicate that Gly or Pro residues are acceptable at the  $\text{P}_2$  position (Morty *et al*, 1999a; Morty *et al*, 2005b). However, in the present study, Z-Pro-Arg-AMC showed the highest  $k_{\text{cat}}/K_m$  value of all substrates indicating that Pro was more acceptable at  $\text{P}_2$  than Gly or Arg. All substrates containing Lys at  $\text{P}_1$  showed lower  $k_{\text{cat}}/K_m$  values regardless of the amino acids present at  $\text{P}_2$  or  $\text{P}_3$ , this reinforced the fact that OPB has a higher preference for Arg over Lys at  $\text{P}_1$  as previously stated by Morty *et al* (1999a) and Rea and Fülöp (2006). *TcoOPB* was unable to hydrolyse H-Gly-AMC indicating it does not have aminopeptidase activity (Morty *et al*, 1999a; Morty *et al*, 2005b). Furthermore, the enzyme did not hydrolyse Z-Gly-Pro-AMC and Cbz-Gly-L-Pro-AMC which are prolyl oligopeptidase substrates, this further confirms

that *TcoOPB* displays trypsin-like activity like that observed by Morty *et al* (1999b) for OPB from *T. brucei* (*TbOPB*) and OPB from *T. vivax* (*TvOPB*) (Huson, 2006).

The reversible competitive inhibitors antipain and leupeptin inhibited *TcoOPB* activity quite efficiently as they displayed low  $K_i$  values of  $7.6 \times 10^3$  mM and  $9.23 \times 10^3$  mM respectively; the same inhibition was observed by Morty *et al* (1999a) and Morty *et al* (1999b) for *TcoOPB* and *TbOPB* respectively and McLuskey *et al* (2010) for *L. major* OPB. Antipain and leupeptin are both peptide aldehydes which have an Arg residue at the P<sub>1</sub> position (Umezawa, 1976). Since antipain displayed the lower  $K_i$ , it proved to be a slightly better inhibitor of *TcoOPB* which further confirmed that *TcoOPB* displays trypsin-like activity as antipain only inhibits trypsin-like proteases (Denker, 1977). Chymostatin, which is a peptide aldehyde with a Phe residue at P<sub>1</sub> (Umezawa, 1976) only slightly inhibited *TcoOPB*, this shows the enzyme's preference for Arg over Phe at P<sub>1</sub> (Morty *et al*, 1999a). Since chymostatin is a cysteine protease inhibitor, it is thought that it is able to inhibit *TcoOPB* as seen by Burleigh *et al* (1997) where OPB from *T. cruzi* was inhibited by chymostatin due to the presence of a cysteine residue near the enzyme active site (Morty *et al*, 1999a). Bastos *et al* (2010) came to the same conclusion when *para*-chloromercuribenzoate (pCMB), a cysteine protease inhibitor slightly inhibited *TbPOP*. Morty *et al* (2005a) also conducted a study to identify cysteine residues within the *TbOPB* sequence that may contribute to catalytic activity. They found that the enzyme was inhibited by iodoacetic acid and thiol-blocking agents due to the alkylation of the C<sup>256</sup> residue which is four residues towards the N-terminus of the active site serine residue. In the present study, *TcoOPB* was also slightly inhibited by E-64, another cysteine protease inhibitor, Morty *et al* (1999a) had the same findings for *TcoOPB* which showed slight inhibition by E-64. *TcoOPB* was not inhibited by bestatin, an aminopeptidase inhibitor, which was expected as *TcoOPB* did not display aminopeptidase activity. The inhibitors 1-10 phenanthroline and EDTA, metalloprotease inhibitors seemed to inhibit *TcoOPB* in the assay against class specific inhibitors, however, in a subsequent assay, the  $K_i$  value for the inhibitors could not be determined, indicating lack of inhibition. This led to the conclusion that the results of the first assay were not accurate since OPB does not display metal ion dependent hydrolysis (Morty *et al*, 1999b).

*TcoOPB* was successfully inhibited by TLCK and TPCK which are irreversible peptide chloromethyl ketones with Arg residues at P<sub>1</sub> (Schoellman & Shaw, 1963; Shaw *et al*, 1965). TLCK, however, a trypsin-like protease inhibitor, was a weaker inhibitor of *TcoOPB*

which was not expected as *TcoOPB* displayed trypsin-like activity. TPCK, a chymotrypsin-like protease inhibitor and AEBSF, a serine protease inhibitor were stronger inhibitors of *TcoOPB*. These results are consistent with those obtained by Morty *et al* (1999a) where peptidyl chloromethyl ketones containing Arg or Lys at the P<sub>1</sub> position inhibited *TcoOPB*, this further reinforced that *TcoOPB* displays trypsin-like activity as it prefers Arg and Lys residues at the P<sub>1</sub> position. The inhibition of *TcoOPB* in the present study by irreversible inhibitors such as AEBSF, TLCK and TPCK suggests that these inhibitors poses anti-trypanosomal activity (Morty *et al*, 2000) and further research can be conducted on their chemotherapeutic properties in an attempt to develop more efficient inhibitors with pharmacological potential and stronger specificity for OPB. Table 3.5 show comparative data from literature of the inhibition of OPB by reversible and irreversible inhibitors. It shows that antipain and TLCK are the most efficient inhibitors of OPB and these results are consistent with those obtained in this study.

**Table 3.5: Comparative data of OPB inhibition by reversible and irreversible inhibitors**

Type of inhibitor	Inhibitor	Inhibition constant	Reference
Competitive reversible serine protease inhibitor	Antipain	2.1 x 10 <sup>-3a</sup>	Morty <i>et al</i> (1999a)
		1.81 x 10 <sup>-3a</sup>	Morty <i>et al</i> (1999b)
		2 ± 0.2 x 10 <sup>-3a</sup>	Morty <i>et al</i> (2005b)
	Leupeptin	21.1 x 10 <sup>-3a</sup>	Morty <i>et al</i> (1999a)
		30.08 x 10 <sup>-3a</sup>	Morty <i>et al</i> (1999b)
		21 ± 4 x 10 <sup>-3a</sup>	Morty <i>et al</i> (2005b)
Irreversible serine protease inhibitor	TLCK	5.23 ± 0.23 <sup>c</sup>	Morty <i>et al</i> (2000)
	AEBSF	22.02 ± 4.05 <sup>b</sup> 14.00 ± 2.07 <sup>b</sup>	Morty <i>et al</i> (1999a) Morty <i>et al</i> (1999b)

<sup>a</sup>  $K_i$  (μM)

<sup>b</sup>  $k_a$  (M<sup>-1</sup>s<sup>-1</sup>)

<sup>c</sup>  $k_a$  (x 10<sup>-5</sup> M<sup>-1</sup>s<sup>-1</sup>)

The development of effective chemotherapeutic agents to treat trypanosomosis is also dependent upon efficient and accurate diagnosis of the disease. There are many methods for the diagnosis of trypanosomosis, these include, the card agglutination test for trypanosomosis (CATT) that detects antibodies against the variant surface coat antigens found on the surface of trypanosome parasites (Sullivan *et al*, 2014). Recombinant

expression of trypanosomal antigens obviates the use of *in vitro* or *in vivo* culturing of trypanosomes to obtain trypanosomal antigens and assay would be more reproducible. In the present study, the antibody detection test on *T. congolense* infected and non-infected cattle sera by indirect ELISA was optimised for the *TcoOPB* antigen coating concentration. The optimum coating concentration was found to be 5 µg/ml, an increase in coating concentration of up to 10 µg/ml showed no improvement in the results obtained. The results from testing various infected and non-infected sera for antibodies were rather inconsistent as it seems false positives were obtained for non-infected serum samples such as 166 (0), 218 (-10) and 163 (-8). Low absorbances were obtained for infected sera such as 48 (+98), 165 (+28) and 47 (+28), these results further suggest that *TcoOPB* may not be a good diagnostic agent in an antibody detection test, Morty *et al* (1999a) also found that *TcoOPB* was not recognised by IgM antibodies produced by *T. congolense* infected cattle sera. These results further confirmed that *TcoOPB* may not have good diagnostic potential in the screening for antibodies in infected sera but could rather be used in an antigen detection test for trypanosomosis.

Antigen detection tests have the advantage of testing for current infections rather than antibodies persisting after cure (Bossard *et al*, 2010; Cabral-Miranda *et al*, 2014). In this test, a sandwich ELISA using species-specific monoclonal antibodies are used (Rebeski *et al*, 1999). The ELISA plate is coated with the monoclonal antibodies, after which the test serum is added, the antigen in the test serum binds to the monoclonal antibodies coating the plate and then finally, a second enzyme-labelled antigen specific antibody is added and the reaction developed with a chromogenic substrate (Nantulya, 1990). This test is easy to conduct, it saves time as it can screen a large number of samples at a time, it can be used to test individual animals and it is highly sensitive (Cabral-Miranda *et al*, 2014). Screening for antigens in infected sera is possibly the best method to diagnose trypanosomosis since these antigens are released by dead or dying parasites. After long periods, parasites lyse and will not be detected microscopically. At this stage, lysed parasites would release antigens into the host's bloodstream and although this would lead to misdiagnosis on account of a lack of parasites in the animal bloodstream, testing for the antigens in infected sera would provide the best method to diagnose trypanosomosis as they can be detected long after parasites have lysed (Cabral-Miranda *et al*, 2014). Monoclonal antibodies against *TcoOPB* could be generated and used to screen for the antigen in infected cattle sera by sandwich ELISA. The work described in this chapter



shows that while OPB may not be a good candidate for antibody detection, it may be a good candidate for antigen detection as highly specific monoclonal antibodies can be generated against the antigen making it a good diagnostic method. This work also showed that certain serine protease inhibitors such as AEBSF have anti-trypanosomal activity and can undergo further studies to develop more efficient chemotherapeutic agents.

# CHAPTER 4

## General Discussion

African animal trypanosomosis (AAT) is a bloodstream disease which is caused by the trypanosome parasites *Trypanosoma congolense*, *T. vivax*, and *T. b. brucei* (Baker *et al*, 2013) and has devastating effects on the farming industry in Africa, with losses of up to billions of dollars (Sanni *et al*, 2013). The development of a vaccine against the disease has been futile due to the antigenic variation phenomenon which allows trypanosomes to evade detection by the hosts' immune system (Haag *et al*, 1998). Therefore, chemotherapy is the only viable option, however, due to the development of drug resistance (Baker *et al*, 2013), this is becoming increasingly ineffective. This led to the conceptualisation of a strategy known as the 'anti-disease' approach, which is the development of drugs against pathogenic factors released extracellularly by dead or dying parasites which lead to disease symptoms such as fever, anaemia, and even paralysis (Morty *et al*, 2006; Steverding, 2008). These pathogenic factors include various classes of peptidases such as the cysteine peptidase cruzipain (*TcrCATL*) from *T. cruzi* which is involved in the invasion of the central nervous system, as well as serine peptidases such as oligopeptidases from *T. b. brucei* and *T. congolense* which are responsible for the degradation of the peptide hormones atrial natriuretic factor and neurotensin (Troeborg *et al*, 1996; Morty *et al*, 2005b; Antoine-Moussiaux *et al*, 2009).

The oligopeptidases from the prolyl oligopeptidase family of serine peptidases were the main focus of the present study. The prolyl oligopeptidase S9 family belongs to clan SC of serine peptidases (Polgar, 2002; Polgar & Szeltner, 2008). The prolyl oligopeptidase S9 family consists of five peptidases, prolyl oligopeptidase, oligopeptidase B, acylaminoacyl peptidase, glutamyl endopeptidase C and dipeptidyl peptidase IV (Polgar, 2002; Rawlings *et al*, 2012). These enzymes were grouped together based on their 3-D secondary structure which is an  $\alpha/\beta$  hydrolase fold, the order of their catalytic residues Ser/Asp/His which is the reverse order of catalytic residues in classic trypsin and subtilisin peptidases (Polgar & Szeltner, 2008) and their ability to hydrolyse substrates of no more than 30 amino acid residues (Bastos *et al*, 2013).

Prolyl oligopeptidase (POP) and oligopeptidase B (OPB) have been implicated as pathogenic factors during trypanosome infection and are involved in the manifestation of

disease symptoms (Coetzer *et al*, 2008). POP from *T. b. brucei* and *T. cruzi* have been shown to hydrolyse type I and type IV collagens to allow parasite movement through the host ECM as well as hydrolyse host peptide hormones such as oxytocin and neuropeptides which can lead to depression (Bastos *et al*, 2010; Bastos *et al*, 2013). OPB from *T. cruzi* has been shown to be involved in host cell invasion via a  $Ca^{2+}$  signaling pathway and OPB from *T. b. brucei* and *T. evansi* has been shown to hydrolyse the peptide hormone atrial natriuretic factor (ANF) which leads to weak blood vessels resulting in uncontrolled bleeding during trypanosomosis infection (Morty *et al*, 2005b; McLuskey *et al*, 2010; Bastos *et al*, 2013). Several studies were conducted in order to find possible inhibitors or drugs against these enzymes, the first effective inhibitor of POP was found to be benzyloxycarbonyl-Pro-prolinal as it was shown to prevent scopolamine-induced amnesia in rats (Yoshimoto *et al*, 1987). The best most highly effective and potent inhibitor of plasmic POP was found to be [(2S,3aS,7aS)-1-[(R,R)-2-phenylcyclopropyl]-carbonyl)-2-([thiazolidin-3-yl]carbonyl)octahydro-1H-indole also known as S17092 where it prevented the hydrolysis of neuroactive peptides (Cacabelos *et al*, 2000; Brandt *et al*, 2007; López *et al*, 2011). OPB from *T. b. brucei* has been identified as a target for the chemotherapeutic drugs suramin, diminazene and pentamidine (Morty *et al*, 1998; Al-Mohammed, 2008). Although some progress has been made in identifying various drugs and inhibitors as inhibitors of these pathogenic factors, more studies need to be conducted to further understand these pathogenic factors' biological functions in order to further develop or create new and improved pathogen targeting agents.

In the present study, full-length POP from *T. b. brucei* (*TbPOP*) and *T. congolense* (*TcoPOP*) were successfully cloned into pET32a expression vectors. *TbPOP* was successfully recombinantly expressed as an approximately 100 kDa protein using the *E. coli* (BL21 DE3) expression system, however, the enzyme was expressed in an insoluble state as inclusion bodies. Inclusion bodies are formed after over expression of protein which accumulates to form insoluble protein known as inclusion bodies (Singh & Panda, 2005). Expression at lower temperatures of 16°C and 27°C was unsuccessful at producing soluble *TbPOP*, this was in contrast to Bastos *et al* (2010) who expressed soluble POP from *T. b. brucei* at 16°C. However, in the present study, *TbPOP* inclusion bodies were successfully solubilised with the denaturant, urea (Tsumoto *et al*, 2003) and refolded by step-wise dialysis, however,  $Ni^{2+}$  affinity purification of soluble refolded *TbPOP* only produced partially purified protein with many residual contaminants and further

purification of the partially purified affinity purification fractions by molecular exclusion chromatography (MEC), was unsuccessful as evidenced by smaller sized or diffuse bands on reducing SDS-PAGE. The banding pattern of desalted *Tb*POP after MEC purification on reducing SDS-PAGE suggested that the protein had degraded during the purification process or was eluted at too low concentration to detect, however, silver staining which is highly sensitive, suggests that there was no protein eluted after MEC.

Since refolding by step-wise dialysis may cause further aggregation of solubilised protein (De Bernardez Clark, 1998), the use of a Sephadex G-25 desalting column was considered, which seemed to remove some impurities during the desalting and refolding process. Purification of *Tb*POP continued to prove to be a quite a challenge, even after refolding with the desalting column. The desalting process resulted in the development of a 20 kDa band which remained after purification by anion exchange chromatography. It was speculated that this could be a degradation product of *Tb*POP or the Trx-His tag since it has a size of approximately 20 kDa. Western blot analysis probing with anti-His tag antibodies also detected the 20 kDa band, further suggesting that it could be the Trx-His tag, although sequencing of the band would provide accurate detail of its identity.

Since further purification of *Tb*POP following Ni<sup>2+</sup> affinity purification proved to be a challenge, an enzymatic assay on semi-purified *Tb*POP against the biological protein substrate collagen type I from rat tail and gelatin was conducted. It revealed that *Tb*POP does hydrolyse collagen which is a proline rich protein as observed by Bastos *et al* (2010) where POP from *T. b. brucei* successfully hydrolysed human type I collagen and native type I collagen from rat mesentery *in vitro*. These observations proved that POP is capable of hydrolysing large substrates as well, as opposed to the theory of only hydrolysing peptides containing no more than 30 amino acid residues. POP from *T. cruzi* (*Tcr*POP) has also been shown to hydrolyse large substrates such as human collagen type I and IV as well as fibronectin (Santana *et al*, 1997; Grellier *et al*, 2001). This collagenolytic activity illustrated by POP strongly suggests that it is involved in trypanosome parasite traversal through hosts' blood vessels and ECM during trypanosome infection. In the present study, *Tb*POP did not hydrolyse gelatin, a collagen derivative.

*Tco*POP was successfully cloned and sub-cloned into pGEM-T<sup>®</sup> and pET32a respectively, however, upon expression and DNA sequencing, it was discovered that *Tco*POP was not

fully expressed. DNA sequencing results revealed that a 'non-sense' mutation had occurred within the recombinant *TcoPOP* DNA sequence resulting in a guanine base within the sequence "TGG" being replaced by an adenine base to form "TAG", a premature stop codon (Gatfield *et al*, 2003). This caused a premature end to protein translation, resulting in the expression of an incomplete protein. Due to this mutation, further work on *TcoPOP* could not be conducted. In future work, the *TcoPOP* gene will once again be cloned and sub-cloned into a T-vector and an expression vector respectively and the resulting recombinant clones sequenced and the recombinant *TcoPOP* protease enzymatically characterised to determine its biochemical properties.

Although further work on *TbPOP* and *TcoPOP* such as determination of kinetic parameters could not be conducted due to *TbPOP* only being partially purified and *TcoPOP* not fully expressed, other work has been conducted on these enzymes which can give possible insight into their biochemical characteristics which can suggest as to why they are regarded as possible drug targets. *TbPOP* and *TcrPOP* have been shown to hydrolyse substrates with Pro/Ala at P<sub>1</sub> with a preference for Pro (Bastos *et al*, 2005). Bastos *et al* (2010) showed that *TbPOP* is highly active at alkaline pH ranging from 7.5 – 8.0; these results along with the hydrolysis of native collagen observed in the present study, strongly suggest that POPs can be active within the hosts' bloodstream. Inhibition studies conducted by Bastos *et al* (2010) on *TbPOP* using the fluorogenic substrate N-Suc-Gly-Pro-Leu-Gly-Pro-AMC showed that *TbPOP* activity was strongly inhibited by diisopropyl fluorophosphates (DFP), TPCK and TLCK which are serine, chymotrypsin-like and trypsin-like protease inhibitors respectively. They also showed that *TbPOP* was inhibited by *para* 4-chloromercuribenzoic acid (pCMB), a cysteine protease inhibitor, this was indicative of the presence of a reduced cysteine residue near the active site. It has also been recently shown that *TcrPOP* may be involved in cell invasion as *TcrPOP* inhibitors blocked the entry of parasites into non-phagocytic cells (Bastos *et al*, 2005; Bastos *et al*, 2013).

In meeting a further aim of the present study, full-length OPB from *T. congolense* (*TcoOPB*) in pET28a was successfully recombinantly expressed using the *E.coli* (BL21 DE3) expression system and successfully purified by Ni<sup>2+</sup> affinity purification. Biochemical studies were conducted on the purified enzyme using various enzyme assays to determine optimum pH, substrate specificity and sensitivity to various class specific inhibitors. Determination of *TcoOPB* optimal pH revealed that the enzyme was highly

active in a pH range of 8 – 9 and retained 79% of maximal activity at physiological pH of 7.4. These results were comparable to those obtained by Morty *et al* (1999b) for OPB from *T. brucei* (*TbOPB*), Morty *et al* (1999a) for OPB from *T. congolense* (*TcoOPB*) and (Huson, 2006) for OPB from *T. vivax* (*TvOPB*). These results are indicative of the fact that OPB is capable of retaining high activity in the bloodstream of infected hosts, this is further reinforced by Morty *et al* (2001) who showed that *TbOPB* was active in the bloodstream of *T. b. brucei* infected rats, they found that *TbOPB* remained highly stable in the rat blood plasma was able to hydrolyse fluorogenic substrates. OPB remains active in the hosts' bloodstream due to the fact that it is not inhibited by host serpins such as  $\alpha_1$ -proteinase inhibitor and  $\alpha_2$ -antiplasmin. Due to their large molecular size, these inhibitors cannot gain access to the proteases' active site that is obscured by the N-terminal  $\beta$ -propeller (Coetzer *et al*, 2008).

Unlike its analogue POP, OPB has an affinity for the basic residues Arg/Lys at P<sub>1</sub>. In the present study, substrate specificity studies revealed that *TcoOPB* displayed high affinity for the substrates Z-Arg-Arg-AMC, Z-Gly-Gly-Arg-AMC and Z-Pro-Arg-AMC. This indicates that *TcoOPB* showed a preference for Arg at P<sub>1</sub> and also favoured Gly and Pro at P<sub>2</sub> (Coetzer *et al*, 2008), a result observed by Morty *et al* (2005b) for OPB from *T. evansi*, Morty *et al* (1999a) for *TcoOPB* and Huson (2006) for *TvOPB*. In the present study, substrates such as H-Ala-Phe-Lys-AMC showed very little hydrolysis, reinforcing the fact that OPB has a higher preference for Arg at P<sub>1</sub> over Lys. The hydrolysis of substrates with basic amino acids at P<sub>1</sub> is indicative of *TcoOPB* displaying trypsin-like activity. The enzyme did not hydrolyse Z-Gly-Pro-AMC, a prolyl oligopeptidase substrate indicating that *TcoOPB* does not have POP-like activity; the same observation was obtained by McLuskey *et al* (2010) for OPB from *Leishmania* (*L. major* OPB).

In the present study, *TcoOPB* was efficiently inhibited by the reversible peptide aldehydes antipain and leupeptin as observed for *TbOPB* and *L. major* OPB (Morty *et al*, 1999b; McLuskey *et al*, 2010). Inhibition of *TcoOPB* by 1-10 phenanthroline and EDTA was surprising as substrate hydrolysis by OPB is not metal ion dependent as observed by Morty *et al* (1999a) for *TcoOPB*. In the present study, *TcoOPB* was inhibited by the irreversible serine protease inhibitors TLCK, TPCK and AEBSF, *TcoOPB*, *TvOPB*, and *L. major* OPB have also been shown to be efficiently inhibited by these inhibitors (Morty *et al*, 1999a; Huson, 2006; McLuskey *et al*, 2010).

In the present study, *TcoOPB* displayed similar biochemical properties to those of previously studied POP. Both enzymes show high activity at physiological pH and susceptibility to the irreversible serine protease inhibitors TPCK and TLCK. The high activity displayed by *TcoOPB* at physiological pH correlates with work previously conducted by Morty *et al* (2005b) where rats were infected with *T. evansi* parasites and OPB was released into the bloodstream of the infected rats. They observed that as blood parasitemia increased, there was a decrease in atrial natriuretic factor (ANF), an OPB substrate. This indicated that OPB is highly active in the infected host's bloodstream. Morty *et al* (2001) obtained similar results with *T. b. brucei* infected rats where *TbOPB* retained full catalytic activity while Bastos *et al* (2010) observed that *TbPOP* retained full catalytic activity in the bloodstream of *T. b. brucei* infected mice. Kangethe *et al* (2012) showed that when the gene coding for *TbOPB* was knocked-down, there was an increase in *TbPOP*-like activity. They concluded that these oligopeptidases may compensate for each other in light of the fact that both *TbOPB* and *TbPOP* hydrolysed several peptide hormones such as ANF,  $\beta$ -Endorphin and Neurotensin which all have either Arg/Lys or Pro/Ala in the sequence. This further suggests that these oligopeptidases share similar functions during trypanosomosis pathogenesis.

Trypanosomosis diagnosis has been conducted using various serological techniques which serve to detect trypanosomal antibodies in the serum of infected animals. One of these techniques is the indirect fluorescent antibody test (IFAT) that has the advantage of being sensitive and specific, however, the technique requires expensive equipment (Luckins & Mehlitz, 1978; Pillay *et al*, 2013). An alternative method is by ELISA; the technique is sensitive, easy to conduct, requires low quantities of antigen and serum and saves time as it can screen samples on a large scale (Luckins, 1977; Bossard *et al*, 2010; Pillay *et al*, 2013). In the present study, *TcoOPB* was also used to screen for antibodies in *T. congolense* infected cattle sera by indirect ELISA since it was thought that OPB would induce the production of antibodies when released into the host bloodstream by dying parasites. However, the inconsistent results obtained strongly suggest that *TcoOPB* may not serve as a good diagnostic agent for detecting antibodies. Similar results were obtained by Morty *et al* (1999a) where *TcoOPB* was not recognised by antibodies produced by *T. congolense* infected cattle sera. Conversely, because OPB is present in the bloodstream of infected hosts, it may be a very good candidate for a diagnostic assay to detect the antigen. This diagnostic assay is known as the antigen detection test, which

is conducted via a sandwich ELISA utilising species-specific monoclonal antibodies to screen for antigens released by parasites in the bloodstream of infected hosts (Rebeski *et al*, 1999). It is the preferred diagnostic test as it is highly sensitive, easy to conduct and since antigens are released into the host's bloodstream will be detectable long after parasite levels diminish (Cabral-Miranda *et al*, 2014). For the antigen detection test, antigen-specific antibodies are required to screen for the antigens in the serum of infected hosts. These antibodies can be generated in chickens where following immunization with OPB or POP the chickens produce antibodies (immunoglobulin Y, IgY) which are easily isolated from the egg yolk (Goldring & Coetzer, 2003). Another method to generate antibodies is by phage display techniques where phages expressing the single chain fragment variable (scFv) regions of antibodies are identified from e.g. the Nkuku<sup>®</sup> semi-synthetic phage display library (Van Wyngaardt *et al*, 2004).

In conclusion, further work needs to be conducted to further purify *TbPOP* as well as clone, express and purify *TcoPOP* for enzymatic characterisation purposes. Alternative expression systems such as the *Pichia pastoris* yeast expression system for *TbPOP* and *TcoPOP* can be attempted or alternative expression vectors to obtain soluble protein. The information obtained from peptidolytic activity studies of *TcoOPB* and *TbPOP* can be applied to the design of inhibitors that may be appropriate drug scaffolds for developing new chemotherapeutic agents for African trypanosomosis.



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## 5. APPENDIX

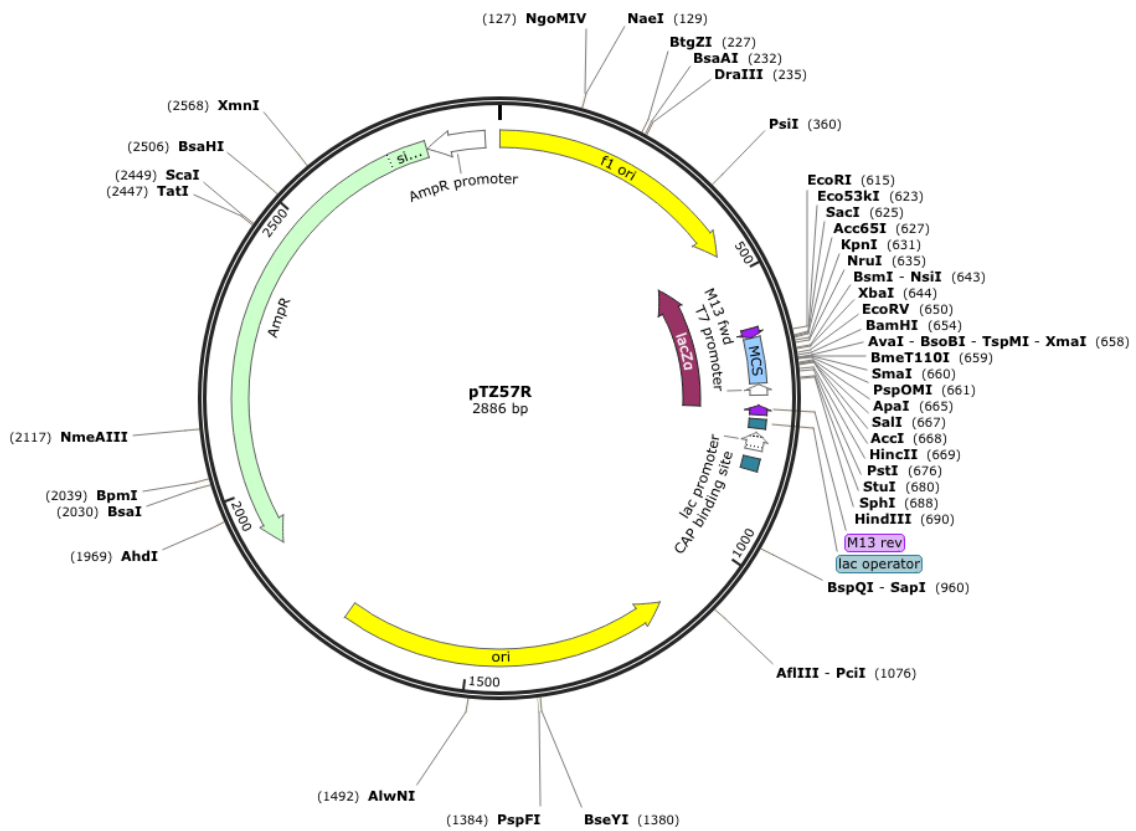
### Appendix 1: Multiple sequence alignment of *TcoOPB*, *TbPOP* and *TcoPOP* from Clan SC subfamily S9 of serine proteases. POP and OPB share 25% identity.

<i>TcoOPB</i>	ATGACATCTGATCGCGGCCCATCGCTGCGCACAAGCCCTATGAGGTG-GTCTTTGGTAA	59
<i>TbPOP</i>	AT----GCGCCTCGCTTACCCCACTGCAAGACGCTCGGCAGCGAGCTACGTGTTGCATAA	56
<i>TcPOP</i>	AT----GCGTCTCGTGTACCCCACTGTCAGGCGTTTCGGCGGCACATACACCCCTTCACAA	56
	** * ** * * * * * * * * *	
<i>TcoOPB</i>	AGTTGAGGGGGAGGACCGTGGGCCAAATCCGATGGATCCCCACGCCGTCGCGTTCGATCC	119
<i>TbPOP</i>	TGTGACGGTACCAGAACC-----TATGA	80
<i>TcPOP</i>	TGTCACGGTATCTGAGCC-----TACGA	80
	** ** * *	
<i>TcoOPB</i>	CCTCTTTTGGCTCCGGGACGACTCCCGCACCAACCCAGACGTCCTCGCTCACCTTCACCT	179
<i>TbPOP</i>	CTACCTTGAAGATCCTGAAAACAACGAAACGAAATCATTCGTGAAAGCCAAAACGATCT	140
<i>TcPOP</i>	CTACCTGGAGGACCCCTGAAGACCCACAAACAAGTCATTTGTACTCGCGAAAACAATTT	140
	* * * * * * * * * * * * * * * *	
<i>TcoOPB</i>	AGAGAGGGACTATTTTCGAGAAGCGCACGGGGACATTAAGGACCTGGCGGAGACGATCTA	239
<i>TbPOP</i>	CTTCGGTGAATA-----CATGAGGTCCACCGAAGCACTTGGGAATAAGTTGTA	188
<i>TcPOP</i>	ATTCACAGAGTA-----TATGACTTCCACAGCAGAGCTTCGTGAGAAAGTCTT	188
	* * * * * * * * * * * * * * *	
<i>TcoOPB</i>	CCAGGAGCATATTTACACATT-GAGGAACTGACATGTCGCCTCCTTACACATACAACC	298
<i>TbPOP</i>	TAACCGC-ATTTCCCAAACGTTTCGACTTCCCCGAACGTCACACCCAGTTTTCGGAACG	247
<i>TcPOP</i>	TAATCGC-ATCACTCAGGTGATGAACTTCCCCGCACATCCAGTCCAAGCTTCCGCAACG	247
	* ** * * * * * * * * * *	
<i>TcoOPB</i>	GCTTTGTGTACTACACGCGCAGGGTGAAGGGACTGTCTTACAAGATCCATTGCCGCGTGC	358
<i>TbPOP</i>	GTCGTTATTACTTTTATCACAATACT--GGTCTACAGAACCAAAGTGTGTTGAAGCGTGC	305
<i>TcPOP</i>	GGCACTATTACTACTTTTAACACC--GGCTACAGAATCAAGACGTTTTAATGCGCGC	305
	* **** * ** *** ** ** *	
<i>TcoOPB</i>	CACTTGGCAAGGTGCCTGGC-----GAAGGCCCGACGAGGAGATTGTGCTGGACGA	410
<i>TbPOP</i>	CACGAGTCTAACAGACTCCAACCCACAACCTTCTTGATCCCAATATCTGAGCAATGA	365
<i>TcPOP</i>	CACGTCCCTTACCGACAAGAACCCCAACACATTCCTGGATCCCAACATTTGAACAGCGA	365
	*** * * * * * * * * * * *	
<i>TcoOPB</i>	GAACAACTTGCAGAGGGAAAGGCATTTTGTGACGTGCGCTCGGTGCCCCGCTCCACC	470
<i>TbPOP</i>	TGGCACAACGGC--CTTGAAGGCCACGGCATGGAG-----TGA	401
<i>TcPOP</i>	TGGAACGAGCGC--ACTGAGGTCCACAGCTGGAG-----CAA	401
	* ** * * * * * * * *	
<i>TcoOPB</i>	GGAACACATGCTGGTGGCGTACTCTGTTGACCACCTCGGAGACGAGTTGTACAGCATTCA	530
<i>TbPOP</i>	GGACGAATCCCTGTTTCGCATACAGTGCCAGTGACAAGGGAAGCGATTGGCAACACATTCA	461
<i>TcPOP</i>	AGACGAATCGTTGTTTGCCTACAGCGTGAGCGATAAGGGAAAGTACTGGCAACAGTTCA	461
	** * * * * * * * * * * * * * *	
<i>TcoOPB</i>	GTTTGTGGGGATGCGTCGCCAGATAAGCTTGAGGGC-----ACC---ACTGG	575
<i>TbPOP</i>	CGTGCGCGGGCAGACACGGCTGAGGACTGAGGATGTTATTGAGTGGGCCAAGTTCAC	521
<i>TcPOP</i>	CGTGCGCGGTGCTGACACCTCCAGGACCTACCGGATGTTGTTGAATGGGCGAAGTTCAC	521
	* * * * * * * * * * * *	
<i>TcoOPB</i>	GAGCATCATATGGGGTACCAACGCTGAGTGCTTCTTCTACGTTACACCAGACTCTACAAA	635
<i>TbPOP</i>	AGGTATCTCATGGTTACACAACACTGGATTCTTCTACA-CGCGCTTCCAGCTCTTAAGG	580
<i>TcPOP</i>	GGGCATCGCATGGTGGCACAGTGATGGATTTTTTATA-CCCGCTACCCGACCTTGAAG	580
	* *** ***** ** * * * * * * * * * *	
<i>TcoOPB</i>	GCGAAGCAACAAGGTGTGGCGTCACATTATTGGCCAATCACAAGCGAGGATGTGTGCCT	695
<i>TbPOP</i>	GGGATGTGGATAAAGGCGCTGAAACAGACGCG-----GCGAAGGATCCTTTTGTGTTGC	633
<i>TcPOP</i>	GGGACGTGGATAAAGGCGCTGAGACGGACACT-----GCAAAGGACCCATTTATATGT	633
	* * * * * * * * * * * * * * * *	
<i>TcoOPB</i>	TTACACAGA-----CGATGACCCACTGTTCTCTGTGTGCTGCGTCC	735
<i>TbPOP</i>	TTCCACCGCCTCGGTACGAAGCAGGATGAGGATGTGGTCGTACTCTCTTACCAGAGCAT	693
<i>TcPOP</i>	TTTCATCGTCTTGGAACGAAGCAGGAGGAAAGATGTTGTTGTTTTCCATGCGCTGAGAAC	693
	** ** * **** * * * * * * * * *	

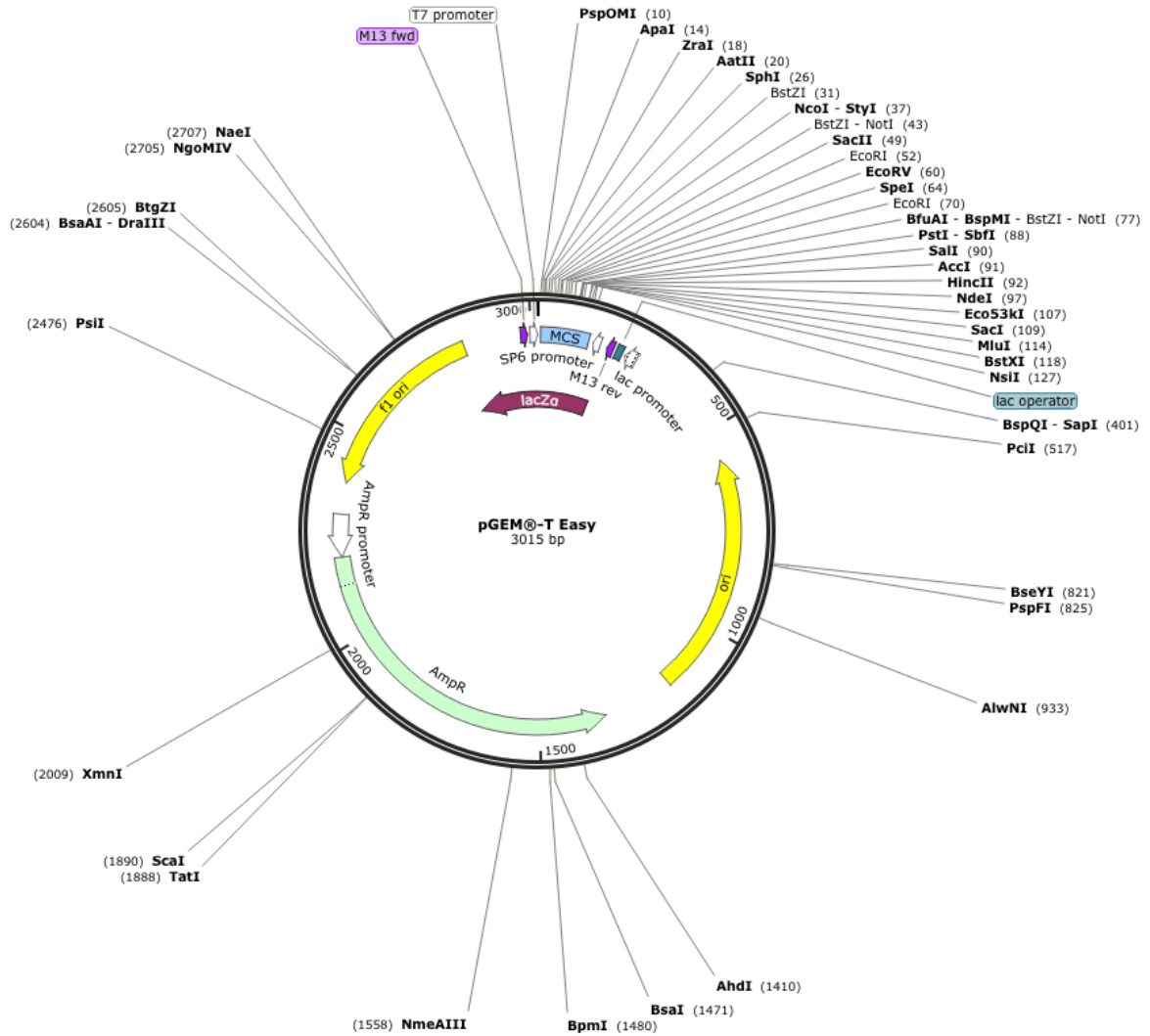
TcOPB	AAGTCTGGTGTGGCCATACGCTGCTAATTAGTCTCCTCCAGTGAAACCACTGAGCTT	795
TbPOP	CCCCAC--TGGGGGGTTT---CTGCTGAGGTATCTAACTGTCACCTCGTACCT-----	740
TcPOP	CCATAT--TGGAGCCTCT---CGGCTGAGGTGTCCGATTGCCAGTCATATCT-----	740
	* * * * *	
TcOPB	CACCTCTTGGATTGCGGAAGGGCCTGAACAACACTACACTCGAGGTGTGCGGAAGCGG	855
TbPOP	-TGTGTTTCTATTACCGATGGTTGTGAGCCAAAGAAC--TTGATATGGATCAC-----	791
TcPOP	-CATTGTTTCTATGACAGAAGGTTGTGAACCGAAAAAT--TTGGTGTGGATCGC-----	791
	* * * * *	
TcOPB	GAGAAGAACGTTTCGATACGAGGTGGAGATGCACGGCACTGAGACACTGGTGATACTAACA	915
TbPOP	CAAGTTACC-----TATCGATGGAGCTGAGAAGCTTTC-----CCTTCTACATTAACA	840
TcPOP	TGAACTGCC-----AAGTGGTGGTGTGAAATCTTACA----TCTGGTGGATTAACA	840
	* * * * *	
TcOPB	AACAAGG-----ACAAATGTATTAACGGTAAGGTCTGTGCTAAGCGGGCTTCA	966
TbPOP	TACAATAAGTTGTTTAAACGAATTTGTCGGTTCGTTTGAATACTTGGGGAATGATGGCACC	900
TcPOP	TTCAATAAATTGGTGGATAAATTTGTTGGTCTAGTATGAGTACCTGGGGAATAATGGGACA	900
	* * * * *	
TcOPB	CCATCGGAGTGGACAATGTTCTCGTACCGCATGACGACAAGGTTTTTATCGATGATATT	1026
TbPOP	ACTTTTTACTTCGTGACAACGCGCGACGCGCGCGAAAGAAGATCATCTCCGTTGACATC	960
TcPOP	GTATTTACTTTGTAAACCACCCGTTGGGGCACCGTGCAAAAATGTTATTTCTATCGATCTT	960
	* * * * *	
TcOPB	GCCGTATTGCGCAAGTTTGTCTCTCAGGACGGCGTGTGACTGACACGCGTCTGG	1086
TbPOP	GCCACTGGGAAGGAGCAGATTGTTGTGGGGGAGAGGGAGCCGTCCTCGACCACGCGCA	1020
TcPOP	TCCACGGGTCAGGAACGGTAATTTGTGGCAGAGGAAGGCTGTACTCGAACACGCGCGC	1020
	* * * * *	
TcOPB	ACGGTTCAGGTGGGTCCA-----GATAATTGTTTCAGTGCC	1122
TbPOP	CTCGTGAAGAACACTCTGATCTCGTCTACCTTGAAGATGTCAAGAGTACTCTTTATTAC	1080
TcPOP	CTTGTGAAGGACACTCTCATTCTTGTGTACTTGAAGACGTTAAAAGTGTTTTATATTAC	1080
	* * * * *	
TcOPB	GGGACGCTGCGTGAGCTACAGTTCGATGAGCCGTTTTTACTGCCACGTAATCACCTCA	1182
TbPOP	TGCTGCTTGGACAACCAGAGTTAAAGAAAATAACCATACCTATCGGAGCGATATCTTCC	1140
TcPOP	TGCCGCTTGGAGAAGCCGAGTTGAAGAACATTGCCATACCCATTGGGACAATATCCTCT	1140
	* * * * *	
TcOPB	CAAATGAAGACGTACGACACATCATCGTGCCTGGAGTATTCCCTCCATGACCACTCCC	1242
TbPOP	CTCTTTGCGGACCGCAAGGTGGACCTCGTGTCCCTTCAAGGTTACATCTTTCTCCTCCCG	1200
TcPOP	CTGTTTACGGACCGGAAGGTGGATTTTGTGTCAATTTAAAATAACATCCTTCTTGTCTCCC	1200
	* * * * *	
TcOPB	ACCACGTGGTTTGGATGAGGACTTGGCGAGTGGGAAGCGC-----ACAGTCGTAAAAGTG	1296
TbPOP	GGGCGATCGTTCGTGTTGGACATCAACGATCCCGAGGGATCACTTCGCGTTTTCAAGGAT	1260
TcPOP	GGACGGTCTTCGTGATGGATTAATGACCCGGAGGTTCACTGAGCATGTTTAAAGGAC	1260
	* * * * *	
TcOPB	TCCAAGGTGGGTGGCGGTTTACTCGAAGAATTACGTTTGCCAACGGCGCCTTGCCACA	1356
TbPOP	GACAATATCGAGGGC---CTCTCTGTGATGACTTTATCACGGAACAGAAGTTCTATAAC	1317
TcPOP	GACATCATTTGAGGGT---GTTTCTGCGGATGACTTCATCACCGAACAAAAGTTTTACAAC	1317
	* * * * *	
TcOPB	GCACCCGACGGGACAACCATCCCCCTTTCGATTTCTCTACGACGTTAGTCTTGACATGAAG	1416
TbPOP	TCAGCTGACGGTACCCGAATTCCAATGTTTCAATATTCACCGGAAGGGGATAGT---GACG	1374
TcPOP	TCGGCCGATGGAACACGAATTCCAATGTTTATTGTCCACCGCAAAGGGATTGT---GACG	1374
	* * * * *	
TcOPB	AAGCCGCATCCGACGATGCTTTACGGTTACGGTTCCATGGGATTTGTGTTGAGCCGACG	1476
TbPOP	TCGGAGTCTCCGGTCTCTCTACGGCTATGGAGGCTTCAACATATCCCTCACACCGTCTG	1434
TcPOP	GCCAACGCGCCATTGCTGCTCTACGGCTACGGTGGATTTAACAATTTCCCTTCAACCGGTC	1434
	* * * * *	
TcOPB	TTCGACATCCGGTCTTGCCGTATGTGG---ACCGTGGTGTGATATACGCCATTGCACAT	1533
TbPOP	TTCAGTCTATCCCGTGTGGTGTCTTACAGCACCTCCGCGGGTTCTCGCGGTGCCGAAC	1494
TcPOP	TTCAGTCTTCTCGTGTGTGTTTTTGCAGCACTTTTGGCGGGTTCTTGCCGTGCCAAAC	1494
	* * * * *	
TcOPB	GTGCGCGGTGGAGGAGAGATGGGCCGAGCATGGTACGAGATCGGAGGGAAGTACTTGACA	1593

TbPOP	ATTCGAGGGGGCGGTGAATACGGAGAGGCTTGGCATA---ACGCTGGTCGTCTGACAAGT	1551
TcPOP	ATCCGAGGTGGCGGTGAGTATGGTGAGGCGTGGCATA---ACGCTGGCCGCCTGAAAACA *	1551
TcOPB	AAGCGAACACCTTCATGGATTTTATTTTCGTGTGCCGAGCATCTTATTTTCGTCTGGTGTG	1653
TbPOP	AAACAAAACGTGTTTACGGACTTCATTGAAGCGGCGAAGTTCCTCCACGGAAATGGCTAC	1611
TcPOP	AAGCAGAAGTGTGTTTACGGACTTCATTGAAGCTGCAAAGTTCTCCACAAAAGTGGATAC *	1611
TcOPB	ACGACTCCCCCGCAGCTTGCCTGCGAGGGGGCGTAGCGCCGGTGGTCTTCTGGTTGGCGCT	1713
TbPOP	GGCAACCCGGCAACAACAGCCATCATGGGGGGCTCAAATGGTGGGTGCTGGTGGCGCG	1671
TcPOP	GGCACACCTGCAACAACAGCCATCATGGGGCGATCCAACGGTGGGTTGCTAGTGGCCGC *	1671
TcOPB	GTAATAAATATGCGCCCTGACTTATTTCCGTGTCGCCGTTGCTGGCGTGGCCTTTGTTGAC	1773
TbPOP	GTTGCAAAATCAAGCAGCTGAACCTCTCCGTTGTGTTATATGCCAGGTGGGTGTGCTGGAC	1731
TcPOP	GTTGCGAACCAAGCTCCTGAACCTCTTCGGTGCCTATTTGTCAGGTGGTGTACTGGAC *	1731
TcOPB	GTCATGACGACTATGTGTGACCCGAGCATTCCGCTCACAACGGGCGAGTGGGAGGAATGG	1833
TbPOP	A---TGTACAAATTCCA-----TAAGTTCACTATCGGCCAC-GCTTGGAGGTCAGATTTT	1782
TcPOP	T---TGTACAAATTCCA-----CAAGTTTACCATTGGCCAT-GCGTGGAGGTCAGACTAT *	1782
TcOPB	GGGAACCCGAACGAATACAAATCTTCGACTATATGAACAGCTACAGTCCGATTGATAAC	1893
TbPOP	GGTGACCCAGATAAAGAGGAGGACTTCAAGGTGTTGCAAAAAGTACAGCCACTACATAAC	1842
TcPOP	GGAGACCCAGACAAAGAGGAAGACTTCAGGGTGTGCAGAAGTACAGTCCGCTGCATAAC *	1842
TcOPB	GTGCGCCCGCAG---GACTACCCGAACCTCATAATCCAAGCTGGACTGCACGATCCCCGC	1950
TbPOP	ATCAAAGCGGGGTTAAATACCCTGCAATTTTGGTGGTAACAGGTGATCAGCAGCAGG	1902
TcPOP	ATCAAGGCTGGCACAAAGTACCCAGCGATATTGGTGTGTGACGGGTGACCAGCAGACCGA *	1902
TcOPB	GTGGCATAATTGGGAGCCAGCGAAGTGGGCCCTCGAAGCTGCGGGAGCTCAAGACGGACAAC	2010
TbPOP	GTGGTGCCACTGCATTCGCTTAAGTACATTGCGGCCTTCAGCACGAAAACCCAACGGAG	1962
TcPOP	GTTGTCCCGCTGCATTTCGTTGAAATACAGCGGACACTTCAGCACGAAAACCCGGAGGAG *	1962
TcOPB	AACGAGGTGTT--GCTGAAGATGGACTTGGACAGTGGCCACTTCTCCGCGAGTGATCGT	2067
TbPOP	GGAGGGCCCTTCCTTGCTCGTGTGAAGTGGCCCGCGGTACGGTGTGGTAAGCCACC	2022
TcPOP	GGAGGGCCATTCTTCGCCCGCATTTGAGGTGGCTGCTGGCCATGGTGTGGGAAGCCAGTA *	2022
TcOPB	TACAAGTACCTGCGAGAGCAGCCATACAACAGGCTTTTGTGTTGAAGCACCTTGGCGTG	2127
TbPOP	AGTAAAATTATGCAGGAGTCGGCGGATATTTACACATTTATT--GCAAAAAATACGAACG	2080
TcPOP	AGCAAAAATAACGCGTGAGGCTGCCGACATTTACACCTTCGTT--GCTAAAAGTATGAACG *	2080
TcOPB	CGCCGGTTGCTGCGGCATTAA	2148
TbPOP	CCCAGTGGACAGA----CTGA	2097
TcPOP	CCCAGTGGACGGA----CTAA *	2097

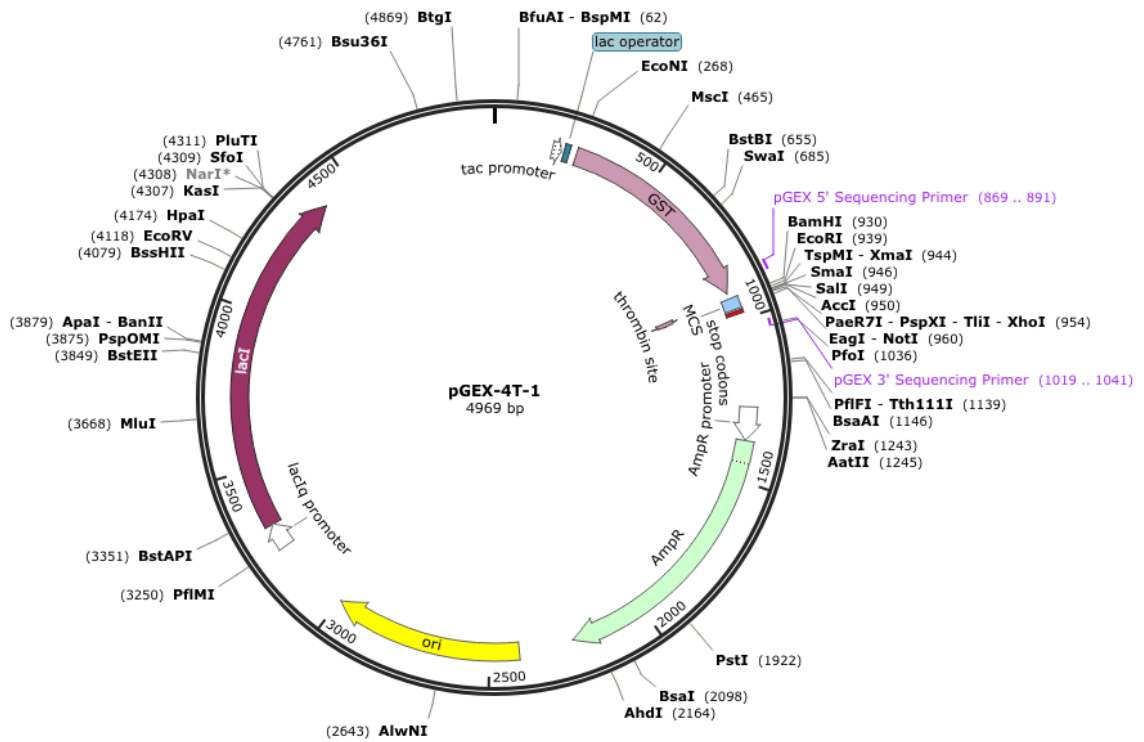
**Appendix 2: Cloning vector pTZ57R/T.** *TbPOP* insert was cloned into the T-vector.  
[http://www.snappgene.com/resources/plasmid\\_files/](http://www.snappgene.com/resources/plasmid_files/) (accessed: 15 August 2014).



**Appendix 3: Cloning vector pGEM® T-easy.** *TcoPOP* insert was cloned into the T-vector.  
[http://www.snappgene.com/resources/plasmid\\_files/](http://www.snappgene.com/resources/plasmid_files/) (accessed: 15 August 2014).

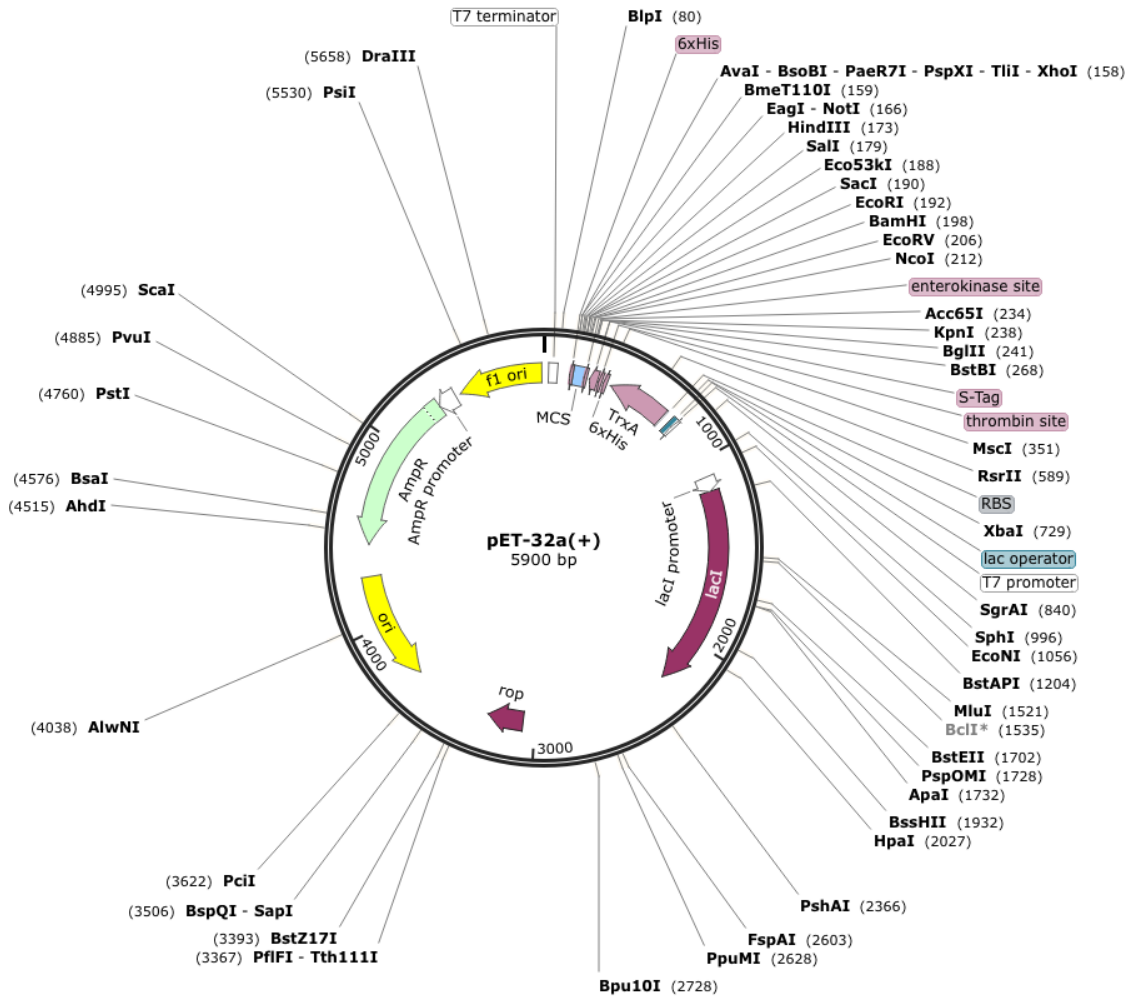


**Appendix 4: Expression vector pGEX-4T-1.** *TbPOP* insert was cloned into the expression vector.  
[http://www.snappgene.com/resources/plasmid\\_files/](http://www.snappgene.com/resources/plasmid_files/) (accessed: 15 August 2014).

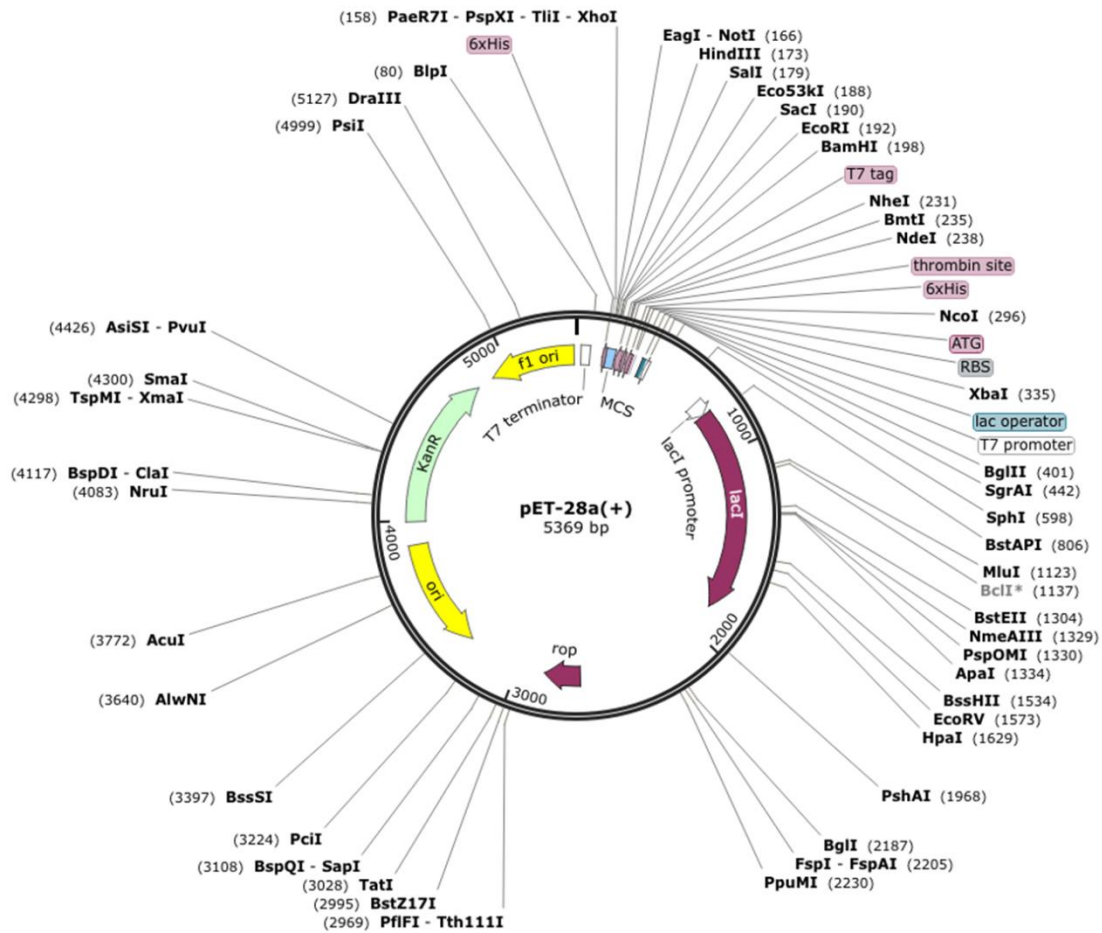




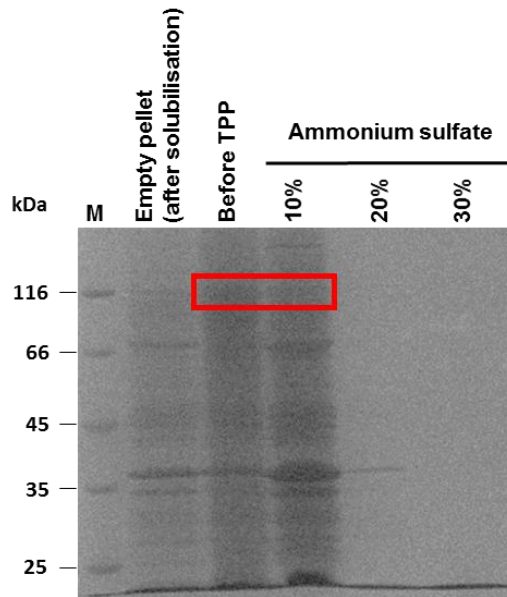
**Appendix 5: Expression vector pET32a.** *TbPOP* and *TcoPOP* inserts were cloned into the expression vector. [http://www.snapgene.com/resources/plasmid\\_files/](http://www.snapgene.com/resources/plasmid_files/) (accessed: 15 August 2014)



**Appendix 6: Expression vector pET28a.** *TcoOPB* inserts was cloned into the expression vector.  
[http://www.snappgene.com/resources/plasmid\\_files/](http://www.snappgene.com/resources/plasmid_files/) (accessed: 15 August 2014)



**Appendix 7: Reducing SDS-PADE (12.5%) analysis of TPP purification of TbPOP.** All proteins precipitated at 10% ammonium sulfate. Gel stained with Coomassie staining solution



**Appendix 8: Reducing SDS-PADE (12.5%) analysis of electro-elution purification of TbPOP.** Excised *TbPOP* band showed degradation after electro-elution. Gel stained with Coomassie staining solution

