RECOMBINANT EXPRESSION, PURIFICATION AND CHARACTERISATION OF *Tvi*CATL FOR ANTIBODY PRODUCTION AND DIAGNOSIS OF AFRICAN ANIMAL TRYPANOSOMIASIS.

By

Phindile Thobeka Ximba

BSc (Hons) Biochemistry

Submitted in fulfilment of the academic requirements for the degree of

MSc degree

in

Biochemistry

School of Life Sciences

University of KwaZulu-Natal

Pietermaritzburg

2014

PREFACE

The experimental work described in this dissertation was carried out in the School of Life Sciences, Department of Biochemistry, University of KwaZulu-Natal, Pietermaritzburg, from January 2013 to December 2014, under the supervision of Prof. Theresa H. T. Coetzer.

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

_____

Phindile Thobeka Ximba (candidate)

_____

Prof. Theresa H.T. Coetzer (supervisor)

DECLARATION-PLAGIARISM

I, Phindile Thobeka Ximba, declare that

1. The research reported in this thesis, except where otherwise indicated, is my original research.

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

3. This thesis does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.

4. This thesis does not contain other persons' writing, unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, then:
   a. Their words have been re-written but the general information attributed to them has been referenced
   b. Where their exact words have been used, then their writing has been placed in italics and inside quotation marks, and referenced.

5. This thesis does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the thesis and in the References sections.

Signed: ……  ……

ABSTRACT

For the effective control of African animal trypanosomiasis, there is a great need for the development of point of care diagnostic tests that are affordable to the end-users. Parasite cysteine proteases are involved in the pathogenesis of African trypanosomiasis, hence serve as a good target for the development of new chemotherapeutics and diagnostics. The aim of this study was to evaluate the potential of the cysteine protease *Tvi*CATL from *Trypanosoma vivax* as a chemotherapeutic agent and diagnostic target for African animal trypanosomiasis. The catalytic domain, *Tvi*CATL, was recombinantly expressed in *Pichia pastoris*, auto-catalytically activated by low pH removal of the proregion, purified by three phase partitioning and molecular exclusion chromatography to homogeneity and its identity confirmed by western blot analysis. Endoglycosidase H treatment of *Tvi*CATL indicated that the protease is Asn-glycosylated. The pH optimum was determined to range between 6.5 and 7.5 suggesting that the enzyme would be active in the host bloodstream following parasite lysis. The protease was able to degrade the host proteins: bovine serum albumin, bovine haemoglobin, gelatin, type I collagen and the endogenous protease inhibitor, bovine α_2-macroglobulin *in vitro* at neutral pH. The peptidolytic specificity of the protease was determined by considering the active site binding pocket S_2- substrate - P_2 interaction. *Tvi*CATL showed high endopeptidase specificity for Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC, suggesting that the hydrophobic residues Phe or Leu are favoured at the P_2 position in the presence of basic Arg or Lys at P_1 position. The *Tvi*CATL peptidolytic activity was inhibited by E-64, iodoacetate, leupeptin, antipain, Z-Gly-Leu-Phe-CMK (albeit at a reduced level) and iodoacetamide inhibitors and this indicated that *Tvi*CATL is a cysteine protease.

Satisfactory anti-*Tvi*CATL antibody levels were produced in chickens as evidenced by good signals in the ELISA and western blot analysis. The specificity and affinity of chicken anti-*Tvi*CATL IgY antibodies for *Tvi*CATL antigen was improved by affinity purification of these antibodies using a *Tvi*CATL-affinity column. The serodiagnostic potential of the *Tvi*CATL antigen and cross-reactivity with anti-*T. congolense* antibodies (in sera) was evaluated in an antibody inhibition ELISA format. These antibodies were able to discriminate between non-infected cattle sera and *T. congolense*-infected sera, thus suggesting some degree of cross-reactivity between *Tvi*CATL antigen and anti-*T. congolense* antibodies. As an alternative to animal-based antibody production, the Nkuku® phage library was used to select for single-chain variable fragment (scFvs) antibodies by panning against *Tvi*CATL antigen. As evaluated by polyclonal and monospecific phage enzyme-linked immunosorbent assay

(ELISA), after the third round of panning, the *Tvi*CATL-scFvs binders were enriched. However, only one clone gave a promising ELISA signal suggesting that further optimisation of panning conditions are required in order to obtain improved monospecific ELISA signals for application of *Tvi*CATL-scFvs in diagnostic assays. *Tvi*CATL therefore shows promise as both a chemotherapeutic and diagnostic target to African animal trypanosomiasis.

ACKNOWLEDGEMENTS

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

First and foremost, I would like to extend my sincere gratitude to my supervisor, Prof THT Coetzer for her expert and phenomenal supervision, patience and understanding, recognising and perfecting my capabilities and constructive criticism during the preparation of this dissertation. Indeed I will be forever grateful for having you as my supervisor, may God bless you abundantly.

To Dr Vukea and Prof Goldring, in one way or another, you each have contributed to my personal growth as well as to my experimental approach and presentation skills.

To staff members of the School of Life Sciences, Pat Joubert (for processing orders), Charmaine, Tanya and Natalie for your help with regard to postgraduate administrative needs.

To my fellow postgraduate colleagues: Omalara Baiyegunhi, Lauren Eyssen, Jacky Viljoen, Kayleen Brien, Sanele Mnkandla, Faith Ndlovu, Wonder Mpaphane, Kelvin Addicott, Robert Krause, Faiaz Shaik, Alex Delport, thank you for all the research ideas we shared and happy times.

To the National Research Foundation and UKZN College of Agriculture, Engineering and Science for their financial support.

To my parents, Mr EJ Ximba and Mrs. NB Ximba for your endless love, support and for allowing me to pursue my academic aspirations without letting the unstable background circumstances pull me down. To my siblings: Nokwethemba for her sisterly advices and my twin brother Sandile for sensing my emotions followed by encouragements and genuine laughter.

To Mr and Mrs. Wanda, for your parenthood during my entire academic life in PMB, indeed you were my shoulder to cry-on.

To my friends Patrice Mkonyeni, Siphelele, Sthandiwe, Thuli, Thobeka, Zama, thank you for all your support and happy times outside the laboratory.

And lastly, all praise and honor goas unto the Almighty Jesus Christ for His grace in my life.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

AMC	7-amino-4-methylcoumarin
AMT	Acetate-Mes-Tris
BCA	bicinchoninic acid
Bis	N, N'-methylenebisacrylamide
Bis-Tris	2-bis (2-hydroxyethyl) amino-2-(hydroxymethyl)-1, 3-propanediol
BMGY	buffered glycerol complex medium
BMM	buffered minimal medium
Boc	butyloxycarbonyl
BSA	bovine serum albumin
C-terminal	carboxy terminal
DMSO	dimethylsulfoxide
DTT	dithiothreitol
$[E]_0$	active enzyme concentration
E-64	L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
Fab	fragment antigen binding
Fv	fragment variable
g	relative centrifugal force
HRPO	horseradish peroxidase
[I]	inhibitor concentration
IAA	iodoacetate
IAM	iodoacetamide
IgY	immunoglobulin Y
k_{ass}	rate of complex association

K_{av}	availability constant
k_{cat}	turnover number
kDa	kilo-Dalton
K_i	inhibition constant
$K_{i(app)}$	apparent inhibition constant
K_m	Michaelis-Menten constant
k_{obs}	pseudo first-order inhibition rate constant
MEC	molecular exclusion chromatography
Mes	acetate-2(N-morpholino) ethanesulfonic acid
N-terminal	amino terminal
OD_{600}	optical density at 600 nm
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline, pH 7.2
PEG	polyethylene glycol
PMSF	phenylmethylsulfonylfluoride
RT	room temperature
[S]	substrate concentration
scFv	single-chain fragment variable
SDS	sodium dodecyl sulfate
Suc	succinyl
$t_{1/2}$	half-life
TBS	tris buffered saline
TEMED	N, N, N', N'-tetramethyl ethylene diamine
Tris	2-amino-2-(hydroxymethyl)-1, 3-propandiol
TU	Transducing units
V_e	elution volume
V_{max}	maximum velocity
V_o	void volume
v_0	initial velocity

VSG	variant surface glycoprotein
V_t	total column volume
YNB	yeast nitrogen base
YP	yeast extract, peptone
YPD	yeast extract, peptone, dextrose
2x YT	2 × yeast extract, tryptone
Z	benzyloxycarbon

CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

African human and animal trypanosomiasis (HAT and AAT) are diseases commonly called sleeping sickness in humans and nagana in domestic livestock. African trypanosomiasis is caused by unicellular flagellated protozoan parasites of the genus *Trypanosoma* (El-Sayed *et al*, 2000; Baral, 2010). Whereas *T. brucei gambiense* (Western Africa) and *T. b. rhodesiense* (Central Africa and East Africa) are the exclusive causative agents of human trypanosomiasis, *T. vivax* and *T. congolense* are the most prevalent pathogenic species for cattle in Africa while *T. brucei brucei* is non-pathogenic in humans since normal human serum contains trypanosomal lytic factor (Pays & Vanhollebeke, 2009). *T. vivax*, *T. congolense* and *T. b. brucei* are mainly transmitted cyclically to their mammalian host by the haematophagous tsetse fly of the genus *Glossina* and to a lesser extent, *T. vivax* is mechanically transmitted by the biting flies most of which are African tabinids (horse flies) and stable flies. Trypanosomes can infect all domesticated animals, but cattle are highly susceptible due to the feeding preferences of tsetse flies. The alternative transmission of *T. vivax* by vectors other than tsetse flies allows this parasite to spread beyond the tsetse fly belt (Moloo *et al*, 2000; Fikru *et al*, 2012). The spread of trypanosomiasis is proportional to the distribution of tsetse vectors which infest more than 10 million km^2 of the continent (Figure 1.1). Consequently, it is estimated that trypanosome infections in livestock negatively influence agricultural-based economies with an estimated total loss of approximately $4.5 billion yearly in Africa (Swallow, 2000; Roditi & Lehane, 2008; Antoine-Moussiaux *et al*, 2009).

1.2 Classification of trypanosomes

The eukaryotic genus *Trypanosoma* is classified as order Kinetoplastida and family Trypanosomatidae (Figure 1.2) (Hoare, 1972). They are called Kinetoplastida due to the presence of a single mitochondrial DNA within a structure called the kinetoplast (Bastin *et al*, 2000). Based on mechanisms of transmission, trypanosomes are divided into two groups: salivaria and stercoraria. The developmental stages of salivarian parasites take place within the stomach of the tsetse vector from where they migrate

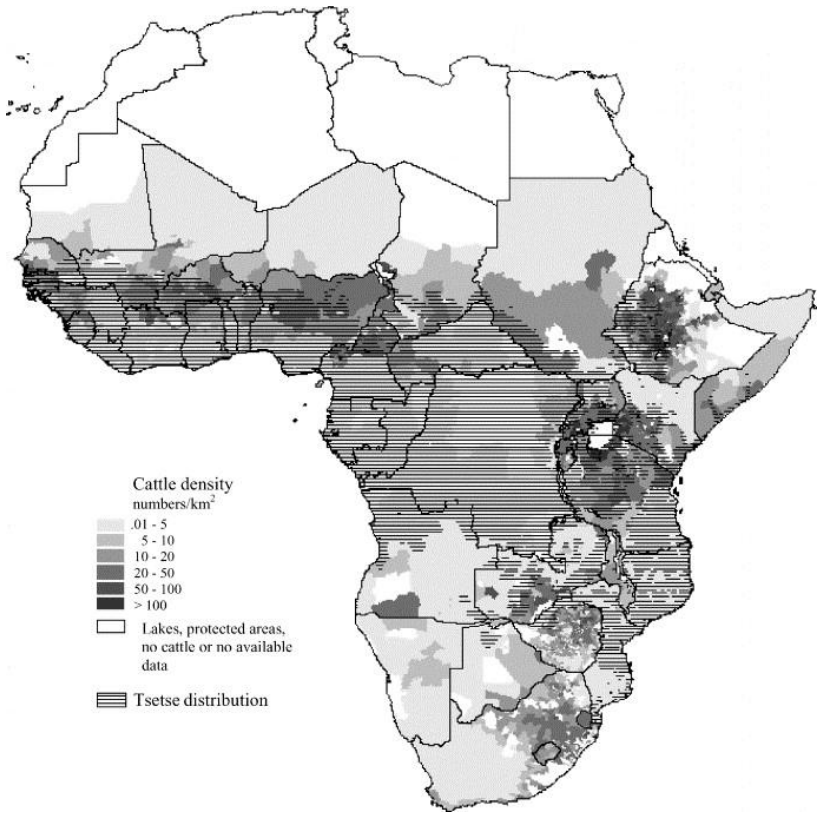

Figure 1.1: The proportional distribution of tsetse flies and cattle in Africa (Naessens *et al*, 2002).

towards the salivary glands where the infectious form of the parasite develops. In contrast, stercorarian parasites develop in the intestinal tract or hind gut, and transmission to the mammalian host is via faeces. Whereas *T. brucei*, *T. congolense, T. evansi* and *T. equiperdum* are examples of salivarian trypanosomes found in the tsetse fly belt of Africa, *T. cruzi*, the causative agent of American Chagas disease, is an example of the stercoraria subgroup (Vickerman, 1985). Other than the mode of transmission being the main distinguishing feature of these two subgroups of trypanosomes, salivarian trypanosomes also differ from the stercoraria in that the former evade the host immune response by antigenic variation due to expression of variant surface glycoproteins (VSGs) (discussed later) which coat the bloodstream

parasites (Borst *et al*, 1996; Haag *et al*, 1998).

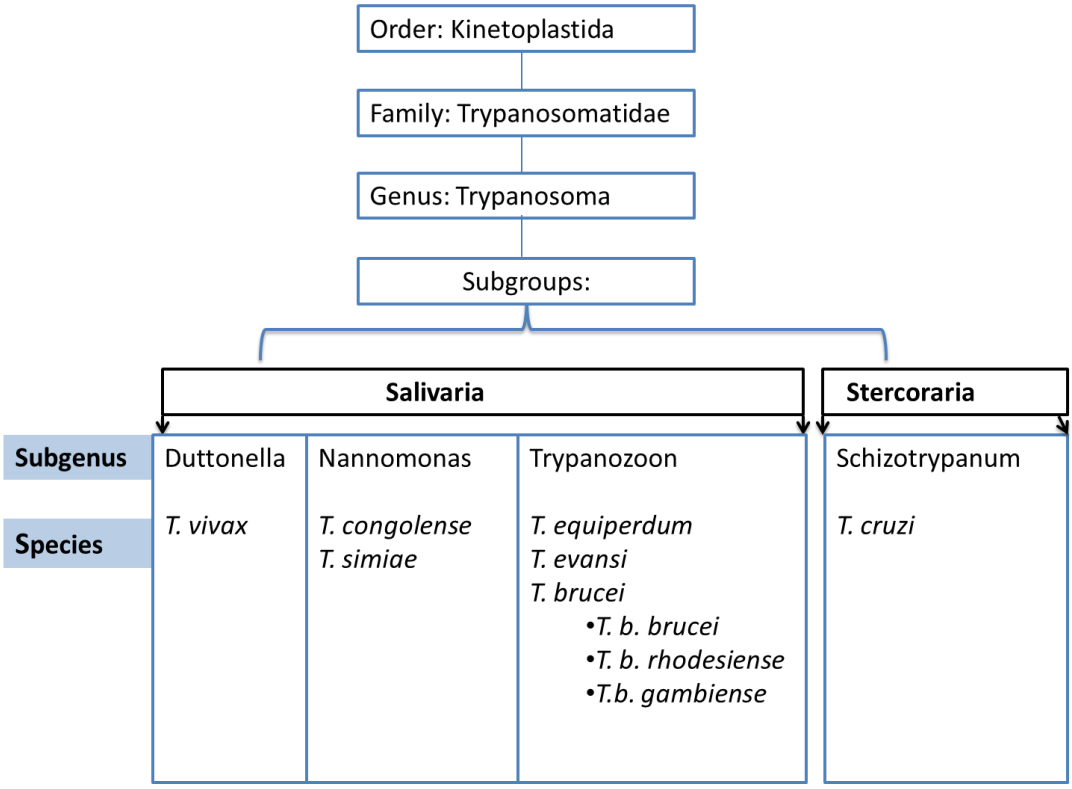

Figure 1.2: Classification of trypanosomes. Adapted from Baral (2010)

1.3 General morphology of trypanosomes

Trypanosomes are single-celled parasites that are elongated (ranging from 8-50 μm) and tapered at both ends (Figure 1.3). Since trypanosomes are eukaryotic, they exhibit typical features such as a nucleus and organelles including an endoplasmic reticulum, Golgi apparatus, a mitochondrion as well as endocytosis and exocytosis systems (Clayton *et al*, 1995; Bastin *et al*, 2000). Trypanosomes have a flagellar pocket, flagellum and mitochondrial DNA. The flagellar pocket (FP) is a protected invagination of the plasma membrane. Due to the concentration of receptors responsible for endocytosis and immune evasion, the FP is considered to be a site for protein trafficking, protein and lipid sorting, and a recycling compartment (Moreira-Leite *et al*, 2001; Gull, 2003; Overath & Engstler, 2004; Gadelha *et al*, 2009). The flagellum which is responsible for parasite motility extends from the flagellar pocket. The basal body at which the flagellum attaches is called the flagellum attachment zone, and it also interacts with a single elongated mitochondrial DNA which is situated within the structure called the kinetoplast (Bastin *et al*, 2000; Kohl *et al*, 2003; Broadhead *et al*, 2006). Mitochondrial DNA consists of a network of maxicircles (22 kb) and minicircles

(1 kb). Maxicircles are responsible for encoding mitochondrial proteins whilst minicircles are responsible for extensive post-transcriptional modification or RNA editing of these regions using guide RNAs (Stuart *et al*, 1997; El-Sayed *et al*, 2000)

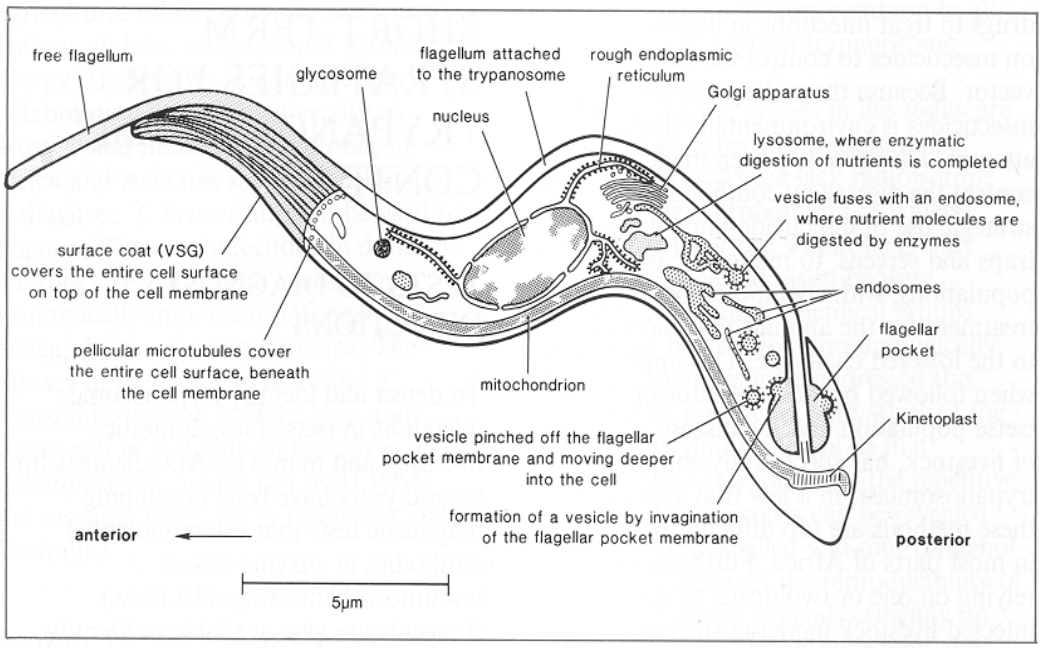

Figure 1.3: Diagrammatic representation of the African trypanosome, *T. brucei* showing associated organelles. Accessed on 20 May 2013 from http://imgarcade.com/1/trypanosoma-brucei-diagram/

1.4 Trypanosome lifecycle

The trypanosome lifecycle is divided into two stages: that in the mammalian host and that in the insect vector (Figure 1.4) (Roditi & Lehane, 2008). In the tsetse vector, trypanosomes undergo preparatory developmental stages (differentiation, proliferation and migration). Different trypanosome species develop in different organs: *T. vivax* develops exclusively in the mouthparts of the vector, while initial infections of both *T. brucei* and *T. congolense* start in the fly midgut and the parasites subsequently migrate into the salivary glands and proboscis of the tsetse fly (Roditi & Lehane, 2008). The transmission cycle begins during a blood meal from a mammalian host, where an infected tsetse fly injects its infective metacyclic trypanosomes into mammalian skin tissue. The metacyclic form is transformed into blood-stage trypomastigotes. The parasite then becomes distributed throughout the body. Insect stages begin when bloodstream trypomastigotes are transformed into procyclic forms which occupy the midgut of the tsetse. Procyclics are heterogeneous comprising both long proliferative

slender forms that can replicate by asexual division and non-replicating stumpy forms (Schnaufer *et al*, 2002; Matthews *et al*, 2004; Roditi & Lehane, 2008; Baral, 2010). When the parasite relocates from the midgut to the salivary gland, epimastigotes multiply by binary fission in the bloodstream, lymph and spinal fluid. The cycle is repeated when susceptible tsetse feed on a new mammalian host (Baral, 2010).

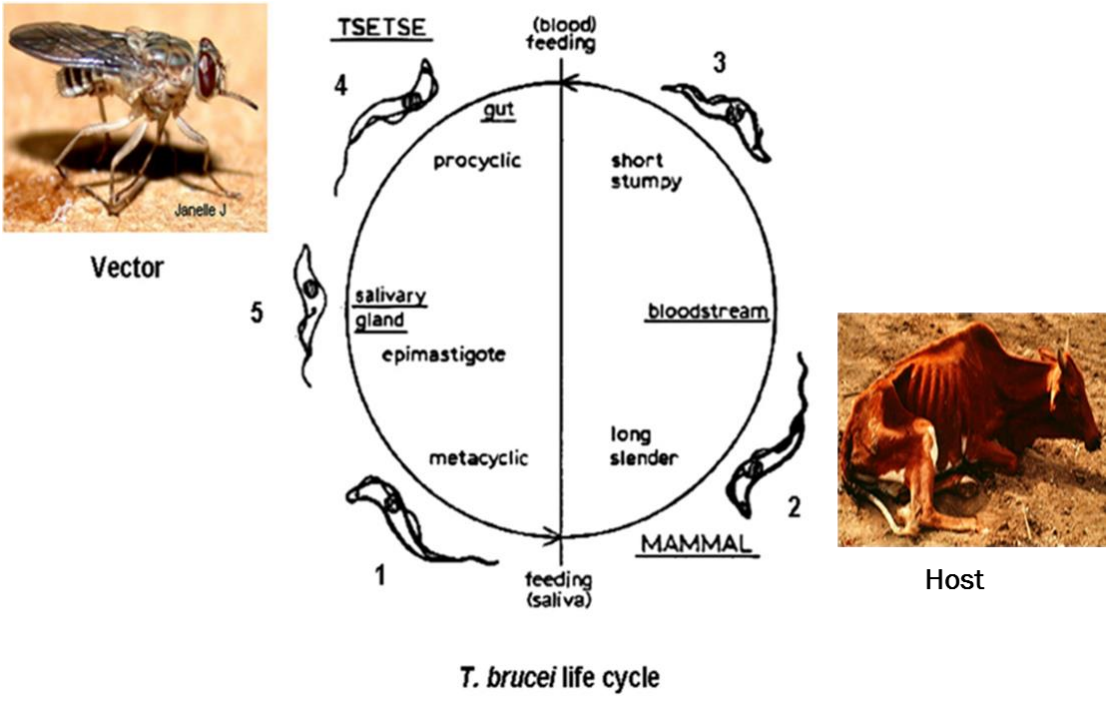

Figure 1.4: Life cycle of *Trypanosoma brucei* in tsetse fly vector and mammalian host. Adapted from Geiger *et al* (2011).

1.5 Antigenic variation

Salivarian trypanosomes exploit a well-documented process called antigenic variation as a defence mechanism against host immune responses (Donelson, 1995; Borst *et al*, 1996; Barry & McCulloch, 2001). Since the trypanosome lifecycle and replication occurs in extracellular compartments such as host tissue fluids and bloodstream, the parasite is susceptible to attack by the host immune system. To circumvent this attack, trypanosomes make use of antigenic variation using VSGs which determine the variant antigen type (VAT). The parasite is coated with more than 10^7 copies of VSGs which are encoded by specific telomeric loci which contain numerous genes for surface proteins (Jackson *et al*, 1985; Pays, 2006). Since the host constantly produces

antibodies against expressed parasite VSGs, the VSGs' antigenic specificity is continuously changed and the parasites proliferate, resulting in a second wave of parasitemia (Donelson *et al*, 1998). Two switching mechanisms are used: the first involves expression of a single gene at the VSG expression site, followed by the subsequent silencing of that gene and activation of the next distinct gene (Morrison *et al*, 2009), while the second mechanism is called recombination involving gene conversion where an active silent gene is copied and transferred to an expression site to compensate for the deleted gene (Barry & McCulloch, 2001; San Filippo *et al*, 2008; Morrison *et al*, 2009). It can be concluded that successive expression of VSGs is critical for chronic infections by trypanosomes and onward transmission by the tsetse vector to a new host (Barry & McCulloch, 2001; Pays, 2006). Due to periodic switching of the parasite VSGs, the development of vaccines capable of preventing trypanosome infection in immunised animals, is unlikely (Lalmanach *et al*, 2002). There are no reports that antigenic variation contribute to the chronic phase of infection by stercorarian trypanosomes, but the tenacity of these parasites inside the host cells is due to the presence of carbohydrate-rich components (glycolipids and glycoproteins) which allow evasion of the host innate immune system (Takle & Snary, 1993; Almeida *et al*, 1994)

1.6 Transmission and pathogenesis of trypanosomes

Livestock such as cattle, pigs and other domestic animals are susceptible to AAT or nagana (Aksoy, 2003). *T. brucei, T. congolense* and *T. vivax* are the most widespread pathogenic trypanosomes causing nagana, and are transmitted to the host by *Glossina* spp. tsetse fly vectors. *T. brucei* and *T. evansi* commonly attack tissues (humoral), while *T. congolense* and *T. vivax* are restricted to the circulation and mainly invade blood (haematic) (Igbokwe, 1994; Mbaya & Ibrahim, 2011). *T. evansi*, mostly distributed in Asia, Africa and South America causes surra, which is transmitted mechanically by tabanic flies (Otto *et al*, 2010). Equines, camels and Asian elephants are more susceptible to surra (Hoare, 1972). *T. equiperdum*, which is closely related to *T. evansi*, causes a venereally transmitted disease called dourine and mainly targets equines (Hoare, 1972; Brun, 1993).

Each trypanosome species shows unique pathogenic properties, but anaemia is the pre-eminent symptom of AAT, the severity of which depends on parasite strain and breed of targeted host (Prowse, 2005; Chamond *et al*, 2010). Due to concurrent infection and susceptibility of the host to more than one trypanosomal parasite species, it is difficult to determine which species is responsible for which clinical symptoms. In

general, injection of the infective metacyclic trypanosomes into the skin of the mammalian host results in inflammation and swelling called a chancre at the site of a fly bite. Since metacyclic trypanosomes divide and multiply rapidly in the chancre, this results in invasive blood forms that invade lymphatics, lymph nodes and the blood stream. The common clinical symptoms of livestock trypanosomiasis are immunosuppression, intermittent fever, anaemia, weight loss and decreased milk yields in cattle. During the chronic condition, which is characterised by fluctuating levels of parasitemia as a result of the competition between the expression of the parasites of the same antigenic type (homotype) and parasites of the variable antigenic type (heterotype), animal reproduction is affected resulting in abortions, premature births and testicular damage in males. Also, working capacity is reduced, cattle become lethargic and emaciated, and subsequently die due to heart failure (Barrett *et al*, 2003; Baral, 2010).

1.7 Control of animal trypanosomiasis

1.7.1 Vector control

The simplest approach to trypanosome control is to reduce transmission by eliminating tsetse vectors (Allsopp, 2001). There are many vector control strategies that have been undertaken including those currently considered as deplorable such as deforestation and slaughtering of wild animals (Dransfield *et al*, 1991). Ground and aerial spraying with residual insecticides such as dichlorophenyltrichloroethane (DDT) has been useful for the reduction of flies but this procedure was limited by quick reinfestation of sprayed areas. Persistent usage of residual insecticides had been associated with a number of pitfalls such as tsetse flies developing resistance to insecticides, imbalances in the ecosystem due to cross-eradication of non-targeted insects, bioaccumulation and potential toxicity of insecticides to individuals spraying them as a result of overexposure. Due to the high cost and environmental unfeasibility, residual insecticide spraying became restricted (Dransfield *et al*, 1991; Holmes, 1997). Residual insecticides were replaced with non-residual insecticides which are aerially sprayed at very low concentration and volume and non-targeted insects are less susceptible. This sequential aerosol technique (SAT) is approved by scientific environmental monitoring groups as an environmentally friendly tsetse control method when used under appropriate supervision (Grant, 2001; Alsopp & Hursey, 2004; Vreysen *et al*, 2013).

Alternative control measures include odour baited traps treated with insecticides to which female flies are attracted over long distances and no-return cages where they die through starvation or heating. Baited traps aim to suppress tsetse population density rather than eradication of the insects (Vale, 1993; Bouyer *et al*, 2010). Baited traps led to the development of "live-bait'' technology in which livestock are treated with "pour-on'' insecticides. The effectiveness of the pour-ons is evidenced by the ability of deltamethrin pour-on used on Ugandan cattle to significantly reduce tsetse populations, thus resulting in a gradual decrease in the prevalence of cattle trypanosomiasis (Okiria *et al*, 2002). The main disadvantages of this technique are the need for repeat treatments, cost of insecticides and potential development of resistance to the insecticides (Bauer *et al*, 1995; Leak, 1999).

The development of sterile insect technique was successful in eradicating populations of *Glossina austeni* from the Island of Zanzibar (Bailey, 1998; Vreysen *et al*, 2000). Sterile insect technique is a genetic suppression approach which releases irradiated sterile male insects over a wide area. If sterile males are dispersed in high numbers that outcompete wild males for wild females over a long time, the reproductive capacity of tsetse fly is severely minimised, thus levels of trypanosome transmission are controlled (Aksoy, 2003; Dyck *et al*, 2005; Vreysen *et al*, 2013). Control measures that utilise molecular genetics are under consideration in an attempt to improve the sterile insect technique. These include the use of genetically produced symbionts like *Wolbachia* with cytoplasmic incompatibility, which induces sterility in their host by introducing reproductive abnormality. If this male strain of tsetse is released, they spread *Wolbachia* infection when mating with wild females, thus resulting in embryonic lethality (Dobson, 2003; Abd-Alla *et al*, 2013).

1.7.2 Trypanotolerant cattle

The use of trypanotolerant cattle is another approach for the control of trypanosomiasis. Trypanotolerance is the genetic trait that makes it possible for cattle to survive and remain productive after trypanosome infection (Naessens, 2006). N'Dama cattle, mainly found in West and Central Africa, is the cattle breed that exhibits this trait (Authié, 1994). However, this breed only represents approximately 6% of the cattle population in Africa and there is a perception that this breed is not economically beneficial to farmers since they are not productive due to their relatively small size.

Genotypic analysis of quantitative trait loci of Boran-N'Dama derived F2 calves indicated that approximately five genes were passed on to calves from the susceptible

Boran grandparent. This led to the perception that trypanotolerance in Boran cattle can be improved by crossbreeding Boran and N'Dama cattle (Hanotte *et al*, 2003). This led to the development of haemopoetic Boran/ N'Dama chimeric twins (Naessens *et al*, 2003). When chimeras were experimentally challenged with *T. congolense*, the chimeras successfully controlled the levels of parasitaemia to a similar extent as N'Dama singletons, but anaemia was severe. Overall, this suggested that trypanotolerance is derived from two independent systems. These include the ability to control anaemia which relies on haematopoietic cells, and an ability to control parasitaemia which is independent of haemopoetic cells (Naessens *et al*, 2003; Naessens, 2006).

1.7.3 Chemotherapeutic approaches

1.7.3.1 Trypanocidal drugs

The treatment of the livestock infected by AAT largely depends on chemotherapy and chemoprophylaxis using three trypanocidal drugs; isometamidium, homidium and diminazine. These drugs generally cease the multiplication of the parasite and this strengthens the capacity of the host immune system to defeat any spread of the ongoing trypanosomal infection (Osman *et al*, 1992). These drugs have been in use for over 50 years, and it is thus not surprising that drug resistance (multiple) has been reported in 18 African countries including South Africa (Delespaux *et al*, 2008b; Chitanga *et al*, 2011). This multiple drug resistance interferes with the idea of using a sanative pair system in which two trypanocidal drugs are used. The first drug is administered until the resistant strain appears, and then the second drug is used to eliminate the accumulated drug resistant strain from both the host and the vector (Mamoudou *et al*, 2008).

A sudden reduction of the effectiveness of isometamidium in Boran cattle infected with *T. congolense* has been reported, and is thought to be associated with drug resistance (Sutherland *et al*, 1991; Codja *et al*, 1993). The prevalence of drug resistance was also reported when *T. congolense* infected cattle were treated with diminazine, and even after withdrawal of treatment with diminazine, there was no possibility of regaining diminazine-sensitive trypanosomal strains (Delespaux *et al*, 2008a; Chitanga *et al*, 2011). Resistance to trypanocidal drugs has been associated with mutated nucleoside transporters which prevent free diffusion of trypanocidals through the parasite membrane (Barrett & Fairlamb, 1999; Baral, 2010). Due to a limited number of drugs that are currently available for the treatment of AAT, improbability of development of

new drugs in the near future, the prevalence of drug resistance and exhibition of severe toxic side effects, there is a high demand for alternative treatments (Barrett *et al*, 2005; Delespaux & de Koning, 2007). The recently synthesised commercial forms of isometamidium (i.e. Veridium® and Samarin®) were tested *in vivo* and *in vitro* to access their trypanocidal capabilities. These drugs were trypanocidal against *T. congolense* and *T. b. brucei* when tested *in vitro*. When mice were treated with 1 mg/kg of these compounds (one drug per mouse), the evaluation of trypanocidal activity two months post treatment showed that these compounds induced prophylactic activity against *T. congolense*. However, more work is needed in order to optimise drug dosage and identify any drug resistant trypanosomal strains (Sahin *et al*, 2014).

1.7.3.2 Targeting cysteine proteases for the development of new drugs

It has been long established that the trypanosomal cysteine proteases, which are involved in parasite invasion, proliferation and differentiation during mammalian lifecycle stages, are involved in pathogenesis by degrading host proteins, and that targeting these proteases with suitable inhibitors results in trypanosomal lifecycle arrest *in vitro* (Troeberg et al, 1999; Bryant et al, 2009). Consequently, research effort is focussed on determining the substrate specificity of each trypanosomal protease for developing specific inhibitors without toxicity towards the treated host. Numerous irreversible inhibitors specific for cysteine proteases have been developed. These include diazomethane, halomethyl ketones, epoxysuccinyl derivatives and vinyl sulfone derivatives. These inhibitors irreversibly bind into the cysteine protease active site and form a covalent adduct that inactivates the enzyme (Otto & Schirmeister, 1997). There are several studies that can be considered as the 'proof of concept' that specific inhibitors result in parasite arrest. Vinyl sulfone inhibitors have been shown to treat mice experimentally infected with *T. cruzi* (Engel *et al*, 1998b; Roush *et al*, 2001). These inhibitors killed *T. cruzi* in culture by inducing morphological abnormalities of the Golgi apparatus due to the accumulation of the unprocessed enzyme as a zymogen. Low micro-molar concentrations of the *Tbb*CATL inhibitors, chloro-, diazo-, and fluoromethyl ketones, were able to alter the growth of *T. b brucei* parasites in culture significantly (Troeberg *et al*, 1999). The vinyl sulfone peptide inhibitor K1117 has also been proven to be a potent inhibitor that rescued animals infected with *T. cruzi* that causes Chagas disease (Doyle *et al*, 2007). Recently, aryl thiosemicarbazone-derived compounds have been synthesised and characterised as anti-*T. cruzi* inhibitors that induce parasitic death through apoptosis, but these compounds did not alter *Tcr*CATL

activity, therefore further studies are necessary for understanding the mechanism involved (Magalhaes Moreira *et al*, 2014).

1.8 Vaccination

There are no vaccines for one of the major animal parasites, African trypanosomes. The first vaccination approach was based on producing anti-VSG antibodies. This vaccination has been fruitless due to antigenic variation that these trypanosomal parasites have deceitfully evolved (Cornelissen *et al*, 1985; Magez *et al*, 2010). It was apparent that vaccination based on anti-VSG antibodies would never eliminate trypanosomiasis due to on-going VSG gene switching and the fact that VSGs predominantly elicit a short lived IgM immune response rather than the preferred long lived IgG isotype (Mitchell & Pearson, 1983). Besides VSGs, the survival of the trypanosomes also depends on the ability to express other invariant surface molecules. The flagellar pocket contains a variety of proteins which have been identified as potential targets for vaccine development (Mkunza *et al*, 1995). Cattle vaccinated with invariant antigen from the flagellar pocket prior to exposure to infection with *T. congolense* and *T. vivax*, were able to partially protect themselves against infection compared to unvaccinated cattle (Mkunza *et al*, 1995). Surprisingly, when mice were subjected to subsequent infections with a high parasite dose, the level of protection was indirectly proportional to parasite dosage. Due to the lack of reproducibility of FP vaccination, this vaccination approach shows little promise (Radwanska *et al*, 2000). The cytoplasm of the trypanosomes contains the microtubules which are made up of tubulins. When the trypanosome tubulin was investigated as a vaccine candidate, mice pre-immunised with tubulin from *T. brucei* were challenged with *T. brucei*, *T. congolense* or *T. rhodesience*. The mice were protected against these parasites, suggesting that tubulin is a promising vaccine candidate (Lubega *et al*, 2002). In order for the host immune system to produce antibodies that are different from antibodies produced as a result of infection, mice were pre-immunised with invariant surface glycoproteins (ISGs). Upon infection with *T. b. brucei*, partial protection was observed. This protection failure was as a result of the vaccinated host's immune system being unable to produce an effective B cell memory response even after successive challenge with incoming *T. b. brucei* ISGs (Radwanska *et al*, 2008; Magez *et al*, 2010; Lança *et al*, 2011). Due to these pitfalls of anti-trypanosome vaccinations, it was necessary to introduce an alternative vaccination approach.

This led to the development of the anti-disease vaccine approach which targets infection-associated pathology rather than the parasite itself (Authié, 1994). This approach targeted parasite cysteine proteases which are thought to play a role in pathogenesis. Experimental studies based on the cathepsin-like cysteine protease from *T. congolense* (*Tco*CATL) showed that *Tco*CATL plays a pathogenic role in trypanosomiasis by degrading host proteins (Authié, 1994). Infections of trypanotolerant cattle with *T. congolense* resulted in an increased IgG response against *Tco*CATL antigen. It was hypothesised that the levels of anti-*Tco*CATL antibodies are directly proportional to levels of resistance in trypanotolerant cattle, and that they lessen the extent of disease in these cattle (Authié *et al*, 1992; Authié, 1994; Authié *et al*, 2001).

1.9 Diagnosis of African animal trypanosomiasis

Successful control of trypanosomiasis depends on reliable detection of the parasites, which is difficult in closely related subspecies (Agbo *et al*, 2003). For a diagnostic test to be considered adequate, it must accomplish a number of criteria such as high diagnostic specificity, sensitivity, simplicity, reproducibility, affordability, availability of expertise and ease of interpretation (Terrestrial, 2013). *T. vivax, T. congolense* and *T. brucei* infection exhibit similar clinical symptoms, hence diagnosis of trypanosomiasis cannot be reliably based only on clinical signs, but require laboratory tests (Holmes, 2013). Diagnosis in the laboratory is achieved through parasitological, molecular and serological techniques.

1.9.1 Parasitological diagnosis

Parasitological diagnosis aims at detection and identification of trypanosome parasites in the infected host blood circulation. The simplest methods include direct visualisation of blood by wet film which identifies motile trypanosomes or by thin or thick smear methods which rely on the detection of trypanosomes based on their morphology using light microscopy. These methods are effective for detecting many animal samples, but are very insensitive (Nantulya, 1990). The insensitivity of these methods was improved by the introduction of haematocrit centrifugation, which concentrates the parasites in the buffy coat layer and this allows simple detection of trypanosomes by microscopy (Ancelle *et al*, 1997; Matovu *et al*, 2012). This method is very sensitive as it effectively detects blood parasites as low as 2.5×10^2 *T. congolense*, 5×10^2 *T. vivax*, and 5×10^3 *T. brucei* parasites per ml of blood (Paris et al, 1988). However, this technique

depends on the availability of electricity, thus cannot be used in the field. Due to lower sensitivity and failure to detect parasites at earlier stages of infection, the development of alternative diagnostic methods such as molecular and serological diagnosis was necessary.

1.9.2 Molecular diagnostic tests

Due to the failure to differentiate two trypanosome species that develop in the same locations in the tsetse e.g. *T. congolense* and *T. simiae,* molecular diagnostic tests have been developed (Gibson, 2009). Effective identification of low parasite copy numbers was enhanced by the introduction of the polymerase chain reaction (PCR) that uses specific oligonucleotide primers to amplify DNA of trypanosome species (Moser *et al*, 1989). The PCR assay showed high sensitivity as it sufficiently identified the mixed genotypes of livestock trypanosomes (including *T. congolense* Savannah, Forest, Kilifi and *T. godfrey*) (Masiga *et al*, 1992). Since the use of several specific primers for PCR is time consuming, a single molecular marker to distinguish different trypanosomal species was attempted. This advanced single PCR utilised multi-copy internal transcribed spacer 1 (ITS1) within ribosomal RNA genes of trypanosomes. This method was able to distinguish a pool of trypanosomal species in a single PCR run because each trypanosomal species constitutes unique sized ITS1 compared to other species, thus the PCR product of each species migrates at a specific size (Desquesnes *et al*, 2001). This method is preferable for diagnosis of trypanosomiasis since this assay is associated with reduced cost and time for conducting a series of species-specific assays. However, this method showed low sensitivity for detection of *T. vivax* (Desquesnes *et* al, 2001), but in a study conducted by Fikru *et al* (2012), a five-fold higher detection efficiency for *T. vivax* as compared to the haematocrit centrifugation technique was reported. The ITS1 PCR assay in a “touchdown’’ format has been identified as a good diagnostic technique for evaluating drug efficacy against *T. congolense* infection in cattle (Tran *et al*, 2014).

A diagnostic assay called PCR-ELISA, which couples PCR for a specific species to ELISA was developed (Masake *et al*, 2002). In PCR-ELISA, nested PCR using specific primers is used for the amplification of DNA, after which the specificity of the PCR products is assessed by coating PCR products on ELISA plates. PCR-ELISA has been effective for the detection of *T. vivax* and *T. brucei,* but not *T. congolense* in peripheral blood of infected cattle. This technique is limited by its high cost that prevents its use in resource poor laboratories in African countries (Masake *et al*, 2002; Cabrera *et al*,

2009). As an alternative to PCR-based diagnostic techniques, the Loop-mediated isothermal amplification (LAMP) has been introduced (Kuboki *et al*, 2003). Although it is an expensive procedure, the LAMP technique amplifies parasite DNA with relatively high specificity, reproducibility and efficiency as compared to PCR-based techniques. Even though this method has been used for the diagnosis of *T. congolense* infections in livestock (Kuboki *et al*, 2003), this method has mainly been used for the diagnosis of human-infecting *T. brucei* parasites (Mitashi *et al*, 2012; Mitashi *et al*, 2013).

1.9.3 Serological diagnosis

Serological diagnosis is an immunodiagnostic technique involving antigen or antibody detection (Luckins, 1977; Nantulya & Lindqvist, 1989). Due to the fact that trypanosome lysis depends on both complement and antibody, it was found that complement fixation is a candidate for trypanosomiasis diagnosis. Indirect immunofluorescence antibody test (IFAT) is another serodiagnostic test. In the IFAT technique, the antigen from parasitised red blood cells is fixed with fixatives such as acetone followed by storage at ultra-low temperatures (Katende *et al*, 1987). The fixed slide is then incubated with antibodies (in serum from cattle with trypanosomal infections). For detection, a secondary antibody such as a rabbit anti-bovine IgG-fluorescein isothiocyanate conjugate is added followed by fluorescence microscopy (Terrestrial, 2013). The IFAT technique is not widely used due to the requirement for several blood smears, ultra-low temperatures for storage and transportation of reagents, the possibility of cross-contamination of samples thus non-specific detection, as well as the need for a sophisticated fluorescence microscope (Nantulya, 1990). In an attempt to improve the IFAT technique, the use of light-emitting diodes (LED) illuminated microscopy has been suggested for replacing fluorescence microscopy. The LED microscope does not require UV light, uses minimal voltage, thus small batteries can be used for field purposes, but this idea is hindered by the need for extensive optimisation of labelling trypanosomal antigens with appropriate fluorophores (Anthony *et al*, 2006; Robertson *et al*, 2009; Mitashi *et al*, 2012). Card agglutination test for trypanosomiasis (CAAT) is a further important diagnostic test and enables detection of surface coat antigens, VSGs. The effectiveness of the CAAT relies on optimal fixing and stabilisation of the parasite using formaldehyde in order to detect the whole trypanosomal parasite during the agglutination test (Nantulya, 1990).

The development of the enzyme linked immunosorbent assay (ELISA) was a major breakthrough since it can be used for large scale testing. The major drawback of

ELISA is that the source of antigen for the test is either whole parasites or crude parasite lysate. ELISA was found to be a suitable diagnostic test for the detection of bovine anti-trypanosome antibodies using both blood stream form and procyclic trypanosomes (Greiner *et al*, 1997). When antigen-capture ELISAs were used for diagnosis of *T. vivax* and *T. congolense* in experimentally infected cattle, the overall specificity was high whilst the sensitivity was very low (Eisler *et al*, 1998). Due to the lower sensitivity of direct ELISA, immunoassay methods for the detection of bovine anti-*T. congolense* and anti-*T. vivax* antibodies were developed. This assay with improved sensitivity and specificity, uses specific ELISA kits, I-TAB ELISA-(*Tc*AGd) and -(*Tv*AGd) where plates were precoated and air-dried with denatured antigen (AGd) from *T. congolense* (*Tc*AGd) and *T. vivax* (*Tv*AGd), respectively. Antigens used in this indirect ELISA were detergent-denatured lysates from bloodstream *T. congolense* and epimastigote *T. vivax* parasites cultured *in vitro* (Rebeski *et al*, 2000; Lejon *et al*, 2003).

To avoid the use of parasite antigens that are cultured *in vitro* and *in vivo*, standardised recombinant expression systems have been used for the production of antigens with diagnostic potential. Targeting non-variant trypanosomal molecules, which are highly antigenic (e.g. heat shock protein 70, HSP70) is associated with improved serodiagnosis (Bannai *et al*, 2003; Bossard *et al*, 2010). The immunodominant antigen HSP70 that is closely related to mammalian immunoglobulin binding protein (BiP), is expressed in all lifecycle stages of *T. congolense*, *T. vivax* and *T. brucei* (Boulangé & Authié, 1994). In an inhibition ELISA format, recombinant HPS70/BiP was used as an antigen for antibody detection in sera from cattle with natural or experimental *T. congolense* infections. The secondary infections were detected at higher sensitivity than primary infections (Boulangé & Authié, 1994; Boulangé *et al*, 2002; Bossard *et al*, 2010). A proteomic approach has been used for identifying trypanosomal antigens with diagnostic potential (Sullivan *et al*, 2013). A flagellar-associated protein, TvGM6 from *T. vivax*, was recombinantly expressed in an *E. coli* expression system and used in an indirect ELISA for the detection of antibodies in sera from cattle with *T. vivax* infections (Pillay *et al*, 2013b). A protein, Tc38630, from *T. congolense* has been identified as an invariant surface glycoprotein with diagnostic potential. This protein was recombinantly expressed in *E. coli* and used in an indirect ELISA for the detection of *T. vivax* infections. High sensitivity and specificity was reported (Fleming *et al*, 2014).

Immunodominant proteases that are involved in the pathogenesis of trypanosomes can serve as serodiagnostic antigens and can be produced using recombinant technology. Cysteine proteases have been considered as virulence factors released in the host

bloodstream upon parasite lysis, and these proteases have diagnostic potential (Caffrey & Steverding, 2009). A cysteine protease *Tco*CATL from *T. congolense* is an example of a virulence factor with diagnostic potential (Authié *et al*, 1992; Authié *et al*, 2001; Lalmanach *et al*, 2002). For effective diagnosis of *T. vivax* infections, it is necessary to evaluate the potential of *Tvi*CATL as a diagnostic antigen. Immunodiagnostic tests based on trypanosomal antigens either detect the antibodies produced in the infected host or the antigen released into the bloodstream following parasite lysis. Both antibody (inhibition ELISA) and antigen detection require specific antibodies produced in experimental animals. For the production of polyclonal antibodies, egg yolk immunoglobulin (IgY) technology represent a preferred method that offers advantages with respect to animal care, cost and antibody yield (Polson *et al*, 1980). Due to the phylogenetic distance between chickens and parasites, parasite antigens are highly immunogenic in chickens, and antibody production in hens is readily stimulated (Bizhanov *et al*, 2004). Recombinant trypanosomal antigens such as ISG65 and ISG75 (Baiyegunhi, 2013), oligopeptidase B, pyroglutamyl peptidase and the cysteine protease *Tco*CATL from *T. congolense* (Eyssen, 2014), have been reported to be very immunogenic in chickens as evidenced by antibodies with high signals in ELISA. The chicken anti-trypanosomal antibodies produced in the study conducted by Eyssen (2014) were useful for diagnosis of AAT using an antibody detection inhibition ELISA format as well as an indirect antibody detection ELISA.

An alternative way of producing antigen detection reagents for use in immunodiagnostics is through identification of single chain variable fragments (scFvs) of antibodies that recognise the diagnostic antigen of interest. Phage display technology is used to this end as will be discussed in Section 1.12.

1.10 Classification of cysteine proteases

Proteases, also called peptidases are peptide-hydrolysing enzymes. Proteases are classified according to the initial position at which they cleave the peptide substrate. The proteases that cleave within the polypeptide chain are called endoproteases, while those cleaving the peptide towards the amino or carboxyl ends are called exoproteases (Sajid & McKerrow, 2002). Based on which reactive residues are found in the active site, these enzymes are further categorised into seven main groups: serine, cysteine, aspartic, metallo, threonine, glutamate and asparagine proteases (Rawlings *et al*, 2011).

Proteases are grouped into specific clans based on their evolutionary relationships. A number of characteristics such as sequence similarity and substrate specificity, are considered in order to assign proteases to a specific clan (Sajid & McKerrow, 2002). Cysteine proteases are divided into ten clans (CA, CD, CE, CF, CH, CL, CM, CN, CO and unassigned clan A-, C-, M-, S-, T-, or U- family) (Rawlings *et al*, 2012). Clan CA is the best documented clan for the parasitic cysteine proteases and belong to the papain superfamily of the proteases (Barrett & Rawlings, 2001; McKerrow *et al*, 2006). Clan CA is further divided into subfamily C1 [i.e. plant cysteine proteases (papain) and mammalian lysosomal cysteine proteases (cathepsin B-like and cathepsin L-like)] and subfamily C2 (calpain-like) (Sajid & McKerrow, 2002). Family C1 is the best characterised since it contains the plant enzyme papain, which was the first cysteine protease to be characterised and its structure solved. An enormous number of recently discovered proteases exhibit features similar to papain, and thus are called papain-like proteases. Trypanosomal cysteine proteases express C1 proteases related to mammalian cathepsin B and L, and are therefore referred to as cathepsin B- and L-like proteases (Mottram *et al*, 2003). However, a new nomenclature for naming cathepsin B-like and cathepsin L-like peptidases has been proposed so that they are now commonly called CATB and CATL, respectively (Clayton *et al*, 1998; Caffrey & Steverding, 2009). This nomenclature replaces the preceding nomenclature proposed by Cazzulo *et al* (1990) whereby the papain-like cysteine proteases were named by adding suffix '-pain' or '-ain'. For example, cruzain from *T. cruzi*, congopain from *T. congolense* and vivapain from *T. vivax*. For the proposed new nomenclature, the first italic capital letter indicates the genus followed by two italic lower case letters indicating the species. For example, *Tco*CATL for the CATL of *T. congolense* and *Tvi*CATL for the CATL of *T. vivax*.

1.11 Structural features and substrate specificity of CATL-like cysteine proteases

CATL-like proteases have structural features common to most papain-like proteases (Lecaille *et al*, 2001). The structure (Figure 1.5, A) of the CATL-like proteases, including *Tcr*CATL and *Tco*CATL homologues, fold into two domains; an α-helical L-domain and R-domain rich in antiparallel sheets forming a β-barrel-like motif. The catalytic triad consisting of conserved Cys25, His159 and Asn175, is found in the cleft between these two domains. The active site Cys25 is located in the central helix of the L-domain opposite to the R-domain containing His159, and these residues are partially aligned to form a nucleophilic ion pair that is stabilised by the third active site residue,

Asn175. Each domain contains two loops connected by disulfide bonds (Lecaille *et al*, 2001).

CATL-like proteases are synthesised as inactive zymogens as characterised by the presence of the proregion or propeptide (Figure 1.5, B). These inactive forms are activated to mature forms by the cleavage of the N-terminal peptide which is conferred through the exposure to the acid endosome. The synthesis of cysteine proteases as precursors is associated with many valuable benefits. These include proregions acting as intramolecular chaperones to enhance protein folding, regulate protease activity by blocking the active site, and as a signal that directs proteases to appropriate intracellular compartments (Lalmanach *et al*, 1998; Wiederanders, 2000). The propeptide contains a highly conserved helical ERFNIN motif found in all cathepsin L-like cysteine proteases. This motif is thought to be crucial for transport and maturation of cysteine proteases (Karrer *et al*, 1993). The trypanosomal propeptide of cysteine proteases also contains a conserved inhibitory YHNGA motif, but this motif is absent in mammalian lysosomal cathepsins (Lalmanach *et al*, 1998). Trypanosomal cysteine proteases such as *Tcr*CATL and *Tco*CATL contain a C-terminal extension which is not present in mammalian CATL proteases (Aslund *et al*, 1991; Rosenthal, 1999). The C-terminal extension of *Tcr*CATL contains two asparagines which are considered as potential Asn-glycosylation sites (Cazzulo *et al*, 1997; Gea *et al*, 2006). The C-terminal extension of *Tco*CATL is linked to a catalytic domain by a proline-rich hinge region while that of *Tcr*CATL is linked by a threonine-rich hinge region (Lalmanach *et al*, 2002). The presence of the C-terminal extension in most trypanosomal CATLs confers relatively high antigenicity and immunogenicity (Cazzulo & Frasch, 1992). The C-terminal extension of plant cysteine proteases has been shown to play a fundamental role in zymogen activation, catalytic activity, folding and stability of the protease (Dutta *et al*, 2011).

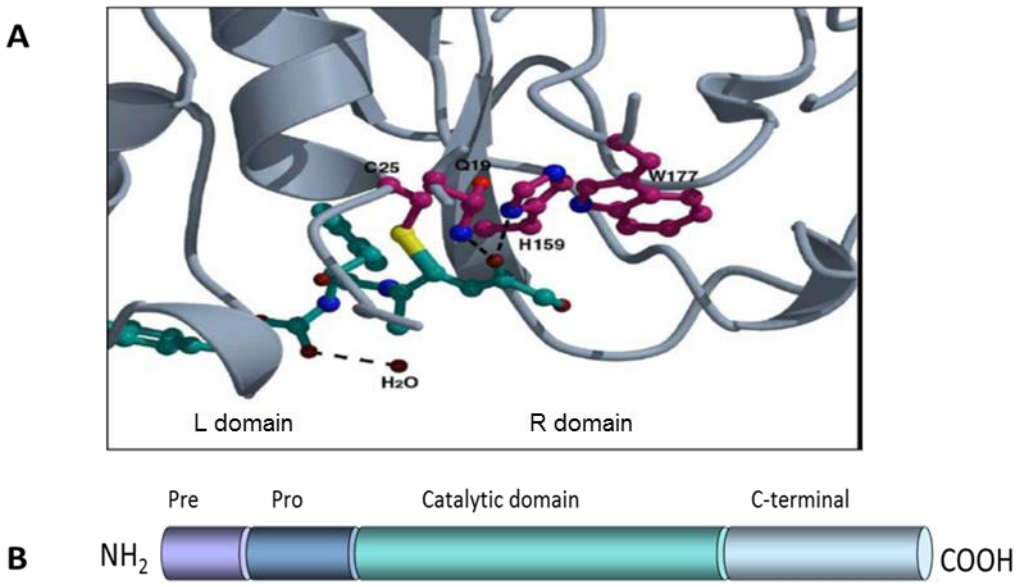

Figure 1.5: A, The ribbon representation of *Tcr*CATL active site in complex with an inhibitor (in ball-and-stick representation). The domains fold into an L-domain rich in α-helices and an R-domain rich in antiparallel β-sheets. The active site Cys25 residue is found in the interior of these domains while His159 is found in the R-domain (Choe *et al*, 2005). **B**, the schematic representation of the CATL-like protease proteins from N to C terminus. The catalytic domain is flanked between the pre-pro and the C-terminal domain, thus forming a zymogen (Rodrigues *et al*, 2014).

The substrate specifically binds in the active site cleft which contains seven subsites (S) each limited to bind only one specific amino acid residue of the peptide substrate (P). There are more than four subsites (…S_4-S_3-S_2-S_1) towards the N-terminal side of the active cleavage site and three subsites towards the C-terminal site (S_1', S_2', S_3'). The protease active N-terminal (…S_4-S_3-S_2-S_1) and primed C-terminal subsites (S_1', S_2', S_3') are complementary to peptide substrate amino acid residues (….P_3-P_2-P_1) and primed peptide substrate residues (P_1', P_2', P_3'), respectively (Schechter & Berger, 1967; Smooker *et al*, 2010) (Figure 1.6). Cysteine proteases must first bind their specific peptide substrates in the active site in order to efficiently hydrolyse the peptide bond (Sajid & McKerrow, 2002). The P_2 residue which is complementary to the active site S_2 pocket, exhibits a very pronounced interaction with both L and R domains (Sajid & McKerrow, 2002; Turk *et al*, 2012). Therefore, the substrate specificity of the majority of CATL-like cysteine proteases is defined by the S_2 pocket. The trypanosomal proteases including *Tcr*CATL and *Tco*CATL prefer bulky hydrophobic amino acids such as phenylalanine in P_2 (Eakin *et al*, 1992; Nery *et al*, 1997; Pillay *et al*, 2010). *Tcr*CATL exhibits a dual specificity for both basic (Arg) and hydrophobic residues (Phe). This is attributed by the presence of Glu205 at S_2 of *Tcr*CATL which is

responsible for adjusting or rotating the conformation of the S_2 pocket in such a way that it is flexible to accommodate both hydrophobic and basic residues (Gillmor *et al*, 1997; Cazzulo, 2013). Conversely, *Tco*CATL exhibits restricted specificity for Phe residues at P_2. This is ascribed to the fact that at the bottom of the S_2 pocket of *Tco*CATL, there is Leu205 (instead of Glu205) which restricts the binding capacity of the S_2 pocket (Chagas *et al*, 1997; Gillmor *et al*, 1997). It has also been reported that the presence of conformational constrained substrate analogs (e.g. Dansyl-Xaa-Arg-Ala-Pro-Trp, where Xaa= nonproteogenic Phe analogs in P_2 position) results in an incorrect binding into the S_2 subsite of *Tco*CATL and this suggested that the hydrophobic Phe group is crucial for efficient binding and hydrolysis of the substrate (Lecaille *et al*, 2001).

1.12 Mechanism of peptide hydrolysis by cysteine proteases

The active sites of cysteine proteases contain Cys25 and His159 residues which are crucial for hydrolysis of the peptide substrate. The active site also contains Asn175 which is responsible for positioning the imazidole ring of histidine so that it is properly orientated for optimal peptide hydrolysis. The active site cysteine residue initiates the peptide hydrolysis by nucleophically attacking the carbonyl carbon of a peptide bond (Figure 1.7). At weak to acid pH, the imazidole group of histidine deprotonates the SH group of cysteine, thus producing a nucleophilic intermediate referred to as a thiolate/imidazoium ion pair. This ion pair intermediate then attacks the carbonyl carbon of the susceptible peptide bond in order to form a tetrahedral intermediate which is stabilised by an oxyanion hole (Otto & Schirmeister, 1997; Lecaille *et al*, 2001). Following the acylation of the enzyme to release $R'NH_2$, an acyl-enzyme is produced. Then, during the deacylation step, the acyl-enzyme reacts with a water molecule to release a second product, thus resulting in a cleaved substrate (as a free acid) and a regenerated enzyme (Lecaille *et al*, 2001; Sajid & McKerrow, 2002).

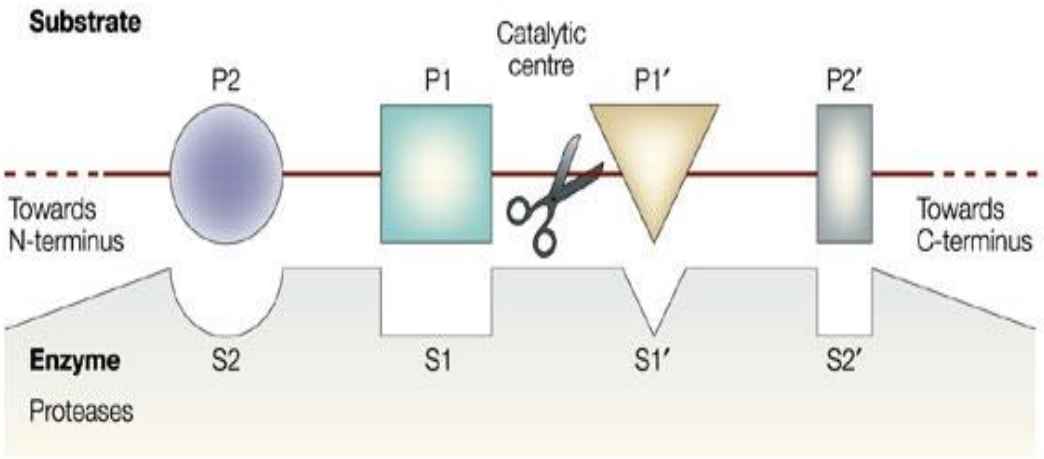

Figure 1.6: The Schechter and Berger schematic representation of the interaction of the peptide substrate with the active site of cysteine protease. Peptide (P) substrate with its N- and C-terminus indicated, interacts with protease sub-sites (S). The peptide substrates are complementary to the sub-sites of the proteases, and this allows correct subsite fitting. The residue in the P_2 position is strictly bulky hydrophobic in the case of *Tco*CATL. Sulfhydryl (SH) of the active site cysteine attacks the peptide bond during hydrolysis. Accessed on 05-June-2014 from http://www.nature.com/horizon/proteases/background/ figs/prespective_f1.html.

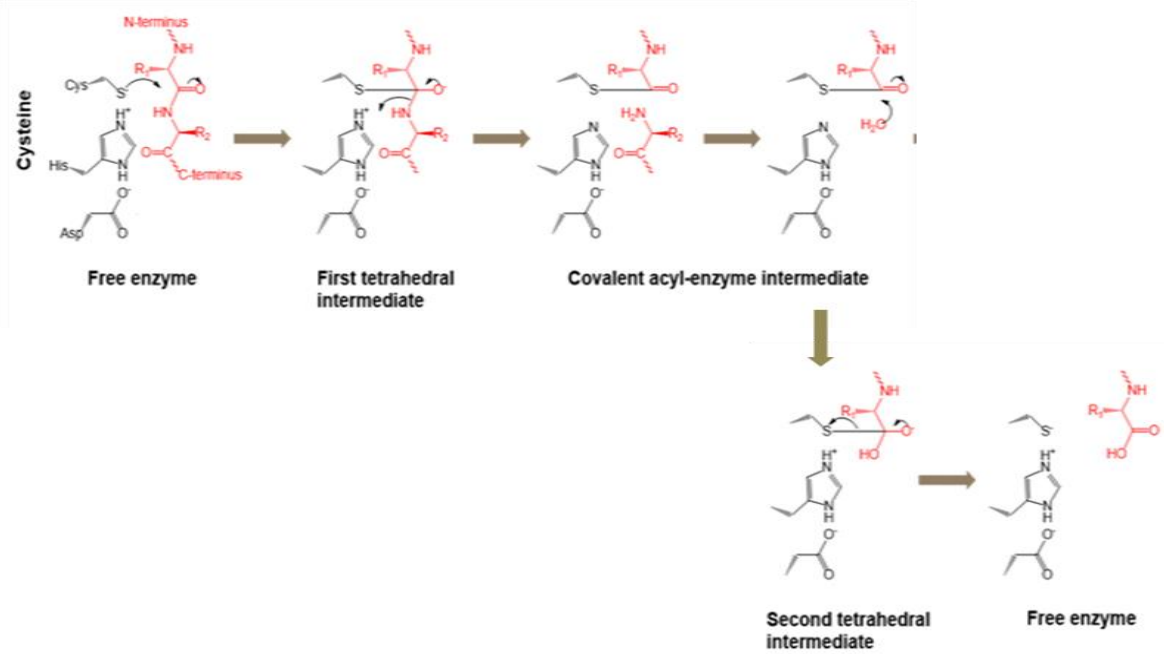

Figure 1.7: Schematic representation of the mechanism of peptide hydrolysis by cysteine proteases. Accessed on 05-June-2014 from http://en.wikipedia. /wiki/Catalytic_triad.

1.13 Phage display technology

Phage display technology is a molecular technique whereby specific libraries (peptides, proteins and antibody fragments) are expressed on the surface of the filamentous phage particle. The specific binding properties allow specific *in vitro* screening of a target (e.g. antibody fragment that recognises a trypanosomal antigen) from a pool of variants (antibody fragments with different affinities or specificities) using a method called bio-panning (Smith, 1985; Winter *et al*, 1994; Kehoe & Kay, 2005).

1.13.1 The biology of the filamentous phage

Filamentous phages are viruses that infect prokaryotic Gram-negative bacteria such as *E. coli* TG1. The fi, M13 and fd phages, collectively called Ff (filamentous) phages share 98% genome identity and are most commonly used for phage display technology. The main feature of the Ff phages is that they are circular with a fixed diameter of 6 nm. The length varies slightly and depends on the genome size. The phage is encapsulated by five coat proteins namely: pIII, pVIII, pVI, pIX and pVII (Figure 1.8), and within these coat proteins, the circular single stranded (ss) DNA is enclosed. The ssDNA is crucial since after replication, it is converted to double stranded DNA which serves as a template for the synthesis of viral coat proteins and the single stranded DNA progeny (Smith, 1985; Sidhu, 2000; Pande *et al*, 2010). These coat proteins are categorised into the major coat protein (pVIII) which extends along the entire length of the phage, and the minor proteins (pIII, pVI, pIX and pVII) which are located at the edges of the phage. Of the five coat proteins, only the minor coat protein pIII (406-residues) and pVIII (50 residues) are commonly used for antibody display libraries (Kehoe & Kay, 2005). However, pVIII is not preferable because it only allows the insertion of short peptides (6-8 residues) and attempts to insert larger peptides results in compromised phage assembly (Greenwood *et al*, 1991; Iannolo *et al*, 1995) .

The pIII coat protein is a more feasible and tolerant coat protein that can allow insertion of proteins longer than 100 residues without compromising phage assembly, and therefore is commonly used for antibody display (Kretzschmar & Geiser, 1995). When a foreign gene coding for the specific peptide was inserted into a virus pIII gene, the immunogenicity of the resultant peptide-pIII fusion was enhanced. The Ff phages initiate their infection by adhering via N-terminal residues of the pIII to the tip of the bacterial F-pili (Deng & Perham, 2002). The Ff phage does not kill the bacteria; instead

it uses a non-lytic mode which ensures that more virions are generated that outcompete the growth of the bacteria, thus resulting in turbid plaques when plated (McCafferty *et al*, 1990; McLaughlin *et al*, 1996).

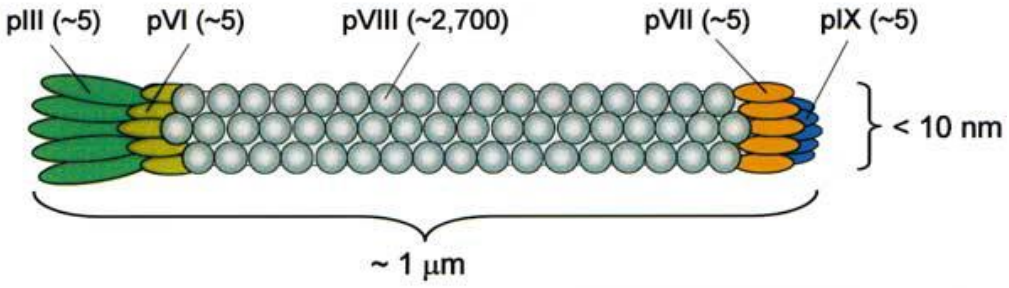

Figure 1.8: The schematic presentation of assembly of the M13 phage coat proteins. The M13 phage single stranded DNA is encapsulated by ~ 2 700 copies of pVIII major coat protein and ~5 copies of each of pIII, pVI, pVII and pIX minor coat proteins (Willats, 2002).

1.13.2 Vectors for phage display technology

Phage and phagemid vectors are commonly used for displaying foreign proteins on the surface of the bacteriophage. Phagemid vectors are preferable for antibody phage display libraries because they exhibit high transformation efficiency, genetic stability and results in monovalent binders (high affinity) (Clausen *et al*, 1992). Phagemids are not complete filamentous phages because they lack other structural and non-structural genes (Lowman *et al*, 1991; Armstrong *et al*, 1996). To generate phagemids which display more of the phage characteristics, and thereby create conditions conducive for bacterial infectivity, a helper phage is introduced (Sahin *et al*, 2014). The M13KO7 helper or 'phage rescue' provides all the coat proteins required for phage assembly. The helper phage is a mutated form of Ff, where a dysfunctional packaging signal and origin of replication is produced. The mutation in its origin of replication ensures limited packaging of the complete genome, i.e. only incorporates a favourable pIII gene (Barbas *et al*, 1991). The dysfunctionality of the packaging signal of the helper phage does not compromise the infectivity of the helper phage towards the bacterial cells. However, once the phagemid is introduced in the system, a phagemid will be assembled in preference to the helper phage because the phagemid carries an optimal packaging signal. The phagemid carries a kanamycin resistance gene which allows growth of helper-infected cells. The phagemid system is considered both phenotypically and genotypically heterologous because the minor coat protein III is

derived either from wild type helper phage or recombinant phagemid (Marks & Bradbury, 2004).

The phagemid vectors are derived from Ff particles and exhibit typical vector characteristics as shown in Figure 1.9 (Qi *et al*, 2012). This includes the coat protein, molecular tags, restriction enzyme recognition sites, origin of replication and a signal peptide. Phagemids are categorised based on the type of coat proteins they exploit for phage display libraries. Therefore, phagemids are mainly divided into type III and type VIII phagemids since these coat proteins are extensively used in phage display. These vectors differ in the length of foreign proteins they can accommodate, resultant copy number and mechanism of infection (Barbas *et al*, 1991).

As mentioned before, type III phagemids accommodate larger proteins than type VIII phagemids. Type III only results in fewer than 5 copies of proteins, whilst type VIII yields more than 1000 copies of foreign proteins and this limits the length of foreign proteins that can be displayed via type VIII phagemids. In addition, these high copy numbers in VIII enhances an avidity effect rather than the desired binding affinity (Smith, 1993; Iannolo *et al*, 1995). Therefore to display antibodies with high affinity, type III phagemid are preferable notwithstanding its low copy numbers (Kretzschmar & Geiser, 1995), while VIII are selected when desiring to display antibodies with multiple affinities (avidity). To ensure the solubility and homogenous purification of the phage displayed foreign proteins, the molecular tags or amber codon tags are included through site-directed mutagenesis in the phagemid between the coat and foreign protein sequences (Qi *et al*, 2012). Restriction enzyme recognition sites incorporated after the foreign protein inserts are there to initiate the digestion of the coat protein from the phagemid in order to ascertain the synthesis of soluble proteins in the periplasm or cytoplasm (Geiger *et al*, 2011). The origin of replication remains inactive until helper phages are introduced in the bacterial cell environment (Iannolo *et al*, 1995).

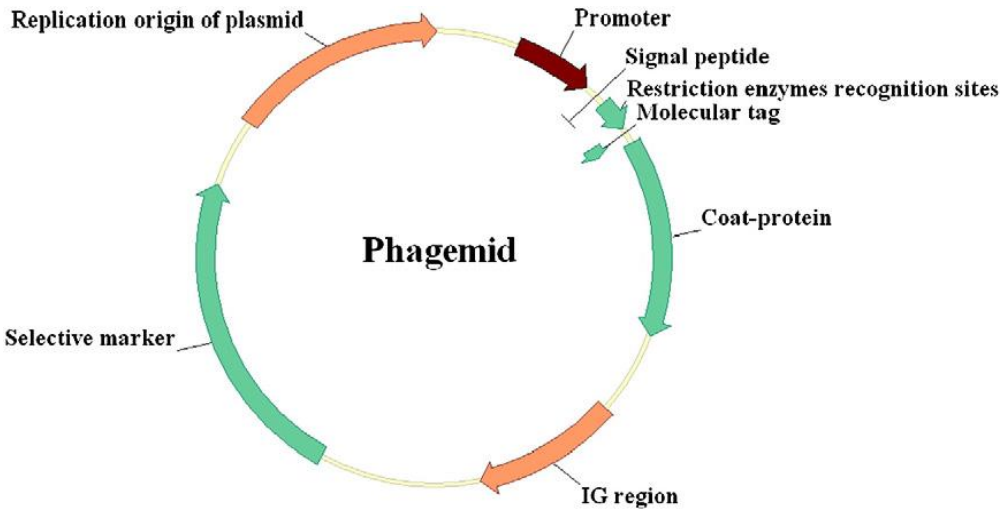

Figure 1.9: Phagemid vector used for displaying foreign proteins on the filamentous bacteriophage (Qi *et al*, 2012)**.**

1.13.3 The concept of antibody scFv fragments in phage display technology

Antibodies comprise two 50 kDa heavy and two 25 kDa light peptide chains (Figure 1.10). Each heavy chain is linked to a light chain via an inter-chain disulfide bond. This linkage forms a fragment antigen binding (Fab). Heavy chains are encoded by variable (V_H), diversity (D), joining (J) and constant (C_H) gene regions, while the light chain is encoded by V_L, J_L, and C_L regions. The interaction between heavy chain V-D-J regions and light chain V-J regions forms an antigen-binding site (paratope) which is specific for a single epitope. The variable regions in both heavy and light chains (V_H and V_L) consist of three hyper variable or complementary determining regions (CDRs) which differ greatly in primary structure. The CDRs form an antigen-binding site where they interact with the antigen.

Following papain digestion, antibody molecules are cleaved into two antigen-binding fragments called Fab and Fv (Porter, 1959). Fab (fragment antigen binding) consists of a V_H-C_H segment disulfide-bonded to its counterpart V_L-C_L segment. The smaller Fv (fragment variable) consists of V_L and V_H regions only. The recombinantly expressed form of Fv is called single-chain variable fragment (scFv). The scFv consist of two variable regions that are joined together by a peptide linker $(Gly_4Ser)_3$. This linkage results in the formation of the antigen-binding site.

For the phage display library construction using phagemid vectors, scFv fragments in-frame with gene III are routinely used compared to Fab fragments (Barbas *et al*, 1991). The scFvs are smaller in size, thus result in libraries that are more stable and efficiently

assembled in the phage particle compared to Fab libraries which encode for a larger sized DNA. Fabs are prone to degradation and yield fewer soluble fragments. However, the main disadvantage of the scFvs is that the short length $(Gly_4Ser)_3$ peptide linker promotes the interaction of V_H of one scFv with V_L of another, thus resulting in higher molecular weight products such as dimers (Holliger *et al*, 1993).

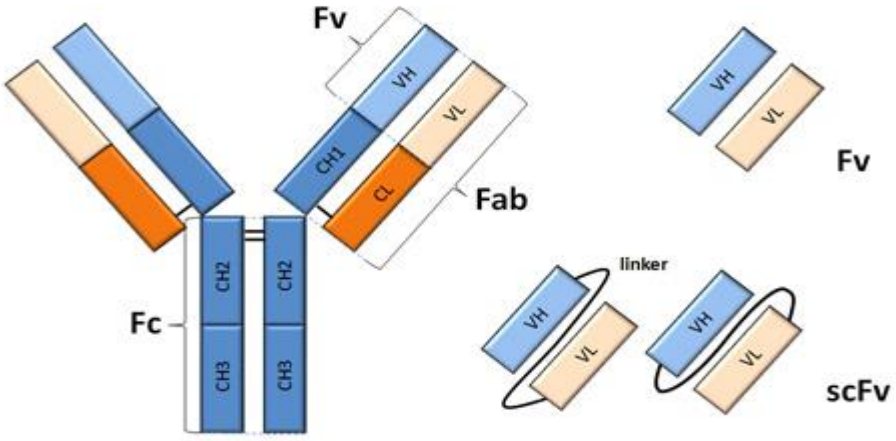

Figure 1.10: Structural representation of Y-shaped IgG antibody molecule with associated fragments commonly used in antibody phage display. The fragment variable [Fv is made up of variable heavy (light blue) and variable light (light pink) chains], fragment crystallisable (Fc is in dark blue), fragment antigen binding [Fab is made up constant light (orange) and variable light (light pink) chains] and single-chain variable fragment (scFv is made up of variable heavy chain disulphide bonded to the variable light chain. Accessed on 30-November 2014 from http://www.abdesignlabs.com/technical-resources/scfv-cloning/

1.13.4 Types of phage antibody display libraries

There are two different types of libraries that have been constructed for antibody production. These include immunised and non-immune libraries. The immunised phage library constitutes monospecific antibodies produced by immunising the animal with a particular target antigen, and therefore, are called immune “reptoires” or antigen biased. These immune libraries have been constructed from a number of species such as mouse (Chester *et al*, 1994), humans (Barbas III, 1993) and rabbit (Lang *et al*, 1996). Antibodies generated through immune phage library are mainly used to treat infectious diseases (Hoogenboom *et al*, 1998; Sun *et al*, 2009). There are two main advantages of immune libraries: they result in antibodies that are antigen-specific regardless of the relatively small library (~10^5 clones) that is generally used for

selection and the donor's immune system enhances affinity maturation thus resulting in a large number of high affinity antibodies (Clackson *et al*, 1991). The main drawbacks of this library are: it is not possible to manipulate the immune response in order to attain antibodies with desired characteristics, due to immune tolerance; it is impossible to produce antibodies against self-antigens and donors are susceptible to toxicity of foreign antigens. Other drawbacks of immune libraries is that it is laborious and time consuming because in order to produce antibodies with high antigen specificity, a new phage-antibody library must be constructed (i.e. repeat animal immunisation) for each and every unique antigen (Azzazy & Highsmith Jr, 2002).

Non-immune libraries are subdivided into naïve, semi-synthetic and synthetic libraries. These libraries are collectively called "single-pot" libraries because they are constructed in such a way that their antibody binding fragments are accessible to almost any antigen (Hust & Dübel, 2004). The naïve and synthetic antibody libraries are differentiated based on the type of immunoglobulin genes they are constructed from. The naïve phage libraries are constructed from rearranged heavy and light chain V genes isolated from IgM mRNA of B cells isolated either from bone marrow, tonsils or blood lymphocytes of non-immunised donors (Marks *et al*, 1991; Hust & Dübel, 2004). The naïve phage libraries are advantageous in that they allow selection of almost any antigen of interest (Holt *et al*, 2000). A variety of antibodies against self-antigens (e.g. tumour necrosis factor) or foreign and toxic antigens (e.g. hapten, bovine serum albumin and doxorubicin) have been quickly (~2 weeks) screened from naïve libraries (Nissim *et al*, 1994; Vaughan *et al*, 1996). The main drawback of these libraries is that in order to obtain antibodies with high affinities, larger libraries ($1x\ 10^{10}$) must be constructed (Vaughan *et al*, 1996).

Synthetic library construction relies on the predetermination of the information regarding antigen-antibody interaction, affinity maturation and arrangement of variable regions (Goletz *et al*, 2002; Barderas *et al*, 2008; Volpe & Kepler, 2008). The complementarity determining regions (CDRs) which determine the specificity of antibodies are used to design synthetic libraries (Knappik *et al*, 2000). The V_H-CDR3 is more variable in amino acid composition, length and is situated in proximity to the antigen binding site as compared to the other two CDRs with limited amino acid variability and located further from the antigen binding site. Therefore, for synthetic library construction, V_H-CDR3 is randomised by site-directed mutagenesis which allows substitution of certain amino acids at specific positions along the CDR3 frame. This randomisation allows the manipulation of the sequence to generate the tertiary structures comprising enhanced specificity-determining residues (Almagro, 2004). The

major advantage of the synthetic library over a naïve library is that it is diverse and adjustable to obtain amino acid properties which favour the production of high affinity antibodies.

Nkuku® phage antibodies are antibodies derived from a large semi-synthetic chicken scFv library (Nkuku® library) (Van Wyngaardt *et al*, 2004). The ease of accessibility of chickens makes them a preferred source of recombinant antibodies compared to donors such as rabbits, camels and mice. In order to clone a gene from other sources like human, substantial effort is needed. For instance, in order to amplify V genes from human donors, a number of PCR primers are required. In chickens, immunoglobulin diversity is generated easily by VJ rearrangements in the light chain and VDJ rearrangements in the heavy chain. Once rearrangement is completed, variability is introduced by the incorporation of the pseudo V gene (Davies *et al*, 1995). The diversity in chicken antibodies attained by pseudo gene conversion means that all chickens contain conserved amino acid sequences at both 3' and 5' termini of light and heavy chains. Therefore, the diverse phage antibody library is constructed using a single set of primers for both the heavy and light chains (Andris-Widhopf *et al*, 2000).

1.13.5 Selection of binders by bio-panning and screening

In order to select for phage clones that specifically recognise the desired antigen, a technique called bio-panning is used. During bio-panning, the phage library is *in vitro* exposed to the immobilised antigen. Just like in ELISA, the non-binding clones are removed from positive binding clones by subsequent washes in appropriate buffers such as HCl or glycine and triethylamine buffers. Bound phages are then amplified in *E. coli* cells. Bio-panning rounds are repeated 2-4 times using precipitated phages from the preceding bio-panning round (e.g. phages obtained from round 1 are used for panning against the immobilised antigen during round two of panning) in order to enhance binding affinity (during the next panning round) to the target and therefore enrich for specific positive phage clones (Azzazy & Highsmith Jr, 2002; Willats, 2002)

There are various *in vitro* methods that can be used to select for specific binders using bio-panning. The common selection method is panning based on binding affinities, i.e. the antigen of interest is immobilised on plastic tubes such as immunotubes, ELISA plates or columns (Marks *et al*, 1991). Selection on immunotubes is associated with limitations such as the need for a pure protein and high avidity limits the selection of specific positive clones (Griffiths & Duncan, 1998). The problems associated with panning using immobilised antigens led to the development of a selection method that

uses biotinylated antigens which are selected in solution as a result of high affinity of biotin for streptavidin-coated beads (Hawkins *et al*, 1992). Panning using biotinylated antigens is advantageous due to its efficacy to select native proteins without disrupting the protein conformation and ability to optimise the concentration of the antigen to use in order to select for high affinity binders without a need to perform many panning rounds (Azzazy & Highsmith Jr, 2002). Panning is also performed by selection on adherent cells in which antigen specific antibodies are selected using cell surface markers containing antigen of interest grown as monolayers or in suspension (Hoogenboom *et al*, 1999). Also, an *in vivo* selection method is used for panning whereby the phage clones are injected in animals in order to allow for the host immune system to raise antibodies against the dominant phage clones.

Once biopanning is completed using one of the methods mentioned above, polyclonal phage ELISA is used to screen for any positive binders. The polyclonal ELISA is then converted to mono-specific phage ELISA by allowing phages from the promising panning round to infect *E. coli* cells. This mixture is then plated on the agar plate followed by picking up single colonies in order to screen for enriched mono-specific antibodies against the antigen of interest (Kretzschmar & von Rüden, 2002).

1.14 Aims and objectives of the present study

The primary aim of the present study is to investigate the potential of a cysteine protease, *Tvi*CATL from *T. vivax*, to serve as a target for the development of chemotherapeutic drugs and a point of care species-specific serodiagnostic tool which are affordable to end-users. *Tvi*CATL shares high sequence identity with *Tco*CATL, the homologue from *T. congolense* that has been shown to participate in pathogenesis of *T. congolense* upon release in the host bloodstream by dying parasites. Presumably, *Tvi*CATL is likewise involved in the pathogenesis of *T. vivax*.

Therefore the first objective is to determine the enzymatic characteristics of *Tvi*CATL. To attain this, it will be necessary to get sufficient quantities of the enzymatically active protein by recombinantly expressing the catalytic domain of *Tvi*CATL in the *P. pastoris* yeast expression system and purifying the protease to homogeneity. The pure protease will be used to determine the enzymatic characteristics such as pH optimum, hydrolysis of host proteins and fluorogenic peptide substrates and the interaction with

both competitive reversible and irreversible inhibitors. These results are presented in Chapter 2 of this dissertation.

The second objective is to raise antibodies for conducting a serological antibody-detection test using an inhibition ELISA format. To this end antibodies will be raised in chickens through immunisation with recombinant *Tvi*CATL. Immunoglobulin Y (IgY) will be isolated from the chicken egg yolks. Antibody production and titres will be tested using enzyme-linked immunosorbent assays (ELISAs), dot and western blots. The antibodies will be affinity purified using a *Tvi*CATL-Aminolink® resin. The species-specificity of the antibodies will be determined using an inhibition ELISA to detect IgG in serum from cattle experimentally infected with *T. congolense*. As an alternative to animal-based antibody production, antibody phage display will be attempted. A large semi-synthetic chicken scFv library called Nkuku® phage library will be used to select positive clones for *Tvi*CATL through biopanning. The polyclonal phage ELISA will be used to monitor the presence of phage pools after each successive round of panning. Lastly, the mono-specific phage ELISA will be used to further screen for enriched specific *Tvi*CATL-scFv antibodies. The results obtained are presented in Chapter 3. Finally, a discussion of all the findings of this study is presented in Chapter 4, the general discussion.

CHAPTER 2

RECOMBINANT EXPRESSION, PURIFICATION AND ENZYMATIC CHARACTERISATION OF THE TRYPANOSOMAL CYSTEINE PROTEASE, *Tvi*CATL, FROM *TRYPANOSOMA VIVAX*.

2.1 Introduction

There is an accumulating evidence for the involvement of *Tco*CATL in the pathogenesis of *T. congolense* (Authié, 1994; Authié *et al*, 2001; Lalmanach *et al*, 2002), but very little has been published about the cysteine protease *Tvi*CATL from *T. vivax*. *Tco*CATL and *Tvi*CATL show 61.81% sequence identity (Figure 2.1); therefore there is a high possibility that *Tvi*CATL is a virulence factor for *T. vivax*. Trypanosomal CATL-like proteases (Figure 2.1) contain a signal peptide, propeptide, a catalytic domain as well as the parasite-specific C-terminal extension. Many trypanosomal cysteine protease recombinant expression constructs are designed in such a way that they contain a propeptide and a catalytic domain (Caffrey *et al*, 2001; Vather, 2010; Boulangé *et al*, 2011). The propeptide is responsible for enzyme stability and folding (Sajid & McKerrow, 2002), while the function of the C-terminal extension has not been determined but it is not required for enzyme activity (Pamer *et al*, 1991; Eakin *et al*, 1993).

Large scale production of recombinant enzymes is of paramount importance for studies focused on determining their biochemical characteristics for the design of specific inhibitors. Both bacterial and eukaryotic expression systems have been exploited for recombinant expression of trypanosomal proteases. Initial attempts at heterologous expression of *Tco*CATL in both *E. coli* and balculovirus expression systems gave poor yields, solubility and protein folding and overall activity were suboptimal (Boulangé *et al*, 2001). Subsequently, high protein yields and activity have been reported when *Tco*CATL was expressed in the *P. pastoris* yeast expression system (Boulangé *et al*, 2011). *Tco*CATL has been shown to be active against the classical cysteine protease peptide substrate, Z-Phe-Arg-AMC, and inhibited by the class-specific inhibitor E-64 and was also shown to digest gelatin substrate. The pH optimum for the hydrolysis of substrates by *Tco*CATL is around physiological pH,

suggesting that the enzyme is active in the host bloodstream upon parasite lysis (Pillay *et al*, 2010; Vather, 2010; Boulangé *et al*, 2011). *Tcr*CATL from *T. cruzi* has been expressed as an inactive, insoluble protein in the *E. coli* expression system. This insoluble fusion protein requires resolubilisation and refolding in urea (Eakin *et al*, 1992; Eakin *et al*, 1993). Inclusion bodies have also been reported when a cysteine protease class B (CPB) from *Leishmania mexicana* is recombinantly expressed in the *E. coli* system (Sanderson *et al*, 2000). Therefore, since *P. pastoris* expression often results in soluble, high yields of active, glycosylated proteins (Caffrey *et al*, 2001; Pillay *et al*, 2010; Vather, 2010; Boulangé *et al*, 2011), this system was used for *Tvi*CATL expression.

In this part of the study, the catalytic domain of *Tvi*CATL (C-terminal extension not included in the construct) from *T. vivax* was recombinantly expressed in the *P. pastoris* yeast system, purified to homogeneity and enzymatically characterised.

Figure 2.1: Sequence alignment of CATL-like proteases from *T. b. rhodesiense* (*Tbr*CATL), *T. congolense* (*Tco*CATL), *T. vivax* Y468 (*Tvi*CATL) *and T. cruzi* (*Tcr*CATL). *Tbr*CATL accession no. CAC67416 (Caffrey *et al*, 2001), *Tco*CATL accession no. CAA81061 (Fish *et al*, 1995), *Tvi*CATL accession no. CCD21670 and *Tcr*CATL accession no. P52779 (Lima *et al*, 2012) sequences were aligned using a Clustal Omega multiple sequence alignment program (Sievers & Higgins, 2014). The N-terminal signal and pro-peptide sequences are grouped within the red-dotted shape; the central catalytic domain residues are grouped within the purple-lined shape; the catalytic residues (C, H and N) are highlighted with a purple arrow: and the C-terminal extension residues (linked to the catalytic domain by a poly-proline sequence) are highlighted within the green-dotted shape. The predicted Asn-glycosylation site (Asn^{288}) for *T. vivax* is blue underlined within the catalytic domain.

2.2 Materials and Methods

2.2.1 Materials

Recombinant expression in *P. pastoris*, protein purification and quantification: Glycerol stocks of *P. pastoris* GS115 cells containing recombinant pPic9-*Tvi*CATL were obtained from Professor Theo Baltz (University of Victor Segalen, Bordeaux, France). HiPrep® 16/60 Sephacryl® S-200 HR pre-packed column and ÄKTA purifier were purchased from GE Healthcare (Sweden). Amicon® Ultra-15 Centrifugal Filter devices were obtained from Merck Millipore (Bedford, USA). Pierce™ BCA Protein Assay Kit and 10 kDa M_r cut-off dialysis tubing were purchased from Pierce (Rockford, IL, USA). Nunc® - Immuno™ 96-well plates and Nunc® Black 96-well plates were purchased from Nunc InterMed (Denmark). The PageRuler prestained or unstained protein ladder was from Thermo Scientific (Lithuania).

Antibodies: Primary antibodies against the recombinant C25A mutant full length inactive form of *Tvi*CATL were previously raised in chickens (Vather, 2010). The rabbit anti-IgY-HPRO conjugate was purchased from Sigma (Munich, Germany).

Protein substrates and inhibitors: Bovine α_2-macroglobulin (α_2-M) was obtained from Boehringer (Mannheim, Germany), bovine haemoglobin, type I collagen from rat tails and bovine serum albumin (BSA) were purchased from Sigma (Munich, Germany).

Peptide substrates and inhibitors: The fluorogenic peptide substrates H-D-Ala-Leu-Lys-7-amino-4-methylcoumarin (AMC), H-D-Val-Leu-Lys-AMC, H-Pro-Phe-Arg-AMC and Z-Gly-Leu-Phe-chloromethylketone (CMK) were purchased from Bachem (King of Prussia, PA, USA). Benzyloxycarbonyl (Z)-Phe-Arg-AMC, Z-Gly-Pro-Arg-AMC, Z-Pro-Arg-AMC, Z-Gly-Gly-Arg-AMC, Z-Arg-Arg-AMC, L-*trans*-epoxysuccinyl-leucylamido (4-guanidino) butane (E64), antipain, chymostatin, leupeptin, bestatin, ethylenediaminetetra-acetic acid (EDTA), iodoacetate (IAA) and iodoacetamide (IAM) were obtained from Sigma (Munich, Germany).

2.2.2 Recombinant expression of *Tvi*CATL in *Pichia pastoris*

*Tvi*CATL was previously cloned into a pPic9 expression vector and the construct preserved as a glycerol stock of *P. pastoris* GS115 cells. The pPic9-*Tvi*CATL glycerol stock was streaked on YPD plates [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v)

dextrose, 15 g/l bacteriological agar] containing tetracycline (50 µg/ml) and incubated at 30°C for 3 days. A single colony of *P. pastoris* was picked from the YPD plate for expansion in liquid YPD (50 ml) containing tetracycline (50 µg/ml). The YPD primary culture was incubated at 30°C in a baffled flask on an orbital shaking incubator (180 rpm) for 2 days. The maximal biomass production was obtained by aseptically transferring YPD primary culture into 500 ml BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6.5, 1.34% (w/v) yeast nitrogen base without amino acids (YNB)]. This secondary culture was grown at 30°C for a further 3 days until an OD_{600} of 4-6 was reached. Cells were collected by centrifugation (6000 x *g*, 10 min, 4°C) and the pellet resuspended in 500 ml buffered minimal medium (BMM) [100 mM potassium phosphate buffer, pH 6.5, 1.34% (w/v) YNB, 0.00004% (w/v) biotin, 5% (v/v) methanol]. The BMM expression culture was grown in a baffled flask, and aeration enhanced by covering the flasks with 3 layers of cheesecloth. The BMM culture was incubated at 30°C in an orbital shaking incubator for 7 days, and expression induced by supplementing the culture with 0.5% (v/v) methanol daily (to compensate for methanol consumption and evaporation) as the sole carbon source. For the duration of expression, samples were taken (1 ml culture) every 24 hours, centrifuged (6000 x *g*, 10 min) and supernatants stored at -20°C for use during the analysis of protein secreted in expression supernatants. After 7 days of expression, the entire volume of expression cultures were centrifuged (6000 x *g*, 10 min, 4°C), and since *Tvi*CATL is secreted into the culture medium, supernatants were stored in plastic vessels at -20°C until further use. Expression supernatants collected on each day of expression were analysed by 12.5% reducing SDS-PAGE and silver stained, Section 2.2.7.

2.2.3 Purification of the recombinant *Tvi*CATL by three phase partitioning (TPP)

The expression supernatants were filtered through Whatman No.1 filter paper and the pH adjusted to pH 4.2 using phosphoric acid to effect autocatalytic processing of *Tvi*CATL (Vernet *et al*, 1991; Eakin *et al*, 1992). In order to concentrate and purify *Tvi*CATL, three phase partitioning (TPP) as described by Pike and Dennison (1989) was used. Tertiary-butanol (t-butanol) was added to the expression supernatant to constitute 30% (v/v) of the total supernatant volume and thoroughly mixed. Ammonium sulfate [40% (w/v) of the final volume of the supernatant plus t-butanol] was added and stirred until entirely dissolved. The mixture was centrifuged (6000 x *g*, 10 min, 4°C) in a swing-out rotor to ensure that all three phases were completely separated. The interfacial precipitate containing *Tvi*CATL was collected and re-dissolved in phosphate

buffered saline (PBS) dialysis buffer [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 1.76 mM KH_2PO_4, pH 7.2] and subjected to dialysis overnight at 4°C using a 10 kDa cut-off dialysis bag (Thermo Scientific Snakeskin® pleated dialysis tubing) against PBS with magnetic stirring. The optimal removal of all traces of the ammonium sulfate and t-butanol was achieved by 3 changes of dialysis PBS buffer. The dialysed sample was concentrated using dialysis against solid polyethylene glycol (PEG) M_r 20 000. The PEG-concentrated TPP samples were quantified using BCA assay (Section 2.2.6) and analysed by a 12.5% reducing SDS-PAGE (Section 2.2.7).

2.2.4 Molecular exclusion chromatography (MEC)

Molecular exclusion chromatography (MEC) was used for further purification of recombinant *Tvi*CATL. The HiPrep® 16/60 Sephacryl® S-300 HR column (16 x 600 mm, flow rate of 0.5 ml/min, 120 ml, GE Healthcare, Sweden) connected to an ÄKTA purifier (GE Healthcare, Sweden) was used. The preparation and storage of the column was as per the manufacturer's instructions. Briefly, the column was equilibrated with two column volumes of MEC buffer [50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0] and calibrated with 1 ml of protein mix containing proteins of known molecular weights. These included 3 mg/ml blue dextran (2000 kDa), 5 mg/ml of each of BSA (68 kDa), ovalbumin (45 kDa) and myoglobin (16.6 kDa). To ensure the optimal resolution of proteins according to size, the sample volume to be loaded on the column never exceeded 2% of the column volume, V_c. The elution volume (V_e) of the higher molecular weight blue dextran was equivalent to the column void volume (V_o). The column calibration curve was constructed by determining the availability constant (K_{av}) of each standard protein using the equation: $K_{av} = (V_e-V_o) / (V_t-V_o)$ and the Fischer's plot (Figure 2.2) to represent the relationship between protein elution volume and its molecular weight in order to estimate the size of the protein with unknown molecular weight (Dennison, 1999).

2.2.5 Concentration of MEC-purified *Tvi*CATL

The pure MEC fractions were pooled and concentrated using Amicon® Ultra-15 Centrifugal Filter devices (with molecular weight cut-off of 10 kDa) (Merck Millipore, Bedford, USA) as per the manufacturer's instructions. Alternatively, dialysis against solid PEG (M_r 20 000) was used to concentrate the pure sample contained within a 10 kDa molecular weight cut-off dialysis bag.

Prior to reducing SDS-PAGE (Section 2.2.7), samples with very low protein concentration were concentrated 5-fold using the SDS-KCl precipitation method. Briefly, sample (100 µl) was mixed with 5% (w/v) SDS (10 µl) followed by 3 M KCl (10 µl). The mixture was inverted and centrifuged (12 000 x *g*, 2 min, RT), the pellet was re-dissolved in stacking gel buffer (10 µl) plus reducing treatment buffer (10 µl).

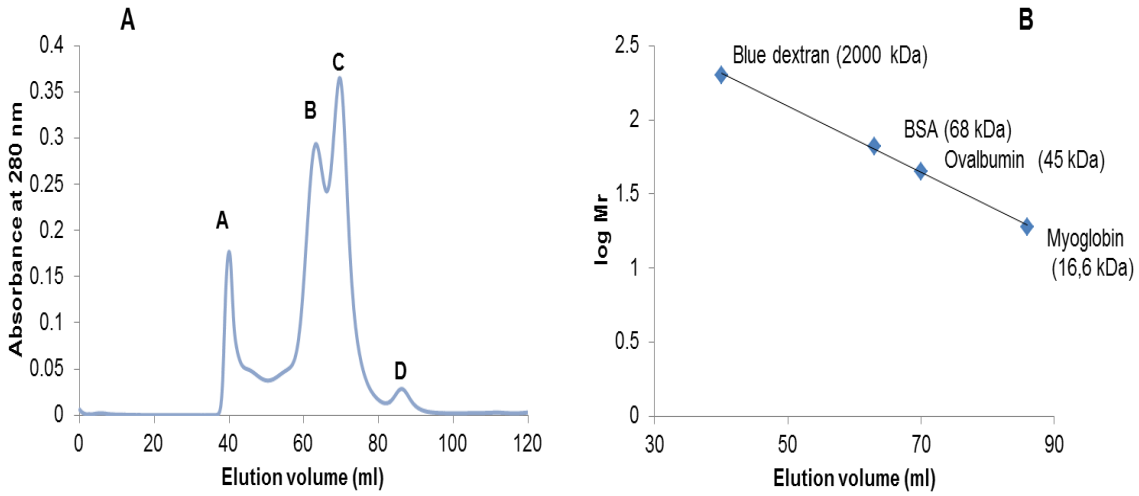

Figure 2.2: Elution profile for the calibration of the MEC column and a Fischer's plot for estimation of protein molecular weights. **A**, the calibration protein mix [Blue dextran (A), BSA (B), ovalbumin (C) and myoglobin (D)] was loaded to the Sephacryl-300 HR column (25 x 650 mm, flow rate of 0.2 cm/min) in MEC buffer [20 mM Tris-HCl-buffer, pH 7.6, 0.02% (w/v) NaN_3]. **B**, Fischer's plot for the estimation of protein molecular weights. The elution of each standard protein was determined and plotted against the protein log M_r in order to construct a calibration curve with the trend line equation y = -0.0223x + 3.2039 and correlation coefficient of 0.9986.

2.2.6 BCA protein quantification assay

The purified recombinant form of *Tvi*CATL was quantified using the bicinchoninic acid (BCA) protein assay (Pierce, Rockford, IL, USA). The BCA assay progresses through a Biuret reaction whereby the Cu^{2+} is reduced to Cu^{+} in the presence of a protein in a basic pH (Lang *et al*, 1996). The rate of formation of the cuprous cation (Cu^{2+}) depends on the number of protein peptide bonds and favours the presence of cysteine, tryptophan and tyrosine residues. This reaction is measured by the formation of a purple-coloured reaction complex that absorbs maximally at 595 nm. Briefly, the Pierce™ BCA Protein Assay Kit comprises BSA standards and reagents A and B. The BSA standards were used to construct a calibration curve (Figure 2.3) by serial diluting BSA (2000-25 µg/ml) in distilled water. The working reagent was prepared by mixing

reagent A and B in a ratio of 50:1 (v/v). Each BSA standard (25 µl) and protein of unknown concentration (25 µl) were pipetted (in duplicate) into a 96 well Nunc® plate, followed by the addition of 200 µl working reagent. The plate was covered and incubated at 37°C for 30 minutes. Colour development was measured at 595 nm using the FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany).

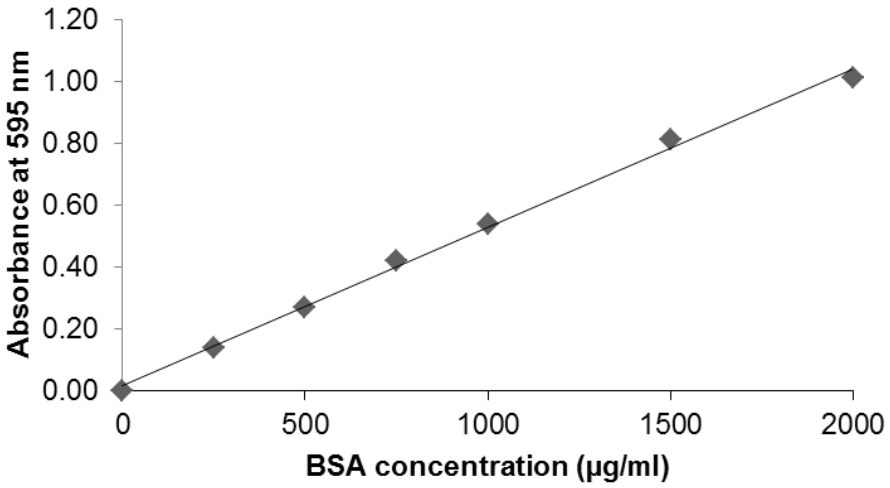

Figure 2.3: The BCA calibration curve for quantification of recombinant *Tvi*CATL. BSA standard solution (2000-25 µg/ml) were combined with BCA working reagent, the absorbance values measured at 595 nm, and a calibration curve was constructed by plotting of absorbance at 595 nm against BSA concentration. The equation of the trend line is y = 0.0005x + 0.0085 with a correlation coefficient of 0.996.

2.2.7 SDS-PAGE analysis

Protein samples were analysed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) described by Laemmli (1970). A discontinuous buffer system was used comprising a running gel buffer (1.5 M Tris-HCl, pH 8.8) and a stacking gel buffer (500 mM Tris-HCl, pH 6.8). The protein samples were combined with the reducing treatment buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol]. SDS-PAGE was conducted at 40 mA per 2 gels in a tank buffer (250 mM Tris-HCl buffer, pH 8.3, 192 mM glycine, 0.1% (w/v) SDS) using the BioRad Mini protein III electrophoresis apparatus (BioRad, CA, USA). After electrophoresis, gels were stained for 4 hours or overnight with Coomassie Blue [0.125% Coomassie Blue R-20, 50% (w/v) methanol, 10% (v/v) acetic acid], destained in destaining solution I [50% (v/v) methanol, 10% acetic acid] followed by complete destaining and gel swelling to original size in several changes of distilled water. In order to determine the molecular weight of protein of unknown size, a set of standard

proteins of known sizes were separated on reducing SDS-PAGE. The measurement of the relative migration distance (R_f) of individual standard proteins, was used to construct a calibration curve (Figure 2.4) in order to determine the molecular weight of protein of unknown size.

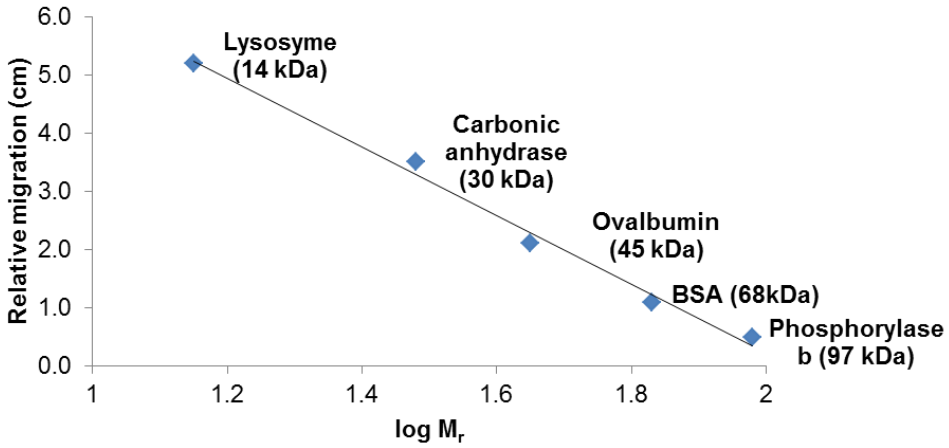

Figure 2.4: Reducing SDS-PAGE relative migration standard curve. In order to estimate the molecular weight of protein of unknown size, the molecular weight marker that comprises proteins of known sizes were separated by 12.5% reducing SDS-PAGE. The relative migration distance was measured and plotted against log M_r. The equation of the standard curve was y = -5.8668x + 11.97 with a correlation coefficient of 0.9916.

In order to visualise very low amounts of protein on SDS-PAGE gels, a very sensitive silver staining method was used (Blum *et al*, 1987). Following reducing SDS-PAGE, the gel was placed in a thoroughly cleaned (with 70% ethanol) glass container and incubated with a fixing solution [50% (v/v) methanol, 12% (v/v) glacial acetic acid, 0.5% (v/v) formaldehyde] for 1 hour or overnight at room temperature on an orbital shaker. The gel was rinsed in 50% (v/v) ethanol (3 x 20 min), and soaked in pre-treatment solution [0.4% (w/v) $Na_2S_2O_3$. $5H_2O$]. After washing in distilled water (3 x 20 min), the gel was incubated with an impregnation solution [0.2% (w/v) silver $AgNO_3$, 0.75% (v/v) 37% formaldehyde] for 20 min. The gel was washed once more in distilled water (3 x 20 min) and incubated in developing solution [0.4% (w/v) $Na_2S_2O_3$. $5H_2O$] until protein bands were visible. The gel was immediately placed in the stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] for 10 min, and subsequently washed in 50% methanol.

2.2.8 Western blot analysis

Western blot analysis of proteins was conducted according to the protocol described by Towbin *et al* (1979). Proteins separated by reducing SDS-PAGE (Section 2.2.7) were electrophoretically transferred from the gel onto a nitrocellulose membrane that was briefly pre-soaked in blotting buffer [45 mM Tris-HCl buffer, pH 8.3, 173 mM glycine and 0.1% (w/v) SDS] using a Sigma Semi-phor® semi-dry blotting apparatus at constant voltage (20V) and maximum current for 50 minutes. Upon completion of protein transfer to the nitrocellulose, the effectiveness of protein transfer to the membrane was checked by transiently staining with Ponceau S [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. After marking the positions of the molecular mass markers using a pencil, the nitrocellulose membrane was destained with several changes of distilled water. All the unoccupied sites on the membrane were blocked with blocking solution [5% (w/v) Elite® fat-free powder milk dissolved in Tris buffered saline (TBS, 20 mM Tris-HCl buffer, pH 7.4, 200 mM NaCl)] for 1 hour, after which the membrane was washed in TBS (3 x 5 min) and subsequently probed overnight at 4°C with antibodies raised against the recombinant full length inactive form of *Tvi*CATL (anti-ΔFLM *Tvi*CATL IgY, 10 µg/ml) diluted in 0.5% (w/v) BSA-TBS. The membrane was washed in TBS (3 x 5 min) and incubated in rabbit anti-IgY conjugated to horseradish peroxidase (HRPO) diluted in 0.5% (w/v) BSA-TBS. Following washing in TBS (3 x 5 min), the membrane was placed in substrate solution [(0.06% (w/v) 4-chloro-1-naphthol, 0.1% (w/v) methanol, 0.0015% (v/v) H_2O_2 in TBS] until bands were visible. Alternatively, an enhanced chemiluminescence detection kit (Pierce, Rockford, USA) was used. The image was captured using the Sys Gene® imaging system (Syngene®, USA).

2.2.9 Determining the glycosylation status of *Tvi*CATL

To assess the glycosylation status of *Tvi*CATL, *Tvi*CATL was treated with endoglycosidase H. Endoglycosidase H is a glycosidase that specifically cleaves Asn-glycosylated proteins. Cleavage is evidenced by size reduction or increased migration on SDS-PAGE as compared to untreated sample (Caffrey *et al*, 2001). The protein was mixed with denaturing and reducing agents [5% (w/v) SDS and 10% (w/v) 2-mercaptoethanol], followed by boiling at 100°C for 5 minutes. After cooling for 5 minutes at room temperature, 1/10 (v/v) 0.5 M sodium citrate, pH 5.5 was added in order to re-adjust the pH of the mixture to the optimal pH for Endoglycosidase H. The sample was split into two equal volumes, of which one was treated with

Endoglycosidase H (500 U), while the other served as an untreated control. The samples were incubated at 37°C for 3 hours, before the reaction was stopped by addition of reducing treatment buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol] and boiling for 5 min. The resulting samples were analysed by 12.5% reducing SDS-PAGE (Section 2.2.7).

2.2.10 Gelatin-substrate SDS-PAGE and *Tvi*CATL interaction with inhibitors

The proteolytic activity of recombinant *Tvi*CATL was analysed on a gelatin containing Laemmli SDS-PAGE gel as described by Heussen and Dowdle (1980). Gelatin [1% (w/v)] was dissolved in 1.5 ml of SDS-PAGE running gel at 37°C, and combined with the rest (2.25 ml) of the running gel buffer for casting a 12.5% SDS-PAGE as described in Section 2.2.7. Samples were treated with equal volumes of non-reducing treatment buffer [125 mM Tris-HCl buffer pH 6.8, 4% (w/v) SDS and 20% (v/v) glycerol]. Following electrophoresis, SDS was removed from the gel by incubation in two changes of 2.5% (v/v) Triton X-100 for an hour at room temperature. The gel was then incubated in *Tvi*CATL assay buffer [100 mM bis-Tris buffer, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 6 mM DTT] for 3 hours at 37°C. The gel was stained with amido black solution [0.1% (w/v) amido black, 30% (v/v) methanol, 10% (v/v) glacial acetic acid, 60% (v/v) water] for 1 hour and subsequently destained in several changes of destaining solution [30% (w/v) methanol, 10% (v/v) acetic acid, 60% (v/v) distilled water]. Also, the effect of inhibitors on proteolytic activity of *Tvi*CATL was investigated by including different inhibitors in the assay buffer. The gels were incubated at 37°C overnight in assay buffer-containing the respective inhibitors [chymostatin (10 µM), bestatin (10 µM), antipain (10 µM), E-64 (10 µM), leupeptin (10 µM), iodoacetate (10 µM), Z-Gly-Leu-Phe-CMK (50 µM), stefin B (80 µM) and EDTA (10 mM)].

2.2.11 Incubation of recombinant *Tvi*CATL with protein substrates

The method described by Dvořák *et al* (2009) was used to monitor the effect of incubating recombinant *Tvi*CATL with different protein substrates. Briefly, recombinant *Tvi*CATL (~1 µg) was combined with 1 mg/ml of each of bovine serum albumin, type I collagen from rat tail, bovine haemoglobin and bovine α_2-macroglobulin in assay buffer [50 mM citrate, 100 mM sodium phosphate buffer, pH 5-10] containing 2 mM DTT. The reaction was stopped by addition of reducing treatment buffer followed by boiling at 100°C for 2 minutes. These samples were analysed on either 12.5% or 15% reducing SDS-PAGE (Section 2.2.7).

2.2.12 Active site titration

Active site titration was conducted according to Barrett *et al* (1982) using the irreversible cysteine protease inhibitor, L-*trans*epoxysuccinylleucylamido (4-guanidino) butane (E-64) in order to determine the active concentration (E_0) of *Tvi*CATL . *Tvi*CATL [1 µM diluted in 0.1% (w/v) Brij-35] was incubated with E-64 (0-1 µM) diluted in 0.1% (w/v) Brij-35 in *Tvi*CATL assay buffer [100 mM Bis-Tris buffer, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 6 mM DTT] at 37°C for 30 minutes. The rate of hydrolysis of Z-Phe-Arg-AMC (20 µM) was measured by reading fluorescence ($Ex_{360\ nm}$; $Em_{460\ nm}$) using the FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany). The plot of fluorescence against E-64 concentrations was used to obtain an estimate of the active concentration of *Tvi*CATL. Approximately 20% of recombinant *Tvi*CATL was active (Figure 2.5), and this is equivalent to 13.8 µM active enzyme.

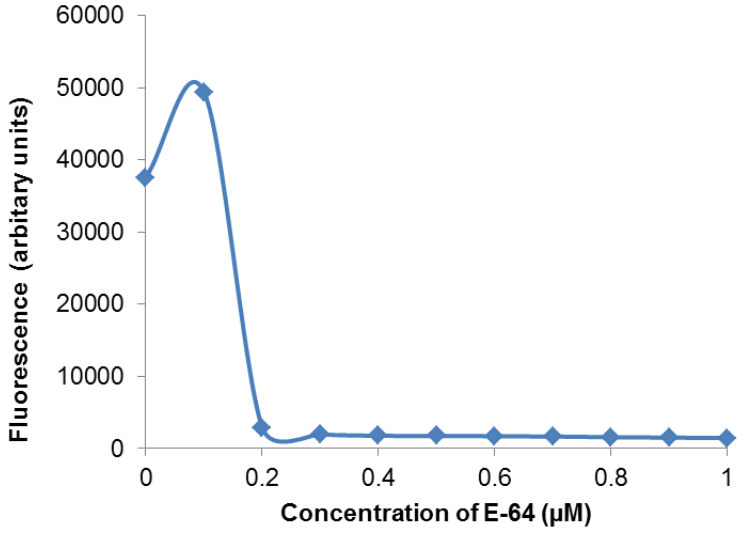

Figure 2.5: Active site titration of *Tvi*CATL. *Tvi*CATL (1 µM) was titrated against different concentrations of E-64 (0-1 µM) and incubated at 37°C for 30 min and residual activity against H-D-Val-Leu-Lys-AMC was measured. Each data point represents the mean activity of the three replicates.

2.2.13 Optimum pH for hydrolysis of H-D-Val-Leu-Lys-AMC by *Tvi*CATL

The optimum pH for hydrolysis of H-D-Val-Leu-Lys-AMC by pure *Tvi*CATL was determined using the constant ionic strength acetate/Mes/Tris (AMT) buffers (100 mM Na-acetate, 200 mM Tris, 100 mM Mes, 8 mM DTT and 4 mM EDTA) titrated from pH 4 to pH 9 (in 1 pH increments) using NaOH or HCl. The enzyme, *Tvi*CATL [1.5 ng diluted in 0.1% (w/v) Brij-35] was incubated with AMT buffers at varying pH-values for

5 minutes at 37°C. The substrate H-D-Val-Leu-Lys-AMC (20 μM) was added and fluorescence measured ($Ex_{360\ nm}$; $Em_{460\ nm}$) using the FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany).

2.2.14 Fluorometric peptide substrate specificity of *Tvi*CATL

The hydrolysis of AMC was followed using the FluorStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany) equilibrated at 37°C in order to calibrate the instrument and determine the relative endpoint for enzymatic hydrolysis of AMC peptide substrates. A series of AMC dilutions (5 $x10^{-3}$-15 μM) were prepared in distilled water and incubated with *Tvi*CATL assay buffer for 5 minutes at 37°C. Fluorescence was measured ($Ex_{360\ nm}$; $Em_{460\ nm}$) using the FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany) and the AMC fluorescence standard curve was constructed, Figure 2.6.

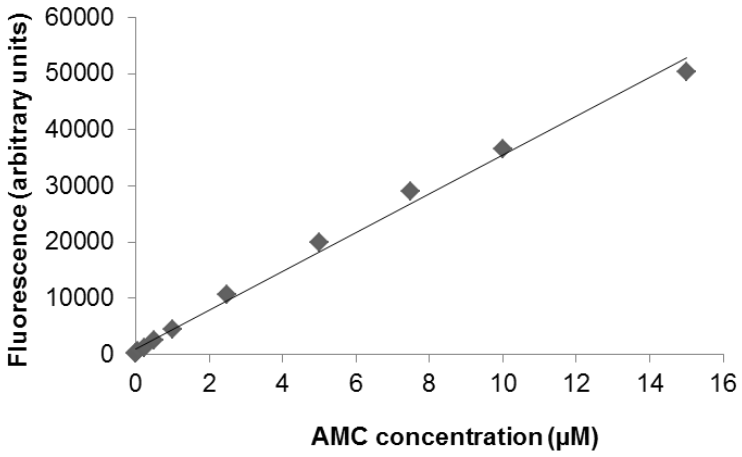

Figure 2.6: The AMC standard curve to determine the relative amount of fluorescence released upon hydrolysis of the AMC substrates. The fluorescence was measured at $Ex_{360\ nm}$; $Em_{460\ nm}$ and the equation of the line was y= 3457.3x + 970.28 with a correlation coefficient of 0.9943.

The kinetic parameters for complete definition of the substrate specificity of the enzyme were determined by measuring the parameters as per revised Michaelis-Menten equation by Briggs and Haldane (1925) and Salvesen and Nagase (1989), respectively:

$v_0 = V_{max}[S]/[S] + K_m$ (1) $k_{cat} = V_{max}/[E]_0$ (2)

Where in equation (1): v_0 is the initial steady-state velocity, V_{max} the maximum velocity of enzyme catalysed reaction, [S] the substrate concentration, K_m the Michaelis-Menten constant; and in equation (2): k_{cat} is the turnover of the enzyme and $[E_o]$ the active concentration of an enzyme.

The substrate specificity of recombinant *Tvi*CATL was experimentally determined by pre-incubation of *Tvi*CATL [1.5 ng diluted in 0.1% (w/v) Brij-35] in *Tvi*CATL assay buffer for 5 minutes at 37°C. The activated enzyme mixture was combined with different substrate concentrations (0-50 µM) and substrate hydrolysis was followed by measuring fluorescence at ($Ex_{360\ nm}$; $Em_{460\ nm}$) using the FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany). The initial steady state velocity (v_0) (gradient) was determined relative to the gradient of the AMC standard curve (Figure 2.6). The substrate concentrations and calculated relative v_0 values were plotted using Hyper32© software (1991 – 2003, Dr. J.S. Easterby, University of Liverpool, Liverpool, UK) in order to construct the Lineweaver-Burk plots for determination of V_{max} and K_m.

2.2.15 Reversible inhibition

The reversible competitive inhibition of an enzyme-substrate reaction occurs in a time-independent manner. As described by Salvesen and Nagase (1989), reversible inhibition is followed by defining the parameters in equations (3) and (4):

$$v_0/v_i = 1 + [I]/K_{i(app)} \qquad (3) \qquad\qquad K_i = K_{i(app)}/1 + [S]/K_m \qquad (4)$$

Where in equation (3): v_0 is an uninhibited rate for the substrate hydrolysis, v_i is the inhibited rate upon addition of the substrate, [I] is the inhibitor concentration and $K_{i(app)}$ is the apparent inhibition constant. In equation (4): K_i is the true equilibrium constant, [S] is the initial substrate concentration used and K_m is the Michaelis-Menten constant for the used substrate.

To experimentally follow the reversible inhibition of the substrate hydrolysis by an enzyme, 1.5 ng active *Tvi*CATL [diluted in 0.1% (w/v) Brij-35] was activated in *Tvi*CATL assay buffer for 15 minutes at 37°C. The specific substrates Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC (20 µM) were added and released fluorescence quickly measured using the FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany) at $Ex_{360\ nm}$ and $Em_{460\ nm}$ in order to determine the uninhibited rate, v_0. The dilution series of inhibitor (2.5-40 µM) was added, followed by measuring fluorescence to determine the new inhibited steady state, v_i. Plotting $v_0/v_i - 1$ against [I] allowed for

the calculation of the $K_{i(app}$ from the slope. The K_i was then calculated by substitution of the parameters as per equation (4).

2.2.16 Irreversible inhibition of *Tvi*CATL

The effect of irreversible inhibitors towards the activity of *Tvi*CATL was followed through pseudo-first-order kinetics at which the inhibitor concentration is kept 10-fold higher than the enzyme concentration (Salvesen and Nagase, 1989). Briefly, the active *Tvi*CATL [1.5 ng = 0.056 nM diluted in 0.1% (w/v) Brij-35] was pre-activated in *Tvi*CATL assay buffer for 15 minutes at 37°C. Aliquots in triplicate were removed and assayed in separate experiments against 20 µM Z-Phe-Arg-AMC or H-D-Ala-Leu-Lys-AMC in order to measure the uninhibited rate, v_0. To the enzyme-assay buffer mixture, the irreversible inhibitor (1.8 nM) was added and incubated at 37°C. At various 5 minute intervals, triplicate aliquots were removed and assayed for residual activity against the two substrates in order to measure the activity after addition of the inhibitor, v_t. The observed rate of inactivation k_{obs} was determined using the equation: $\ln v_t/v_0 = -k_{obs} \times t$. The apparent rate of association k_{ass} was calculated using the relationship: $k_{ass} = k_{obs}/[I]$, where [I] is the concentration of the inhibitor. In order to determine the inhibitor concentration required for 50% inhibition (IC_{50}) of the enzyme during the incubation time, the assumption that for the pseudo-first-order, IC_{50} is equivalent to half-life $t_{1/2}$ was used in the following equation: $t_{1/2} = 0.693/ k_{ass}$ (Salvesen and Nagase, 1989).

2.3 Results

2.3.1 Recombinant expression and purification of the catalytic domain of *Tvi*CATL

The *P. pastoris* yeast expression system was used to recombinantly express *Tvi*CATL from *T. vivax*. Silver stained reducing SDS-PAGE analysis of expression supernatants indicated that *Tvi*CATL is expressed in two forms as evidenced by two bands at 29 and 33 kDa on reducing SDS-PAGE (Figure 2.7, panel A). This figure also revealed that there was an increase in intensity of bands from day 3 to day 6, signifying a time dependent increase in expression levels. However, at day 7, there was a decrease in intensity of bands as compared to day 3-6 samples, thus signifying a decrease in expression levels. Thus for the next expression, induction was allowed to proceed for only 6 days. The expression medium yielded mainly the desired protein, with very few contaminating proteins, and this simplified the purification steps needed. The lower

molecular weight bands of *Tvi*CATL (29 kDa) indicates that autocatalytic processing of the propeptide occurred in the expression supernatants during the autocatalytic maturation process of the pro-enzymes at pH 4.2 to release a processed and mature active enzyme, as is the case for other cysteine proteases (Vernet *et al*, 1991; Boulangé *et al*, 2001).

Three phase partitioning using 40% ammonium sulfate allowed optimal precipitation of *Tvi*CATL and partial purification of *Tvi*CATL from the expression medium as evidenced by two prominent bands at 29 and 33 kDa on reducing SDS-PAGE (Figure 2.7, panel B).

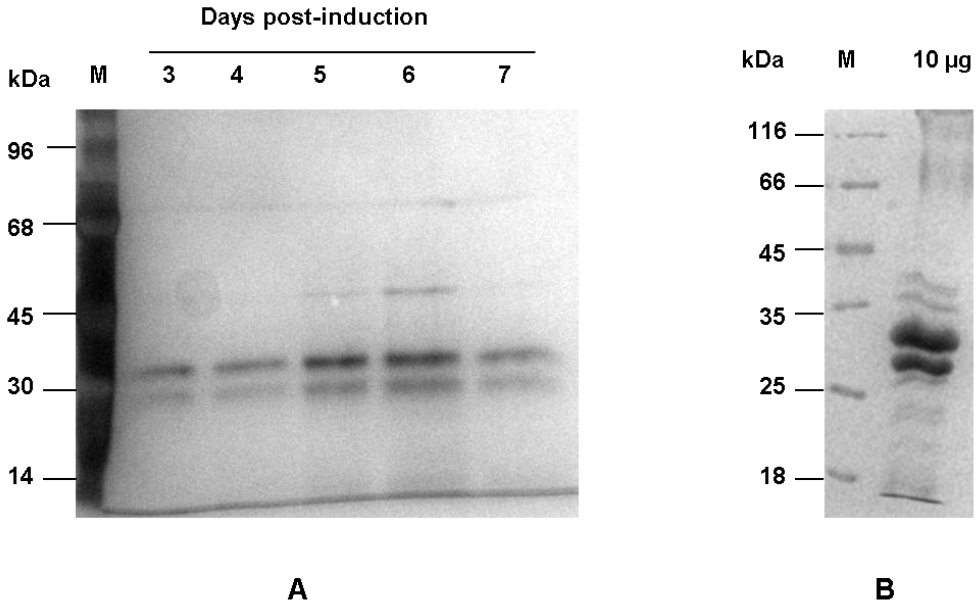

Figure 2.7: Reducing SDS-PAGE (12.5%) analysis of expression and purification of recombinant *Tvi*CATL by TPP. A, aliquots were collected for the duration of expression. Supernatants collected from day 3-7 were concentrated by the SDS-KCl method prior to loading on a reducing gel that was afterwards silver stained. **B**, the final supernatant volume at day 7 of expression was concentrated by three phase partitioning using 40% ammonium sulfate. Dialysed and concentrated sample (10 µg) was separated on the gel and stained with Coomassie blue R-250.

Three phase partitioning did not adequately remove all contaminating proteins; hence further purification was necessary to remove the higher molecular weight contaminants. Molecular exclusion chromatograph (MEC) using an AKTA Sephacryl-300 column was used to further purify *Tvi*CATL (Figure 2.8, panel A). According to the MEC calibration curve (Figure 2.2, panel B), the retention volumes for *Tvi*CATL were predicted to be 75.57 ml for a 33 kDa protein and 78.1 for a 29 kDa protein. The first peak (at around 39.60 ml) that appeared around the void volume (V_0, 40 ml)

corresponded to the higher molecular weight contaminants. The last two peaks at around 80.7 ml and 104 ml corresponded to the co-eluted 33 and 29 kDa bands respectively, representing *Tvi*CATL; these retention volumes were larger than those predicted (75.57 ml for a 33 kDa protein and 78.1 for a 29 kDa protein). Higher retention volumes of *Tvi*CATL could be due to the glycosylated nature of this protein.

The residual activity of each MEC eluted fraction was measured against the specific substrate, Z-Phe-Arg-AMC. The activity plot shows three peaks that overlap with the elution profile peaks. Interestingly, it is evident that the last two *Tvi*CATL peaks [which correspond to 33 and 29 kDa fractions on the SDS-PAGE gel (Figure 2.8, panel B)] had the highest enzymatic activity as compared to the first peak which corresponds to the higher molecular weight contaminants. The eluted fractions corresponding to the two peaks with the highest purity and activity were pooled and concentrated. This activity shows that successive purification methods did not compromise the activity of the enzyme.

Analysis by reducing SDS-PAGE of MEC purified samples (Figure 2.8, panel B), indicates that the higher molecular weight contaminates were eluted from 72 to 80 ml. At 80 ml, there was a mixture of a hardly visible higher molecular weight contaminant and a prominent 33 kDa band corresponding to the *Tvi*CATL form. From fractions 82 to 96, the 33 kDa and 29 kDa bands co-eluted at almost the same intensity. Lower molecular weight degradation products were visible in the latter fractions.

Table 2.1 shows that TPP adequately removed contaminating proteins as indicated by an increase in specific activity as compared to that of the expression lysate. The TPP sample further subjected to purification by MEC indicates an increase in specific activity while the concentration and total protein decreased significantly due to removal of contaminants. The final yield is very high but that is in line with findings of other workers in the field who used TPP to purify proteins from expression supernatants. These values may not be precise due to the possibility of over estimation of the total protein presence after each purification step.

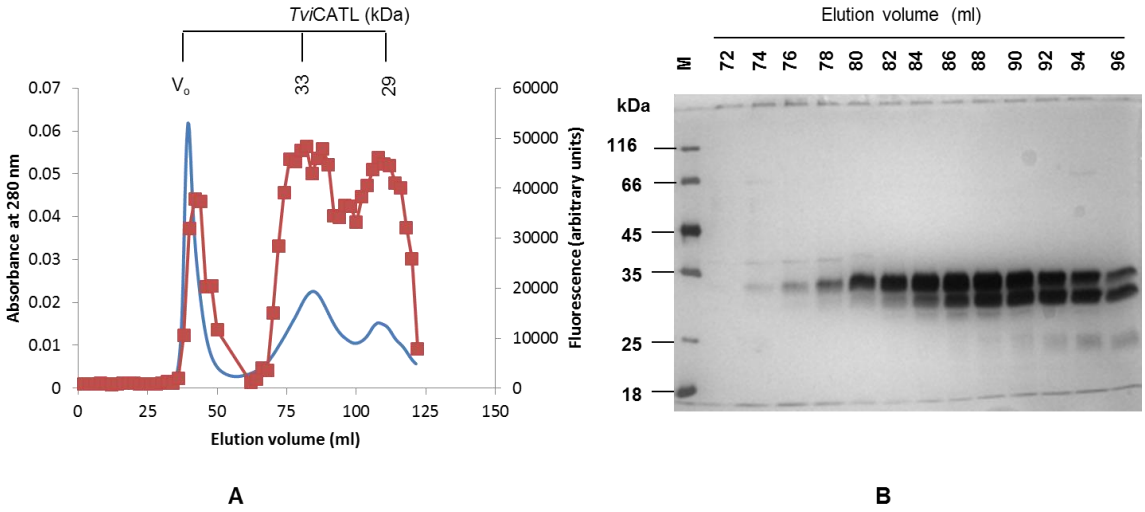

Figure 2.8: Purification of *Tvi*CATL on a Sephacryl® S-300 HR MEC column. *Tvi*CATL was subjected to MEC (column: 16 x 600 mm, flow rate of 0.5 ml/min, 120 ml, 2 ml/fraction) in MEC buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8). Panel **A**, elution profile (blue, $A_{280\ nm}$) and the approximate calibration sizes and their corresponding retention volumes are diagrammatically represented at the top of panel A. For all fractions, residual activity was measured against Z-Phe-Arg-AMC peptide substrate (red). **B**, MEC fractions (including those eluted from 72-96 ml) were analysed by 12.5% reducing SDS-PAGE followed by silver staining.

Table 2.1: Purification of recombinant expression supernatant of *Tvi*CATL by TPP and MEC

Steps	Volume (ml)	Concentration (mg/ml)[a]	Total protein (mg)	Total activity (units)[b]	Specific activity (units/mg)	Purification (fold)	Yield (%)
Supernatant	480	0.538	258	7528	29	1	100
TPP	5.5	8.28	45.54	42915	942	32.5	570
MEC	1.5	6.63	10.02	44368	4428	153	589

[a] BCA assay was used to quantify protein and [b] residual activity was determined by measuring the hydrolysis of Z-Phe-Arg-AMC (20 µM) by *Tvi*CATL pre-activated at 37°C in assay buffer (100 mM Bis-Tris, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 8 mM DTT)

Western blot analysis was carried out using chicken anti-full length inactive mutant *Tvi*CATL IgY antibodies in order to confirm the identity of the recombinantly expressed and purified *Tvi*CATL. Both bands observed at approximately 29 and 33 kDa (before and after purification by TPP and MEC), were faintly detected using this antibody (Figure 2.9). Despite extensive optimisation of the antigen concentration, and dilution of the primary and secondary antibodies used, no significant improvement of the signal

was achieved (results not shown). In subsequent work (see Chapter 3) improved results were obtained by using enhanced chemiluminescence for western blots.

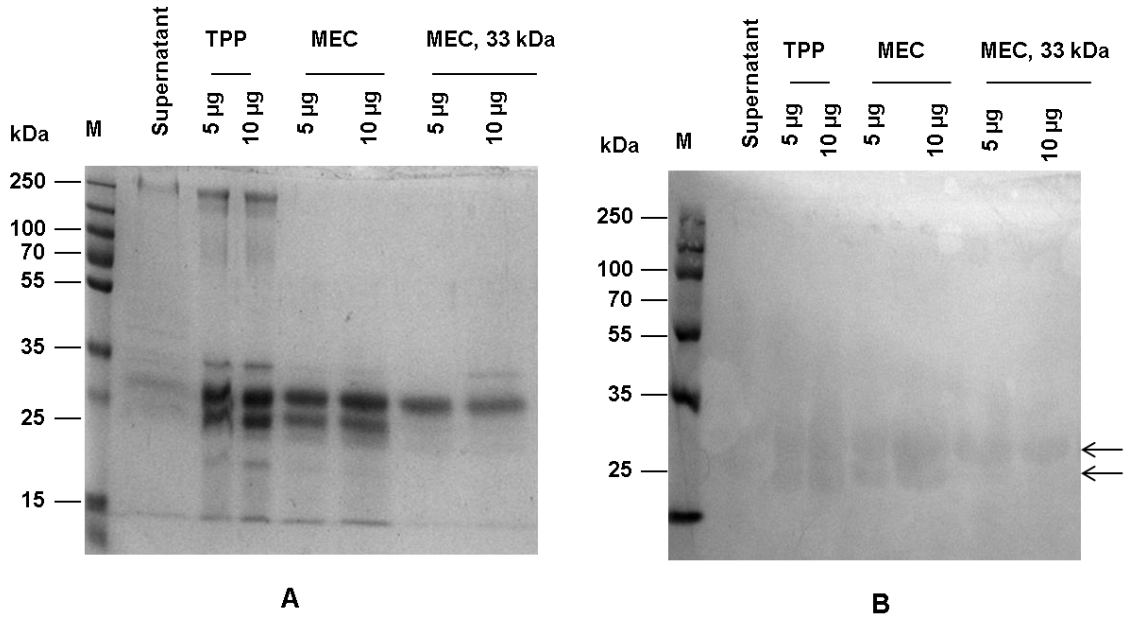

Figure 2.9: Western blot analysis of expression and purification (TPP and MEC) of *Tvi*CATL. Purified expression supernatants were analysed by 12.5% reducing SDS-PAGE. Panel **A,** *Tvi*CATL from expression supernatants (SDS-KCl concentrated), TPP, MEC and MEC-33 kDa fractions were loaded at 5 and 10 µg. The gel was Coomassie blue R-250 stained to serve as a reference gel. Panel **B**, duplicate gel as in panel A was transferred to a nitrocellulose membrane, probed with anti- chicken anti-full length inactive mutant *Tvi*CATL IgY (30 µg/ml) and detected by rabbit anti-IgY-HRPO conjugate (1: 1000 dilution) followed by colour development using a 4-chloro-1-naphthol and H_2O_2. *Tvi*CATL bands are indicated by arrows.

2.3.2 Glycosylation status of recombinant *Tvi*CATL

The electrophoretic migration of *Tvi*CATL as two bands at 33 and 29 kDa even after purification by MEC suggests that *Tvi*CATL may contain one or more glycosylation sites in its sequence. The glycosylation status of *Tvi*CATL was assessed by treatment (prior to electrophoresis) of the sample containing both 33 and 29 kDa *Tvi*CATL bands that co-eluted from MEC as well as the 33 kDa fraction that partially separated from the 29 kDa fraction. Figure 2.10, shows that for the MEC (29 and 33 kDa) sample treated with endoglycosidase H (+ Endo H), the 33 kDa band disappeared as compared to the untreated sample (- Endo H) which migrated as both 29 and 33 kDa. When a 33 kDa eluted fraction was treated with endo H (+ Endo H), there was a decrease in electrophoretic migration as compared to an untreated sample (- Endo H). Overall,

endoglycosidase treatment proved that the 33 kDa of *Tvi*CATL is as a result of Asn-glycosylation.

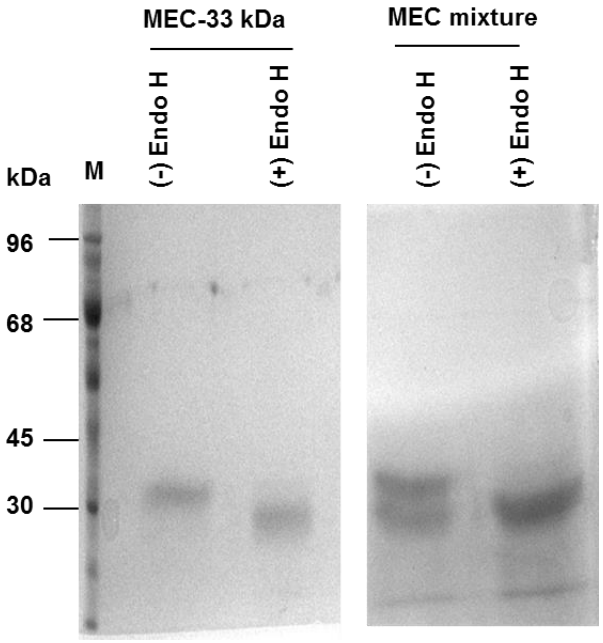

Figure 2.10: Glycosylation status of recombinant *Tvi*CATL. MEC eluted fractions containing 29 and 33 kDa or 33 kDa forms of *Tvi*CATL only, were treated with endoglycosidase H prior to analysis by 12.5% reducing SDS-PAGE that was Coomasie blue R-250 stained. (+) Endo H refers to endoglycosidase H treated samples while (-) Endo H refers to the untreated control samples. Molecular marker (M) lane was loaded as size reference.

2.3.3 Gelatin SDS-PAGE

The gelatinase activity of *Tvi*CATL was compared to that of *Tco*CATL, which is the cysteine protease that shares approximately 62% sequence identity with *Tvi*CATL. Both enzymes efficiently and comparably hydrolysed the gelatin substrate as evidenced by clear zones over a darky stained background (Figure 2.11). Non-reducing conditions resulted in migration of the enzymes at molecular sizes higher than their migration under reducing conditions (2-mercaptoethanol plus boiling of samples, Figure 2.8). The approximate molecular weights for *Tvi*CATL are 50 and 33 kDa, while that of *Tco*CATL is 39 kDa on non-reducing gelatin SDS-PAGE.

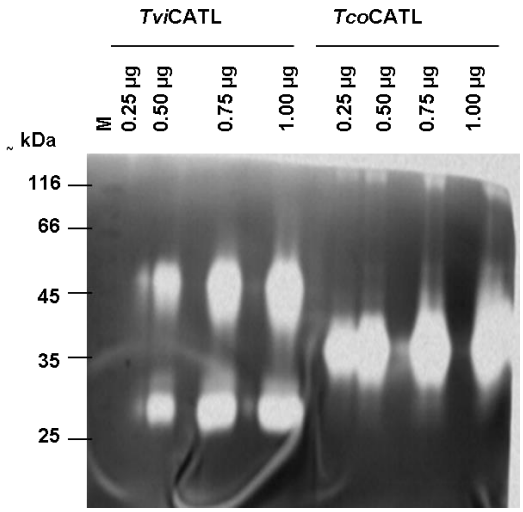

Figure 2.11: Analysis of proteolytic activity of *Tvi*CATL and *Tco*CATL by 12.5% non-reducing SDS-PAGE copolymerised with a gelatin substrate. During sample preparation, neither reducing agent nor boiling of sample was performed prior to gel loading. Approximate molecular mass, M, are presented on the first left lane. The gel was stained with Amido black.

To evaluate how different inhibitors affect the gelatinase activity of *Tvi*CATL, different inhibitors were included in the *Tvi*CATL assay buffer for incubation of the gelatin SDS-PAGE gels. Figure 2.12 shows that as compared to the no inhibitor control, the gelatinase activity of *Tvi*CATL was completely inhibited by the specific cysteine protease inhibitor, E-64. Complete inhibition was also seen when cysteine/serine inhibitors, antipain, leupeptin and iodoacetate were used. The irreversible cysteine protease inhibitors, Z-Gly-Leu-Phe-CMK, chymostatin and stefin B partially inhibited the gelatinase activity of *Tvi*CATL. Gelatinase activity was neither inhibited by a specific aminopeptidase inhibitor (bestatin) nor by a specific metalloprotease inhibitor (EDTA). Therefore these results prove that *Tvi*CATL is a cysteine protease.

2.3.4 pH optimum

The pH optimum of the purified *Tvi*CATL was determined by measuring the rate of hydrolysis of H-D-Val-Leu-Lys-AMC [a substrate also pre-determined to be specific for this enzyme (Vather, 2010)] in constant ionic strength AMT buffers set at different pH values. Results shown in Figure 2.13 indicate that the recombinant *Tvi*CATL was active at a pH range between 6.5 and 7.5 suggesting that the enzyme would be active in the host blood stream following parasite lysis.

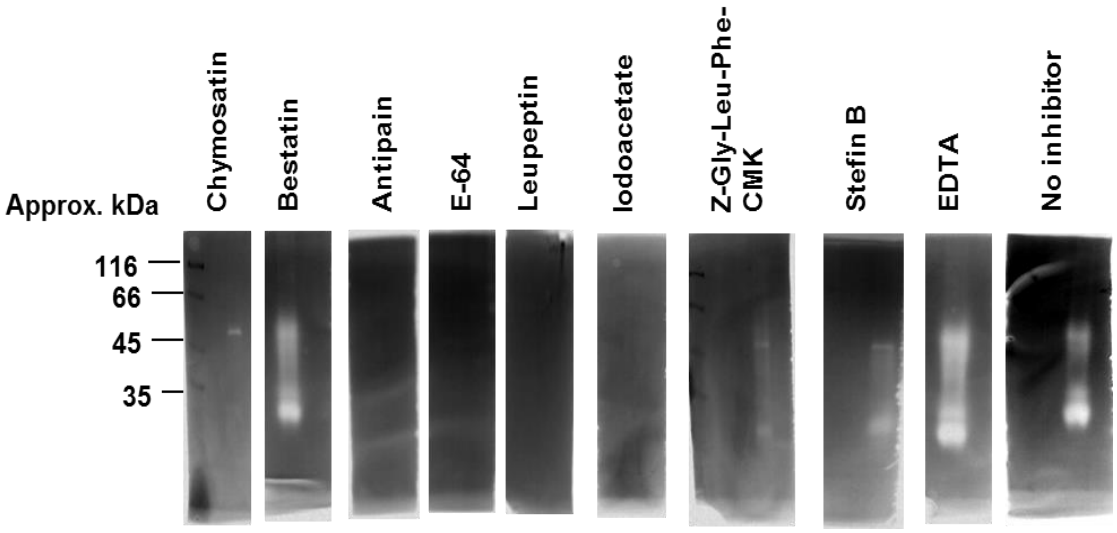

Figure 2.12: The effect of inhibitors on gelatinase activity of *Tvi*CATL. *Tvi*CATL (0.25 µg) was separated by a 12.5% non-reducing SDS-PAGE containing gelatin. During sample preparation, neither reducing agent nor boiling of the sample was performed prior to gel loading. Upon removal of SDS by Triton X100, a number of gel strips were incubated overnight at 37°C in *Tvi*CATL assay buffer supplemented with the respective inhibitors at the indicated concentrations. In one strip, inhibitor was not added to serve as a positive control. Inhibitor concentrations: chymostatin (10 µM), bestatin (10 µM), antipain (10 µM), E-64 (10 µM), leupeptin (10 µM), iodoacetate (10 µM), Z-Gly-Leu-Phe-CMK (50 µM), stefin B (80 µM) and EDTA (10 mM). All gelatin-SDS-PAGE strips were stained with Amido black.

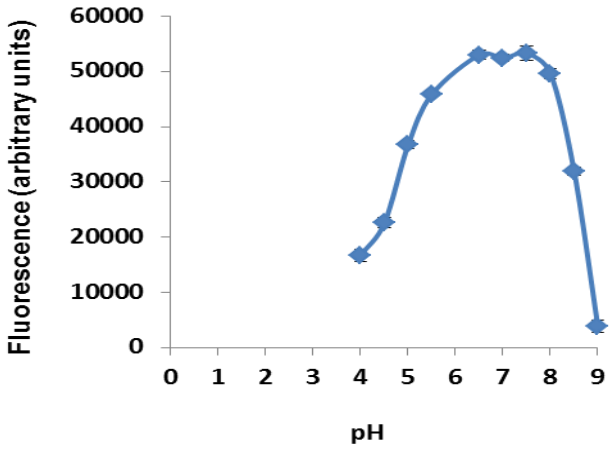

Figure 2.13: The pH optimum of *Tvi*CATL. Constant ionic strength Acetate-Mes-Tris buffers (AMT), pH 4-9, were combined with purified *Tvi*CATL (1.5 ng) at 37°C for 5 minutes, followed by measuring residual activity against the peptide substrate, H-D-Val-Leu-Lys-AMC (20 µM). The error bars represent an average of three independent readings.

2.3.5 Incubation of *Tvi*CATL with protein substrates

To evaluate if the proteolytic enzyme *Tvi*CATL could potentially be involved in pathogenesis of trypanosome parasites by degrading host proteins and tissues, and to evaluate at which pH this hydrolysis occurs optimally, *Tvi*CATL was incubated with different protein substrates, i.e. bovine serum albumin, bovine haemoglobin, type I collagen and bovine α_2-macroglobulin (Figure 2.14). Panel A for hydrolysis of BSA (68 kDa) by *Tvi*CATL, shows that there was optimal hydrolysis at pH 7 and 7.5. At pH-values below 7, no noticeable hydrolysis occurred while at pH 8 and 9, hydrolysis was partial with observed degradation products at around 35 and 40 kDa. At pH 10, no hydrolysis occurred since the band at 68 kDa appears exactly as that seen in the lane for an untreated control sample, U.

Bovine haemoglobin migrated as a major band at 15 kDa which is a representative of haemoglobin alpha and beta monomers and a faint band at around 30 kDa representing the alpha-beta dimers (Foot *et al*, 2009). Panel B shows that haemoglobin was completely hydrolysed in the pH range 6-7.5 as evidenced by complete disappearance of the major 15 kDa and a minor 30 kDa band. At pH-values below and above this range, no hydrolysis occurred since the intensity of the 15 kDa and the 30 kDa bands appeared almost the same as the untreated control sample, U.

Collagen separates on SDS-PAGE as doublets at 115 and 130 kDa and another doublet at 215 and 235 kDa. Panel C shows that at pH 5-7, there was partial hydrolysis of a lower molecular doublet with pH 6.5 and 7 being optimal for this particular hydrolysis. However, the higher molecular weight doublet was more resistant to hydrolysis in the pH range 5-7 as evidenced by the minor decrease in the prominence of these bands. At pH 7.5-10, there was no hydrolysis as the band intensity of these doublets stained similarly as that of an untreated control sample, U.

Reducing SDS-PAGE analysis of the interaction of *Tvi*CATL with α_2-macroglobulin is presented in panel D. The untreated control (lane 1), showed that α_2-macroglobulin migrates as a “slow” 170 kDa band, and this is the expected α_2-macroglobulin monomer size under reducing conditions. At pH 5- 7.5 there was an accumulation of cleavage bands called the “fast” α_2-macroglobulin form which corresponds to the cleaved bait region of α_2-macroglobulin under reducing conditions. These degraded bands are at approximately 95 and 85 kDa and the intensity of these bands was more pronounced at pH 5, meaning this was a partial hydrolysis of α_2-macroglobulin. At pH 6-7 and 8, these cleavage products were less pronounced thus suggesting that optimal

hydrolysis occurred in these pH ranges. Surprisingly, at pH 7.5, no hydrolysis was evidenced. At pH 9 and 10, no significant hydrolysis occurred as the protein bands separated the same as an untreated control sample, U.

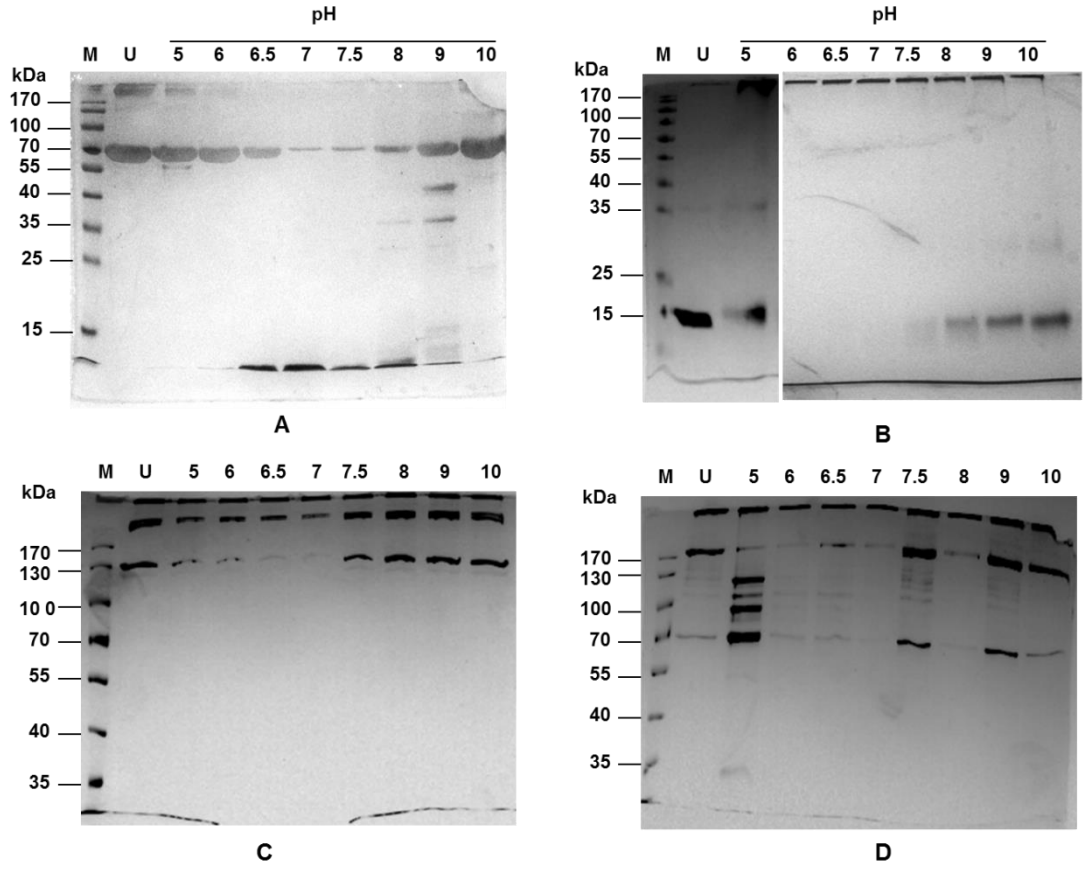

Figure 2.14: The hydrolysis of protein substrates by *Tvi*CATL. Recombinant *Tvi*CATL (1 µg) was incubated with bovine serum albumin (**A**), bovine haemoglobin (**B**), type I collagen (**C**) and bovine α_2-macroglobulin (**D**) overnight at 37°C in assay buffer [50 mM citrate, 100 mM sodium phosphate containing 2mM DTT] (pH 5-10). For **A** and **B**, samples were separated on a 15% reducing SDS-PAGE gel, while for **C** and **D** samples were separated on a 10% reducing SDS-PAGE gel. The untreated protein substrate (U, no enzyme added and protein diluted in distilled water) was included to serve as a control. All gels were silver stained.

2.3.6 Substrate specificity of *Tvi*CATL

The ability of *Tvi*CATL to hydrolyse different fluorogenic peptide substrates was assessed by reacting *Tvi*CATL with a range of fluorogenic peptide substrates that differed in the amino acid residues in P_1, P_2 and P_3. Table 2.2 indicates that Z-Phe-Arg-AMC is the best substrate for *Tvi*CATL as indicated by the lower K_m value of 4.125 µM and higher catalytic efficient (k_{cat}/K_m) value of 29.39 s^{-1} mM^{-1}. Results also show that H-D-Ala-Leu-Lys-AMC (k_{cat}/K_m = 16.95 s^{-1} mM^{-1}) and H-D-Val-Leu-Lys-AMC (k_{cat}/K_m =

3.81 s^{-1} mM^{-1}) are good substrates for *Tvi*CATL. H-D-Val-Leu-Lys-AMC has been previously described as the best substrate for recombinant *Tvi*CATL (Vather, 2010; Jackson, 2011), but in this case, low catalytic efficiency was obtained. The reason for this discrepancy is not clear. These results indicate that pronounced hydrolysis of the peptides by *Tvi*CATL occur if the basic amino acids Lys or Arg occupies the P_1 substrate position (which binds to the S_1 of the active site cleft) and large hydrophobic amino acids Leu or Phe at the P_2 substrate position (which binds to the S_2 of the active site cleft). From these observations, it can be concluded that Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC or H-D-Val-Leu-Lys-AMC are the best substrates for the recombinant form of *Tvi*CATL. Consistent with all cysteine proteases, S_2 is the primary determinant of substrate specificity.

As evidenced by a relatively high k_{cat}/K_m of 14.69 s^{-1} mM^{-1}, *Tvi*CATL also hydrolysed a substrate with proline at the P_2 position of the endopeptidase substrate (Z-Pro-Arg-AMC), however, the presence of small hydrophobic amino acids Gly or Val at P_3 compromised the potential of *Tvi*CATL to hydrolyse substrates containing Pro at P_2 (Z-Gly-Pro-Arg-AMC and Boc-Val-Pro-Arg-AMC). *Tvi*CATL was unable to hydrolyse a substrate lacking a P_2 residue (H-Leu-AMC) and an arginine rich substrate, Z-Arg-Arg-AMC.

Overall, *Tvi*CATL preferentially cleaved peptide bonds at which the P_2 position is occupied by hydrophobic amino acid residues and this preference is best described by the following order: Phe>Leu. The preference at P_1 is Arg>Lys. Due to the promiscuity of the P_3 position, no preference trend could be deduced since a variety of amino acid residues were tolerated this position.

Table 2.2: The kinetic parameters for the cleavage of the peptide substrates by recombinant *Tvi*CATL. *Tvi*CATL (1.5 ng) pre-activated at 37°C in assay buffer (100 mM Bis-Tris, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 8 mM DTT) was incubated with a series of fluorogenic substrates ranging from a concentration of 0-50 µM. Hydrolysis was monitored by measuring fluorescence ($Ex_{360\ nm}$; $Em_{460\ nm}$). Hyper32© software was used to determine K_m.

Substrates	K_m (µM)	V_{max}	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1} mM^{-1})
Z-Phe-Arg-AMC	4.125	1.673	0.1212	29.39
H-D-Ala-Leu-Lys-AMC	19.83	4.638	0.3361	16.95
H-D-Val-Leu-Lys-AMC	56.01	2.945	0.2134	3.81
Z-Pro-Arg-AMC	11.48	2.328	0.1687	14.69
H-Pro-Phe-Arg-AMC	26.38	0.4770	0.03456	1.310
Z-Gly-Gly-Arg-AMC	55.55	0.3189	0.02311	0.4161

Substrates with no activity detected even after long time incubation at 37°C: Z-Gly-Pro-Arg-AMC, Boc-Val-Pro-Arg-AMC, H-Leu-AMC and Z-Arg-Arg-AMC

2.3.7 Competitive reversible and irreversible inhibition of *Tvi*CATL

*Tvi*CATL was reacted with a series of reversible inhibitors to determine their inhibitory effect on the hydrolysis of the substrates, Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC. Results in Table 2.3 indicate that leupeptin is the best reversible inhibitor of *Tvi*CATL in the presence of a preferred substrate (Z-Phe-Arg-AMC) since it has the lowest K_i (3.34) as compared to other inhibitors. There is no significant difference between K_i values of chymostatin and antipain. When reversible inhibition was assayed in the presence of H-D-Ala-Leu-Lys-AMC, antipain and chymostatin inhibited substrate hydrolysis to comparable levels, but their K_i values did not differ much from the K_i value of leupeptin. Bestatin and EDTA did not yield any inhibitory effect on the activity of *Tvi*CATL against either substrates and this is consistent with results obtained for the evaluation of gelatinase inhibition.

*Tvi*CATL was also reacted with irreversible protease inhibitors and residual activity assayed against specific substrates Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC (Table 2.4). Both iodoacetate and iodoacetamide showed very similar kinetic values against both Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC and are considered to be better inhibitors as compared to Z-Gly-Leu-Phe-CMK since they have large values of association constants with the lowest half-lives. The serine protease inhibitors, PMSF and TPCK had no effect on the hydrolysis of the specific substrates Z-Phe-Arg-AMC by *Tvi*CATL.

Table 2.3: The inhibition of recombinant *Tvi*CATL by competitive reversible inhibitors. *Tvi*CATL (1.5 ng) pre-activated at 37°C in assay buffer (100 mM Bis-Tris, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 8 mM DTT) was incubated with competitive reversible inhibitors (2.5 – 40 µM). The hydrolysis of the specific substrates Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC was monitored by measuring fluorescence ($Ex_{360\ nm}$; $Em_{460\ nm}$).

Substrate	**Z-Phe-Arg-AMC**	**H-D-Ala-Leu-Lys-AMC**
Inhibitor	K_i (µM) x 10^3	K_i (µM) x 10^3
Leupeptin	3.34	1.8
Chymostatin	4.06	1.18
Antipain	4.02	1.14
Bestatin	ND	ND
EDTA	ND	ND

ND: Refers to bestatin (10 µM) and EDTA (10 mM) for which no reversible inhibition towards hydrolysis of peptides was observed.

Table 2.4: Competitive irreversible inhibition of recombinant *Tvi*CATL. *Tvi*CATL (1.5 ng) pre-activated at 37°C in assay buffer (100 mM Bis-Tris, pH 6.5, 4 mM EDTA, 0.02% (w/v) NaN_3, 8 mM DTT) was incubated with competitive irreversible inhibitors (28 nM).The hydrolysis of the specific substrates Z-Phe-Arg-AMC and H-D-Ala-Leu-Lys-AMC was monitored by measuring fluorescence ($Ex_{360\ nm}$; Em_{460}).

Substrate	**Z-Phe-Arg-AMC**		**H-D-Ala-Leu-Lys-AMC**	
Inhibitor	k_{ass} ($mM^{-1}.s^{-1}$)[a]	$t_{1/2}$ (s)	k_{ass} ($mM^{-1}.s^{-1}$)[a]	$t_{1/2}$ (s)
Iodoacetate	71.4±0.025	319	71.4±0.025	319
Iodoacetamide	35.70±0.01	638	35±0.025	639
Z-Gly-Leu-Phe-CMK	28.57±0.02	798	7.1±0.01	3192

[a] k_{ass} ± SD (n=3)

No inhibition was detected using PMSF (phenylmethanesulfonyl) and TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone).

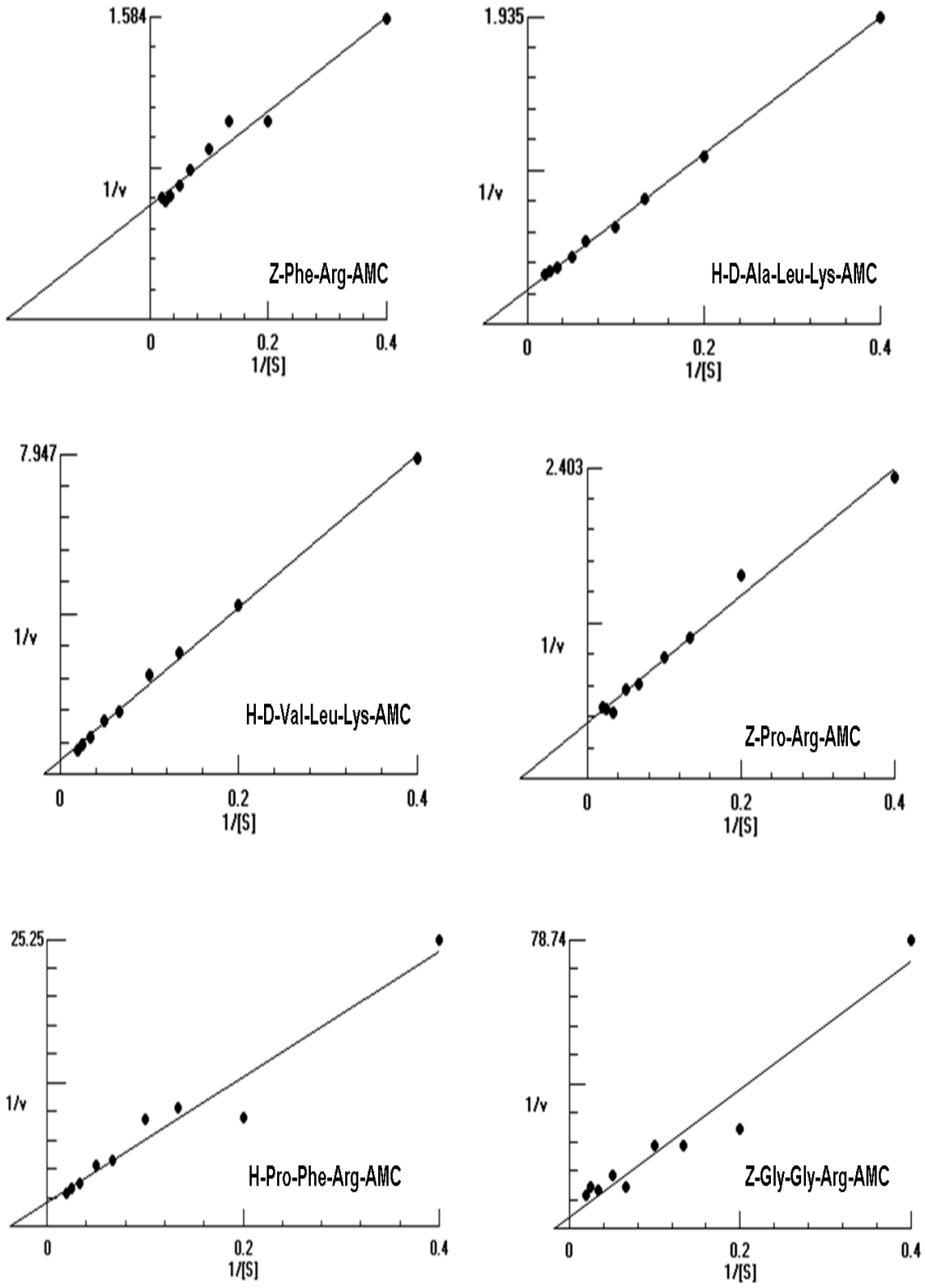

Figure 2.15: Lineweaver-Burk plots for determination of the substrate specificity of *Tvi*CATL. *Tvi*CATL was reacted with a series of peptide substrates over a range of concentrations. The substrate concentrations and relative steady state velocity, v_0 values were subjected to Hyper32© analysis for determination of K_m values (Table 2.2).

2.4 Discussion

The effective control of the neglected tropical disease, African animal trypanosomiasis depends on an accurate species-specific diagnostic tool at an early developmental stage of the parasitic infection, followed by treatment with trypanosome-specific chemotherapeutic drugs. A cysteine protease *Tvi*CATL from *T. vivax* is thought to be involved in pathogenesis, therefore serves as a good target for the development of effective diagnostic tools. In the present study, *Tvi*CATL was recombinantly expressed, purified and enzymatically characterised for possible use as a diagnostic target.

There is a wide variety of prokaryotic and eukaryotic expression systems available for the production of recombinant proteins. Each system is associated with advantages and disadvantages, therefore, recombinant expression of proteins remain a matter of trial-and-error. Factors such as resultant protein stability, folding, solubility, posttranslational modifications and production yields, are considered when choosing an expression system for recombinant protein production (Goeddel, 1990). The prokaryotic *Escherichia coli* is usually the first choice for heterologous expression of proteins (Baneyx, 1999). The *E. coli* expression system is preferable due to rapid growth in inexpensive media and availability of a variety of cloning vectors and host strains (Terpe, 2006). The shortcomings associated with the *E. coli* expression system include production of unfolded or inactive proteins or inclusion of the expressed protein in the insoluble inclusion bodies, lack of posttranslational modifications and overexpression of proteins is associated with accumulation of endotoxins that are detrimental to bacterial cell growth and biomass production which in turn results in altered protein expression patterns (Makrides, 1996; Hoffmann & Rinas, 2004).

The first papain-like protease to be expressed in an *E. coli* system containing a T7 promoter was a human cysteine protease, cathepsin L, for which expression levels were very low and the resultant protein was included in inactive insoluble aggregates (Smith & Gottesman, 1989). An attempt to express *Tco*CATL from *T. congolense* in an *E. coli* system was unsuccessful as it yielded incorrectly folded and inactive enzyme. However, expression of an active enzyme at marginal yields was achieved through a more expensive and complicated Balculovirus expression system (Boulangé *et al*, 2001). A cysteine protease from *Leishmania braziliensis* was also expressed in *E. coli* mostly as an insoluble protein where denaturation followed by on column re-naturation steps were the only options to harvest the refolded and active enzyme (Lanfranco *et al*, 2008). In an attempt to evaluate the potential of alanyl aminopeptidases from *T. congolense* as trypanosomal drug targets, an *E. coli* expression system was used.

These proteases were expressed as insoluble fusion proteins which required solubilisation and refolding steps to attain the enzyme activity (Pillay *et al*, 2013a).

Due to the shortcomings of the expression systems mentioned above, the eukaryotic methylotrophic yeast *Pichia pastoris* has been identified as an alternative expression system for heterologous proteins (Macauley-Patrick *et al*, 2005; Cregg, 2007; Li *et al*, 2007). The common advantages of the *P. pastoris* expression system over bacterial expression include posttranslational modifications such as glycosylation, disulfide bond formation, proteolytic processing, folding, and maximal biomass production on a defined medium with minimal salt content (Cregg & Higgins, 1995; Wu & Letchworth, 2004). This expression system also constitutes a leader or signal sequence (prepro-peptide α-factor of *Saccharomyces*) which allows the secretion of the heterologous protein into expression media. Another remarkable advantage of the *Pichia* system is its insignificant secretion of endogenous proteins and this promotes high levels of expression of heterologous proteins in expression media, and this significantly simplifies the purification procedure (Laroche *et al*, 1994). Examples of successful expression of trypanosomal cysteine proteases in the *Pichia* system include: *Tco*CATL variants that were expressed at maximal yields as a soluble and active enzyme (Pillay *et al*, 2010; Boulangé *et al*, 2011) and *Tbr*CATL from *T. b. rhodesiense* (Cazzulo *et al*, 1990). Also, the surface antigens called invariant surface glycoprotein (ISG75) from *T. b gambiense* as well as the variant surface glycoprotein (VSG) RoTat 1.2 from *T. evansi*, were successfully expressed in the *Pichia* system (Rogé *et al*, 2013).

In the present study, the successful expression and enzymatic characterisation of the catalytic domain, *Tvi*CATL, from *T. vivax* was reported. *Tvi*CATL was successfully expressed at maximal yields and activity in the *P. pastoris* GS115 system. The overall yield obtained was significantly higher than that reported by Vather (2010). *Tvi*CATL migrated on reducing SDS-PAGE as two bands at 29 and 33 kDa. The presence of two bands suggested that the secreted protein may be asparagine-glycosylated as expected when using the *P. pastoris* expression system (Macauley-Patrick *et al*, 2005). Upon treatment of *Tvi*CATL with endoglycosidase H, which cleaves between a chitobiose (two linked N-acetyl glucosamine residues) core of high mannose-type N-linked glycoproteins (Maley *et al*, 1989), the double band was transformed into a single band at approximately 29 kDa, and the band at 33 kDa disappeared. This suggests that the potential glycosylation site (Figure 2.1), Asn^{288} (as predicted by NetNGlc 1.0 server, www.cbs.dtv.dk/services/NetNglyc), was deglycosylated. The Asn-glycosylation of *Tbr*CATL was also reported in the study conducted by Caffrey *et al* (2001). The glycosylation status of *Tbr*CATL recombinantly expressed in *P. pastoris*

was determined by treatment with Asn-glycosidase PNGase F prior to electrophoresis. It was noted that the higher molecular weight band disappeared. This suggested that the glycosylation site (Asn^{295}-Ala) was deglycosylated.

When comparing the recombinant *Tvi*CATL to native *Tvi*CATL, native *Tvi*CATL was distinguished using chicken anti-*Tvi*CATL C-terminal extension peptide antibodies, as a band at 56 kDa (Jackson, 2011), while in the present study, the recombinant *Tvi*CATL was expressed as 29 and 33 kDa forms of protein. The reason for the higher molecular weight for the native protein is due to the fact that the native protein had an intact C-terminal extension, while in the recombinant catalytic domain form of *Tvi*CATL, the C-terminal extension was truncated. Beside the fact that the C-terminal domain was truncated, the pro-region of *Tvi*CATL was included in the expression constructs since it has been largely associated with correct folding, stability and secretion of many expressed cathepsins (Smith & Gottesman, 1989; Vernet *et al*, 1991). The importance of the prodomain for activity of CATL proteases was verified by the fact that when a variant of *Tcr*CATL in which the prodomain was deleted, and expressed, the variant was inactive, unstable and prone to precipitation upon storage at cold temperatures (Eakin *et al*, 1992). The same phenomenon was observed for a variant of cathepsin L in which a prodomain was truncated (Smith & Gottesman, 1989).

As is the case of other cysteine proteases, the autocatalytic activation of *Tvi*CATL (removal of the pro-region) into a mature active enzyme, was facilitated by acidification of the expression supernatant (Vernet *et al*, 1991; Eakin *et al*, 1992). This activation is similar to that observed for recombinant *Tcr*CATL, in which auto-processing of the proenzyme resulted in activation of the catalytic domain (Eakin *et al*, 1992). The fact that the recombinant catalytic domain of *Tvi*CATL can be expressed as a functional enzyme, proves and is in accordance with previous findings that the C-terminal domain is not essential for enzymatic activity of trypanosomal cysteine proteases (Eakin *et al*, 1993). The successful expression of the catalytic domain of *Tvi*CATL is consistent with the expression of its homologue, *Tco*CATL, which was relatively stable and expressed at five-fold higher levels than the native full-length form (Boulangé *et al*, 2011).

The TPP purification technique was the first choice for the concentration of the large volumes of expression supernatants and the purification of the recombinant protease. Three phase partitioning is similar to conventional salting out, since both techniques employ ammonium sulfate as a precipitating agent. As compared to conventional salting out, TPP incorporates tertiary butanol which lessens the proteins' density, thus allowing them to float on the aqueous phase compared to highly dense proteins which

sink with ammonium sulfate in salting out. Therefore, the protein-rich disc appears between the ammonium sulfate and t-butanol liquid phases (Pike & Dennison, 1989). The two bands of recombinant *Tvi*CATL observed in reducing SDS-PAGE analysis prior to purification were also observed after purification by TPP. This suggests that activation of the propeptide at pH 4.2 prior to TPP did not disrupt the glycosylation status of *Tvi*CATL.

As the higher molecular weight contaminants were not sufficiently removed by TPP, molecular exclusion chromatography was used for purifying *Tvi*CATL to homogeneity. Notably, the 29 and 33 kDa bands were not fully separated, implying that chromatographic separation posed no effect on the glycosylation status of *Tvi*CATL. The final protein yield (589%) is higher than that obtained by a previous researcher, 143% (Vather, 2010). The higher yields and specific activity after purification by TPP are consistent with the observation that many proteases' activity is not altered by the presence of t-butanol. When proteinase K was purified by TPP, the specific activity was 210% as compared to 100% obtained for protease that was not TPP-purified. To understand the factors contributing to enhancement of the protease activity after TPP, the crystal structure of the TPP-purified protease was resolved. From the structure, it was concluded that activity enhancement is attributed to the rearrangement in the active site in which an acetate ion was introduced and that the protease appeared to exist in an excited state (Singh *et al*, 2001). The bands of the recombinantly expressed *Tvi*CATL, before purification and after purification by TPP and MEC, were efficiently recognised by antibodies raised against the full length inactive form of *Tvi*CATL.

Consistent with a requirement shared by all cysteine proteases, *Tvi*CATL activity was stimulated by the presence of the reducing agent DTT, which is responsible for maintaining the active site cysteine residue in a reduced state (Mbawa *et al*, 1992; Troeberg *et al*, 1996). The pre-activated recombinant *Tvi*CATL enzyme, at very low amounts, showed high proteolytic activity in gelatin substrate SDS-PAGE. This activity was comparable to that observed for recombinant *Tco*CATL. However, the high level of gelatinase activity of recombinant *Tvi*CATL outweighs the activity reported for native *Tvi*CATL, for which no substantial gelatin proteolysis was observed under standard conditions (Jackson, 2011). The *Tvi*CATL gelatinase activity was inhibited in the presence of classical clan CA protease inhibitors such as E-64 and leupeptin.

*Tvi*CATL in the constant ionic strength AMT buffer, had an optimum pH at 6.5 and 7.5 against a peptide substrate. This pH optimum (6.5 and 7.5) is comparable to that reported for the native *Tvi*CATL except that the native protein exhibited some activity

even at acidic pH-values of 4-5 (Jackson, 2011). The neutral optimal pH of recombinant *Tvi*CATL makes it convincing that this parasitic protease could remain active in the bloodstream upon parasite lysis, and that *Tvi*CATL has potential to be a virulence factor. Recombinant *Tco*CATL is also active at pH-values of 6 or 6.5 (Mbawa *et al*, 1992; Pillay *et al*, 2010).

The involvement of *Tvi*CATL in pathogenesis is supported by the fact that, at a pH around psychological pH, *Tvi*CATL was able to degrade endogenous protein substrates such as bovine haemoglobin, bovine serum albumin, bovine α_2-macroglobulin which is an endogenous inhibitor found in the host bloodstream, as well as type I collagen from rat tails (a model substrate). Also, the protein substrate fibrinogen, was degraded by native *Tvi*CATL (Jackson, 2011). The potential for hydrolysis of protein substrates by *Tvi*CATL is in accordance with the limited proteolysis of fibrinogen and bovine serum albumin reported for *Tco*CATL isolated from the bloodstream form of the *T. congolense* life-cycle (Mbawa *et al*, 1992) as well as the hydrolysis of α_2-macroglobulin by *Tco*CATL (Huson *et al*, 2009). The collagenolytic activity has been also reported for a prolyl oligopeptidase from *T. brucei* and is thought to allow the parasite to penetrate through the endothelial cells, cross blood barriers and multiply within the host (Bastos *et al*, 2010).

The knowledge of the substrate specificity of trypanosomal proteases is essential for the development of inhibitors with anti-trypanosomal potential. The substrate specificity of all proteases depends on the characteristic of the amino acid (whether hydrophobic, basic or non-charged) on the substrate peptide sequence which is susceptible to hydrolysis by an enzyme. The position of the peptide bond to be cleaved also matters. This is called positional specificity, and is divided into: endopeptidases, aminopeptidases and carboxypeptidases (Nägler *et al*, 1999). *Tvi*CATL was reacted with fluorogenic substrates in order to find its substrate preference. For all CATL-like endoproteases, the P_2 is known as a specificity determining position (Turk *et al*, 1998). The significance of the presence of an amino acid residue in P_2 of the substrate for hydrolysis is supported by the inability of the enzyme to hydrolyse the aminopeptidase substrate, H-Leu-AMC, which lacks a P_2 site. The recombinant *Tvi*CATL showed a pronounced preference for hydrophobic residues Phe or Leu at P_2 position. This preference also relies on the presence of the basic residues Arg or Lys in the P_1 position. Therefore, Z-Phe-Arg-AMC, H-D-Ala-Leu-Lys-AMC and H-D-Val-Leu-Lys-AMC were the substrates with a relatively high k_{cat}/K_m ratio, therefore high affinity for the *Tvi*CATL active site for hydrolysis. The presence of structurally constrained residues such as proline in P_3 of the substrates largely decreases the hydrolysing

potential of *Tvi*CATL even in the presence of Phe or Leu at the P_2 position. This is evidenced by the relatively slow rate of hydrolysis of H-Pro-Phe-Arg-AMC. This preference for hydrophobic residues at P_2 was also reported for the native *Tvi*CATL (Jackson, 2011), recombinant *Tco*CATL (Pillay *et al*, 2010; Vather, 2010), recombinant *Tcr*CATL (Eakin *et al*, 1992; Nery *et al*, 1997) and *Tbb*CATL (Lonsdale-Eccles & Grab, 1987). These substrates considered as best substrates for recombinant *Tvi*CATL were able to somewhat discriminate the preferences between Phe and Leu at the P_2 position. This is evidenced by the fact that the k_{cat}/K_m of an endopeptidase substrate (Z-Phe-Arg-AMC) containing Phe at P_2 position is larger than that of the substrate containing Leu at P_2 (H-D-Ala-Leu-Lys-AMC). This preference for Phe over Leu at P_2 was also reported for *Tcr*CATL and *Tco*CATL (Caffrey *et al*, 2001; Jackson, 2011). However, *Tbr*CATL showed an equal preference for Phe and Leu at P_2, and this is attributed to the presence of an Ala residue at the bottom of the S_2 position, making it difficult to hydrolyse arginine-containing substrates (Caffrey *et al*, 2001).

The ability of *Tvi*CATL to cleave Z-Pro-Arg-AMC was a new characteristic observed for this protease. Z-Pro-Arg-AMC is the specific substrate for mammalian cathepsin K, which has a marked preference for proline residues at the P_2 position (Lecaille *et al*, 2003). The ability of *Tvi*CATL to hydrolyse a cathepsin K substrate could be explained by the fact that the residues at the bottom of the S_2 subsite of *Tvi*CATL and *Tco*CATL are identical to residues of the S_2 of cathepsin K which contains Leu205 (papain numbering). The ability to hydrolyse a proline containing peptide substrate is consistent with the finding that *Tvi*CATL is able to cleave a protein substrate, collagen which is a proline-rich substrate. This preference is in contrast with the specificity of cathepsin B proteases which has a glutamyl residue in position 205 (papain numbering) at the bottom of the S_2 subsite. The presence of a glutamyl residue accounts for dual specificity of cathepsin B and *Tcr*CATL at which both Arg and Phe are preferentially accommodated at the P_2 position (Musil *et al*, 1991; McGrath *et al*, 1995; Chagas *et al*, 1997; Gillmor *et al*, 1997). Therefore the inability of *Tvi*CATL to cleave Z-Arg-Arg-AMC suggests that this protease lacks the cathepsin B-like activity.

The enzyme was effectively inhibited by the reversible aldehyde inhibitors antipain, chymostatin and leupeptin, which contain a basic arginine residue at P_1. This inhibition further confirms that the enzyme prefers a basic Arg residue at P_1. This inhibition is similar to that reported for native *Tvi*CATL (Jackson, 2011), native and recombinant *Tco*CATL (Pillay *et al*, 2010; Vather, 2010) and *Tcr*CATL (Garcia *et al*, 1998). The lack of inhibition of the *Tvi*CATL peptidase and gelatinase activity by bestatin which contains Phe at P_1, suggests that Phe is not a favoured residue at P_1. This also

suggests that *Tvi*CATL is not an aminopeptidase; therefore the synthesis of specific potent inhibitors against *Tvi*CATL requires that the N-terminus of the peptide is blocked. The enzyme was insensitive towards the addition of the metalloprotease inhibitor, EDTA, but it rather activated the gelatinase activity of the enzyme probably by chelating all the ions that might have been reactive towards the SH group in the active site.

The effective inhibition of *Tvi*CATL hydrolytic activity against specific fluorogenic peptide and gelatin substrates by thiol-specific irreversible inhibitors such as E-64, iodoacetate and iodoacetamide, further confirm that *Tvi*CATL is a cysteine protease. The recombinant *Tvi*CATL was partially inhibited by Z-Gly-Leu-Phe-CMK. The native *Tvi*CATL was also inhibited by this inhibitor as well as by H-D-Ala-Phe-Lys-CMK (Jackson, 2011). These inhibition patterns further show that in P_2, Phe and Leu residues are important for efficient substrate hydrolysis.

In the work reported in this chapter, recombinant *Tvi*CATL was successfully expressed and purified to homogeneity. Enzymatic characterisation of *Tvi*CATL was conducted in order to evaluate the pH optimum, substrate specificity, interaction with reversible, irreversible and host protease inhibitors. It can be concluded that recombinant *Tvi*CATL have a pronounced preference for hydrophobic residues Phe or Leu at P_2 position, and that its activity is inhibited by the class specific cysteine protease inhibitors. *Tvi*CATL also have the activity against host serum protein at physiological pH. Further studies such as RNA interference (RNAi) gene silencing are necessary steps towards determining if *Tvi*CATL is a pathogenic factor. In work described in the next chapter, recombinant *Tvi*CATL was used for antibody production and to find scFvs binders in the Nkuku® semi-synthetic phage display library in order to evaluate the potential of *Tvi*CATL as a diagnostic tool in an antigen and antibody species-specific serological diagnosis.

CHAPTER 3

ANTIBODY PRODUCTION IN CHICKENS AND SELECTION OF SINGLE-CHAIN VARIABLE FRAGMENTS (SCFVS) FROM THE NKUKU® LIBRARY AGAINST RECOMBINANT *Tvi*CATL

3.1 Introduction

To effectively control African animal trypanosomiasis, it is of paramount importance to develop sensitive and species-specific point-of-care serological diagnostic tests that are affordable to the end-users. Antigen and antibody detection enzyme-linked immunosorbent assays (ELISAs), in an indirect or inhibition format, are the commonly used serological tests that are affordable, and relatively simple procedures for large scale screening (Luckins, 1977; OIE, 2012). Both antigen and antibody detection ELISAs have been used for diagnosis of *T. vivax*, *T. congolense* and *T. brucei* infection in cattle (Nantulya & Lindqvist, 1989; Eisler *et al*, 1998; Bossard *et al*, 2010; Pillay *et al*, 2013b; Fleming *et al*, 2014; Zhou *et al*, 2014) as well as *T. evansi* infections in buffaloes and horses (Singh *et al*, 1995; Lejon *et al*, 2005; Tran *et al*, 2009). The efficacy of some of these antigen and antibody detection ELISAs is limited by the requirement of the whole or crude parasite lysates which are unstable. Furthermore there is a lack of standardisation for antigen preparations, need for ethical clearance for culturing parasites *in vivo*, as well as the lack of reproducibility of the procedure (Greiner *et al*, 1997; Rebeski *et al*, 2000; Desquesnes *et al*, 2009).

Therefore, to circumvent the challenges mentioned above, it is necessary to screen for antigens with immunodiagnostic potential. Research has focused on serological antibody tests using trypanosomal antigens that are recombinantly produced. These antigens are non-variant and immunogenic trypanosomal antigens which have been identified as suitable candidates for serodiagnostic purposes. These antigens include: heat shock protein 70 (HSP70) closely related to a mammalian immunoglobulin binding protein (BiP) (i.e. BiP/HSP70) in sera of *T. congolense* infected cattle (Boulangé & Authié, 1994; Boulangé *et al*, 2001; Boulangé *et al*, 2002; Bannai *et al*, 2003; Bossard *et al*, 2010), recombinant invariant surface glycoprotein (rISG75) from *T. b. gambiense*

(Tran *et al*, 2008) and flagellar associated-protein, GM6 from *T. vivax* (Pillay *et al*, 2013b) and TcP46 from *T. congolense* (Fleming *et al*, 2014; Zhou *et al*, 2014). Further antigens serve as potential diagnostic antigens because they express tandem repeat domain which largely elicit B-cell response in trypanosomal parasites such as *T. evansi* (Goto *et al*, 2011; Thuy *et al*, 2012).

Trypanosomal cysteine proteases are virulence factors involved in the pathogenesis of African animal trypanosomiasis hence may serve as plausible immunodominant antigens for the development of serodiagnostic tools for AAT (Authié *et al*, 1992; Authié *et al*, 2001; Lalmanach *et al*, 2002). Serological diagnosis through an inhibition ELISA format has an advantage that it can be used to diagnose trypanosomiasis in different species without the need for different anti-species enzyme-labelled detection antibodies. The inhibition ELISA format requires that enough antibodies against the target recombinant antigen are produced. Egg yolk immunoglobulin (IgY) technology is a preferred method for the generation of antibodies. Immunoglobulin IgY which is equivalent to mammalian IgG, is isolated from chicken egg yolks (Polson *et al*, 1980). Immunoglobulin IgY antibodies are advantageous in that they are produced at high concentrations and titres against parasite antigens (Morty *et al*, 1999; Eyssen, 2014), lack cross-reactivity with mammalian IgGs, therefore serve as an inexpensive source of antibodies for diagnostic purposes (Hädge & Ambrosius, 1984; Larsson *et al*, 1993; Kricka, 1999; Dias da Silva & Tambourgi, 2010). Alternatively, the need for animal immunisation for antibody production can be replaced by panning with *Tvi*CATL for single-chain variant fragments (scFvs) acquired from the semi-synthetic phage display library called Nkuku® chicken antibody reptoire as described by Van Wyngaardt *et al* (2004). The phage-produced scFvs are considered as renewable immunoreagents for diagnostic purposes using an easily standardised ELISA assay. The phage-produced scFvs can be sequenced to confirm their identity and the soluble scFvs can be produced in large quantities by heterologous expression systems (Verma *et al*, 1998; Fehrsen *et al*, 2005b; Chiliza *et al*, 2008; Rakabe *et al*, 2011; van Wyngaardt *et al*, 2013).

In this chapter, the production, purification and characterisation of IgY antibodies against the recombinant cysteine protease, *Tvi*CATL is reported. These antibodies were characterised by ELISA and western blot analysis and used in an antibody detection inhibition ELISA format for diagnosis of trypanosomiasis in sera from experimentally infected cattle. The initial Nkuku® phage display trials for the production of monospecific scFvs against *Tvi*CATL are also reported.

3.2 Production of anti-*Tvi*CATL IgY antibodies in chickens

3.2.1 Materials

Chicken IgY antibodies: Freund's complete and incomplete adjuvants and rabbit anti-chicken IgY-HRPO conjugate were obtained from Sigma (Munich, Germany). Nunc®-Immuno™ 96-well plates and Nunc® Black 96-well plates were purchased from Nunc (Intermed, Denmark). Filter paper was obtained from Whatman (Middlesex, UK). Aminolink® matrix was purchased from Pierce (Rockford, IL, USA). ABTS was from Roche (Mannheim, Germany). The PageRuler™ prestained or unstained protein ladders (M) were from Thermo Scientific (Lithuania). Novex® ECL Chemiluminescent Substrate Reagent Kit was obtained from Invitrogen™ (Van Allen Way, California).

***T. congolense*-infected sera**: Sera from infected (labelled as +) and non-infected (labelled as -) cattle were obtained from ClinVet International (PTY) LTD, Bloemfontein, South Africa.

Nkuku® phage display library: TG1 *E. coli* cells, M13KO7 helper phage and Nkuku® library stocks were obtained from Prof. Dion du Plessis, Immunology Division, Onderstepoort Veterinary Institute, Onderstepoort, 0110, Republic of South Africa. Immuno™ Tubes Nunc® Maxisorp were purchased from Nunc Intermed (Roskilde, Denmark). Corning® Costar® cell culture plates, 96 well with low evaporation lid were from Corning (USA). HRP-labelled-anti-M13 mAb was purchased from Amersham (Pharmacia, Biotech, USA). Sheep or goat anti-mouse IgG-HRPO conjugates were obtained from Sigma (Munich, Germany).

3.2.2 Immunisation of chickens

The MEC-purified *Tvi*CATL (Section 2.2.4) was used for immunisation of chickens to raise anti-*Tvi*CATL IgY antibodies. Briefly, three chickens were injected intramuscularly into the breast muscle with 50 µg antigen emulsified in Freund's complete adjuvant [1:1 (v/v)]. The chickens received three immunisation boosts (at weeks 2, 4 and 6) with 50 µg antigen emulsified in Freund's incomplete adjuvant [1:1 (v/v)]. Eggs were collected daily for 16 weeks and stored at 4°C.

3.2.3 Isolation of IgY from egg yolks

The polyethylene glycol (PEG) precipitation method described by Polson *et al* (1980) and modified by Goldring and Coetzer (2003) was used for the isolation and purification of IgY from egg yolks. Briefly, under running water, the egg yolk was separated from the egg white and and then poured into a measuring cylinder. The yolk volume was determined, and mixed with 2 volumes of IgY isolation buffer [100 mM Na-phosphate buffer, pH 7.6, 0.02% (w/v) NaN_3], followed by the addition of 3.5% (w/v) solid polyethylene glycol (PEG) M_r 6 000. After gently dissolving the PEG, the mixture was centrifuged (4 420 x *g*, 30 min, RT) and the lipid fraction removed from the supernatant by filtering through absorbent cotton wool. The volume of the filtered supernatant was determined and 8.5% (w/v) PEG dissolved in this supernatant. After centrifugation (12 000 x *g*, 10 min, RT), the pellet was resuspended in IgY isolation buffer in a volume equivalent to that obtained after filtration. Polyethylene glycol [12.5% (w/v)] was added, dissolved by magnetic stirring and centrifuged (12 000 x *g*, 10 min, RT). The IgY buffer containing [0.1% (w/v)] sodium azide was used to resuspended the final resultant antibody pellet in 1/6 of the original yolk volume. The concentration of each IgY pool was determined by measuring the $A_{280\ nm}$ of IgY diluted 1/40 in IgY buffer, and the concentration of the undiluted IgY was calculated using the extinction coefficient, $1.25 = \mathrm{E}^{1\ \mathrm{mg/ml}}_{280\ \mathrm{nm}}$ (Goldring & Coetzer, 2003).

3.2.4 Enzyme-linked immunosorbent assays (ELISA) and cross-reactivity of trypanosomal antibodies

The ELISA was used to evaluate the antibody production and cross-reactivity of these antibodies with other trypanosomal antigens. The 96-well ELISA plates were coated overnight at 4°C with *Tvi*CATL antigen (0.5, 1 or 2 µg/ml, 150 µl per well) diluted in PBS, pH 7.2. The unoccupied sites on the wells were blocked by incubating with blocking buffer [0.5% (w/v) BSA-PBS; 200 µl per well] for 1 hour at 37°C. The wells were washed 3x with PBST [PBS-0.1% (v/v) Tween-20] using a BIOTEK® ELx50™ Microplate washer (BioTek® Instruments, Inc., USA) and incubated for 2 hours at 37°C with chicken anti-*Tvi*CATL IgY diluted in blocking buffer (100 µl per well). After washing 3x with PBST, the wells were incubated for 1 hour at 37°C with rabbit anti-chicken IgY-HRPO conjugate (1:10 000, 100 µl per well) diluted in the blocking buffer. Colour development in the dark followed addition of the chromogen-substrate solution [0.05% (w/v) ABTS; 0.015% (v/v) H_2O_2 in 150 mM citrate phosphate buffer, pH 5; 145 µl per well]. The FLUOStar Optima microplate reader (BMG Labtech, Offenburg, Germany)

was used to monitor the absorbance at 405 nm every 15 min until an absorbance at 405 nm was as close as possible to 1.

3.2.5 Western blot analysis

Western blot analysis was conducted as described in Section 2.2.8

3.2.6 Preparation of *Tvi*CATL affinity column for purification of IgY antibodies

Aminolink® resin (2 ml of 50% resin slurry) was added to the affinity chromatography column to provide 1 ml gel after settling. The resin storage solution was drained, but at all times, the resin bed was never allowed to get dry. The column was equilibrated by adding 3-resin-bed volumes (3 ml) of pH 10 coupling buffer [0.1 M sodium citrate dihydrate, 0.05 M sodium carbonate, pH 10]. After draining the pH 10 coupling buffer, the column was capped at the bottom, followed by addition of 1 ml MEC-purified *Tvi*CATL protein (3 mg/ml) diluted in pH 10 coupling buffer. Protein sample (100 µl) was kept for determination of the coupling efficiency. The resin and the protein were allowed to mix for 4 hours at room temperature using an end-over-end rotor. This continual rotation allowed the resin aldehydes to react with the primary amine of the proteins through the formation of unstable Schiff base bonds. The unbound protein was drained into a new collection tube, and the column washed with 3 ml pH 7.2 coupling buffer. To form stable secondary amine bonds between the resin aldehydes and the protein, 1 ml of pH 7.2 coupling buffer containing 50 mM sodium cyanoborohydride was added to the column reaction slurry and mixed on an end-over-end rotor overnight at 4°C. The unoccupied sites were then blocked by passing through 2 ml quenching buffer (1 M Tris-HCl, pH 7.2), followed by addition of 1 ml quenching buffer supplemented with 50 mM sodium cyanoborohydride to form stable secondary amine bonds between aldehydes and the Tris, and mixed by end-over-end rotating for 30 minutes at room temperature. The column was washed with approximately 5 ml wash solution (1 M sodium chloride) until $A_{280\ nm} = 0$ was reached. The column resin was then washed with 3 ml PBS containing 0.05% (w/v) sodium azide and the column stored upright at 4°C with at least 1 ml of this buffer covering the resin bed. To determine the coupling efficiency, the concentrations of *Tvi*CATL prior to coupling and the eluted unbound protein were measured. The coupling efficiency was found to be approximately 72%.

3.2.7 *Tvi*CATL affinity purification of chicken anti-*Tvi*CATL IgY antibodies

The ELISA was used to identify antibody pools (from eggs from each of the three chickens) with high antibody titres. The antibody pools were filtered through Whatman no.1 filter paper before passing through the *Tvi*CATL affinity column pre-equilibrated with 3 ml PBS. The antibody pool was allowed to pass through the resin in a forward direction and the flow rate was controlled using a Micro Tube pump MP-3 (Tokyo Rikakikai, Japan), followed by reversing the direction of flow and this reverse cycling was allowed to continue for overnight at RT. The unbound antibodies were eluted (through forward direction of flow) and the column washed with 10 ml PBS. The bound antibodies were eluted using IgY elution buffer [0.1 M glycine-HCl, pH 2.8]. These antibodies (900 µl per fraction) were eluted into collection tubes containing 100 µl neutralisation buffer [1 M NaH_2PO_4, 0.02% (w/v) NaN_3, pH 8.5]. Antibody elution was monitored by measuring $A_{280\ nm}$. The fractions with the highest $A_{280\ nm}$ values were pooled, and the concentration determined by dividing the $A_{280\ nm}$ values by the IgY extinction coefficient, 1.25.

3.2.8 Antibody detection in an inhibition ELISA format

The ability of *T. congolense* infected sera to prevent the binding of chicken anti-*Tvi*CATL IgY antibodies to the immobilised capture *Tvi*CATL antigen on the plate was investigated in an inhibition ELISA format (Figure 3.1) as described by Bossard *et al* (2010). The 96-well ELISA plates were coated at 4°C for 16 hours with *Tvi*CATL (5 µg/ml, 100 µl per well) diluted in PBS, pH 7.2. Each serum was tested in triplicate and the corresponding uncoated triplicate wells were included as a no coat control. After blocking the nonspecific sites with 200 µl per well blocking buffer [0.5% (w/v) BSA-PBS-0.1% (v/v) Tween-20] for 1 hour at 37°C, the wells were washed 3x with PBST [PBS-0.1% (v/v) Tween-20] using a BIOTEK® ELx50™ Microplate washer. The wells were then incubated for 1 hour at 37°C with the respective bovine serum samples (1:100, diluted in blocking buffer). The no serum control quadruplicate wells were included and incubated with blocking buffer instead of serum. The wells were washed 3x with PBST and incubated for 1 hour at 37°C with affinity purified chicken anti-*Tvi*CATL IgY (5 µg/ml, 100 µl per well) diluted in blocking buffer. After washing 3x with PBST, the wells were incubated for 1 hour at 37°C with rabbit anti-chicken IgY-HRPO conjugate (1:10 000, 100 µl per well) diluted in blocking buffer. The plate was washed 3x and incubated with the substrate solution [0.05% (w/v) ABTS; 0.015% (v/v) H_2O_2 in 150 mM citrate phosphate buffer, pH 5] for 15 min in the dark, followed by measuring

absorbance values at 405 nm using a FLUOStar Optima microplate reader (BMG Labtech, Offenburg, Germany).

The average absorbance (triplicate samples, antigen-coated wells) for each serum was calculated and corrected by subtracting the average absorbance (triplicate samples, non-coated wells) of the corresponding uncoated control wells. The average (quadruplicate samples, antigen-coated wells) for no serum control (0% inhibition) was determined and also corrected by subtracting the average of the uncoated wells. The percentage (%) inhibition was determined using the following equation:

$$\%\ \text{inhibition} = \frac{A_{405\ \text{nm}}\ (\text{no serum control}) - A_{405\ \text{nm}}\ (\text{serum})}{A_{405\ \text{nm}}\ (\text{no serum control})} \times 100$$

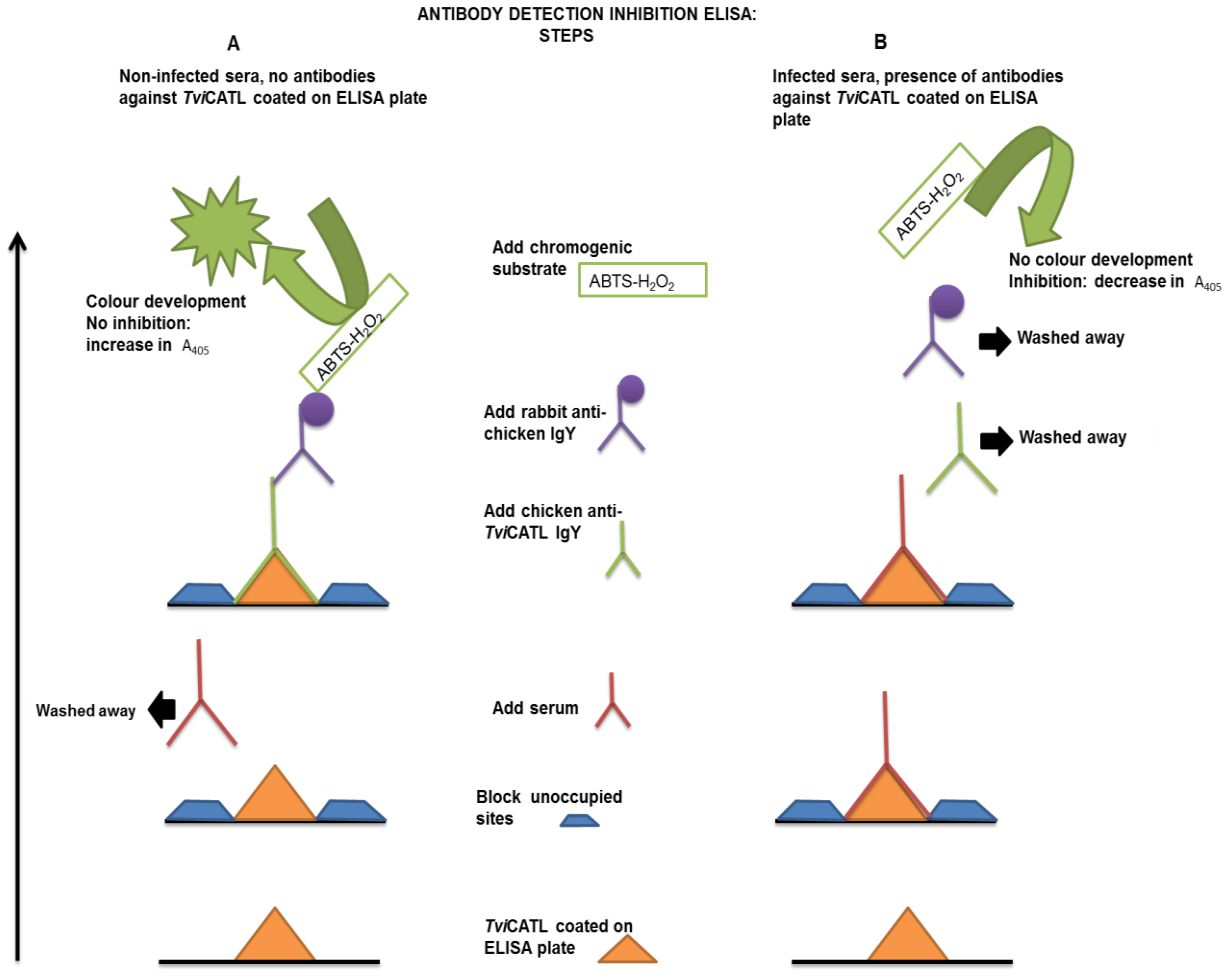

Figure 3.1: Schematic diagram summarising the steps in an inhibition ELISA format. There is a final colour development which in turn results in a signal at A_{405} when a non-infected serum is used **(A)** while there is no colour development which in turn results in no signal at A_{405} when the infected serum is used **(B).**

3.2.9 Assay for immunoinhibition of *Tvi*CATL activity by chicken anti-*Tvi*CATL antibodies

The influence of chicken anti-*Tvi*CATL antibodies on the activity of *Tvi*CATL was investigated as described by Troeberg *et al* (1997) and Huson *et al* (2009). *Tvi*CATL (100 ng active enzyme) diluted in 0.1% (w/v) Brij-35 was incubated for 15 minutes at 37°C with anti-*Tvi*CATL IgY (1000-15 µg/ml) serially diluted in phosphate buffer [200 mM sodium phosphate buffer, pH 7.2, 4 mM Na_2EDTA and 0.1% (v/v) Tween-20]. The incubation mixture at each respective concentration was aliquoted (50 µl in triplicate) into 96-well Nunc® Black plates and activated with *Tvi*CATL assay (25 µl) buffer containing 8 mM DTT for 1 min at 37°C. This was followed by the addition of 25 µl Z-Phe-Arg-AMC (20 µM) and further incubation at 37°C for 5 min. The rate of hydrolysis of Z-Phe-Arg-AMC was measured by reading fluorescence ($Ex_{360\ nm}$; $Em_{460\ nm}$) using the FLUORStar Optima spectrophotometer (BMG Labtech, Offenburg, Germany).

3.3 Nkuku® phage display for production of scFvs monospecific antibodies

3.3.1 Culturing the *E. coli* TG1 cells

The *E. coli* TG1 glycerol stock was streaked on TYE agar [15 g agar, 8 g NaCl, 1 g tryptone, 5 g yeast in 1L double distilled deionised water]. After allowing the colonies to grow at 37°C overnight, a single colony was inoculated in 5 ml 2xYT medium [16 g tryptone, 10 g yeast extract, 5 g NaCl dissolved in 1L double distilled deionised water] and left to grow overnight at 37°C (220 rpm shaking incubator). One ml of the overnight culture was diluted in 100 ml 2xYT medium and allowed to grow at 37°C until the log phase was reached (OD_{600} = 0.5), followed by storage at 4°C and used within 5 days (or as fresh as possible). The TG1 glycerol stock was made by adding glycerol [15% (v/v) final concentration] to the overnight cultured TG1 cells and kept at -70°C.

3.3.2 Titering and culturing M13KO7 helper phage

To determine the concentration (plaque forming units per ml) of the initial M13KO7 glycerol stock, serial dilutions (10^{-2}-10^{-10}) were made in sterile 2xYT medium. To induce the bacterial infection by phages, 100 µl of the respective dilutions were mixed with 100 µl of the log phase TG1 cells for 5 min at room temperature. This mixture together with 3 ml top agar [44°C 2xYT containing 0.7% (w/v) agar], were sprinkled on the set pre-warmed 2xYT plates containing 1.5% (w/v) agar, and swirled gently to allow even distribution along the entire plate. The top agar was allowed to set, plates

inverted and incubated at 37°C overnight. The pfu/ml was determined for plates with 30 ≥300 plaques (single distributed plaques).

To culture the M13KO7 cells, a single isolated plaque was punched out (from the titered plate) using the reverse end of a yellow pipette tip, inoculated into 4 ml 2xYT medium containing 40 µl overnight grown TG1 cells and finally incubated at 37°C (100 rpm orbital shaking incubator for 2 hours). This primary culture was inoculated into 400 ml 2xYT medium for 1 hour at 37°C (100 rpm shaking) followed by the addition of 50 µg/ml kanamycin and left for further growth overnight. The overnight culture was centrifuged (10 800 x *g*, 15 min, 4°C) and the supernatant was precipitated with ¼ volume of phage precipitating buffer [20% (w/v) PEG M_r 6000, 2.5 M NaCl] for 30 minutes on ice or 4°C. This was followed by two successive centrifugation steps: (10 800 x *g*, 15 min, 4°C followed by 2000 x *g*, 2 min, 4°C). The final pellet was suspended in sterile PBS, filter-sterilised (0.22 µM) and the titer (pfu/ml) determined by measuring the absorbance at 268 nm as described by Tonikian *et al* (2007).

3.3.3 Culturing the Nkuku® phagemid library

The Nkuku® phagemid glycerol stock (~250 µl) was inoculated into 500 ml 2xYT medium containing 0.22 µm filter sterilised 2% (w/v) glucose and 100 µg/ml ampicillin. The volume of phagemid added was adjusted until the initial OD_{600} was as close as possible to 0.05. This culture was incubated at 37°C with shaking at 240 rpm until OD_{600} = 0.5. Once the log phase was reached, 100 ml of this phagemid culture was phage rescued with M13KO7 helper phage (8x 10^9 pfu/ml) at 37°C (30 minutes standing, 30 minutes shaking at 100 rpm). The rest of the phagemid culture was further grown for 3 hours, centrifuged (3300 x *g*, 15 min, 4°C), the pellet suspended in 1/100 2xYT medium, followed by addition of 50% (v/v) glycerol to form a glycerol stock at a final glycerol concentration of 15% (v/v). The glycerol stock was kept at -70°C. The portion that was rescued by M13KO7 helper phage, was centrifuged (3300 x *g*, 15 min, 4°C), pellet suspended in 1L 2xYT medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin in a 5L flask. This culture was left overnight at 30°C (240 rpm orbital shaking incubator) overnight. The overnight culture was centrifuged (3300 x *g*, 20 min, 4°C) and the supernatant precipitated in 1/4 (v/v) phage precipitating buffer [20% (w/v) PEG M_r 6000, 2.5 M NaCl] for 1 hour on ice, followed by two successive centrifugation steps: (3300 x *g*, 30 min, 4°C followed by 2000 x *g*, 2 min, 4°C). The final pellet was suspended in 20 ml PBS and re-centrifuged (11 000 x *g*, 2 min, 4°C). The supernatant was filter-sterilised (0.22 µm) and stored at 4°C for use during the first panning round.

3.3.4 Titering the Nkuku® phagemid library

In order to determine the titre of the cultured library, the cultured Nkuku® phagemid library was titered through serial dilution (10^{-2}-10^{-6}) in 2xYT medium. The respective dilutions (100 µl) were infected for 5 minutes at room temperature with 100 µl log phase *E. coli* TG1 cells. The entire mixture (200 µl) was then spread onto pre-warmed TYE A/G plates [15 g agar, 8 g NaCl, 1 g tryptone, 5 g yeast in 1L double distilled deionised water; 2% (w/v) glucose and 100 µg/ml ampicillin]. The uninfected phagemid and TG1 cells alone were also spread on separate plates in order to monitor for contamination. Plates were inverted and incubated at 30°C overnight. The colony forming units (cfu/ml) was determined for the plates with 30 ≥300 colonies. The absence of colonies on the TG1 cells alone plate and the Nkuku® phagemid alone plate implied the absence of contamination.

3.3.5 Selection/ panning of scFvs against *Tvi*CATL from the Nkuku® library

The scFvs against recombinant *Tvi*CATL were selected from the Nkuku® phagemid library using an affinity selection method called bio-panning (Figure 3.2). *Tvi*CATL (100-10 µg/ml) diluted in PBS was coated into the immunotube and left overnight at 4°C. The unbound antigen was removed by washing 3x with PBS. The unoccupied sites were blocked for 1 hour at 37°C with 5 ml 2% (w/v) Elite® fat-free milk powder in PBS. The immunotube was washed 2x with PBST [PBS-0.2% (v/v) Tween-20] followed by 2x washes with PBS alone. Pre-incubated (30 min, RT) Nkuku® phagemid library (3.5 ml; 1 x 10^{13} TU, diluted in PBST containing 2% (w/v) milk powder) was added. The immunotube was rotated for 30 min at RT using an end-over-end rotor, followed by standing for 90 minutes. After washing 20x with PBST followed by 20x washes with PBS alone, log phase TG1 cells (3.5 ml) were added to this immunotube and incubated for 30 min at 37°C, and placed on ice. This was centrifuged (3300 x *g*, 10 min, 4°C) and the pellet suspended in 1 ml 2xYT medium. This 1 ml phage culture was plated onto 3 TYE/AG agar plates (~333 µl per plate) and incubated at 30°C overnight. The resultant colonies were suspended in ~10 ml 2xYT medium, and the glycerol stock made by adding glycerol to a final concentration of 15% (v/v). To obtain a large amount of phagemid from each panning round, ~500 µl of suspended colonies or prepared glycerol stock were inoculated into 50 ml 2xYT/AG medium and ensuring that the initial OD_{600} was as close as possible to 0.05. This phagemid culture was grown at 37°C until OD_{600} = 0.5. Five ml of this log phase phagemid culture was phage rescued with M13KO7 helper phage (8x10^9 pfu/ml) at 37°C for 30 minutes with occasional agitation

at 100 rpm. This culture was then centrifuged 2x (3300 x *g*, 10 min, 4°C) and the final pellet suspended in 25 ml 2xYT medium containing 100 µg/ml ampicillin and 25 µg/ml kanamycin. The culture was grown overnight at 30°C in a 240 rpm shaking incubator. The overnight culture was centrifuged (3300 x *g*, 20 min, 4°C) and the supernatant precipitated in 1/5 (v/v) phage precipitating buffer [20% (w/v) PEG M_r 6000, 2.5 M NaCl] for 1 hour at 4°C, followed by two successive centrifugation steps (3300 x *g*, 30 min, 4°C). The final pellet was suspended with 1 ml PBS, followed by centrifugation (1100 x *g*, 2 min, RT). The supernatant was filter-sterilised through a 0.22 µm filter. Sterilised respective panning round phages (0.5 ml) were used for the next panning round at which the immunotubes were coated with *Tvi*CATL (100-10 µg/ml). The successive panning rounds were done up to a total of four rounds.

3.3.6 Screening for binders by polyclonal phage ELISA

The polyclonal ELISA was conducted in a similar way as the ELISA described in Section 3.2.4 except that 50 µl of *Tvi*CATL diluted in PBS was used for coating wells at concentrations of: 100, 75, 50, 25, 10 and 1 µg/ml. The control wells were coated with PBS. Wells were blocked with 300 µl 3% (w/v) BSA-PBS for 1 hour at 37°C. From each panning round, phage dilutions (1:200, 1:100, 1:50, 1:25) were made in 6% (w/v) BSA-PBS-0.2% (v/v) Tween-20, followed by further dilution in an equal volume of 6% (w/v) BSA-PBS-0.2% (v/v) Tween-20. Each respective dilution (25 µl) was added to the respective wells and incubated at 37°C for 1 hour. After washing 3x with PBST [PBS-0.1% (v/v) Tween-20], the plate was incubated with 50 µl mouse-anti-M13 monoclonal antibody (1:8000 diluted in 3% (w/v) BSA-PBS-0.2% (v/v) Tween-20) for 1 hour at 30°C. After washing 3x with PBST, the plate was incubated with 50 µl sheep anti-mouse IgG-HRPO secondary antibody (1:1000 diluted in 3% (w/v) BSA-PBS-0.2% (v/v) Tween-20) for 1 hour at 37°C. The ELISA was completed as described in Section 3.2.4

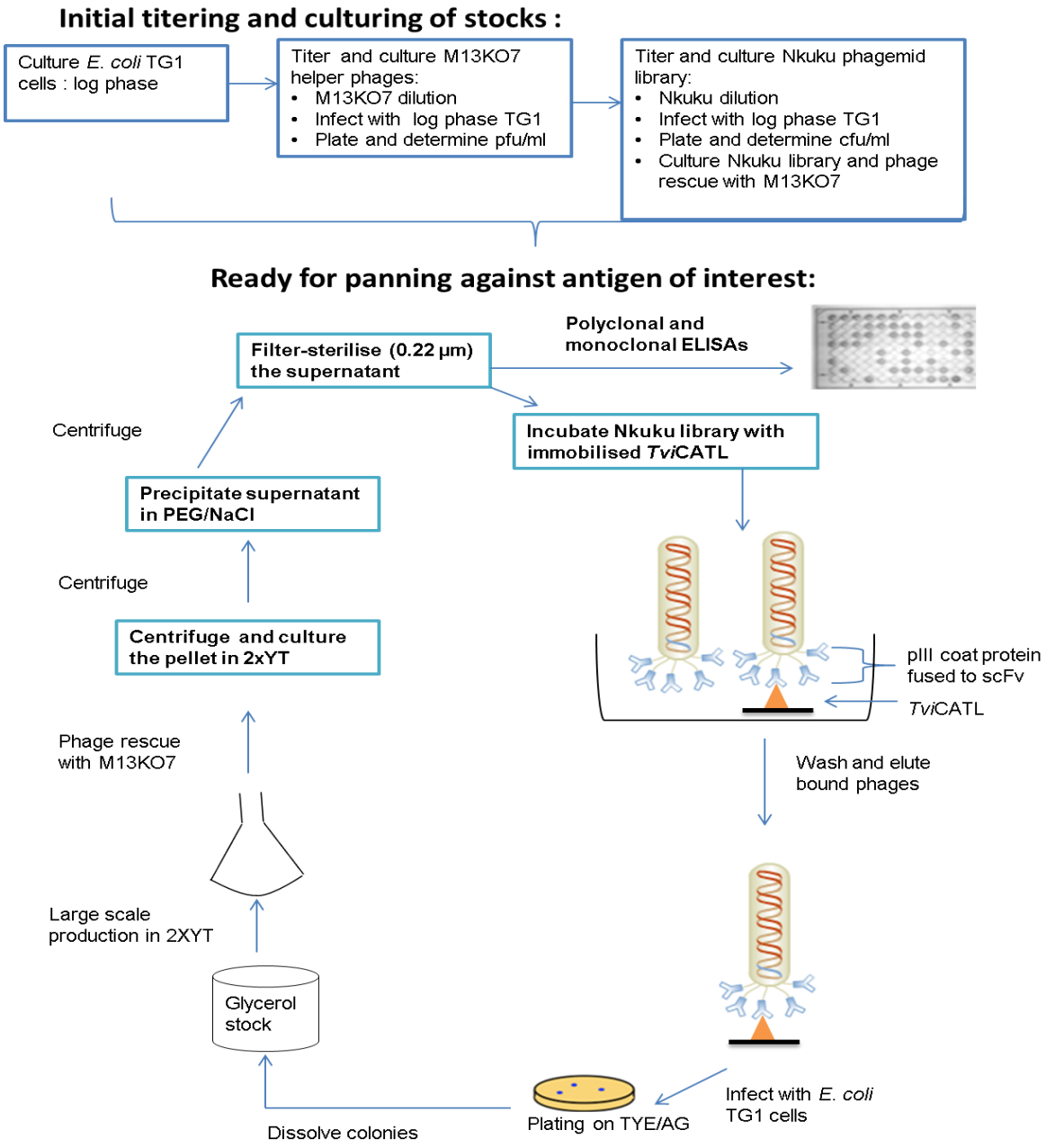

Figure 3.2: Flow diagram summarising the panning of Nkuku® phage scFvs against recombinant *Tvi*CATL.

3.3.7 Screening for phage-displayed binders by monospecific ELISA

For each panning round with detectable polyclonal antibodies, the phage stock was titered as described in Section 3.3.4. Under sterile conditions near the flame, 48 single colonies were randomly picked and inoculated into individual wells of 96-well Costar® cell culture plates containing 100 µl 2xYT medium and incubated at 30°C overnight in

the incubator with shaking at 220 rpm. This plate was called the master plate. The glycerol stock was made by adding 50 µl 60% (v/v) glycerol and stored at -70°C. From the master plate, 5 µl inoculum per well was inoculated into a new individual plate wells containing 150 µl 2xYT A/G medium, and grown at 37°C for 2 hours at 220 rpm. This plate was called the clone plate. The phages in each well of the clone plate were rescued by adding 100 µl M13 helper phage (2 x 10^9 pfu/ml) diluted in 2xYT medium, followed by incubation at 37°C for 30 minutes. The plate was centrifuged (600 x *g*, 10 min, RT), and all the supernatants were decanted. To each well containing the pellets, 150 µl 2xYT medium (containing 100 µg/ml ampicillin and 25 µg/ml kanamycin) was added, and the plate incubated at 30°C overnight with shaking at 220 rpm. After centrifugation (600 x *g*, 10 min, 4°C), the supernatant was mixed with an equal volume of 4% (w/v) milk-PBS-0.2% (v/v) Tween-20. These phages were used for monospecific ELISA as described in Section 3.2.4, and uncoated wells were included as a control for milk binders.

3.4 Results

3.4.1 Evaluation by ELISA of chicken anti-*Tvi*CATL IgY antibody production

A checkerboard ELISA (Crowther, 1995) was performed (results not shown) for the optimisation of *Tvi*CATL coating concentrations and primary antibody concentrations. The checkerboard titration curve indicated that 0.5 µg/ml *Tvi*CATL coating concentration probed with 75 µg/ml chicken anti-*Tvi*CATL IgY resulted in an optimal A_{405} signal. The progress of anti-*Tvi*CATL antibody production in eggs collected from 3 chickens for a 16 week period was determined by ELISA (Figure 3.3). This ELISA shows that for chicken 1 there was a linear increase in antibody production from weeks 1-8, after which a decrease was seen up to week 14, followed by an insignificant increase in signal at weeks 15 and 16. For chicken 1, two prominent peaks were observed at weeks 4 and 8. Chicken 2 and 3 showed a similar trend in that there was an increase in antibody production from weeks 1-6, followed by a drastic decrease at weeks 7-8, followed by a brief recovery at week 9 and a fluctuating pattern of antibody production from weeks 10-16. Chicken 2 showed prominent peaks of antibody production at week 6, 9 and 13, while chicken 3 showed prominent peaks of antibody production at weeks 6 and 9. Overall, chickens 1 and 3 showed the best antibody production when compared to that by chicken 2.

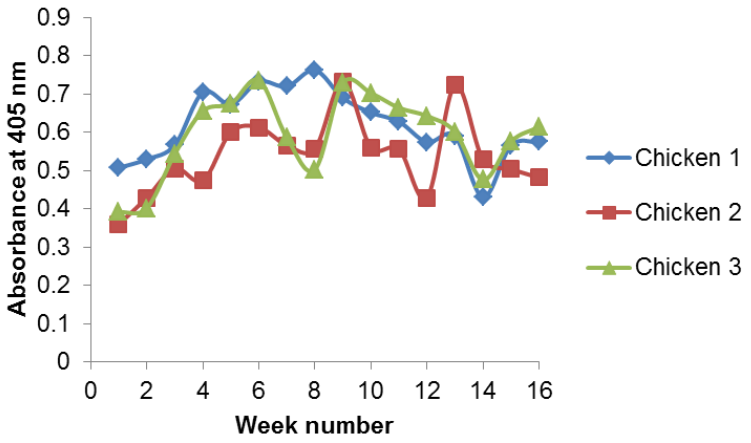

Figure 3.3: Evaluation by ELISA of antibody production in chickens (1, 2 and 3) immunised with recombinant *Tvi*CATL. The ELISA plates were coated with 0.5 µg/ml *Tvi*CATL. The immobilised antigen was probed with the primary antibodies isolated from each egg yolk (1 egg/chicken/week) over a period of 16 weeks. The antibodies were used at a concentration of 75 µg/ml as pre-determined by a checkerboard ELISA. Rabbit anti-chicken IgY-HRPO conjugate (1:15 000) was used for the detection of the interaction between *Tvi*CATL and anti-*Tvi*CATL antibodies. The colour development upon addition of ABTS-H_2O_2 substrate was monitored over 45 minutes (15 min intervals) at 405 nm and the average of duplicate samples plotted against week number.

After determining the antibody production using 1 egg per week, the antibodies were isolated as pools based on results from the ELISA presented in Figure 3.3. For chicken 1, pools were as follows: weeks 4-9, 10-13, and 14-16; chicken 2: weeks 5-9, 10-13, and 14-16; and chicken 3: weeks 4-8, 9-13, and 14-16. The antibody titre of each IgY pool for each chicken was determined by primary antibody titration ELISA (Figure 3.4). This ELISA shows that the highest levels of antibody were found in pool 4-9 for chicken 1 (Panel A), 4-8 for chicken 2 (Panel B) and 5-9 for chicken 3 (Panel C). The signals were lower than those reported in Figure 3.3, suggesting that pooling together different pools compromised the overall antibody titers. All anti-*Tvi*CATL IgY pools showed higher antibody responses than the pre-immune IgY. The IgY pools obtained from all chickens showed similar levels of anti-*Tvi*CATL antibodies, but chickens 1 and 3 antibody pools showed higher levels compared that of chicken 2.

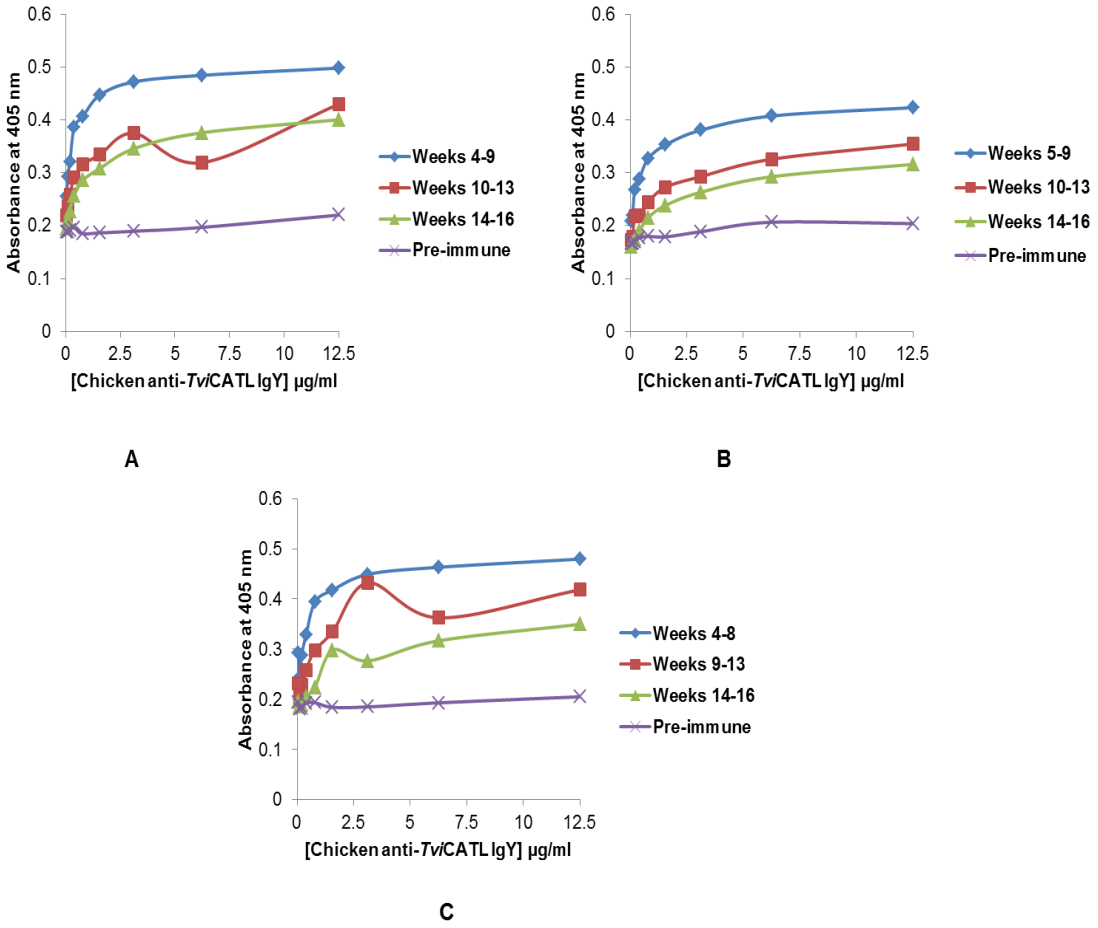

Figure 3.4: The titration ELISA showing the antibody levels for chickens immunised with recombinant *Tvi*CATL. The ELISA plates were coated with *Tvi*CATL (0.5 µg/ml). The primary antibodies used were pools from each chicken (chicken 1: A, chicken 2: B, and chicken 3: C) at concentrations ranging from 0.05-12.5 µg/ml. Rabbit anti-chicken IgY-HRPO conjugate (1:15 000) was used for detection. The colour development upon addition of ABTS-H_2O_2 substrate was monitored over 45 minutes (15 min intervals) at 405 nm and the average of duplicate samples plotted against week number.

In an attempt to improve the absorbance measured in the ELISA (A_{405} less than 1, Figure 3.4), further optimisation of the *Tvi*CATL coating concentrations was performed. Higher coating concentrations, 1 and 2 µg/ml were used and probed with 0.05-12.5 µg/ml anti-*Tvi*CATL IgY pools from each of the 3 chickens which previously showed relatively high antibody levels (Figure 3.4). However, results showed no significant improvement in signal when plates were coated with 1 and 2 µg/ml *Tvi*CATL, Figure 3.5. Once again for 1 µg/ml (Figure 3.5 A), chicken 1 weeks 4-9 pool showed the highest signal, while for 2 µg/ml (Figure 3.5 B), all the pools showed very similar signals as indicated by their curves bunched together around the same level. Improved distinction between the antibodies and pre-immune control samples were observed. A

further ELISA was conducted in order to optimise the secondary antibody concentrations (results not shown). The A_{405} signal improved (up to ~0.8), but it was noted that nonspecific background was accumulating since the pre-immune signal was very high (~0.35).

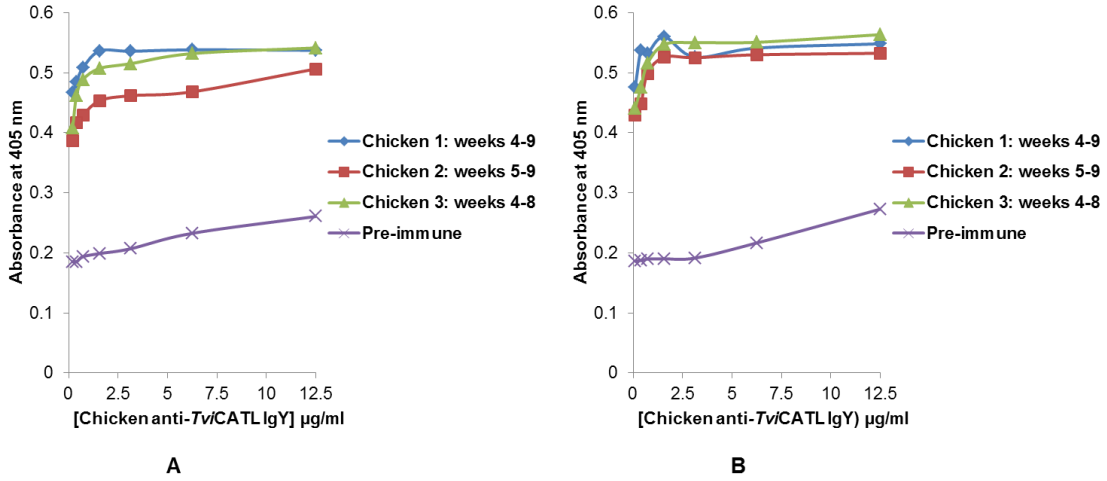

Figure 3.5: Re-optimisation of *Tvi*CATL coating concentrations in order to improve signal at A_{405}. ELISA plates were coated with 1 µg/ml (A) and 2 µg/ml (B) *Tvi*CATL. The primary antibodies used were IgY pools at the concentrations ranging from 0.05-12.5 µg/ml. Rabbit anti-chicken IgY-HRPO conjugate (1:15 000) was used for detection. The colour development upon addition of ABTS-H_2O_2 substrate was monitored over 45 minutes (15 min intervals) at 405 nm and the average of duplicate samples plotted against week number.

To improve the specificity of the chicken anti-*Tvi*CATL antibodies, the crude IgY antibody pools which showed relatively high levels of antibody isolated from each chicken's eggs, were subjected to affinity purification using an affinity column prepared by immobilising *Tvi*CATL on an Aminolink® coupling resin. A satisfactory coupling efficiency of 72% (equivalent to 2.2 mg/ml) was obtained. The individual pools of chicken anti-*Tvi*CATL were separately cycled through the affinity column and specific antibodies eluted using low pH buffer (Figure 3.6). The elution profiles indicate that the pooled IgY from chicken 1 gave the highest absorbance (therefore highest antibody concentration) but only in one fraction, after which there was a drastic decrease in absorbance. The IgY pool from chicken 3 showed two fractions with an absorbance close to 1. In comparison to the IgY from chickens 1 and 3, the IgY from chicken 2 had the lowest absorbance values (less than 0.5) consistent with the results shown in previous Figures 3.3 and 3.4. Figure 3.6 also showed that even though the absorbance values were not the same for the antibodies affinity purified from the IgY from eggs

collected from three chickens, the elution profile was the same in the sense that at fraction 2, there was a sudden increase in absorbance values for IgY pools from all three chickens.

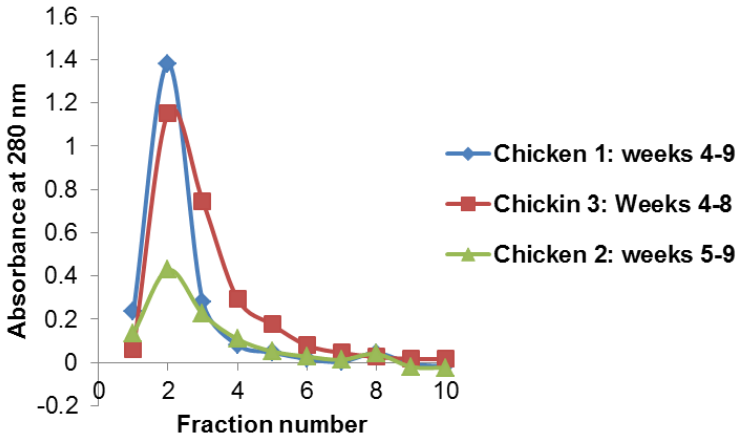

Figure 3.6: Elution profile for affinity purification of chicken anti-*Tvi*CATL IgY pools. The crude IgY was purified with *Tvi*CATL coupled to an Aminolink® resin. The elution of pure IgY fractions was achieved by low pH buffer [0.1 M glycine HCl, pH 2.8] and monitored by measuring absorbance at 280 nm.

An ELISA was conducted to evaluate the affinity purification of the chicken anti-*Tvi*CATL IgY antibodies (Figure 3.7). The antibodies were successfully affinity purified as evidenced by the increase in A_{405} signals for the three affinity purified IgY preparations when compared to those obtained for the pre-purified crude IgY as well as the pre-immune IgY. It was noted that even though the A_{405} signal of the affinity purified antibodies was higher than that for the unbound IgY fraction, the signal of the unbound IgY was relatively high. This indicated that a large pool of antibodies did not bind to the resin, hence the unbound fraction was reapplied to the column to obtain as much affinity purified anti-*Tvi*CATL antibodies as possible from the unbound fraction.

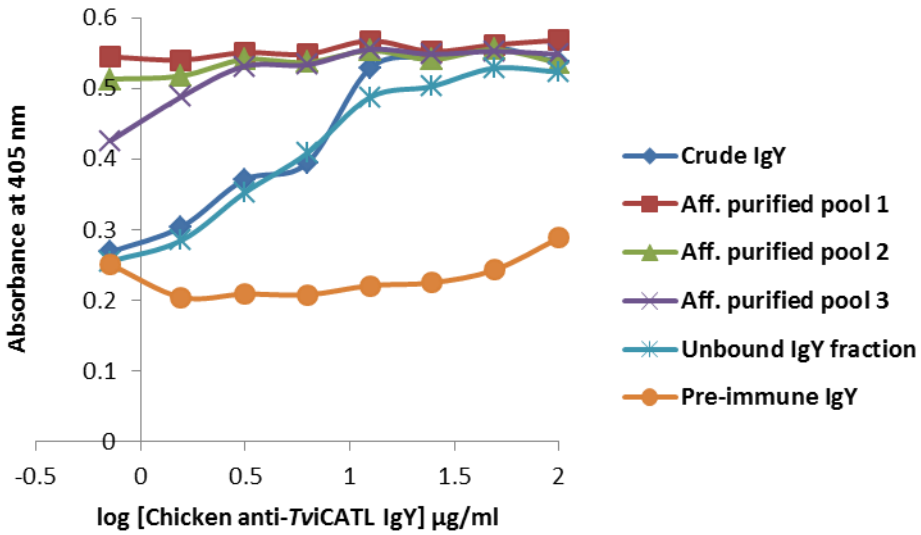

Figure 3.7: Evaluation by ELISA of affinity purification of anti-*Tvi*CATL IgY antibodies. The ELISA plates were coated overnight at 4°C with 0.5 µg/ml *Tvi*CATL in carbonate coating buffer, pH 9.6 and probed with antibodies (pre-purified crude IgY, affinity purified pools from 3 chickens, unbound IgY from the affinity column and pre-immune IgY) serial diluted from 100-0.718 µg/ml. Rabbit anti-chicken IgY-HRPO conjugate (1:10 000) was used for detection. The colour development upon addition of ABTS-H_2O_2 substrate was monitored over 45 minutes (15 min intervals) at 405 nm and the average of duplicate samples plotted against log chicken IgY concentrations.

3.4.2 Cross-reactivity of anti-trypanosomal antigen antibodies

An ELISA was conducted to check the possibility of cross-reactivity of antibodies against trypanosomal antigens with similar characteristics. In this ELISA, *Tvi*CATL and *Tco*CATL antigens were probed with chicken anti-*Tvi*CATL, chicken anti-*Tco*CATL, chicken anti-*Tco*CATL N-terminal peptide (*Tco*$CATL_{pept}$) and chicken anti-full length *Tco*CATL (*Tco*$CATL_{FL}$) antibodies. Figure 3.8 indicates that each antibody showed best recognition of its specific antigen and some cross-reactivity with the homologue was observed. This was evidenced by the lower signals obtained where the chicken anti-*Tco*CATL IgY and chicken anti-*Tco*$CATL_{pept}$ IgY were titrated against *Tvi*CATL or where chicken anti-*Tvi*CATL IgY was titrated against *Tco*CATL. However, it was noted that the chicken anti-*Tco*$CATL_{FL}$ IgY showed substantial cross-reaction with *Tvi*CATL. The pre-immune IgY antibodies shows a dose dependent increase in binding, however, the level of binding is less than the level at which the antibodies were used to detect their specific antigens. Overall, these results indicate that the cross-reactivity of the antibodies was quite low.

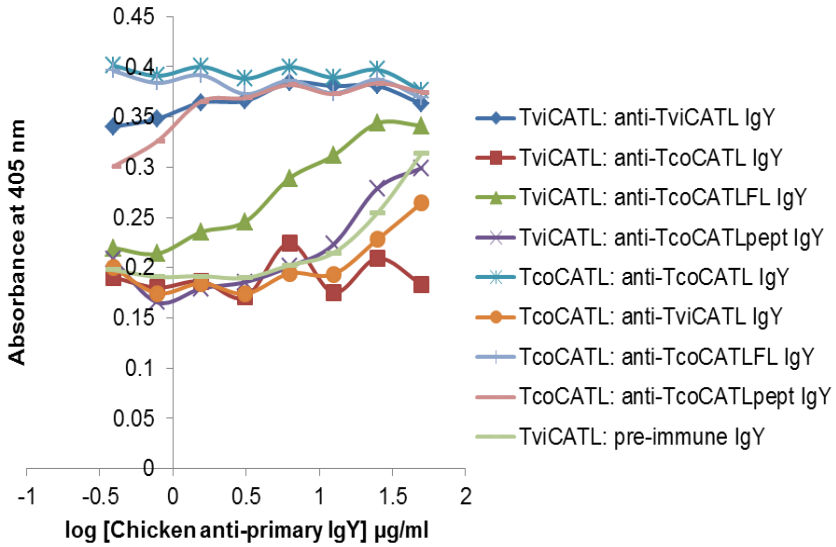

Figure 3.8: Evaluation by ELISA of cross-reactivity of anti-trypanosomal antigen antibodies. The ELISA plates were coated overnight at 4°C with 1 µg/ml *Tvi*CATL or *Tco*CATL and probed with chicken antibodies (affinity purified anti-*Tvi*CATL, anti-*Tco*$CATL_{FL}$, and anti-*Tco*$CATL_{pept}$, non-affinity-purified anti-*Tco*CATL and the pre-immune IgY) serial diluted from 100-0.718 µg/ml. Rabbit anti-chicken IgY-HPRO conjugate (1:10 000) was used for detection. The colour development upon addition of ABTS-H_2O_2 substrate was monitored over 45 minutes (15 min intervals) at 405 nm and the average of duplicate samples plotted against log chicken IgY concentrations.

3.4.3 Serological antibody-detection in an inhibition ELISA format

The diagnostic potential of *Tvi*CATL was assayed in an inhibition ELISA using sera from cattle experimentally infected with *T. congolense*. The *T. vivax* infected sera were not available, hence only *T. congolense* sera were used. The sera from cattle prior to experimental infection were called [185 (-6), 109 (-7) and 183 (-10)] and sera collected post-infection were labelled as [181 (+19), 18 (+23), 149 (+28) and 152 (+45)]. Figure 3.9 indicates that in the presence of uninfected cattle serum, there was no inhibition as expected. This is because pre-infection sera do not contain antibodies against the coated *Tvi*CATL antigen on the ELISA plate; therefore there is no antigen-serum interaction, hence the chicken anti-*Tvi*CATL IgY binds to the coated antigen which in turn results in binding of HRPO labelled rabbit anti-chicken IgY. When the substrate is added, there will be colour development, thus absorbance at 405 nm. When a positive serum is added that contains anti-*Tvi*CATL antibodies, these would bind to the coated *Tvi*CATL antigen and hence the chicken anti-*Tvi*CATL IgY will not be able to bind, resulting in decreased binding of HRPO labelled rabbit anti-chicken IgY and lower A_{405}-values (inhibition of the signal). In the presence of a positive serum [181 (+19), 18

(+23), 149 (+28) and 152 (+45)], inhibition up to approximately 22% was observed for positive sera as expected since the sera could contain antibodies against the coated antigen. The results show discrimination between negative and positive sera and suggests cross-reactivity between *Tco*CATL (in the sera) and the chicken anti-*Tvi*CATL IgY antibodies.

To validate the observed results where there was some cross-reactivity between *Tco*CATL (in sera) and chicken anti-*Tvi*CATL antibodies, it was necessary to compare these findings with results obtained for the positive control [i.e. ELISA plates coated with *Tco*CATL/ *Tco*CATL$_{FL}$, probed with *Tco*CATL sera (same sera used for *Tvi*CATL inhibition ELISA), followed by chicken anti-*Tco*CATL/ anti-*Tco*CATL$_{FL}$ antibodies and detection with rabbit anti-chicken IgY-HRPO). Due to the limitation of these antigens and the sera, these results were obtained from Eyssen (2014) for comparative purposes only. The means to optimise the *Tco*CATL inhibition ELISA was unsuccessful, only the indirect antibody ELISA was successfully optimised. Therefore, the *Tvi*CATL inhibition ELISA findings of the present study were compared with those obtained by Eyssen (2014) for *Tco*CATL$_{FL}$ inhibition ELISA. Moderate cross-reactivity between *Tvi*CATL antigen and chicken anti-*Tco*CATL$_{FL}$ IgY was reported in Section 3.4.2, thus making *Tco*CATL$_{FL}$ inhibition ELISA a suitable positive control. Figure 3.9 panel B showed that no inhibition was obtained for non-infected serum [185 (-6)] as expected, however the non-infected sera [109 (-7) and 183 (-10)] unexpectedly showed some inhibition; 21 and 32%, respectively. All the positive sera expectedly resulted in satisfactory inhibition, with 149 (+28) giving highest % inhibition (48%). This inhibition ELISA was not able to discriminate between infected and non-infected sera, since two out of three negative sera unexpectedly showed some inhibition equivalent or greater than some of the infected sera. The discrepancy between *Tvi*CATL and *Tco*CATL inhibition ELISAs might be contributed by the fact that the sera dilution used were not the same, 1:100 for *Tvi*CATL inhibition ELISA while 1:20 sera dilution was used for *Tco*CATL$_{FL}$ inhibition ELISA. The discrepancies between the two inhibition ELISAs made the extent of cross-reactivity between *Tco*CATL antibodies (in sera) and *Tvi*CATL, an inconclusive matter.

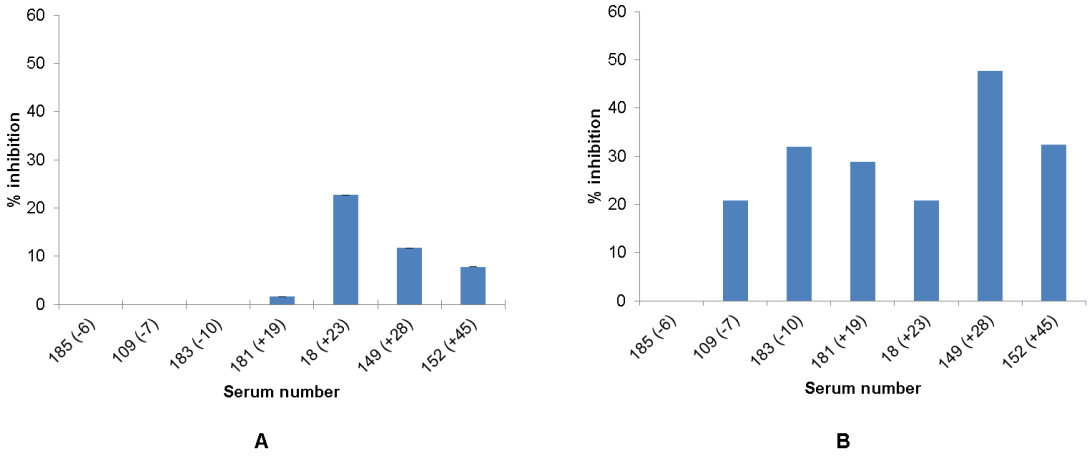

Figure 3.9: Serological antibody detection in an inhibition ELISA format. Panel **A:** the ELISA plates were coated with *Tvi*CATL (5 µg/ml) in and probed with sera (1:100) from cattle experimentally infected with *T. congolense* or with negative sera collected prior to infection. For a 0% serum inhibition control, the quadruplicate wells received blocking buffer instead of serum. This was followed by the addition of the affinity purified chicken anti-*Tvi*CATL IgY (5 µg/ml) and detected with secondary rabbit anti-chicken IgY-HRPO conjugate (1:10 000). The colour development upon addition of ABTS-H_2O_2 substrate was monitored over 45 minutes (15 min intervals) at 405 nm and the average of triplicate samples plotted against serum number. The no serum control was included in quadruplicate. This inhibition ELISA was expressed as a percentage inhibition and the error bars represent an average of three independent readings. Panel **B**: **The *Tco*CATL$_{FL}$ inhibition ELISA obtained from Eyssen (2014) for comparative purposes only**. The ELISA plates were coated with *Tco*CATL$_{FL}$ (1 µg/ml), probed with sera (1:20) from cattle experimentally infected with *T. congolense* or with negative sera collected prior to infection, followed by the addition of the affinity purified chicken anti-*Tco*CATL$_{FL}$ IgY (0.5 µg/ml) and detected with secondary rabbit anti-chicken IgY-HRPO conjugate (1:15 000). The colour development upon addition of ABTS-H_2O_2 substrate was monitored over 45 minutes (15 min intervals) at 405 nm and the average of triplicate samples plotted against serum number. This inhibition ELISA was expressed as a percentage inhibition.

3.4.4 Western and dot blot analysis of antibodies

The crude IgY pools with high titres were further used in a dot blot assay for the optimisation of antigen and antibody concentrations for use in western blot analysis. A dot blot (Figure 3.10) showed that 10 µg/ml chicken anti-*Tvi*CATL IgY resulted in the best detection of *Tvi*CATL antigen. The recognition pattern of the antigen at different concentrations (10-100 µg/ml) was similar, therefore for a western blot analysis, it was concluded that as little as 10 µg/ml *Tvi*CATL would be optimally recognised by 10 µg/ml chicken anti-*Tvi*CATL IgY antibodies. The ability to get the highest signal at lowest antibody concentration and no signal at higher antibody concentrations indicates that these antibodies are prone to the phenomenon called the prozone effect. No signal was obtained for controls at which the primary and secondary antibodies were omitted. Also, the pre-immune IgY was unable to recognise the *Tvi*CATL antigen.

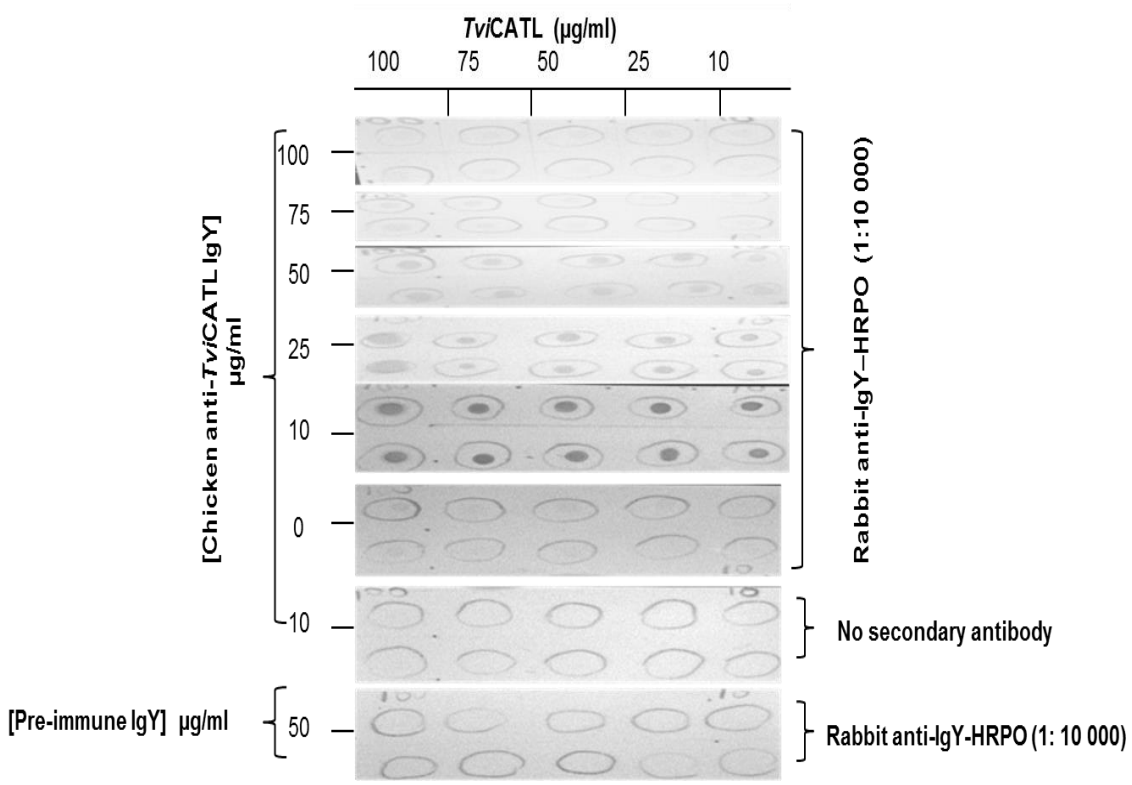

Figure 3.10: Dot blot of *Tvi*CATL antibodies. Nitrocellulose strips were each spotted with *Tvi*CATL (10-100 µg/ml in duplicate). Each strip was then incubated with the respective chicken anti-*Tvi*CATL IgY antibody concentration (0-100 µg/ml). The negative controls were: omission of primary or secondary antibodies as well as the use of a pre-immune IgY.

The optimised primary antibody concentrations were used for western blot analysis. Figure 3.11 Panel B shows that the 29 and 33 kDa bands of *Tvi*CATL were strongly recognised by this optimised antibody concentrations. However, it was noted that the antibodies were not specific since they were able to recognise the higher molecular weight contaminants in the TPP-purified sample. The pre-immune antibodies were unable to recognise this antigen (results not shown).

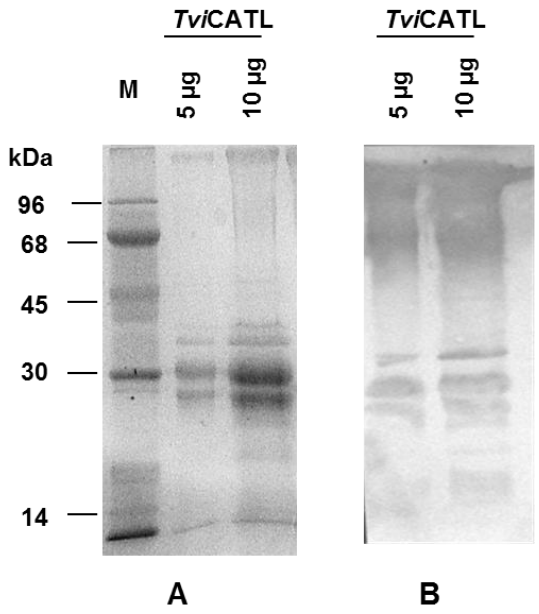

Figure 3.11: Characterisation of chicken anti-*Tvi*CATL antibodies by western blot analysis. The TPP-purified *Tvi*CATL samples were separated by 12.5% reducing SDS-PAGE. Panel **A,** SDS-PAGE of *Tvi*CATL TPP samples (5 and10 µg) followed by Coomassie blue R-250 staining to serve as a reference. Panel **B**, same gel as panel **A** was transferred onto a nitrocellulose membrane, probed with chicken anti-*Tvi*CATL IgY (10 µg/ml) and detected with rabbit anti- chicken IgY-HRPO conjugate (1:5 000 dilution) followed by colour development using 4-chloro-1-naphthol and H_2O_2.

The non-specificity observed for the antibodies necessitated further purification using the *Tvi*CATL-Aminolink® affinity resin. Western blot analysis of affinity purified antibodies indicated that all nonspecific reactive epitopes were removed (Figure 3.12). This is evidenced by the inability of the purified antibodies to recognise the higher molecular weight contaminants on the TPP-purified sample. This antibody recognised the 29 and 33 kDa bands of *Tvi*CATL in the expression supernatant, TPP and MEC samples. There was an increase in prominence of bands in the MEC sample compared to those in the expression supernatant and TPP samples. However, it was noted that the antibody fairly cross-reacted with *Tco*CATL and that is expected due to the high levels of sequence identity of these antigens. Cross-reactivity of these antibodies was thus expected and is consistent with results reported in Sections 3.4.2 and 3.4.3. The pre-immune (negative control) antibodies were unable to recognise these antigens as expected (results not shown).

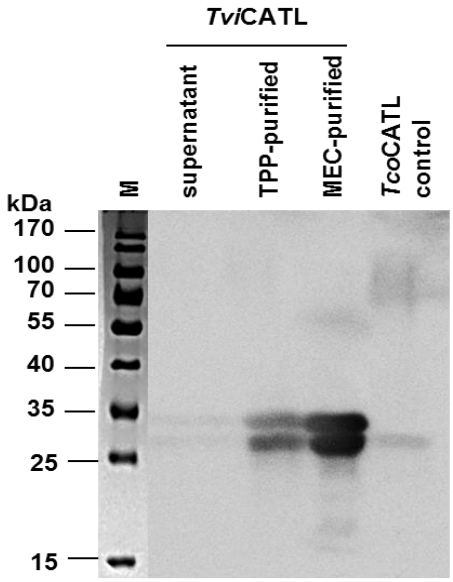

Figure 3.12: Characterisation of affinity purified chicken anti-*Tvi*CATL IgY by western blot analysis. Expression supernatants, TPP and MEC-purified *Tvi*CATL as well as *Tco*CATL control samples were analysed by 12.5% reducing SDS-PAGE and transferred onto the nitrocellulose membrane, probed with affinity purified chicken anti-*Tvi*CATL IgY (1 µg/ml) and detected with rabbit anti-chicken IgY-HRPO conjugate (1:5 000 dilution) followed by incubation with ECL substrate.

3.4.5 Immunoinhibition of *Tvi*CATL activity by chicken anti-*Tvi*CATL IgY antibodies

The effect of chicken anti-*Tvi*CATL IgY antibodies on the activity of *Tvi*CATL against Z-Phe-Arg-AMC substrate hydrolysis was followed by assaying this enzyme with chicken anti-*Tvi*CATL IgY with high titres. Samples where no antibody was added or pre-immune IgY was added served as negative controls. Figure 3.13 indicates that as the concentration of chicken anti-*Tvi*CATL IgY antibody increases, there was a proportional decrease in activity. The activity in the presence of chicken anti-*Tvi*CATL IgY is less than the activity in the presence of the pre-immune antibodies, but the trend line are effectively identical indicating that the inhibition of *Tvi*CATL activity was not quite specific in this assay.

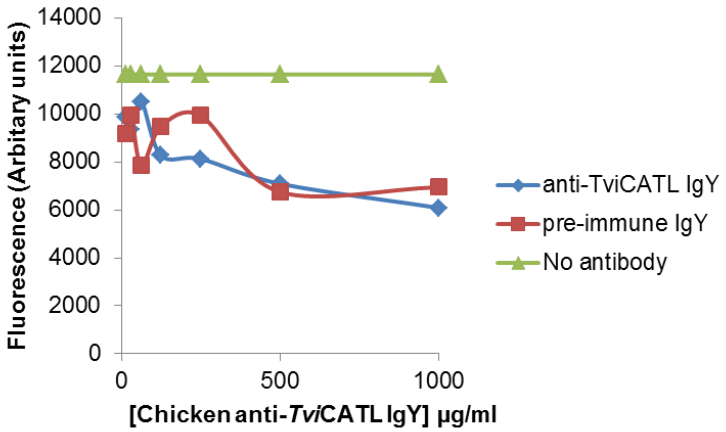

Figure 3.13: The inhibition of *Tvi*CATL activity by varying concentrations of chicken anti-*Tvi*CATL IgY antibodies. *Tvi*CATL (100 ng) was incubated with chicken anti-*Tvi*CATL IgY (1000-15 µg/ml) and residual activity measured against Z-Phe-Arg-AMC. The data represent an average of three independent assays.

3.5 Selection of scFvs against recombinant *Tvi*CATL

The large semi-synthetic Nkuku® phage display library was used to select for scFvs against recombinant *Tvi*CATL. These phages were subjected to four consecutive rounds of panning against *Tvi*CATL (100-10 µg/ml). The phage titering followed by polyclonal ELISA was used to monitor the enrichment of scFv binders against *Tvi*CATL. Overall, the signals (after subtracting the signal for non-antigen-coated milk binders) were very weak and any means to optimise the antigen coating, phage and secondary antibody concentrations were all futile (Figure 3.14). Panning round 1 (100 µg/ml), 2 (75 µg/ml) and 4 (10 µg/ml) had very low signals compared to panning round 3 (25 µg/ml), suggesting that only at panning round 3 (25 µg/ml) the polyclonal phage populations against *Tvi*CATL were enriched. In this ELISA, the *Tvi*CATL coating concentrations (10 and 1 µg/ml) probed with a 1:25 phage dilution resulted in the highest signal at ~0.2 for panning round 3, Figure 3.14, panel C.

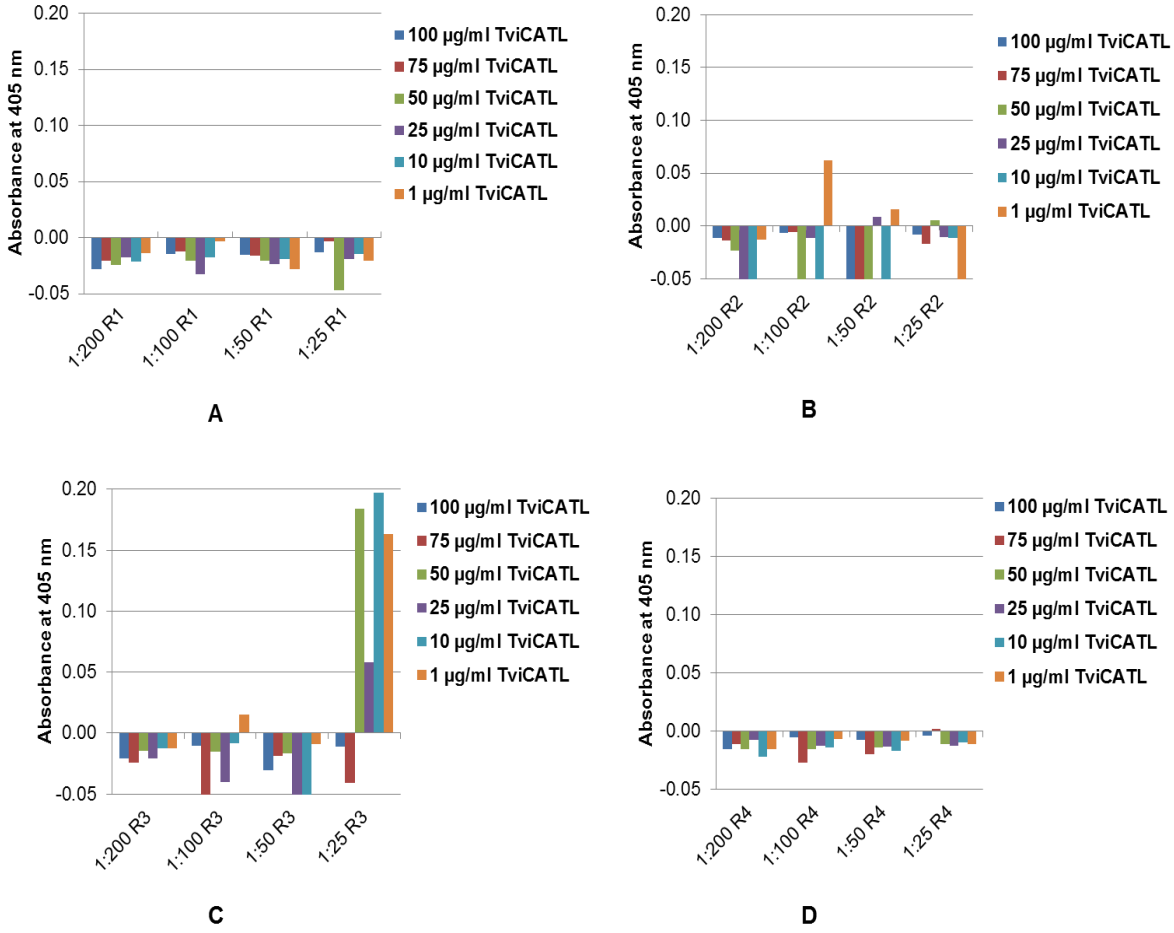

Figure 3.14: Polyclonal phage ELISA for monitoring the enrichment of polyclonal *Tvi*CATL-scFvs populations after four consecutive rounds of panning. The wells were coated with *Tvi*CATL (1-100 µg/ml) in PBS, pH 7.4 and no antigen control wells were coated with PBS only. After blocking the nonspecific sites, the wells were incubated with phage from each respective panning round [R1 (A), R2 (B), R3 (C) and R4 (D)] at respective phage dilutions (1:200, 1:100, 1:50 and 1:25) and probed with the primary HRPO-anti-M13 monoclonal antibody (1:8 000). The sheep anti-mouse IgG- HRPO conjugate (1:1 000) was used for detection and the colour development upon addition of ABTS-H_2O_2 substrate was monitored by taking $A_{405\ nm}$ readings. Plots are averages of duplicate experiments.

It was identified that the polyclonal populations were mostly enriched during round 3 of biopanning. These phage populations were titered through bacterial infection. Each bacteriophage colony or clone from panning round 3 was further screened to identify possible monospecific phage antibodies. Fourty eight colonies (clones) were randomly chosen for screening by monospecific phage ELISA in order to identify any fusion phages specific for *Tvi*CATL antigen. As according to Van Wyngaardt *et al* (2004), $A_{405\ nm}$ (after subtracting the signal for milk binders) that is greater than 0.2 was used as a criterion for identifying positive *Tvi*CATL-scFv fusion clones. It is shown in Figure 3.15 that when 10 µg/ml *Tvi*CATL was used for coating wells, only clone 38

had the highest signal (~0.4) which was 2-fold more than the signals obtained for polyclonal ELISA (Figure 3.14). Clones 15 and 28 also had promising signals at ~0.2. However, when the higher concentration of *Tvi*CATL (75 µg/ml) was used for coating, there was a reduction in signals (to ~0.2) of the promising clones (results not shown). Further optimisation of the ELISA conditions is required in order to improve the signal of the promising clone 38.

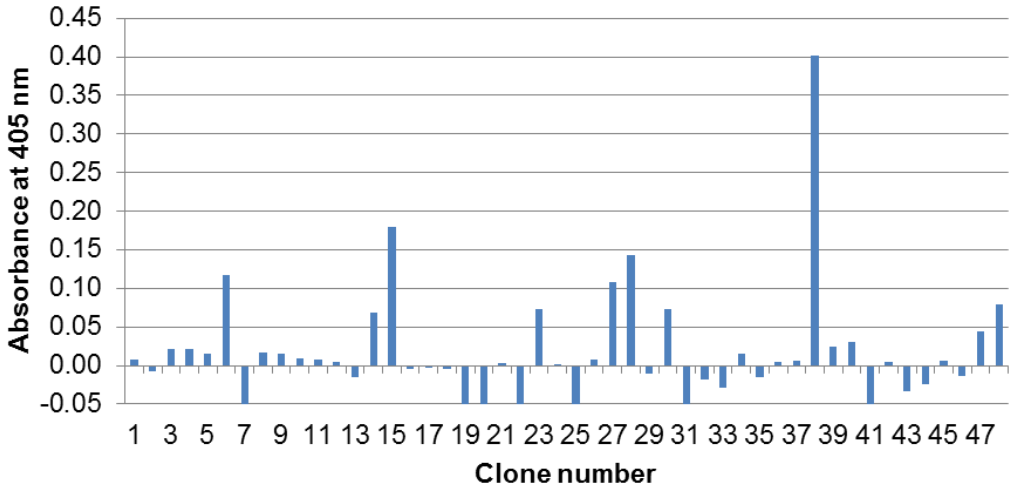

Figure 3.15: Monospecific phage ELISA for monitoring the enrichment of monospecific *Tvi*CATL-scFvs antibodies after round 3 (R3) of panning. The wells were coated with *Tvi*CATL (10 and 75 µg/ml) in PBS, pH 7.4 and no antigen control wells were coated with PBS only. After blocking the nonspecific sites, wells were incubated with the respective phage clones and probed with primary anti-M13 monoclonal antibody (1:8000). Goat anti-mouse IgG-HRPO conjugate (1:1 000) was used for detection and colour development upon addition of ABTS-H_2O_2 substrate was monitored by taking $A_{405\ nm}$ readings. Plots are averages of duplicate experiments.

3.6 Discussion

Monospecific antibodies have important applications in diagnosis of African animal trypanosomiasis. In this study, we report the production of anti-*Tvi*CATL antibodies for use in a serological diagnosis of AAT. Animal hosts such as rabbits, mice, goats and chickens are available for antibody production. The choice of the host depends on the ability to meet the ethical welfare of the animal to be immunised as well as an ability to obtain antibodies with high titres and affinity for the targeted antigen. In chickens, the B cell response triggers the continuous production of IgY into the blood, which is then transferred to egg yolks in order to provide protective immunity against any possible

attacking pathogens to their offspring (Schade *et al*, 1996; Tini *et al*, 2002). The chicken antibodies are collected from egg yolks; therefore providing a more humane way of harvesting antibodies than by bleeding of the animals. In addition, due evolutionary distance, chickens results in the pronounced immune response to parasite antigens (Morty *et al*, 1999; Eyssen, 2014) as well as the lack of cross-reactivity of IgY antibodies with mammalian IgG antibodies. Chicken IgY does not cross-react with mammalian IgG antibodies since it does not activate the rheumatoid and complement factors in mammalian serum. The lack of IgY cross-reactivity with IgG is associated with improved sensitivity and decreased background in an immunological assay such as the ELISA. Therefore, chickens are the preferred hosts for the production of antibodies for diagnostic purposes (Larsson *et al*, 1993; Tini *et al*, 2002).

There are many precipitation and isolation procedures that have been introduced for the precipitation of IgY. These include: water dilution method (Akita & Nakai, 1992), PEG precipitation (Polson *et al*, 1980; Goldring & Coetzer, 2003), ammonium sulfate (Svendsen *et al*, 1995), dextran sulfate (Jensenius *et al*, 1981) and chromatographic techniques (Schade *et al*, 1996). All these methods share one commonality in that they all require a delipidation step to remove all traces of insoluble lipids and lipoproteins (Hansen *et al*, 1998). The choice of a method for IgY isolation depends on the availability of technical equipment or reagents, scale of purification, impact on environment and cost. In this study, PEG precipitation was chosen since it is a simple, short and effective method that results in relatively high yields of electrophoretically homogenous antibodies (Goldring & Coetzer, 2003).

In this study, the analysis by ELISA of anti-*Tvi*CATL IgY production in chickens showed that a satisfactory levels of antibody was obtained. These high titres are as a result of the pronounced immune response of this antigen in chickens upon immunisation possibly as a result of the evolutionary distance between the parasite immunogen and the chicken immune system. This immune response over a long time is attributed to the use of adjuvants during immunisations. Freund's adjuvant forms an antigen-depot at the site of injection so that the antigen emulsion is slowly released to the lymph nodes. This prolongs the host B-cell immune response, thus resulting in prolonged antibody production (Leroux-Roels, 2010). The relevance of including adjuvants for immunising with trypanosomal antigens is supported by the fact that rabbits immunised with *Tco*CATL alone (no adjuvant) produced very low titres of anti-*Tco*CATL compared to that produced when adjuvants were included (Huson *et al*, 2009). In addition, these antibodies had no immunoinhibition potential against the activity of *Tco*CATL.

Immunoinhibition was an important factor necessary for the design of anti-disease vaccines (Huson *et al*, 2009). The pattern for antibody production in chickens against *Tvi*CATL is similar to that reported for antibody production against the inactive full length form of *T. vivax* (Vather, 2010). The antibody titres for *Tvi*CATL peaked from weeks 4-9, while they peaked from weeks 5-9 for the inactive full length mutant of *T. vivax* (Vather, 2010). As observed for *Tco*CATL in Freund's adjuvant (Huson *et al*, 2009), *Tvi*CATL in Freund's adjuvant also elicited antibodies with moderate ability to inhibit the activity of *Tvi*CATL against the hydrolysis of the cysteine protease substrate, Z-Phe-Arg-AMC. However, in order to confirm that the observed inhibition is as a result of chicken anti-*Tvi*CATL IgY, it will be necessary to design a *Tvi*CATL peptide corresponding to the active site sequence. This peptide is expected to compete with *Tvi*CATL for binding to the chicken anti-*Tvi*CATL IgY epitopes, thus decreasing the inhibitory effect of these antibodies. This phenomenon was reported by Coetzer *et al* (1992) where the cathepsin L active site-associated peptide was able to hinder the immunoinhibition of cathepsin L by chicken anti-cathepsin L IgY. Therefore, it was concluded that the active-site region of cathepsin L is responsible for the production of chicken anti-cathepsin L IgY with immunoinhibitory properties (Coetzer *et al*, 1992). The nonspecific peptides can also be used as a negative control; therefore if the antibody inhibitory effect is specific, the substrate hydrolysis will not be altered by the presence of the unrelated peptides. Since *Tvi*CATL is able to hydrolyse bovine α_2-macroglobulin (see Chapter 2), the neutralising potential of anti-*Tvi*CATL antibodies can be improved by exploiting α_2-macroglobulin as an adjuvant. Thus, in order to produce anti-disease trypanosomal vaccines, it is very likely that antibodies produced against *Tvi*CATL coupled with α_2-macroglobulin would be inhibitory towards the activity of *Tvi*CATL *in vitro.* This inhibitory phenomenon against recombinant and native (trypanosomal lysate) *Tco*CATL was observed when antibodies were raised in rabbits against a *Tco*CATL- α_2-macroglobulin complex (Huson *et al*, 2009). The ability to inhibit native *Tco*CATL was a very promising feature in the context of an anti-disease vaccine since when these antibodies are used as vaccines; they would be able to trigger the immunoinhibition mechanism against the native trypanosomal *Tco*CATL protease they encounter in the host upon infection by the parasite.

The affinity (as measured by ELISA) and specificity of the anti-*Tvi*CATL for *Tvi*CATL antigen was improved by affinity purification of these antibodies using a *Tvi*CATL-affinity column. In most cases, protein A and G immobilised on the affinity columns are used to purify mammalian IgG since they are capable of binding to the Fc-region of IgG in crude sera. However, protein A and G cannot be used for IgY purification since they

lack IgY binding sites (Schade *et al*, 2005). Therefore, the crude IgY antibodies were purified by coupling the pure recombinant *Tvi*CATL onto the Aminolink® resin. Analysis by ELISA of the affinity purified IgY indicated a slight increase in signals compared to the pre-purified IgY pools. The reason for the low signals even after optimising the coating buffer, antigen coating and primary antibody concentrations, is not clear, but it is suspected that the effectiveness of the secondary antibody (rabbit anti-chicken IgY-HRPO conjugate) used has been lowered. These low ELISA signals when using the same secondary antibody used in the present study, were also reported by Mnkandla (2013).

The chicken anti-*Tvi*CATL antibodies were able to strongly detect *Tvi*CATL antigen on dot and western blots analysis using both 4-chloro-1-naphthol (colorimetric) and ECL (chemiluminescent) chromogens. On the dot blot, the prozone effect was observed, i.e. the lower antibody concentrations showed a higher signal than the higher antibody concentrations. The prozone effect is a common feature for many trypanosomal antibodies including the chicken anti-nLiTat 1.3 IgY antibodies (Mnkandla, 2013). The signals were strong when the ECL substrate was used. The use of ECL instead of 4-chloro-1-naphthol offers advantages such as sharp, intense or prominent bands even at lower antigen and primary antibody concentrations, blots can be stripped and reprobed to optimise blot conditions and is a relatively fast procedure (Novex® ECL chemiluminescent substrate reagent Kit, Invitrogen™).

The chicken anti-trypanosomal antibodies were assayed by ELISA for their cross-reactivity with homologous antigens from different trypanosomal species. In all cases, the antibodies significantly recognised their respective antigens. However, the exception was that the chicken anti-full length *Tco*CATL IgY (anti-*Tco*CATLC_{FL}) antibodies showed some of recognition of the *Tvi*CATL antigen suggesting that the chicken anti-*Tco*CATLC_{FL} antibodies were able to recognise the conserved sequences or epitopes between *Tco*CATL and *Tvi*CATL. However, in all cases, cross-detection signals were lower than those observed for the specific antigen. Due to the fact that *Tvi*CATL is not a surface protein like VSGs and due to the unavailability of *T. vivax* parasite lysates in our laboratory, it was not possible to use these antibodies in order to conduct localisation studies through immunofluorescence microscopy. This could be used to establish if *Tvi*CATL is developmentally regulated (i.e. if *Tvi*CATL predominates in the procyclics, epimastigotes, metacyclics or blood stream forms). Since *Tvi*CATL shares high sequence identity with *Tco*CATL which is highly expressed in the blood stream form (insect-infective stage) (Pillay *et al*, 2010), *Tvi*CATL likewise *Tco*CATL, is expressed in blood stream form as confirmed by greater hydrolysis of Z-

Phe-Arg-AMC by the blood stream form of *Tvi*CATL (Mbawa *et al*, 1991; Mbawa *et al*, 1992). The presence of *Tvi*CATL in the bloodstream form is also confirmed by an ability to isolate native *Tvi*CATL from blood stream form of the *T. vivax* strain Y486 stabilate (Jackson, 2011).

The immunodiagnostic potential of the *Tvi*CATL antigen was tested through an antibody inhibition ELISA format for the detection of any cross-reactivity in the sera from cattle experimentally infected with *T. congolense*. The upper and lower limits were set as 0-100% in order to distinguish between the trypanosome-infected and non-infected bovine sera (Eyssen, 2014). Serum (1:100 dilution) were found optimal and this is consistent with conditions reported by Pillay *et al* (2013b). Although the % inhibition values obtained were very low even for trypanosome-infected sera, the antibodies were able to distinguish between infected and non-infected sera. Although the sera used were from *T. congolense* infected cattle and not *T. vivax* cattle, cross-reactivity was expected because of the level of sequence identity between *Tvi*CATL and *Tco*CATL. For comparative purposes, *Tco*CATL$_{FL}$ inhibition ELISA was previously performed by Eyssen (2014). The chicken anti-*Tco*CATL$_{FL}$ antibodies were able to compete (inhibit) with sera collected post infection. However it was also noted that some inhibition was obtained with negative sera. Therefore, the extent of cross-reactivity between the two species remains an inconclusive matter. It is therefore necessary to further optimise *Tvi*CATL and *Tco*CATL inhibition ELISAs in order to further investigate the cross-reactivity of *T. vivax* and *T. congolense* sera, therefore improve the reliability of the pan-trypanosome point-of-care diagnostic tool. It is also important to test the antigen against sera from cattle infected with *Anaplasma*, *Babesia* and *Theileria* to confirm the specificity of these antibodies by checking if there is any cross-reactivity (Bossard *et al*, 2010). Due to the limited sera, we were unable to further investigate the repeatability and reproducibility of the test. This is a necessary to validate the obtained experimental results.

The cross-reactivity between antibodies produced against *T. vivax* and *T. congolense* antigens is a common phenomenon. When the anti-*T. congolense* HSP70/BiP antibody was used for serological diagnosis, cross-reactivity within the *Trypanosoma* genus was reported. The anti-*T. congolense* HSP70/BiP antibodies were able to cross-react (therefore lower A_{405} values due to inhibition of the signal) with sera from *T. vivax* and *T. brucei* infected cattle (though at a very low extent) (Bossard *et al*, 2010). Also when *T. vivax* GM6 antigen was used for diagnostic assays, cross-reactivity (albeit very low sensitivity) with *T. congolense*-infected sera was reported (Pillay *et al*, 2013b). In that study, an antibody detection indirect ELISA format was able to distinguish between

infected and non-infected sera. These types of ELISAs (antibody detection inhibition or indirect antibody detection) are disadvantageous in that it is unable to differentiate between current and past infections in cattle sera since antibodies may persist in the host immune system for a long time after recovery (Van den Bossche *et al*, 2000; Lejon *et al*, 2003). Therefore this can be substituted by an antigen capture ELISA which is advantageous in that it detects circulating trypanosomal antigens in the blood of the infected cattle, which is similar to parasitological diagnosis which detects existing infections (Nantulya, 1990; Bengaly *et al*, 1995; Eisler *et al*, 1998).

In an attempt to find more convenient, cheap and reusable immunodiagnostic reagents, *Tvi*CATL binders from a large semi-synthetic Nkuku® phage display library was partially optimised. In this study, the recombinant scFvs against pure *Tvi*CATL adsorbed on immunotubes were screened through four consecutive panning rounds. During these rounds of panning, the large pool of Nkuku® scFv reptoires were screened by enriching for paratopes that are highly accessible and complementary to the immobilised *Tvi*CATL antigen. In general, the specific binders for the antigen of interest should be enriched relative to the number of panning rounds. In this study, as determined by ELISA, it was observed that the polyclonal *Tvi*CATL-scFv fusions were enriched up to round 3, followed by a large decrease in polyclonal phage at round 4. Though the polyclonal fusion phages were enriched at panning round 3, the signal was very low and it was unfortunate that the unpanned Nkuku® library was not included as a control for comparison in the polyclonal ELISA for monitoring the enrichment. There are many factors that may have attributed to poor screening for polyclonal *Tvi*CATL-scFv fusions. It has been reported that the conformational changes of some proteins when immobilised on polystyrene wells could compromise the ease of accessibility to all antigen epitopes (Butler *et al*, 1993). The coating efficiency of VP7, the structural protein of bluetongue virus, was improved by using the poly-histidine-Grab Ni^{2+} chelate-coated plates which bind antigen of interest through poly-histidine tails. The main disadvantage of this protocol is that there was a need for eluting fusion scFvs using the anti-bluetongue virus hyperimmune rabbit and guinea pig sera followed by concentrated triethylamine at high pH (Rakabe *et al*, 2011).

The four panning rounds were titered through exposure to bacterial TG1 cells and plating on TYE A/G plates. The randomly picked bacterial colonies (called clones) were tested in a monospecific ELISA. The specificity of these scFv clones was confirmed by the ability of one clone to sufficiently recognise the *Tvi*CATL antigen immobilised on an ELISA plate. It will be necessary to further select for soluble scFvs using an ELISA in which the detergents are omitted or used in minimal amounts. In this ELISA, *Tvi*CATL

will be coated on ELISA plate, probed with monospecific *Tvi*CATL-scFv, followed by detection of scFvs with mouse anti-c-myc tag monoclonal antibody 9E10 (myc-tag is fused to the C-terminus of the scFv) and rabbit anti-mouse IgG-HRPO conjugate (Van Wyngaardt *et al*, 2004). The identity of each soluble clone can be determined by sequencing using suitable primers. The soluble scFvs will be then expressed on large scale by IPTG induction (Kramer, 2002). The temperature stability of expressed scFvs can be evaluated. The specificity of the soluble scFvs can be further confirmed by investigating its usability in a serodiagnostic scFvs inhibition ELISA format.

In the present study, good levels of chicken anti-*Tvi*CATL IgY were produced in chickens as evaluated by ELISAs, dot and western blot analysis. These antibodies showed moderate immunoinhibition of the hydrolysis of Z-Phe-Arg-AMC by *Tvi*CATL *in vitro*. *Tvi*CATL immobilised on ELISA plate for antibody detection inhibition ELISA, also showed some cross-reactivity with *Tco*CATL antibodies (in sera from *T. congolense* infected cattle) as evidenced by the ability to discriminate between the *T. congolense*-infected and non-infected cattle sera. Further optimisation of inhibition ELISA conditions are necessary to investigate the extent of cross-reactivity between antibodies produced against *T. vivax* and *T. congolense* antigens. The initial trials of Nkuku® phage display library were conducted through panning against the recombinant *Tvi*CATL antigen. Monospecific *Tvi*CATL-scFvs were enriched as evidenced by a signal (albeit very low) on a monospecific phage ELISA. The optimisation of panning conditions is a necessary step in order to enhance the enrichment of specific *Tvi*CATL-scFvs, thus improve the ELISA signal.

CHAPTER 4

GENERAL DISCUSSION

African animal trypanosomiasis (AAT) or nagana is a neglected tropical disease that is a major constraint to agricultural-based economies with losses estimated to approximate $4.75 billion yearly on the African continent (Swallow, 2000; Van den Bossche & Delespaux, 2011). This disease is caused by the parasites of the genus *Trypanosoma*, in which the prevalent species include *Trypanosoma congolense*, *T. vivax* and *T. brucei brucei*. These parasites are mainly transmitted from host to host by the haematophagous tsetse flies which infest approximately 10 million km^2 of the African continent (Rogers & Randolph, 2002; Van den Bossche & Delespaux, 2011).

The major control methods for AAT include the use of chemotherapeutic drugs, trypanotolerant cattle and vector control strategies. The Trypanocidal drugs isometamidium, homidium and diminizine have been in use for more than 50 years to treat animal trypanosomiasis, but there is an alarming increase of drug resistance which hampers the drug-based control strategies (Geerts *et al*, 2001; Delespaux *et al*, 2008a; Delespaux *et al*, 2008b). Vector control strategies including spraying of insecticides using a sequential aerosol technique (Vreysen *et al*, 2013), odour-baited traps as well as the sterile insect technique had variable success (Vreysen *et al*, 2000; Abd-Alla *et al*, 2013). Trypanotolerant cattle (N'Dama) breeds are distributed only in West and Central Africa and this breed is relatively small in size and thus less attractive to farmers (Holmes, 1997; d'Ieteren *et al*, 1998). Due to antigenic variation in which the parasite constantly switches expression of its surface variable surface glycoproteins (VSGs), no anti-parasite vaccines have been successfully developed (Donelson *et al*, 1998). There is therefore an urgent need to develop new AAT control methods such as new chemotherapeutic agents.

As an alternative to the anti-parasite vaccination approach, the feasibility of an anti-disease vaccination approach which targets the infection-associated pathology, rather than the parasite, was investigated (Authié, 1994; Authié *et al*, 2001). When trypanotolerant and trypanosusceptible cattle were immunised with the cysteine protease, *Tco*CATL, and exposed to primary *T. congolense* infections, it was observed that there was an increase in the IgG antibody response in trypanotolerant cattle as opposed to susceptible cattle. Therefore, this led to the conclusion that *Tco*CATL is a virulence factor released by the parasite in the host bloodstream, whereupon it initiates

the pathogenesis of the parasite and that anti-*Tco*CATL antibodies may regulate trypanosome-associated pathologies. These antibodies correlate with the ability to tolerate infections in trypanotolerant cattle.

For the effective control of AAT, the development of definitive species-specific serodiagnostic tests is of utmost importance to ensure appropriate administration of drugs (Brun *et al*, 2010). Serological tests are considered to be specific and sensitive (Ndao, 2009). More specifically, enzyme-linked immunosorbent assay (ELISA) is considered as a sensitive, affordable and simple procedure for diagnosis of trypanosomiasis (OIE, 2012). ELISA is subdivided into antibody capture ELISA (AbELISA) which detects anti-trypanosomal antigen antibodies in the parasite lysate; and antigen capture ELISA (AgELISA) in which circulating antigens in the host bloodstream are detected. Both tests are limited by the fact that crude or whole parasite lysates are the source antigen; a procedure that lacks standardisation and requires culturing of the parasite *in vivo* or *in vitro* (Reid & Copeman, 2002).

To avoid the use of unstable native parasite antigens for diagnosis, several antigens with diagnostic potential have been identified and produced using optimisable and standardised recombinant technology. The ideal trypanosomal antigens with diagnostic potential are the immunodominant antigens in the bloodstream developmental stage of trypanosomal species (Hutchinson *et al*, 2004; Goto *et al*, 2011; Thuy *et al*, 2012). Trypanosomal cysteine proteases are considered as immunodominant antigens with ideal potential for the development of serodiagnostic tools against AAT (Authié *et al*, 1992; Authié *et al*, 2001; Lalmanach *et al*, 2002). Therefore, the aim of the present study was to recombinantly express and enzymatically characterise the immunodominant cysteine protease, *Tvi*CATL from *T. vivax* and produce antibodies for serodiagnosis of AAT using the antibody detection inhibition ELISA format.

Sequence alignment of *Tvi*CATL from *T. vivax* (Accession no. CCD21670) with *Tco*CATL from *T. congolense* (Accession no. CAA81061) showed high sequence identity (61.8%). *Tco*CATL has been elucidated as a pathogenic factor and an immunodominant antigen with diagnostic potential (Authié *et al*, 1992; Authié *et al*, 2001; Lalmanach *et al*, 2002). Hence, the findings about recombinant *Tvi*CATL in the present study were mainly compared to those obtained for recombinant *Tco*CATL.

The production of recombinant antigen over isolation of native proteins was considered since recombinant technology is a standardised procedure which eliminates the use of *in vivo* methods for antigen production (Rogé *et al*, 2014). In the present study, the *Pichia pastoris* yeast expression system was used for recombinant expression of

*Tvi*CATL. In the expression construct, the pro-region (which is important for protein stability, folding and secretion) as well as the catalytic domain (responsible for the activity of cysteine proteases) were included (Vernet *et al*, 1991). The C-terminal extension was not included since it is not crucial for the activity of trypanosomal cysteine proteases (Eakin *et al*, 1993). *Tvi*CATL was recombinantly expressed as a soluble protein and at yields significantly higher than those reported by Vather (2010). The expression of *Tvi*CATL as a soluble, glycosylated protein and at higher yields, is in accordance with the advantages associated with the *Pichia* system (Macauley-Patrick *et al*, 2005; Cregg, 2007). Such success has also been reported for recombinant *Tco*CATL variants expressed in the *Pichia* system (Pillay *et al*, 2010; Boulangé *et al*, 2011).

*Tvi*CATL migrated on reducing SDS-PAGE as two bands at 29 and 33 kDa. As is the case for all trypanosomal cysteine proteases, *Tvi*CATL was synthesised as an inactive pro-enzyme or a zymogen. This inactivity is as result of the presence of the propeptide which is considered to be a potent inhibitor of its associated protease's catalytic domain (Fox *et al*, 1992; Yamamoto *et al*, 2002). This inactive pro-enzyme was transformed to an active enzyme through autocatalytic cleavage of the propeptide which was effected by acidification (pH 4.2) of the expression supernatants (Boulangé *et al*, 2011). Three phase partitioning followed by molecular exclusion chromatography were used to concentrate and purify *Tvi*CATL to homogeneity. The identity of the purified *Tvi*CATL was confirmed by western blot analysis by probing with chicken anti-full length mutant *Tvi*CATL antibodies produced in a previous study by Vather (2010). The treatment of *Tvi*CATL with endoglycosidase H proved that *Tvi*CATL is an N-glycosylated protein. Glycosylation is associated with advantages such as protein folding and stability (Dwek, 1998). Glycosylation of *Tvi*CATL is as a result of the presence of the N-glycosylation site (Asn^{288}) in the catalytic domain. The sequence alignment of the two closely related cysteine proteases, *Tvi*CATL and *Tco*CATL, showed that the glycosylation site of *Tvi*CATL (*T. vivax,* accession no. NCBI CCD2167.0) is located within the active site while the potential glycosylation sites for *Tco*CATL (*T. congolense*: NCBI accession no. CAA81061.1) are found in the proregion. *Tco*CATL may exist as a non-covalent dimer at neutral pH (Boulangé *et al*, 2011), however, glycosylation is not possible as its glycosylation sites are found in the propeptide which are cleaved off during spontaneous activation of the pro-enzyme *in vivo*. *Tcr*CATL from *T. cruzi* shares the same glycosylation feature with *Tvi*CATL since it also contains three asparagine-glycosylation sites: two sites located in the catalytic domain and one in the C-terminal extension (Eakin *et al*, 1992). The presence of two

bands does not always mean that the protein is glycosylated, in other proteases, some bands are due to the presence of several cleavage sites during activation of the enzyme *in vitro* (Vernet *et al*, 1990; Eakin *et al*, 1992). Papain and human procathepsin L are examples of proteases that contain several possible cleavage sites for autocatalytic processing at lower pH (Vernet *et al*, 1990; Ménard *et al*, 1998). The cleavage sites for papain are found between Gly^{108} and Asp^{107} within the propeptide, while the cleavage sites for procathepsin L are found between the propeptide and the central domain at Phe^{89}-Gln^{90} and Gln^{90}-Glu^{91} (Vernet *et al*, 1990; Ménard *et al*, 1998).

In-depth biochemical characterisation of trypanosomal cysteine proteases is of eminent importance for designing anti-trypanosomal chemotherapeutic drugs. This is necessary in order to identify unique and similar biochemical features shared amongst host and pathogen CATL-like proteases. Biochemical characterisations include: determination of pH optimum and stability, substrate specificity including hydrolysis of host proteins and interaction with synthetic and host endogenous inhibitors. In the present study, recombinant *Tvi*CATL exhibited high gelatinase activity as compared to that reported for a native form of *Tvi*CATL (Jackson, 2011). After incubation with thiol cysteine protease inhibitors (E-64, antipain, leupeptin, iodoacetate and iodoacetamide), gelatinase activity was inhibited proving that *Tvi*CATL is a cysteine protease. Cysteine proteases are lysosomally located in the parasite, and are released into the host bloodstream upon parasite death and lysis. In the host, cysteine proteases' hydrolysing potential is usually controlled by endogenous inhibitors such as cystatins, kininogens and stefins (Turk *et al*, 1997). However, in the present study, endogenous inhibitors cystatin and stefin B (including previous results from W. Mpaphane, personal communication), did not fully inhibit gelatinase activity, some activity was observed. Previous studies also reported that instead of endogenous inhibitors (kininogens, serpins and stefin B) posing inhibitory effects towards the parasitic cysteine proteases, active *Tb*CATL protease-inhibitor complexes were formed (Lonsdale-Eccles *et al*, 1995). The formation of the proteolytic active-inhibitor complexes were also reported for mammalian cathepsin L and the bloodstream forms of *Leishmanial* proteases *in vitro* (Pike *et al*, 1992; Mottram *et al*, 1998). However, *Tbb*CATL was inhibited by kininogens and cystatins while a serine oligopeptidase from *T. b brucei* was not inhibited by serpins or α_2-macroglobulin (Troeberg *et al*, 1996). The failure of host protease inhibitors to inhibit released proteases leads to an inevitable progression of the parasite pathologies as the host inhibitors fail to trigger any protective mechanisms (Dubin, 2005).

In the constant ionic strength AMT buffers, the hydrolysis of Z-Phe-Arg-AMC by recombinant *Tvi*CATL had an optimum pH at 6.5 and 7.5, and this is consistent with findings reported by Vather (2010). This pH is similar to the pH optimum (6 or 6.5) reported for *Tco*CATL (Mbawa *et al*, 1992; Pillay *et al*, 2010). The pH optima around physiological pH suggest that these proteases will remain active when released into the host bloodstream upon parasite lysis and could therefore be pathogenic factors. At an acidic pH (pH 4), the recombinant *Tvi*CATL was inactive. This contradicts with the lysosomal localisation of trypanosomal cysteine proteases within the parasite (Mbawa *et al*, 1992). However, this suggests that although the enzyme is located within the lysosomes of the parasite; its extracellular functionality in the host bloodstream differs from its functionality within the parasite. This phenomenon was reported for other cysteine proteases such as *Tco*CATL (Sajid & McKerrow, 2002; Serveau *et al*, 2003).

A notable finding from the present study is that, at around physiological pH, *Tvi*CATL was able to hydrolyse endogenous protein substrates (BSA, type I collagen and bovine haemoglobin) as well as the general plasma protease inhibitor bovine α_2-macroglobulin *in vitro*. The ability to hydrolyse collagen, a prolyl-rich protein, was a new feature observed for a trypanosomal cysteine protease. This prolyl-digesting ability of *Tvi*CATL was further substantiated by an ability of *Tvi*CATL to efficiently hydrolyse a peptide substrate (Z-Pro-Arg-AMC) with Pro in P_2. Collagenolytic activity is a common feature for trypanosomal serine prolyl oligopeptidases (Santana *et al*, 1997; Bastos *et al*, 2010). Collagenolytic activity facilitates the distribution of the protease within the host extracellular matrix and invasion of the host tissues (Piña-Vázquez *et al*, 2012). The ability to hydrolyse protein substrates such as collagen and BSA has been reported to be optimal at an acidic pH range for an epimastigote-stage isolated 30 kDa *T. cruzi* cysteine protease (Garcia *et al*, 1998). The triple helical collagenolytic activity was also reported at 37°C for mammalian cathepsin L. Upon incubation of the protease with glucosaminoglycan, the protease collagenolytic activity was potently inhibited (Li *et al*, 2004).

The recombinant *Tvi*CATL exhibits optimal haemoglobinolytic activity at pH around 6-7. This haemoglobinolytic activity is similar to that reported for a thiol protease from *Schistosoma mansoni* (Dvořák *et al*, 2009). It was noted that the pattern on SDS-PAGE for haemoglobinolytic activity of *Tvi*CATL suggest that the haemoglobinolytic activity of *Tvi*CATL is very rapid in that hydrolysis does not yield any detectable cleavage products on SDS-PAGE. This is unfortunate because the presence of quantifiable cleavage products is of particular interest since the resultant cleavage peptides can be used for prediction of the cleavage specificity (or positions) on protein

substrates. The use of a lower amount of *Tvi*CATL in the assay may result in partial haemoglobinolytic activity which in turn could provide cleavage products on SDS-PAGE for N-terminal sequencing analysis.

Recombinant *Tvi*CATL was able to cleave bovine α_2-macroglobulin, a promiscuous proteinase-inhibiting glycoprotein. Alpha-2-macroglobulin inhibits a large range of proteases due to the presence of multiple binding sites (Barrett & Starkey, 1973). Alpha-2-macroglobulin consists of four subunits linked together by thioester bonds. Each subunit consists of a "bait" region susceptible to cleavage by proteases. As a result of conformational changes of α_2-macroglobulin upon "bait" cleavage by the protease, the protease remains entrapped within the "bait" region. However, the protease active site is not blocked, thus is capable of interacting with small peptide substrates and inhibitors (Borth, 1992). The cleavage of the "bait" region exposes residues which have a high affinity specific receptors on antigen presenting cells. Therefore, protease-α_2-macroglobulin complexes are efficiently recognised by macrophages and this results in pronounced antibody production (Chu & Pizzo, 1993; Chu *et al*, 1994).

At pH 6-7, *Tvi*CATL optimally cleaved the "bait" region of α_2-macroglobulin as evidenced by the presence of a "fast" α_2-macroglobulin form (Barrett *et al*, 1979) on reducing SDS-PAGE. The ability of *Tvi*CATL to cleave the "bait" region of α_2-macroglobulin is similar to that reported for *Tco*CATL (Huson *et al*, 2009), and therefore, *Tvi*CATL can also be used to cleave the "bait" region which can then be conjugated to another high molecular weight proteases such as oligopeptidase B that itself is unable to cleave the "bait" region of α_2-macroglobulin (Pizzo & Grøn, 2002; Huson *et al*, 2009). High antibody titres have been reported when rabbits were immunised with recombinant *Tco*CATL in complex with bovine α_2-macroglobulin; and these antibodies were inhibitory towards *Tco*CATL activity, a property necessary for designing anti-disease vaccines against AAT (Huson *et al*, 2009). The ability of *Tvi*CATL to hydrolyse bovine α_2-macroglobulin suggests that α_2-macroglobulin can be used as a nontoxic adjuvant associated with pronounced antibody responses (Chu & Pizzo, 1993; Singh & O'Hagan, 2003).

For CATL-like cysteine proteases, the P_2 substrate binding site which is complementary to the S_2 subsite of the protease, is the primary determinant of the substrate specificity of the protease (Turk *et al*, 1998). In the present study, based on k_{cat}/K_m values, it was found that *Tvi*CATL preferentially hydrolysed substrates with hydrophobic (Phe or Leu) residues at P_2. Substrate specificity further required that the

P_1-postion is occupied by basic (Arg or Lys) residues. These findings are consistent with findings reported for recombinant and native *Tvi*CATL (Vather, 2010; Jackson, 2011), *Tco*CATL (Mbawa *et al*, 1992; Pillay *et al*, 2010) and *Tbr*CATL (Caffrey *et al*, 2001). This subsite preference was further validated by the observed pronounced enzymatic inhibition when the recombinant (in the present study) and native (Jackson, 2011) forms of *Tvi*CATL were incubated with reversible inhibitors (Z-Gly-Leu-Phe-CMK and H-D-Val-Leu-Phe-Lys-CMK) containing Leu or Phe in P_2. These inhibition patterns further show that in P_2, Phe and Leu residues are important for efficient substrate hydrolysis. Therefore, if these residues are the constituents of the P_2 position of the inhibitor, access to the active site of the enzyme is blocked in such a way that the addition of the specific substrate will not reverse the inhibitor binding. An irreversible inhibitor, K11777 (N-methyl-piperazine-Phe-homoPhe-vinylsulfone-phenyl), which is a vinyl sulfone-pseudo peptide, has been classified as a potent inhibitor of *Tcr*CATL. This is validated by the fact that in a mice model chemotherapeutic trials, *T. cruzi* infected mice were protected against epimastigote-infection associated pathology (Engel *et al*, 1998a). However, the k_{cat}/K_m ratio further indicated that Phe (Z-Phe-Arg-AMC) is preferred over Leu (H-D-Ala-Leu-Lys-AMC) in P_2. Recombinant *Tvi*CATL was further proved to be a cysteine protease since it was inhibited by thiol inhibitors: E-64, antipain, leupeptin, iodoacetate and iodoacetamide. Although the effect of inhibition by antipain and leupeptin on the parasitic lifecycle was not investigated in the present study, it has been reported that these inhibitors killed *T. cruzi* parasites through inhibiting the transition from the epimastigote stage to the metacyclic stage (Bonaldo *et al*, 1991). Recombinant *Tvi*CATL had no activity towards the aminopeptidase (H-Leu-AMC) and a cathepsin B substrate (Z-Arg-Arg-AMC) nor was it inhibited by metalloprotease (EDTA), aminopeptidase (bestatin) and serine (TPCK and PMSF) protease inhibitors.

The second objective of the present study was to raise antibodies in chickens and test the potential of chicken anti-*Tvi*CATL IgY antibodies and *Tvi*CATL antigen as suitable serodiagnostic candidates in an antibody-detection inhibition ELISA format. As an alternative to animal immunisation for antibody production, the Nkuku® phage display library was evaluated for the production of mono-specific scFvs antibodies.

Egg-laying chickens were chosen as the hosts for antibody production due to the associated advantages as described by Polson *et al* (1980), Larsson *et al* (1993) and Tini *et al* (2002). Chickens were immunised with the purified recombinant *Tvi*CATL and chicken anti-*Tvi*CATL IgY antibodies were isolated from egg yolks using the PEG precipitation method described by Polson *et al* (1980) and modified by Goldring and

Coetzer (2003). These antibodies were further affinity purified by passing through a *Tvi*CATL affinity column. High antibody titres were obtained following immunisation of chickens as evidenced by measureable signals in an ELISA. This high immunogenicity may be attributed by the evolutionary distance between the trypanosomal antigen source (parasite) and the immune system of the immunised host (chicken). Though *Tvi*CATL and *Tco*CATL share high sequence identity, minimal cross-reactivity of chicken anti-*Tvi*CATL IgY antibodies with *Tco*CATL antigen was observed. On the dot blot for optimisation of concentration of antigen, primary and secondary antibodies, the prozone effect was observed. A prozone effect was also observed with the chicken anti-*T. b. gambiense* LiTat 1.3 and chicken anti-*T. b. gambiense* LiTat 1.5 IgY antibodies as reported by Mnkandla (2013). In the present study, high signals were obtained in the western blots, however, ECL detection kit proved to be more effective than a colorimetric 4-chloro-1-naphthol chromogen. The anti-*Tvi*CATL antibodies were also shown to inhibit the activity of *Tvi*CATL which is important in the context of an anti-disease vaccine for *T. vivax* induced AAT as reported by Authié *et al* (2001) for *Tco*CATL for *T. congolense* induced AAT.

In the present study, the next step was to use the recombinantly expressed *Tvi*CATL and produced chicken anti-*Tvi*CATL antibodies for the optimisation of a serodiagnostic test for African animal trypanosomiasis. Due to the unavailability of sera from cattle experimentally infected with *T. vivax*, sera from cattle experimentally infected with *T. congolense* were used in order to optimise the antibody detection inhibition ELISA format while at the same time gaining information about the extent of cross-reactivity between these two trypanosomal antigens. This antibody detection inhibition ELISA format was able to discriminate between infected and non-infected sera. Notably, the percentage inhibition was very low (less than 25%) suggesting that some degree of cross-reactivity of *T. congolense* serum antibodies with *Tvi*CATL antigen was observed. In the present study, antibody detection inhibition ELISA was chosen over an antibody detection indirect ELISA since the latter requires species-specific detection antibodies (i.e. anti-rabbit/goat/sheep/bovine IgG-HRPO conjugates) depending on the species being tested. An antibody detection inhibition ELISA format is cost effective since one detection system is used such as rabbit anti-chicken IgY-HRPO conjugate for testing e.g. infected cattle, goats or camels (Bossard *et* al, 2010).

A broad range of recombinant trypanosomal antigens have been identified as potential candidates for diagnostic purposes (Boulangé *et al*, 2001; Boulangé *et al*, 2002; Bannai *et al*, 2003; Tran *et al*, 2008; Bossard *et al*, 2010; Pillay *et al*, 2013b; Fleming *et al*, 2014; Zhou *et al*, 2014). In the study conducted by Boulangé *et al* (2002), the

antigen BiP/HSP70 from *T. congolense* was recombinantly expressed in a bacterial system and used as an antigen in an indirect ELISA and probed with *T. congolense*-infected sera. It was noted that this test was more sensitive for detection of secondary infections than primary infections. Also, when BiP/HSP70 from *T. congolense* was probed with sera from cattle with natural or experimental *T. congolense* infections and screened in an antibody detection inhibition ELISA format, high sensitivity was also reported for secondary infections (Bossard *et al*, 2010). Recombinant invariant surface glycoprotein (ISG75) from *T. b. gambiense* has also been used for serodiagnosis of human trypanosomiasis. This antigen was recombinantly expressed in an *E. coli* system as a double tagged (thioredoxin reductase and glutathione oxidoreductase) fusion protein. Its diagnostic potential was evaluated in an indirect ELISA in which this antigen was probed with sera from goats experimentally infected with *T. b. brucei*. The antibodies in the sera collected post-infection resulted in maximal signals as compared to sera collected prior to infection (Tran *et al*, 2008). More recently, the proteomic selection method as described by Sullivan *et al* (2013), has been a helpful tool for the selection of immunodiagnostic antigens for *T. vivax* and *T. congolense* infections (Pillay *et al*, 2013b). This includes a flagellar associated-protein, GM6 antigen of *T. vivax* (TvGM6) that was recombinantly expressed in *E. coli*. In an indirect ELISA, TvGM6 was probed with sera from naturally and experimentally *T. vivax* infected cattle. High sensitivity (91.4%) was obtained for sera collected 10-days post-infection. However, sensitivity was reduced to ten-fold (9.1%) after an elongated infection period (one month post infection) (Pillay *et al*, 2013b). This low diagnostic sensitivity was thought to be attributed to the persistence of trypanosomal antibodies. This antibody persistence in the host might have resulted in false positive infection status of the cattle sera as pre-predicted by polymerase chain reaction (PCR). The persistence of trypanosomal antibodies in the host has been reported following 10-13 months post-treatment of cattle with diminazine trypanocidal drug (Authié *et al*, 1993; Van den Bossche *et al*, 2000). Also, cross-reactivity of TvGM6 antigen with *T. congolense*-infected sera was observed and was thought to be contributed by the presence of conserved epitopes amongst these two species (Pillay *et al*, 2013b).

An invariable antigen from *T. congolense* (TcP46) has also been proteomatically identified as a diagnostic antigen (Zhou *et al*, 2014). This antigen was recombinantly expressed in *E. coli* as a glutathione S-transferase-tagged protein. In an indirect ELISA, this antigen was optimally recognised by serum antibodies collected from mice as early as eight days post-infection with *T. congolense*. No cross-reactivity was observed with serum collected from non trypanosomal species such as *Babesia*

bigemina (Zhou *et al*, 2014). In another related study, an invariant surface glycoprotein Tc38630 was identified as a potential serodiagnostic antigen for detection of *T. congolense* infections (Fleming *et al*, 2014). In an indirect antibody ELISA, high sensitivity (87.2%) and specificity (97.4%) were obtained. Interestingly, it was noted that no signals were obtained soon after treatment with trypanosomal drugs, thus suggesting that there was no persistence of trypanosomal antibodies in the host soon after treatment and this is an important feature which will enable accurate monitoring of the drug treatment using an AbELISA (Fleming *et al*, 2014).

In order to find a standardised, cheap and renewable method other than animal immunisation for the production of mono-specific antibody against trypanosomal antigens, initial attempts were made to optimise a semi-synthetic chicken antibody library called Nkuku® phage display library (Van Wyngaardt *et al*, 2004). The Nkuku® phage display library allows for the selection of recombinant scFvs against the immobilised antigen of interest *in vitro*. Several optimisation studies have been reported using bluetongue (BTV) and African horse sickness virus (AHSV) antigens and the resultant recombinant scFvs were used for serodiagnostic purpose in an inhibition ELISA format (Van Wyngaardt *et al*, 2004; Fehrsen *et al*, 2005a; Rakabe *et al*, 2011; van Wyngaardt *et al*, 2013). In the study conducted by Van Wyngaardt *et al* (2004) in which the Nkuku® scFvs were panned against BTV and AHSV antigens, the analysis by ELISA of phage pools showed that the AHSV signals for 1-4 panning rounds were very low (less than 0.2). For BTV, the signal obtained for panning round 3 was about 0.5 while for panning round 4, the signal was about 1.4 thus suggesting the enrichment of the BTV-specific phage pools with successive rounds of panning. For further characterisation, the phage clones against BTV and AHSV were randomly picked and tested in a mono-specific phage ELISA. As a criterion for antigen-specific scFvs, clones with $A_{492\ nm}$ greater than 0.2 were considered as specific binders. For BTV, 76% phage clones were specific binders while for AHSV, 61% phage clones were specific binders. No cross-reactivity was observed for enriched scFvs, i.e. BTV-specific scFvs did not recognise other antigens such as the keyhole limpet haemocyanin.

The antigen VP7, a major structural protein conserved in all 24 serotypes of BTV, is highly immunogenic and thus a suitable antigen for diagnosis of all serotypes in the competitive ELISA (Eaton *et al*, 1991; Martyn *et al*, 1991). Therefore, in the study conducted by Rakabe *et al* (2011) it was necessary to screen for VP7-specifc scFvs for serodiagnostic purpose. Nkuku® phage display scFvs were panned against VP7 directly coated in Polysorp mitrotitre plates as well as VP7 coated in Ni^{2+} chelate

coated-plates. The phage pools enriched against panning on VP7 coated on Polysorp plates were eluted with triethylamine at pH 9.5 while the phage pools enriched by panning against VP7 immobilised in Ni^{2+} chelate coated-plates were eluted by competition with anti-VP7 hyper immune rabbit and guinea pig sera in an attempt to enrich for the phage pools with serodiagnostic potential. After four rounds of panning, enriched phage pools and negative control (pre-panned Nkuku® library) were analysed by ELISA. The ELISA signal for the pre-panned library was less than 0.3 and the signal was 1.2 for phage pools eluted with immune sera. The phage pools enriched against VP7 coated on Ni^{2+} chelate-coated plates gave ELISA signals at 3.6 and 2.5, respectively. For further characterisation of enriched phage clones, single phage clones were randomly picked and screened by EISA for soluble VP7-specific scFv binders. The negative controls i.e. *E. coli* Origami™ proteins and milk binders were included. The signals for soluble VP7-specific scFvs enriched on VP7 immobilised on Ni^{2+} chelate coated-plates were greater than those enriched on VP7 directly immobilised on plastic Polysorp plates. This suggested that VP7 epitopes on Ni^{2+} chelate coated-plates were highly accessible as compared to those on plastic Polysorp plates at which protein conformational changes are very likely (Butler *et al*, 1997).

In the present study, mono-specific scFvs were selected against *Tvi*CATL antigen (directly coated on plastic immunotubes) through four successive rounds of panning as illustrated in Figure 3.2. The phage pools were enriched during panning round three as evidenced by moderate signals in the first phage pools ELISA. Then, it was necessary to screen for the mono-specific scFvs from the precipitated phage pools in order to further select for *Tvi*CATL-specific scFvs. For this ELISA, forty eight phage clones were randomly picked and tested. The signals of the mono-specific suggested that only one clone (with signal at 0.4) was a specific binder. The signal for this clone is very low as compared to those obtained by Rakabe *et al* (2011) for VP7 and Van Wyngaardt *et al* (2004) for BTV and AHSV. Therefore, this suggests that in order to obtain satisfactory ELISA signals for *Tvi*CATL-specific scFvs, it will be necessary to further optimise coating plates and antigen coating concentrations for panning and optimise the elution of the bound phages by exploiting strategies described by Rakabe *et al* (2011).

In the present study, the initial optimisations of Nkuku® phage display were achieved. However, for future work, it will be necessary to further optimise initial panning conditions to ensure that more specific *Tvi*CATL-scFvs fusion clones are enriched, therefore resulting in satisfactory signals when analysed by ELISA. It will also be necessary to further select for soluble scFvs. In the study conducted by Van

Wyngaardt *et al* (2004), to obtain soluble scFvs, heterologous expression was conducted in which the *E. coli* TG1 cells together with antigen-specific scFvs were induced with IPTG (Kramer, 2002) in order to produce large quantities of soluble scFvs. These soluble scFvs were then analysed using the soluble phage ELISA in which the detergents were omitted or used at minimal amounts. This ELISA indicated that only two clones (F3 and F10) sufficiently recognised BTV serotype 10. In order to confirm that the enriched scFvs were indeed screened from Nkuku® phage display library (i.e. if they were enriched from the library in which the chicken immunoglobulins were synthetically randomised into the heavy chain CDR3s), the DNA of randomly picked phage clones were sequenced and compared to the CDR sequence of the primers which were used for amplification of the chicken immunoglobulin genes. Results showed that some of the BTV and AHSV scFv clones were originating from this library. Therefore, once *Tvi*CATL-scFvs are enriched, sequencing will be necessary to confirm their identity. To test the usability of BTV-specific and AHSV-specific phage displayed scFvs for immunodiagnostic of BTV and AHSV in rabbit or sheep sera, sandwich ELISAs were performed and moderate signals were obtained (0.5). This was followed by the inhibition ELISA to further evaluate the specificity of the BTV-specific and AHSV-specific scFv clones. Some degree of inhibition was observed but depended on serum dilution and no inhibition was observed when the non-immune sera were used.

In the study conducted by Fehrsen *et al* (2005a), the BTV-specific scFv clones (F3 and F10) produced in the study conducted by Van Wyngaardt *et al* (2004) were further evaluated for their diagnostic potential in an inhibition ELISA format. In this ELISA, BTV serotypes (1-24) were coated on Polysorp ELISA plates, probed with guinea-pig sera or sheep serum. This was followed by incubation with soluble scFvs (F3 and F10) pre-incubated with mouse monoclonal antibody (mAb) 9E10 (recognising the scFv-c-Myc-tagged Ab). For detection, anti-mouse IgG-HRPO conjugate was added followed by addition of the chromogen. Results indicated that when F3-scFvs were used, serotype-specific inhibition was observed i.e. the signal for serotype 10 coated wells was lower than the signals for other serotypes. When F10-scFvs were used, serogroup cross-inhibition was observed i.e. signals of the other serotypes were inhibited. It was concluded that though F3 and F10 were both screened by panning against serotype 10, their behaviour is different in an inhibition ELISA. F10 was stable for almost 3 months at -20°C while F3 was unstable after one freeze-thaw cycle (Fehrsen *et al*, 2005a). Therefore, once *Tvi*CATL-scFv specific clones are produced as soluble fragments, it will be necessary to test their usability in an inhibition ELISA for diagnosis

of AAT. To ascertain specificity of the *Tvi*CATL-scFvs specific antibodies, cross-reactivity with other trypanosomal antigens such as *Tco*CATL could be tested in an inhibition ELISA format.

In the present study, the cysteine protease, *Tvi*CATL from *T. vivax*, was successfully expressed in the *Pichia* yeast system. For future studies, it will be necessary to get larger quantities of ultra-pure *Tvi*CATL in order to optimise crystallisation conditions to resolve the crystal structure of *Tvi*CATL using the Rigaku CrystalTrak™ available in our department. Previous crystallisation trials for *Tco*CATL as optimised by Jackson (2011) will be helpful for this purpose. Conditions such as pH, protein, PEG 3350 and PEG 6000 concentrations were optimised. It was observed that broader needle-like crystals were obtained in PEG 6000 buffer than in PEG 3350 buffer. In order to test if the crystals obtained were able to diffract under X-ray beam, crystals were grown using the hanging drop method (in order to transfer them into sealable capillary tubes) for three days and the protein: crystallant ratio was 80:20. One crystal diffracted though diffraction was weak.

Enzymatic characterisation studies were conducted as a first step towards evaluating different inhibitors as possible drug targets for trypanosomiasis. However, to further get convincing evidence that *Tvi*CATL is involved in pathogenesis, RNAi studies are necessary (LaCount & Donelson, 2001). This can be used to down regulate the expression of *Tvi*CATL *in vitro* in order to evaluate the resulting effect on parasite growth *in vitro*. In addition, this approach will indicate whether activation of *Tvi*CATL could result in modulation of *T. vivax* pathogenesis. When *Tbb*CATL gene expression was down regulated by RNAi, i.e. parasites were eletroporated with recombinant vector containing *Tbb*CATL transgenes, prolonged survival of the mice infected with the parasite in which *Tbb*CATL was down regulated was observed as compared to mice infected with the parasite in which *Tbb*CATL was not down regulated (Abdulla *et al*, 2008). Gene down regulation of alanyl aminopetidases from *T. congolense* resulted in slight inhibition of growth of the procyclic *T. congolense in vitro* (Pillay *et al*, 2013a).

In the present study, *Tvi*CATL and chicken anti-*Tvi*CATL IgY antibodies were tested in preliminary antibody detection inhibition ELISA format for their potential as a species-specific diagnostic tool. For future work, it will be necessary to conduct antibody detection inhibition ELISA in parallel with an indirect antibody detection ELISA in order to compare the specificity, sensitivity and reproducibility of the two procedures. In these tests it will be necessary to include non-related parasitic species that are found

in areas in which AAT is endemic such as *Anaplasma*, *Babesia* and *Theileria* as negative controls.

In order to ensure accurate serodiagnosis of current infections by detecting circulating trypanosomal antigens in the host bloodstream, it is also necessary to develop an antigen detection ELISA (Nantulya & Lindqvist, 1989; Eisler *et al*, 1998). Chicken anti-*Tvi*CATL IgY or alternatively *Tvi*CATL-scFv specific antibodies can be used to detect sera with *T. vivax* infections. The steps for the proposed antigen detection ELISA are: coat with chicken anti-*Tvi*CATL, probe with sera from cattle with *T. vivax* infections (with circulating antigen) and detect with rabbit anti-IgY-HRPO conjugate followed by addition of the chromogen.

This study laid the foundation for *Tvi*CATL as a chemotherapeutic target and as antigen for antibody or antigen detection ELISA for serodiagnosis of AAT.

REFERENCES

Abd-Alla AMM, Bergoin M, Parker AG, Maniania NK, Vlak JM, Bourtzis K, Boucias DG and Aksoy S (2013) Improving Sterile Insect Technique (SIT) for tsetse flies through research on their symbionts and pathogens. *Journal of Invertebrate Pathology* **112, Supplement 1:** S2-S10

Abdulla M-H, O'Brien T, Mackey ZB, Sajid M, Grab DJ and McKerrow JH (2008) RNA interference of *Trypanosoma brucei* cathepsin B and L affects disease progression in a mouse model. *PLoS neglected tropical diseases* **2:** 1-6

Agbo EC, Majiwa PAO, Büscher P, Claassen E and Pas M (2003) *Trypanosoma brucei* genomics and the challenge of identifying drug and vaccine targets. *Trends in Microbiology* **11:** 322-329

Akita E and Nakai S (1992) Immunoglobulins from egg yolk: isolation and purification. *Journal of food science* **57:** 629-634

Aksoy S (2003) Control of tsetse flies and trypanosomes using molecular genetics. *Veterinary Parasitology* **115:** 125-145

Allsopp R (2001) Options for vector control against trypanosomiasis in Africa. *Trends in parasitology* **17:** 15-19

Almagro JC (2004) Identification of differences in the specificity-determining residues of antibodies that recognize antigens of different size: implications for the rational design of antibody repertoires. *Journal of Molecular Recognition* **17:** 132-143

Almeida IC, Ferguson MAJ, Schenkman S and Travassos LR (1994) Lytic anti-alpha-galactosyl antibodies from patients with chronic Chagas' disease recognize novel O-linked oligosaccharides on mucin-like glycosyl-phosphatidylinositol-anchored glycoproteins of *Trypanosoma cruzi*. *Biochemical Journal* **304:** 793-802

Alsopp R and Hursey BH (2004) Insecticidal control of tsetse. *In: Maudlin, I, Holmes, PH, Miles, MA (Eds), The Trypanosomiasis Cabi Publishing, Oxfordshire, UK***:** 491-507

Ancelle T, A., Paugam F, Bourlioux A, Merad A and Vigier JP (1997) Detection of trypanosomes in blood by the Quantitative Buffy Coat (QBC) technique: experimental evaluation. *Médicine tropicale: reveu du Corps de Santé colonial* **57:** 245-248

Andris-Widhopf J, Rader C, Steinberger P, Fuller R and Barbas III CF (2000) Methods for the generation of chicken monoclonal antibody fragments by phage display. *Journal of Immunological Methods* **242:** 159-181

Anthony R, Kolk A, Kuijper S and Klatser P (2006) Light emitting diodes for auramine O fluorescence microscopic screening of *Mycobacterium tuberculosis* [Technical Note]. *The International Journal of Tuberculosis and Lung Disease* **10:** 1060-1062

Antoine-Moussiaux N, Buescher P and Desmecht D (2009) Host-Parasite Interactions in Trypanosomiasis: on the Way to an Antidisease Strategy. *Infection and Immunity* **77:** 1276-1284

Armstrong N, Adey NB, McConnel SJ and Kay BK (1996) *Vectors for Phage Display. In Phage Display of Peptides and Proteins: a laboratory manual*, Vol. 1., J. (eds) edn. San Diego, CA: Academic Press Inc. 35-54.

Aslund L, Henriksson J, Campetella O, Frasch AC, Petterson U and Cazzulo JJ (1991) The C-terminal extension of the major cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. *Molecular & Biochemical Parasitology* **45:** 345-347

Authié E (1994) Bovine trypanosomiasis and trypanotolerance: a role for congopain? *Parasitology Today* **10:** 360–364

Authié E, Boulangé A, Muteti D, Lalmanach G, Gauthier F and Musoke AJ (2001) Immunisation of cattle with cysteine proteinases of *Trypanosoma congolense*: targetting the disease rather than the parasite. *International Journal for Parasitology* **31:** 1429-1433

Authié E, Muteti DK, Mbawa ZR, Lonsdaleeccles JD, Webster P and Wells CW (1992) Identification of a 33-kilodalton Immunodominant Antigen of *Trypanosoma-congolense* as a Cysteine Protease. *Molecular and Biochemical Parasitology* **56:** 103-116

Authié E, Muteti DK and Williams DJ (1993) Antibody responses to invariant antigens of *Trypanosoma congolense* in cattle of differing susceptibility to trypanosomiasis. *Parasite immunology* **15:** 101-111

Azzazy HME and Highsmith Jr WE (2002) Phage display technology: clinical applications and recent innovations. *Clinical Biochemistry* **35:** 425-445

Bailey S (1998) Tsetse fly eradicated on the Island of Zanzibar. *Nuclear News Lanuary***:** 56-61

Baiyegunhi OO (2013) Heterologous expression of invariant surface glycoproteins, ISG75 of *Trypanosoma brucei brucei* and *T.b. gambiense,* for antibody production and diagnosis of African Trypanosomiasis. MSc dissertation. University of KwaZulu-Natal (Pietermaritzburg campus).

Baneyx F (1999) Recombinant protein expression in *Escherichia coli*. *Current Opinion in Biotechnology* **10:** 411-421

Bannai H, Sakurai T, Inoue N, Sugimoto C and Igarashi I (2003) Cloning and expression of mitochondrial heat shock protein 70 of *Trypanosoma congolense* and potential use as a diagnostic antigen. *Clinical and Diagnostic Laboratory Immunology* **10:** 926-933

Baral TN (2010) Immunobiology of African trypanosomes: need of alternative interventions. *Journal of biomedicine & biotechnology* **2010:** 389153

Barbas CF, Kang AS, Lerner RA and Benkovic SJ (1991) Assembly of combinatory antibody libraries on phage surfaces - The gene-III site. *Proceedings of the National Academy of Sciences of the United States of America* **88:** 7978-7982

Barbas III CF (1993) Recent advances in phage display. *Current Opinion in Biotechnology* **4:** 526-530

Barderas R, Desmet J, Timmerman P, Meloen R and Casal JI (2008) Affinity maturation of antibodies assisted by in silico modeling. *Proceedings of the National Academy of Sciences of the United States of America* **105:** 9029-9034

Barrett AJ, Brown MA and Sayers CA (1979) The electrophoretically 'slow' and 'fast' forms of the alpha 2-macroglobulin molecule. *Biochemical Journal* **181:** 401-418

Barrett AJ, Kembhavi AA, Brown MA, Kirschke H, Knight CG, Tamai M and Hanada K (1982) L-Trans-Epoxsuccinyl-leucylamido (4-guanidino) Butane (E-64) and its analogs as inhibitors of cysteine proteinases including cathepsin B, H and L. *Biochemical Journal* **201:** 189-198

Barrett AJ and Rawlings ND (2001) Evolutionary lines of cysteine peptidases. *Biological Chemistry* **5:** 727-733

Barrett AJ and Starkey PM (1973) The interaction of α2-macroglobulin with proteinases. Characteristics and specificity of the reaction, and a hypothesis concerning its molecular mechanism. *Biochem J* **133:** 709-724

Barrett MP, Burchmore RJS, Stich A, O Lazzari J, Frasch AC, Cazzulo JJ and Krishna S (2003) The trypanosomiases. *Lancet* **362:** 1469–1480

Barrett MP, Coombs GH and Mottram JC (2005) *Future prospects in chemotherapy for trypanosomiasis*. 445-460.

Barrett MP and Fairlamb AH (1999) The biochemical basis of arsenical-diamidine crossresistance in African trypanosomes. *Parasitology Today* **15:** 136-140

Barry JD and McCulloch R (2001) Antigenic variation in trypanosomes: Enhanced phenotypic variation in a eukaryotic parasite. *Advances in Parasitology* **49:** 1-70

Bastin P, Pullen TJ, Moreira-Leite FF and Gull K (2000) Inside and outside of the trypanosome flagellum:a multifunctional organelle. *Microbes and Infection* **2:** 1865-1874

Bastos I, Motta FN, Charneau S, Santana JM, Dubost L, Augustyns K and Grellier P (2010) Prolyl oligopeptidase of *Trypanosoma brucei* hydrolyzes native collagen, peptide hormones and is active in the plasma of infected mice. *Microbes and Infection* **12:** 457-466

Bauer B, Amsler de la Fosse S, Clausen PH, Kabore I and Petrich-Bauer J (1995) Successful application of deltamethrin pour-on to cattle in a campaign against tsetse flies (*Glossina* spp.) in the pastoral zone of Samorogouan, Burkina Faso. *Tropical Medicine and Parasitology* **46:** 183- 189

Bengaly Z, Kanwe A and Duvallet G (1995) Evaluation of an antigen detection-ELISA test for the diagnosis of trypanosomiasis in naturally infected cattle. *Tropical medicine and parasitology: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ)* **46:** 284-286

Bizhanov G, Jonauskiene I and Hau J (2004) A novel method, based on lithium sulfate precipitation for purification of chicken egg yolk immunoglobulin Y, applied to immunospecific antibodies against Sendai virus. *Scandinavian Journal of Laboratory Animal Science* **31:** 121-130

Blum H, Beier H and Gross HJ (1987) Improved silver staining of plant proteins, RNA and DNA in polyacrylamide gels. *Electrophoresis* **8:** 93-99

Bonaldo MC, d'Escoffier LN, Salles JM and Goldenberg S (1991) Characterization and expression of proteases during *Trypanosoma cruzi* metacyclogenesis. *Experimental parasitology* **73:** 44-51

Borst P, Rudenko G, Taylor MC, Blundell PA, van Leeuwen F, Bitter W, Cross M and McCulloch R (1996) Antigenic variation in trypanosomes. *Archives of Medical Research* **27:** 379-388

Borth W (1992) Alpha 2-macroglobulin, a multifunctional binding protein with targeting characteristics. *The FASEB Journal* **6:** 3345-3353

Bossard G, Boulange A, Holzmuller P, Thevenon S, Patrel D and Authie E (2010) Serodiagnosis of bovine trypanosomosis based on HSP70/BiP inhibition ELISA. *Veterinary Parasitology* **173:** 39-47

Boulangé A and Authié E (1994) A 69 kDa immunodominant antigen of *Trypanosoma* (*Nannomonas) congolense* is homologous to immunoglobulin heavy chain binding protein (BiP). *Parasitology* **109:** 163-173

Boulangé A, Katende J and Authié E (2002) *Trypanosoma congolense*: Expression of a Heat Shock Protein 70 and Initial Evaluation as a Diagnostic Antigen for Bovine Trypanosomosis. *Experimental Parasitology* **100:** 6-11

Boulangé A, Serveau C, Brillard M, Minet C, Gauthier F, Diallo A, Lalmanach G and Authié E (2001) Functional expression of the catalytic domains of two cysteine proteinases from *Trypanosoma congolense*. *International Journal for Parasitology* **31:** 1435-1440

Boulangé AF, Khamadi SA, Pillay D, Coetzer THT and Authié E (2011) Production of congopain, the major cysteine protease of *Trypanosoma (Nannomonas) congolense*, in *Pichia pastoris* reveals unexpected dimerisation at physiological pH. *Protein Expression and Purification* **75:** 95-103

Bouyer J, Seck MT, Sall B, Ndiaye EY, Guerrini L and Vreysen MJB (2010) Stratified entomological sampling in preparation for an area-wide integrated pest management program: the example of *Glossina palpalis gambiensis* (Diptera: Glossinidae) in the Niayes of Senegal. *Journal of medical entomology* **47:** 543-552

Briggs GE and Haldane JBS (1925) A note on the kinetics of enzyme action. *Biochemical Journal* **19:** 338-339

Broadhead R, Dawe HR, Farr H, Griffiths S, Hart SR, Portman N, Shaw MK, Ginger ML, Gaskell SJ, McKean PG and Gull K (2006) Flagellar motility is required for the viability of the bloodstream trypanosome. *Nature* **440:** 224-227

Brun R (1993) Advanses in chemotherapy of African trypanosomiasis. *Acta Tropica* **54:** 1-9

Brun R, Blum J, Chappuis F and Burri C (2010) Human African trypanosomiasis. *The Lancet* **375:** 148-159

Bryant C, Kerr ID, Debnath M, Ang KKH, Ratnam J, Ferreira RS, Jaishankar P, Zhao D, Arkin MR, McKerrow JH, Brinen LS and Renslo AR (2009) Novel non-peptidic vinylsulfones targeting the S2 and S3 subsites of parasite cysteine proteases. *Bioorganic & Medicinal Chemistry Letters* **19:** 6218-6221

Butler J, Navarro P and Sun J (1997) Adsorption-induced antigenic changes and their significance in ELISA and immunological disorders. *Immunological investigations* **26:** 39-54

Butler J, Ni L, Brown W, Joshi K, Chang J, Rosenberg B and Voss Jr E (1993) The immunochemistry of sandwich ELISAs—VI. Greater than 90% of monoclonal and 75% of polyclonal anti-fluorescyl capture antibodies (CAbs) are denatured by passive adsorption. *Molecular immunology* **30:** 1165-1175

Cabrera L, De Witte J, Victor B, Vermeiren L, Zimic M, Brandt J and Geysen D (2009) Specific detection and identification of African trypanosomes in bovine peripheral blood by means of a PCR-ELISA assay. *Veterinary Parasitology* **164:** 111-117

Caffrey CR, Hansell E, Lucas KD, Brinen LS, Alvarez Hernandez A, J. Cheng, S.L. G, Roush WR, Stierhof YD, Bogyo M, Steverding D and McKerrow JH (2001) Active site mapping, biochemical properties and subcellular localization of rhodesain, the major cysteine protease of *Trypanosoma brucei rhodesiense*. *Molecular & Biochemical Parasitology* **118:** 61-73

Caffrey CR and Steverding D (2009) Kinetoplastid papain-like cysteine peptidases. *Molecular and Biochemical Parasitology* **167:** 12-19

Cazzulo JJ (2013) Chapter 438 - Cruzipain. In *Handbook of Proteolytic Enzymes*, Rawlings ND, Salvesen G (eds). Academic Press, pp. 1913-1918

Cazzulo JJ, Cazzulo Franke MC, Martínez J and Franke de Cazzulo BM (1990) Some kinetic properties of a cysteine proteinase (cruzipain) from *Trypanosoma cruzi*. *Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology* **1037:** 186-191

Cazzulo JJ and Frasch AC (1992) SAPA/trans-sialidase and cruzipain: two antigens from *Trypanosoma cruzi* contain immunodominant but enzymatically inactive domains. *Federation of American Societies for Experimental Biology* **6:** 3259-3264

Cazzulo JJ, Stoka V and Turk B (1997) Cruzipain, the major cysteine proteinase from the protozoan parasite *Trypanosoma cruzi*. *Biological Chemistry* **378:** 1-10

Chagas JR, Authié E, Serveau C, Lalmanach G, Juliano L and Gauthier F (1997) A comparison of the enzymatic properties of the major cysteine proteinases from *Trypanosoma congolense* and *Trypanosoma cruzi*. *Molecular and Biochemical Parasitology* **88:** 85-94

Chamond N, Cosson A, Blom-Potar MC, Jouvion G, D'Archivio S, Medina M, Droin-Bergere S, Huerre M, Goyard S and Minoprio P (2010) *Trypanosoma vivax* Infections: Pushing Ahead with Mouse Models for the Study of Nagana. I. Parasitological, Hematological and Pathological Parameters. *Plos Neglected Tropical Diseases* **4:** 1-10

Chester K, Begent R, Robson L, Keep P, Pedley R, Boden LIBiol J, Boxer G, Green A, Winter G and Cochet O (1994) Phage libraries for generation of clinically useful antibodies. *The Lancet* **343:** 455-456

Chiliza TE, Van Wyngaardt W and Du Plessis DH (2008) Single-chain antibody fragments from a display library derived from chickens immunized with a mixture of parasite and viral antigens. *Hybridoma* **27:** 413-421

Chitanga S, Marcotty T, Namangala B, Van den Bossche P, Van Den Abbeele J and Delespaux V (2011) High prevalence of drug resistance in animal trypanosomes without a history of drug exposure. *PLoS Negleted Tropical Diseases* **5:** 1-5

Choe Y, Brinen LS, Price MS, Engel JC, Lange M, Grisostomi C, Weston SG, Pallai PV, Cheng H and Hardy LW (2005) Development of α-keto-based inhibitors of cruzain, a cysteine protease implicated in Chagas disease. *Bioorganic & Medicinal Chemistry* **13:** 2141-2156

Chu CT, Oury TD, Enghild JJ and Pizzo SV (1994) Adjuvant-free in vivo targeting. Antigen delivery by alpha 2-macroglobulin enhances antibody formation. *The Journal of Immunology* **152:** 1538-1545

Chu CT and Pizzo SV (1993) Receptor-mediated antigen delivery into macrophages. Complexing antigen to alpha 2-macroglobulin enhances presentation to T cells. *The Journal of Immunology* **150:** 48-58

Clackson T, Hoogenboom HR, Griffiths AD and Winter G (1991) Making antibody fragments using phage display libraries. *Nature* **352:** 624-628

Clausen PH, Sidibe I, Kabore I and Bauer B (1992) Development of multiple-drug resistance of *trypanosoma-congolense* in zebu cattle under high natural tsetse-fly

challenge in the pastoral zone of Samorogouan, Burkina-Faso. *Acta Tropica* **51:** 229-236

Clayton C, Adams M, Almeida R, Baltz T, Barrett M, Bastien P, Belli S, Beverley S, Biteau N, Blackwell J, Blaineau C, Boshart M, Bringaud F, Cross G, Cruz A, Degrave W, Donelson J, El-Sayed N, Fu G, Ersfeld K, Gibson W, Gull K, Ivens A, Kelly J, Lawson D, Lebowitz J, Majiwa P, Matthews K, Melville S, Merlin G, Michels P, Myler P, Norrish A, Opperdoes F, Papadopoulou B, Parsons M, Seebeck T, Smith D, Stuart K, Turner M, Ullu E and Vanhamme L (1998) Genetic nomenclature for *Trypanosoma* and *Leishmania*. *Molecular and Biochemical Parasitology* **97:** 221-224

Clayton C, Hausler T and Blattner J (1995) Protein Trafficking in Kinetoplastid Protozoa. *Microbiological Reviews* **59:** 325-344

Codja V, Majiva PAO, Leak SGA, Rowlands GJ, Authie E, d'Heven GDM and Peregrine AS (1993) Epidemiology of bovine trypanosomiasis in the Ghibe valley, southwest Ethiopia. 3. Occurrence of populations of *Trypanosoma congolense* resistant to diminazene, isometamidium and homidium. *Acta Tropica* **53:** 151–163

Coetzer TH, Pike RN and Dennison C (1992) Localization of an immunoinhibitory epitope of the cysteine proteinase, cathepsin L. *Immunological investigations* **21:** 495-506

Cornelissen AW, Bakkeren GA, Barry JD, Michels PA and Borst P (1985) Characteristics of trypanosome variant antigen genes active in the tsetse fly. *Nucliec acids research* **13:** 4661-4676

Cregg JM (2007) Introduction: Distinctions Between *Pichia pastoris* and Other Expression Systems. *Methods in Molecular Biology* **389:** 1-10

Cregg JM and Higgins DR (1995) Production of foreign proteins in the yeast *Pichia pastoris*. *Canadian Journal of Botany* **73:** 891-897

Crowther JR (1995) *ELISA: theory and practice*, Vol. 42: Springer, Humana Press Inc., Towana, NJ.

d'leteren GD, Authié E, Wissocq N and Murray M (1998) Trypanotolerance, an option for sustainable livestock production in areas at risk from trypanosomosis. *Revue Scientifique et Technique* **17:** 154-175

Davies EL, Smith JS, Birkett CR, Manser JM, Anderson-Dear DV and Young JR (1995) Selection of specific phage-display antibodies using libraries derived from chicken immunoglobulin genes. *Journal of immunological methods* **186:** 125-135

Delespaux V and de Koning HP (2007) Drugs and drug resistance in African trypanosomiasis. *Drug Resistance Updates* **10:** 30-50

Delespaux V, Dinka H, Masumu J, Van den Bossche P and Geerts S (2008a) Five-fold increase in Trypanosoma congolense isolates resistant to diminazene aceturate over a seven-year period in Eastern Zambia. *Drug Resistance Updates* **11:** 205-209

Delespaux V, Geysen D, Van den Bossche P and Geerts S (2008b) Molecular tools for the rapid detection of drug resistance in animal trypanosomes. *Trends in parasitology* **24:** 236-242

Deng L-W and Perham RN (2002) Delineating the Site of Interaction on the pIII Protein of Filamentous Bacteriophage fd with the F-pilus of *Escherichia coli*. *Journal of Molecular Biology* **319:** 603-614

Dennison C (1999) *A guide to protein isolation*, Dordrecht: Kluwer Publishers. 71-114.

Desquesnes M, Kamyingkird K, Pruvot M, Kengradomkij C, Bossard G, Sarataphan N and Jittapalapong S (2009) Antibody-ELISA for *Trypanosoma evansi*: Application in a serological survey of dairy cattle, Thailand, and validation of a locally produced antigen. *Preventive Veterinary Medicine* **90:** 233-241

Desquesnes M, McLaughlin G, Zoungrana A and Davila AMR (2001) Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *International Journal for Parasitology* **31:** 610-614

Dias da Silva W and Tambourgi DV (2010) IgY: a promising antibody for use in immunodiagnostic and in immunotherapy. *Veterinary immunology and immunopathology* **135:** 173-180

Dobson SL (2003) Reversing Wolbachia-based population replacement. *Trends in parasitology* **19:** 128-133

Donelson JE (1995) Mechanisms of Antigenic Variation in *Borrelia hermssii* and African Trypanosomes. *The Journal of Biological Chemistry* **270:** 7783-7786

Donelson JE, Hill KL and El-Sayed NMA (1998) Multiple mechanisms of immune evasion by African trypanosomes. *Molecular and Biochemical Parasitology* **91:** 51-66

Doyle PS, Zhou YM, Engel JC and McKerrow JH (2007) A Cysteine Protease Inhibitor Cures Chagas' Disease in an Immunodeficient-Mouse Model of Infection. *Antimicrobial agents and chemotherapy* **51:** 3932-3939

Dransfield RD, Williams BG and Brightwell R (1991) Control of tsetse flies and trypanosomiasis: Myth or reality? *Parasitology Today* **7:** 287-291

Dubin G (2005) Proteinaceous cysteine protease inhibitors. *Cellular and molecular life sciences* **62:** 653-669

Dutta S, Choudhury D, Dattagupta JK and Biswas S (2011) C-Terminal extension of a plant cysteine protease modulates proteolytic activity through a partial inhibitory mechanism. *The FEBS journal* **278:** 3012-3024

Dvořák J, Mashiyama ST, Sajid M, Braschi S, Delcroix M, Schneider EL, McKerrow WH, Bahgat M, Hansell E and Babbitt PC (2009) SmCL3, a gastrodermal cysteine protease of the human blood fluke *Schistosoma mansoni*. *Plos Neglected Tropical Diseases* **3:** e449

Dwek RA (1998) Biological importance of glycosylation. In *Molecular Recognition and Inclusion*. Springer, pp. 1-6

Dyck VA, Hendrichs JP and Robinson AS (2005) The Sterile Insect Technique: Principles and Practice in Area-Wide Intergrated Pest Management. *Springer, Dodrecht***:** 69-146

Eakin AE, McGrath ME, McKerrow JH, Fletterick RJ and Craik CS (1993) Production of Crystallizable Cruzain, the Major Cysteine Protease from *Trypanosoma cruxi*. *The Journal of Biological Chemistry* **268:** 6115-6118

Eakin AE, Mills A, Harth G, McKerrow JH and Craik CS (1992) The sequence, organization, and expression of the major cysteine protease (cruzain) from *Trypanosoma cruzi*. *Journal of Biological Chemistry* **267:** 7411-7420

Eaton BT, Gould AR, Hyatt AD, Coupar BE, Martyn JC and White JR (1991) A bluetongue serogroup-reactive epitope in the amino terminal half of the major core

protein VP7 is accessible on the surface of bluetongue virus particles. *Virology* **180:** 687-696

Eisler MC, Lessard P, Masake RA, Moloo SK and Peregrine AS (1998) Sensitivity and specificity of antigen-capture ELISAs for diagnosis of *Trypanosoma congolense* and *Trypanosoma vivax* infections in cattle. *Veterinary Parasitology* **79:** 187-201

El-Sayed NM, Hegde P, Quackenbush J, Melville SE and Donelson JE (2000) The African trypanosome genome. *International Journal for Parasitology* **30:** 329-345

Engel J, Doyle P and McKerrow J (1998a) Trypanocidal effect of cysteine protease inhibitors in vitro and in vivo in experimental Chagas disease. *Medicina* **59:** 171-175

Engel JC, Doyle PS, Hsieh I and McKerrow JH (1998b) Cysteine protease inhibitors cure an experimental *Trypanosoma cruzi* infection. *The Journal of experimental medicine* **188:** 725-734

Eyssen LE (2014) Studying trypanosomal peptidase antigen targets for the diagnosis of animal African trypanosomiasis. MSc dissertation. University of KwaZulu-Natal (Pietermaritzburg campus).

Fehrsen J, Van Wyngaardt W, Mashau C, Potgieter A, Chaudhary V, Gupta A, Jordaan F and Du Plessis D (2005a) Serogroup-reactive and type-specific detection of bluetongue virus antibodies using chicken scFvs in inhibition ELISAs. *Journal of virological methods* **129:** 31-39

Fehrsen J, van Wyngaardt W, Mashau C, Potgieter AC, Chaudhary VK, Gupta A, Jordaan FA and du Plessis DH (2005b) Serogroup-reactive and type-specific detection of bluetongue virus antibodies using chicken scFvs in inhibition ELISAs. *Journal of Virological Methods* **129:** 31-39

Fikru R, Goddeeris BM, Delespaux V, Moti Y, Tadesse A, Bekana M, Claes F, De Deken R and Büscher P (2012) Widespread occurrence of *Trypanosoma vivax* in bovines of tsetse- as well as non-tsetse-infested regions of Ethiopia: A reason for concern? *Veterinary Parasitology* **190:** 355-361

Fish WR, Nkhungulu ZM, Muriuki CW, Ndegwa DM, Lonsdale-Eccles JD and Steyaert J (1995) Primary structure and partial characterization of a life-cycle-regulated cysteine protease from *Trypanosoma (Nannomonas) congolense*. *Gene* **161:** 125-128

Fleming JR, Sastry L, Crozier TW, Napier GB, Sullivan L and Ferguson MA (2014) Proteomic Selection of Immunodiagnostic Antigens for *Trypanosoma congolense*. *PLoS neglected tropical diseases* **8:** 1-9

Foot JS, Lui FE and Kluger R (2009) Hemoglobin bis-tetramers via cooperative azide–alkyne coupling. *Chemical Communications***:** 7315-7317

Fox T, De Miguel E, Mort JS and Storer AC (1992) Potent slow-binding inhibition of cathepsin B by its propeptide. *Biochemistry* **31:** 12571-12576

Gadelha C, Rothery S, Morphew M, McIntosh JR, Severs NJ and Gull K (2009) Membrane domains and flagellar pocket boundaries are influenced by the cytoskeleton in African trypanosomes. *Proceedings of the National Academy of Sciences of the United States of America* **106:** 17425-17430

Garcia MP, Nóbrega OT, Teixeira AR, Sousa MV and Santana JM (1998) Characterisation of a *Trypanosoma cruzi* acidic 30 kDa cysteine protease. *Molecular and biochemical parasitology* **91:** 263-272

Gea S, Guiñazu N, Pollegrini A, Carrera Silver EA, Giordanengo L, Cano R and Aoki MP (2006) Cruzipain, a major *Trypanosoma cruzi* cystein protease in the host-parasite interplay. *immunologia* **25:** 225-238

Geerts S, Holmes PH, Diall O and Eisler MC (2001) African bovine trypanosomiasis: the problem of drug resistance. *Trends in parasitology* **17:** 25-28

Geiger A, Simo G, Grébaut P, Peltier J-B, Cuny G and Holzmuller P (2011) Transcriptomics and proteomics in human African trypanosomiasis: Current status and perspectives. *Journal of Proteomics* **74:** 1625-1643

Gibson W (2009) Species-specific probes for the identification of the African tsetse-transmitted trypanosomes. *Parasitology* **136:** 1501-1507

Gillmor SA, Craik CS and Fletterick RJ (1997) Structural determinants of specificity in the cysteine protease cruzain. *Protein Science* **6:** 1603-1611

Goeddel DV (1990) Systems for heterologous gene expression. In *Methods in Enzymology*, David VG (ed), Vol. 185. Academic Press, pp. 3-7

Goldring J and Coetzer TH (2003) Isolation of chicken immunoglobulins (IgY) from egg yolk*. *Biochemistry and Molecular Biology Education* **31:** 185-187

Goletz S, Christensen PA, Kristensen P, Blohm D, Tomlinson I, Winter G and Karsten U (2002) Selection of large diversities of antiidiotypic antibody fragments by phage display. *Journal of Molecular Biology* **315:** 1087-1097

Goto Y, Duthie MS, Thu-Thuy N, Asada M, Kawazu S-I, Carter D and Inoue N (2011) Serological characterizations of tandem repeat proteins for detection of African trypanosome infection in cattle. *Parasitology International* **60:** 538-540

Grant IF (2001) Insecticides for tsetse and trypanosomiasis control: is the environmental risk acceptable? *Trends in parasitology* **17:** 10-14

Greenwood J, Willis AE and Perham RN (1991) Multiple display of foreign peptides on a filamantous bacteriophage - Peptides from *Plasmodium-falciparum* circumsporozoite protein as antigens. *Journal of Molecular Biology* **220:** 821-827

Greiner M, Kumar S and Kyeswa C (1997) Evaluation and comparison of antibody ELISAs for serodiagnosis of bovine trypanosomosis. *Veterinary Parasitology* **73:** 197-205

Griffiths AD and Duncan AR (1998) Strategies for selection of antibodies by phage display. *Current Opinion in Biotechnology* **9:** 102-108

Gull K (2003) Host-parasite interactions and trypanosome morphogenesis: a flagellar pocketful of goodies. *Current Opinion in Microbiology* **6:** 365-370

Haag J, O'HUigin C and Overath P (1998) The molecular phylogeny of trypanosomes: evidence for an early divergence of the Salivaria. *Molecular and Biochemical Parasitology* **91:** 37-49

Hädge D and Ambrosius H (1984) Evolution of low molecular weight immunoglobulins—IV. IgY-like immunoglobulins of birds, reptiles and amphibians, precursors of mammalian IgA. *Molecular immunology* **21:** 699-707

Hanotte O, Ronin Y, Agaba M, Nilson P, Gelhaus A, Horstmann R, Sugimoto Y, Kemp S, Gibson J, Korol A, Soller M and Teale A (2003) Mapping of quantitative trait loci controlling trypanotolerance in a cross of tolerant West African N'Dama and susceptible East African Boran cattle. *Proceedings of the National Academy of Sciences of the United States of America* **100:** 7443-7448

Hansen P, Scoble JA, Hanson B and Hoogenraad NJ (1998) Isolation and purification of immunoglobulins from chicken eggs using thiophilic interaction chromatography. *Journal of Immunological Methods* **215:** 1-7

Hawkins RE, Russell SJ and Winter G (1992) Selection of phage antibodies by binding affinity: Mimicking affinity maturation. *Journal of Molecular Biology* **226:** 889-896

Heussen C and Dowdle EB (1980) Electrophoretic analysis of plasminogen activators in polyacrylamide gels containing sodium dodecyl sulfate and copolymerized substrates. *Analytical Biochemistry* **102:** 196-202

Hoare (1972) The trypanosomes of mammals. A zoological monograph. *Oxford: Blackwell***:** 749

Hoffmann F and Rinas U (2004) Stress induced by recombinant protein production in *Escherichia coli*. In *Physiological Stress Responses in Bioprocesses*. Springer, pp. 73-92

Holliger P, Prospero T and Winter G (1993) Diabodies - Small bivalent and bispecific antibody fragments. *Proceedings of the National Academy of Sciences of the United States of America* **90:** 6444-6448

Holmes P (2013) Tsetse-transmitted trypanosomes – Their biology, disease impact and control. *Journal of Invertebrate Pathology* **112, Supplement 1:** S11-S14

Holmes PH (1997) New approaches to the integrated control of trypanosomosis. *Veterinary Parasitology* **71:** 121-135

Holt LJ, Bussow K, Walter G and Tomlinson IM (2000) By-passing selection: direct screening for antibody-antigen interactions using protein arrays. *Nucleic acids research* **28:** 1-5

Hoogenboom HR, de Bruïne AP, Hufton SE, Hoet RM, Arends J-W and Roovers RC (1998) Antibody phage display technology and its applications. *Immunotechnology* **4:** 1-20

Hoogenboom HR, Lutgerink JT, Pelsers MM, Rousch MJ, Coote J, van Neer N, de Bruïne A, van Nieuwenhoven FA, Glatz JF and Arends JW (1999) Selection-dominant and nonaccessible epitopes on cell-surface receptors revealed by cell-panning with a large phage antibody library. *European Journal of Biochemistry* **260:** 774-784

Huson LEJ, Authié E, Boulangé AF, Goldring JPD and Coetzer THT (2009) Modulation of the immunogenicity of the *Trypanosoma congolense* cysteine protease, congopain, through complexation with α2-macroglobulin. *Veterinary research* **40:** 1-12

Hust M and Dübel S (2004) Mating antibody phage display with proteomics. *Trends in Biotechnology* **22:** 8-14

Hutchinson OC, Webb H, Picozzi K, Welburn S and Carrington M (2004) Candidate protein selection for diagnostic markers of African trypanosomiasis. *Trends in parasitology* **20:** 519-523

Iannolo G, Minenkova O, Petruzzelli R and Cesareni G (1995) Modifying filamantious phage capsid - Limits in the size of the major capsid protein. *Journal of Molecular Biology* **248:** 835-844

Igbokwe IO (1994) Mechanisms of cellular injury in African trypanosomiasis. . *Veterinary Bulletin* **64:** 611-620

Jackson DG, Owen MJ and Voorheis HP (1985) A new method for the rapid purification of both the membrane-bound and released forms of the variant surface glycoprotein from *Trypanosoma brucei*. *Biochemical Journal* **230:** 195-202

Jackson L (2011) Enzymatic and crystallisation studies of CATL-like trypanosomal cysteine peptidases. MSc dissertation. University of KwaZulu-Natal (Pietermaritzburg campus).

Jensenius JC, Andersen I, Hau J, Crone M and Koch C (1981) Eggs: conveniently packaged antibodies. Methods for purification of yolk IgG. *Journal of immunological methods* **46:** 63-68

Karrer KM, Peiffer SL and DiTomas ME (1993) Two distinct gene subfamilies within the family of cysteine protease genes. *Proceedings of the National Academy of Sciences* **90:** 3063-3067

Katende J, Musoke A, Nantulya V and Goddeeris B (1987) A new method for fixation and preservation of trypanosomal antigens for use in the indirect immunofluorescence antibody test for diagnosis of bovine trypanosomiasis. *Tropical medicine and parasitology: official organ of Deutsche Tropenmedizinische Gesellschaft and of Deutsche Gesellschaft fur Technische Zusammenarbeit (GTZ)* **38:** 41-44

Kehoe JW and Kay BK (2005) Filamentous phage display in the new millennium. *Chemical Reviews* **105:** 4056-4072

Knappik A, Ge L, Honegger A, Pack P, Fischer M, Wellnhofer G, Hoess A, WoÈlle J, Plückthun A and Virnekäs B (2000) Fully synthetic human combinatorial antibody libraries (HuCAL) based on modular consensus frameworks and CDRs randomized with trinucleotides. *Journal of Molecular Biology* **296:** 57-86

Kohl L, Robinson D and Bastin P (2003) The flagellum: from cell motility to morphogenesis. *Journal de la Societe de Biologie* **197:** 379-387

Kramer K (2002) Evolutionary affinity and selectivity optimization of a pesticide-selective antibody utilizing a hapten-selective immunoglobulin repertoire. *Environmental science & technology* **36:** 4892-4898

Kretzschmar T and Geiser M (1995) Evaluation of antibodies fused to minor coat protein-III and major coat protein-VIII of bacteriophage M13. *Gene* **155:** 61-65

Kretzschmar T and von Rüden T (2002) Antibody discovery: phage display. *Current Opinion in Biotechnology* **13:** 598-602

Kricka LJ (1999) Human anti-animal antibody interferences in immunological assays. *Clinical chemistry* **45:** 942-956

Kuboki N, Inoue N, Sakurai T, Di Cello F, Grab DJ, Suzuki H, Sugimoto C and Igarashi I (2003) Loop-Mediated Isothermal Amplification for Detection of African Trypanosomes. *Journal of Clinical Microbiology* **41:** 5517-5524

LaCount DJ and Donelson JE (2001) RNA interference in African trypanosomes. *Protist* **152:** 103-111

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *nature* **227:** 680-685

Lalmanach G, Boulangé A, Serveau C, Lecaille F, Scharfstein J, Gauthier F and Authié E (2002) Congopain from *Trypanosoma congolense*: Drug target and vaccine candidate. *Biological Chemistry* **383:** 739-749

Lalmanach G, Lecaille F, Chagas JR, Authié E, Scharfstein J, Juliano MA and Gauthier F (1998) Inhibition of trypanosomal cysteine proteinases by their propeptides. *Journal of Biological Chemistry* **273:** 25112-25116

Lança ASC, de Sousa KP, Atouguia J, Prazeres DMF, Monteiro GA and Silva MS (2011) *Trypanosoma brucei*: Immunisation with plasmid DNA encoding invariant surface glycoprotein gene is able to induce partial protection in experimental African trypanosomiasis. *Experimental Parasitology* **127:** 18-24

Lanfranco MF, Loayza-Muro R, Clark D, Núñez R, Zavaleta AI, Jimenez M, Meldal M, Coombs GH, Mottram JC, Izidoro M, Juliano MA, Juliano L and Arévalo J (2008) Expression and substrate specificity of a recombinant cysteine proteinase B of *Leishmania braziliensis*. *Molecular and Biochemical Parasitology* **161:** 91-100

Lang IM, Barbas III CF and Schleef RR (1996) Recombinant rabbit Fab with binding activity to type-1 plasminogen activator inhibitor derived from a phage-display library against human α-granules. *Gene* **172:** 295-298

Laroche Y, Storme V, De Meutter J, Messens J and Lauwereys M (1994) High- level secretion and very efficient isotopic labeling of tick anticoagulant peptide (TAP) expressed in the methylotrophic yeast, *Pichia pastoris*. *Nature Biotechnology* **12:** 1119-1124

Larsson A, Bålöw R-M, Lindahl TL and Forsberg P-O (1993) Chicken antibodies: taking advantage of evolution—a review. *Poultry Science* **72:** 1807-1812

Leak SGA (1999) *Tsetse Biology and Ecology: Their role in the Epidemiology and Control of Trypanosomosis*, 10 edn.: CABI publishing, Wallingford. 179-224.

Lecaille F, Authié E, Moreau T, Serveau C, Gauthier F and Lalmanach G (2001) Subsite specificity of trypanosomal cathepsin L-like cysteine proteases - Probing the S2 pocket with phenylalanine-derived amino acids. *European Journal of Biochemistry* **268:** 2733-2741

Lecaille F, Weidauer E, Juliano M, Bromme D and Lalmanach G (2003) Probing cathepsin K activity with a selective substrate spanning its active site. *The Biochemical Journal* **375:** 307-312

Lejon V, Claes F, Verloo D, Maina M, Urakawa T, Majiwa P and Büscher P (2005) Recombinant RoTat 1.2 variable surface glycoprotein as antigen for diagnosis of *Trypanosoma evansi* in dromedary camels. *International journal for parasitology* **35:** 455-460

Lejon V, Rebeski DE, Ndao M, Baelmans R, Winger EM, Faye D, Geerts S and Buscher P (2003) Performance of enzyme-linked immunosorbent assays for detection of antibodies against *T. congolense* and *T. vivax* in goats. *Veterinary Parasitology* **116:** 87-95

Leroux-Roels G (2010) Unmet needs in modern vaccinology: adjuvants to improve the immune response. *Vaccine* **28:** C25-C36

Li P, Anumanthan A, Gao XG, Ilangovan K, Suzara VV, Duzgunes N and Renugopalakrishnan V (2007) Expression of Recombinant Proteins in *Pichia Pastoris*. *Applied Biochemestry Biotechnology* **142:** 105-124

Li Z, Yasuda Y, Li W, Bogyo M, Katz N, Gordon RE, Fields GB and Brömme D (2004) Regulation of collagenase activities of human cathepsins by glycosaminoglycans. *Journal of Biological Chemistry* **279:** 5470-5479

Lima L, Ortiz PA, da Silva FM, Alves JMP, Serrano MG, Cortez AP, Alfieri SC, Buck GA and Teixeira MM (2012) Repertoire, genealogy and genomic organization of cruzipain and homologous genes in *Trypanosoma cruzi*, *T. cruzi*-like and other trypanosome species. *PloS one* **7:** 1-15

Lonsdale-Eccles J, Mpimbaza G, Nkhungulu Z, Olobo J, Smith L, Tosomba O and Grab D (1995) Trypanosomatid cysteine protease activity may be enhanced by a kininogen-like moiety from host serum. *Biochemical Journal* **305:** 549-556

Lonsdale-Eccles JD and Grab DJ (1987) Lysosomal and non-lysosomal peptidyl hydrolases of the bloodstream forms of *Trypanosoma brucei brucei*. *European Journal of Biochemistry* **169:** 467-475

Lowman HB, Bass SH, Simpson N and Wells JA (1991) Selecting high-affinity binding proteins by monovalent phage display. *Biochemistry* **30:** 10832-10838

Lubega GW, Byarugaba DK and Prichard RK (2002) Immunization with a tubulin-rich preparation from *Trypanosoma brucei* confers broad protection against African trypanosomosis. *Experimental parasitology* **102:** 9-22

Luckins AG (1977) Detection of antibodies in trypanosome-infected cattle by means of a microplate enzyme-linked immunosorbent assay. *Tropical Animal Health and Production* **9:** 53-62

Macauley-Patrick S, Fazenda ML, McNeil B and Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* **22:** 249-270

Magalhaes Moreira DR, de Oliveira ADT, Teixeira de Moraes Gomes PA, de Simone CA, Villela FS, Ferreira RS, da Silva AC, dos Santos TAR, Brelaz de Castro MCA and Pereira VRA (2014) Conformational restriction of aryl thiosemicarbazones produces potent and selective anti-*Trypanosoma cruzi* compounds which induce apoptotic parasite death. *European journal of medicinal chemistry* **75:** 467-478

Magez S, Caljon G, Tran T, Stijlemans B and Radwanska M (2010) Current status of vaccination against African trypanosomiasis. *Parasitology* **137:** 2017-2027

Makrides SC (1996) Strategies for achieving high-level expression of genes in *Escherichia coli*. *Microbiological reviews* **60:** 512-538

Maley F, Trimble RB, Tarentino AL and Plummer Jr TH (1989) Characterization of glycoproteins and their associated oligosaccharides through the use of endoglycosidases. *Analytical biochemistry* **180:** 195-204

Mamoudou A, Delespaux V, Chepnda V, Hachimou Z, Andrikaye JP, Zoli A and Geerts S (2008) Assessment of the occurrence of trypanocidal drug resistance in trypanosomes of naturally infected cattle in the Adamaoua region of Cameroon using the standard mouse test and molecular tools. *Acta Tropica* **106:** 115-118

Marks JD and Bradbury A (2004) Selection of human antibodies from phage display libraries. In *Antibody Engineering*. Springer, pp. 161-176

Marks JD, Hoogenboom HR, Bonnert TP, McCafferty J, Griffiths AD and Winter G (1991) By-passing immunization - Human-antibodies from V-gene libraries displayed on phage. *Journal of Molecular Biology* **222:** 581-597

Martyn JC, Gould AR and Eaton BT (1991) High level expression of the major core protein VP7 and the non-structural protein NS3 of bluetongue virus in yeast: use of expressed VP7 as a diagnostic, group-reactive antigen in a blocking ELISA. *Virus research* **18:** 165-178

Masake RA, Njuguna JT, Brown CC and Majiwa PAO (2002) The application of PCR-ELISA to the detection of *Trypanosoma brucei* and *T-vivax* infections in livestock. *Veterinary Parasitology* **105:** 179-189

Masiga DK, Smyth AJ, Hayes P, Bromidge TJ and Gibson WC (1992) Sensitive detection of trypanosomed in tsetse-flies by DNA amplification. *International Journal for Parasitology* **22:** 909-918

Matovu E, Kazibwe AJ, Mugasa CM, Ndungu JM and Njiru ZK (2012) Towards Point-of-Care Diagnostic and Staging Tools for Human African Trypanosomiaisis. *Journal of Tropical Medicine* **2012:** 1-9

Matthews KR, Ellis JR and Paterou A (2004) Molecular regulation of the life cycle of African trypanosomes. *Trends in parasitology* **20:** 40-47

Mbawa ZR, Gumm ID, Fish WR and Lonsdale-Eccles JD (1991) Endopeptidase variations among different life-cycle stages of African trypanosomes. *European journal of biochemistry* **195:** 183-190

Mbawa ZR, Gumm ID, Shaw E and Lonsdaleeccles JD (1992) Characterization of a Cysteine Protease from Blood-Stream Forms of *Trypanosoma congolense*. *European Journal of Biochemistry* **204:** 371-379

Mbaya AW and Ibrahim UI (2011) *In-vivo* and *in-vitro* activities of medicinal plants on haemic and humoral trypanosomes: A Review. *International Journal of Pharmacology* **7:** 1-12

McCafferty J, Griffiths AD, Winter G and Chiswell DJ (1990) Phage antibodies: filamentous phage displaying antibody variable domains. *Nature* **348:** 552-554

McGrath ME, Eakin AE, Engel JC, McKerrow JH, Craik CS and Fletterick RJ (1995) The crystal structure of cruzain: a therapeutic target for Chagas' disease. *Journal of Molecular Biology* **247:** 251-259

McKerrow JH, Caffrey C, Kelly B, Loke Pn and Sajid M (2006) Proteases in parasitic diseases. In *Annual Review of Pathology-Mechanisms of Disease* Vol. 1. pp. 497-536

McLaughlin GL, Ssenyonga SS, Nanteza E, Rubaire-Akiki WO, Hansen RD, Vodkin MH, Novak RJ, Gordon VR, Montenegro-JamesS., James M, Avile H, Armijos R, Santrich C, Weigle K, Saravia N, Wozniak E, Gaye O, Mdachi R, Shapiro SZ, Chang K-P and Kakoma I (1996) PCR-based detection and typing of parasites. *Azcel, MA,Alkan, MZ (Eds), Parasitology for the 21 Century CAB International,Wallingford Oxon***:** 261-287

Ménard R, Carmona E, Takebe S, Dufour É, Plouffe C, Mason P and Mort JS (1998) Autocatalytic Processing of Recombinant Human Procathepsin L contribution of both intermolecular and unimolecular events in the processing of procathepsin l in vitro. *Journal of Biological Chemistry* **273:** 4478-4484

Mitashi P, Hasker E, Lejon V, Kande V, Muyembe J, Lutumba P and Boelaert M (2012) Human African Trypanosomiasis Diagnosis in First-Line Health Services of Endemic Countries, a Systematic Review. *Plos Neglected Tropical Diseases* **6:** 1-11

Mitashi P, Hasker E, Ngoyi DM, Pyana PP, Lejon V, Van der Veken W, Lutumba P, Bu¨ scher P, Boelaert M and Deborggraeve S (2013) Diagnostic Accuracy of Loopamp *Trypanosoma bruce*i Detection Kit for Diagnosis of Human African Trypanosomiasis in Clinical Samples. *Plos Neglected Tropical Diseases* **7:** 1-5

Mitchell LA and Pearson TW (1983) Antibody responses induced by immunization of inbred mice susceptible and resistant to African trypanosomes. *Infection and Immunity* **40:** 894-902

Mkunza F, Olaho WM and Powell CN (1995) Partial protection against natural trypanosomiasis after vaccination with a flagellar pocket antigen from *Trypanosoma brucei rhodesiense*. *Vaccine* **13:** 151-154

Mnkandla SM (2013) Recombinant expression of, and characterisation of antibodies against variable surface glycoproteins : LiTat 1.3 and LiTat 1.5 of *Trypanosoma brucei gambiense.* MSc dissertation. University of KwaZulu-Natal (Pietermaritzburg campus).

Moloo SK, Kabata JM and Gitire NM (2000) Study on the mechanical transmission by tsetse fly Glossina morsitans centralis of *Trypanosoma vivax, T. congolense* or *T. brucei brucei* to goats. *Acta Tropica* **74:** 105-108

Moreira-Leite FF, Sherwin T, Kohl L and Gull K (2001) A Trypanosome Structure Involved in Transmitting Cytoplasmic Information During Cell Division *Science* **294:** 610-612

Morrison LJ, Marcello L and McCulloch R (2009) Antigenic variation in the African trypanosome: molecular mechanisms and phenotypic complexity. *Cellular Microbiology* **11:** 1724-1734

Morty RE, Authié E, Troeberg L, Lonsdale-Eccles JD and Coetzer TH (1999) Purification and characterisation of a trypsin-like serine oligopeptidase from *Trypanosoma congolense*. *Molecular and biochemical parasitology* **102:** 145-155

Moser DR, Cook GA, Ochs DE, Bailey CP, McKane MR and Donelson JE (1989) Detection of *Trypanosoma congolense* and *Trypanosoma brucei* subspecies by DNA amplification using polymerase chain reaction. *Parasitology* **99:** 57-66

Mottram JC, Brooks DR and Coombs GH (1998) Roles of cysteine proteinases of trypanosomes and leishmania in host-parasite interactions. *Current Opinion in Microbiology* **1:** 455-460

Mottram JC, Helms MJ, Coombs GH and Sajid M (2003) Clan CD cysteine peptidases of parasitic protozoa. *Trends in parasitology* **19:** 182-187

Musil D, Zucic D, Turk D, Engh R, Mayr I, Huber R, Popovic T, Turk V, Towatari T and Katunuma N (1991) The refined 2.15 A X-ray crystal structure of human liver cathepsin B: the structural basis for its specificity. *The EMBO journal* **10:** 2321

Naessens J (2006) Bovine trypanotolerance: A natural ability to prevent severe anaemia and haemophagocytic syndrome? *International Journal for Parasitology* **36:** 521-528

Naessens J, Leak SGA, Kennedy DJ, Kemp SJ and Teale AJ (2003) Responses of bovine chimeras combining trypanosomosis resistant and susceptible genotypes to experimental infection with *Trypanosoma congolense*. *Veterinary Parasitology* **111:** 125-142

Naessens J, Teale AJ and Sileghem M (2002) Identification of mechanisms of natural resistance to African trypanosomiasis in cattle. *Veterinary Immunology and Immunopathology* **87:** 187-194

Nägler DK, Tam W, Storer AC, Krupa JC, Mort JS and Ménard R (1999) Interdependency of sequence and positional specificities for cysteine proteases of the papain family. *Biochemistry* **38:** 4868-4874

Nantulya VM (1990) Trypanosomiasis in domestic animals: the problems of diagnosis. *Revised Science Technology* **9:** 357-367

Nantulya VM and Lindqvist KJ (1989) Antigen-detection enzyme immunoassays for the diagnosis of *Trypanosoma vivax*, *T. congolense* and *T. brucei* infections in cattle. *Tropical Medicine and Parasitology* **40:** 267-272

Ndao M (2009) Diagnosis of parasitic diseases: old and new approaches. *Interdisciplinary perspectives on infectious diseases* **2009:** 1-15

Nery Ed, Juliano M, Meldal M, Svendesen I, Scharfstein J, Walmsley A and Juliano L (1997) Characterization of the substrate specificity of the major cysteine protease (cruzipain) from *Trypanosoma cruzi* using a portion-mixing combinatorial library and fluorogenic peptides. *The Biochemical Journal* **323:** 427-433

Nissim A, Hoogenboom HR, Tomlinson IM, Flynn G, Midgley C, Lane D and Winter G (1994) Antibody fragments from a single pot phage display library as immunochemical reagents. *Embo Journal* **13:** 692-698

OIE (2012) Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7eth ed. OIE, Paris.

Okiria R, Okuna NM, Magona JW and Mayende JS (2002) Sustainability of tsetse control by subsequent treatment of 10% of a previously treated Ugandan cattle population with 1% w/v deltamethrin. *Tropical Animal Health and Production* **34:** 105-114

Osman AS, Jennings FW and Holmes PH (1992) The rapid development of drug-resistance by *Trypanosoma evansi* in immunosuppressed mice. *Acta Tropica* **50:** 249-247

Otto H and Schirmeister T (1997) Cysteine Proteases and Their Inhibitors. *Chemical Reviews* **97:** 133-171

Otto MA, Silva ASd, Gressler LT, Farret MH, Tavares KCS, Zanette RA, Miletti LC and Monteiro SG (2010) Susceptibility of *Trypanosoma evansi* to human blood and plasma in infected mice. *Veterinary Parasitology* **168:** 1-4

Overath P and Engstler M (2004) Endocytosis, membrane recycling, and sorting of GPI-anchored proteins:*Trypanasoma brucei* as a model system. *Molecular Microbiology* **53:** 735-744

Pamer E, Davis C and So M (1991) Expression and deletion analysis of the *Trypanosoma brucei rhodesiense* cysteine protease in *Escherichia coli*. *Infection and immunity* **59:** 1074-1078

Pande J, Szewczyk MM and Grover AK (2010) Phage display: Concept, innovations, applications and future. *Biotechnology Advances* **28:** 849-858

Pays E (2006) The variant surface glycoprotein as a tool for adaptation in African trypanosomes. *Microbes and Infection* **8:** 930-937

Pays E and Vanhollebeke B (2009) Human innate immunity against African trypanosomes. *Curr Opin Immunol* **21:** 493-498

Pike RN, Coetzer TH and Dennison C (1992) Proteolytically active complexes of cathepsin L and a cysteine proteinase inhibitor; purification and demonstration of their formation *in vitro*. *Archives of biochemistry and biophysics* **294:** 623-629

Pike RN and Dennison C (1989) Protein fractionation by three phase partitioning (TPP) in aqueous/t-butanol mixtures. . *Biotechnology and Bioengineering* **33:** 221-228

Pillay D, Boulangé AF and Coetzer THT (2010) Expression, purification and characterisation of two variant cysteine peptidases from *Trypanosoma congolense* with active site substitutions. *Protein Expression and Purification* **74:** 264-271

Pillay D, Boulangé AFV, Coustou V, Baltz T and Coetzer THT (2013a) Recombinant expression and biochemical characterisation of two alanyl aminopeptidases of *Trypanosoma congolense*. *Experimental Parasitology* **135:** 675-684

Pillay D, Izotte J, Fikru R, Büscher P, Mucache H, Neves L, Boulangé A, Seck MT, Bouyer J and Napier GB (2013b) *Trypanosoma vivax* GM6 Antigen: A Candidate Antigen for Diagnosis of African Animal Trypanosomosis in Cattle. *PloS one* **8:** 1-10

Piña-Vázquez C, Reyes-López M, Ortíz-Estrada G, De la Garza M and Serrano-Luna J (2012) Host-parasite interaction: parasite-derived and-induced proteases that degrade human extracellular matrix. *Journal of parasitology research* **2012**

Pizzo S and Grøn H. (2002) Immune response modulator alpha-2 macroglobulin complex. Google Patents.

Polson A, von Wechmar MB and Van Regenmortel M (1980) Isolation of viral IgY antibodies from yolks of immunized hens. *Immunological Investigations* **9:** 475-493

Prowse E (2005) Trypanosomosis, the disease and its control. **46:** 1-40

Qi H, Lu H, Qiu H-J, Petrenko V and Liu A (2012) Phagemid Vectors for Phage Display: Properties, Characteristics and Construction. *Journal of Molecular Biology* **417:** 129-143

Radwanska M, Guirnalda P, De Trez C, Ryffel B, Black S and Magez S (2008) Trypanosomiasis-induced B cell apoptosis results in loss of protective anti-parasite antibody responses and abolishment of vaccine-induced memory responses. *PLoS pathogens* **4:** 1-11

Radwanska M, Magez S, Dumont N, Pays A, Nolan D and Pays E (2000) Antibodies raised against the flagellar pocket fraction of *Trypanosoma brucei* preferentially recognize HSP60 in cDNA expression library. *Parasite Immunology* **22:** 639-650

Rakabe M, Van Wyngaardt W and Fehrsen J (2011) Chicken single-chain antibody fragments directed against recombinant VP7 of bluetongue virus. *Food and Agricultural Immunology* **22:** 283-295

Rawlings ND, Barrett AJ and Bateman A (2011) Asparagine Peptide Lyases A seventh catalytic type of proteolytic enzymes. *Journal of Biological Chemistry* **286:** 38321-38328

Rawlings ND, Barrett AJ and Bateman A (2012) *MEROPS*: the database of proteolytic enzymes, their substrates and inhibitors. *Nucliec acids research* **40:** 343-350

Rebeski DE, Winger EM, Okoro H, Kowalik S, Burger HJ, Walters DE, Robinson MM, Dwinger RH and Crowther JR (2000) Detection of *Trypanosoma congolense* antibodies with indirect ELISAs using antigen-precoated microtitre plates. *Veterinary Parasitology* **89:** 187-198

Reid S and Copeman D (2002) Evaluation of an antibody-ELISA using five crude antigen preparations for the diagnosis of *Trypanosoma evansi* infection in cattle. *Veterinary parasitology* **104:** 79-84

Robertson JB, Zhang Y and Johnson CH (2009) Light-emitting diode flashlights as effective and inexpensive light sources for fluorescence microscopy. *Journal of microscopy* **236:** 1-4

Roditi I and Lehane MJ (2008) Interactions between trypanosomes and tsetse flies. *Current Opinion in Microbiology* **11:** 345-351

Rodrigues AC, Ortiz PA, Costa-Martins AG, Neves L, Garcia HA, Alves JM, Camargo EP, Alfieri SC, Gibson W and Teixeira MM (2014) Congopain genes diverged to become specific to Savannah, Forest and Kilifi subgroups of *Trypanosoma congolense*, and are valuable for diagnosis, genotyping and phylogenetic inferences. *Infection, Genetics and Evolution* **23:** 20-31

Rogé S, Van Nieuwenhove L, Meul M, Heykers A, de Koning AB, Bebronne N, Guisez Y and Büscher P (2014) Recombinant Antigens Expressed in *Pichia pastoris* for the Diagnosis of Sleeping Sickness Caused by *Trypanosoma brucei gambiense*. *PLoS neglected tropical diseases* **8:** 1-9

Rogé S, Van Reet N, Odiwuor S, Tran T, Schildermans K, Vandamme S, Vandenberghe I, Vervecken W, Gillingwater K, Claes F, Devreese B, Guisez Y and Büscher P (2013) Recombinant expression of trypanosome surface glycoproteins in *Pichia pastoris* for the diagnosis of *Trypanosoma evansi* infection. *Veterinary parasitology* **197:** 571-579

Rogers DJ and Randolph SE (2002) A response to the aim of eradicating tsetse from Africa. *Trends in Parasitology* **18:** 534-536

Rosenthal PJ (1999) Proteases of Protozoan Parasites. In *Advances in Parasitology*, J.R. Baker RM, Rollinson D (eds), Vol. Volume 43. Academic Press, pp. 105-159

Roush WR, Cheng J, Knapp-Reed B, Alvarez-Hernandez A, McKerrow JH, Hansell E and Engel JC (2001) Potent second generation vinyl sulfonamide inhibitors of the trypanosomal cysteine protease cruzain. *Bioorganic & Medicinal Chemistry Letters* **11:** 2759-2762

Sahin A, Asencio C, Izotte J, Pillay D, Coustou V, Karembe H and Baltz T (2014) The susceptibility of Trypanosoma congolense and Trypanosoma brucei to isometamidium chloride and its synthetic impurities. *Veterinary Parasitology* **203:** 270-275

Sajid M and McKerrow JH (2002) Cysteine proteases of parasitic organisms *Molecular and Biochemical Parasitology* **121:** 159-159

Salvesen G and Nagase H (1989) Inhibition of proteolytic enzymes. In *Proteolytic Enzymes*, Benynon RJ, Bond JS (eds). Oxford University Press, pp. 83-104

San Filippo J, Sung P and Klein H (2008) Mechanism of eukaryotic homologous recombination. *Annual Review of Biochemistry* **77:** 229-257

Sanderson S, Pollock K, Hilley J, Meldal M, Hilaire P, Juliano M, Juliano L, Mottram J and Coombs G (2000) Expression and characterization of a recombinant cysteine proteinase of *Leishmania mexicana*. *Biochem J* **347:** 383-388

Santana J, Grellier P, Schrevel J and Teixeira A (1997) A *Trypanosoma cruzi*-secreted 80 kDa proteinase with specificity for human collagen types I and IV. *Biochem J* **325:** 129-137

Schade R, Calzado EG, Sarmiento R, Chacana PA, Porankiewicz-Asplund J and Terzolo HR (2005) Chicken egg yolk antibodies (IgY-technology): a review of progress in production and use in research and human and veterinary medicine. *Altern Lab Anim* **33:** 129-154

Schade R, Staak C, Hendriksen C, Erhard M, Hugl H, Koch G, Larsson A, Pollmann W, Van Regenmortel M and Rijke E (1996) The production of avian (egg yolk) antibodies: IgY. *ATLA-NOTTINGHAM-* **24:** 925-934

Schechter I and Berger A (1967) On the size of the active site in proteases. I. Papain. *Biochemical and Biophysical Research Communications* **27**

Schnaufer A, Domingo GJ and Stuart K (2002) Natural and induced dyskinetoplastic trypanosomatids: how to live without mitochondrial DNA. *International Journal for Parasitology* **32:** 1071-1084

Serveau C, Boulangé A, Lecaille F, Gauthier F, Authié E and Lalmanach G (2003) Procongopain from *Trypanosoma congolense* is processed at basic pH: an unusual feature among cathepsin L-like cysteine proteases. *Biological chemistry* **384:** 921-927

Sidhu SS (2000) Phage display in pharmaceutical biotechnology. *Current Opinion in Biotechnology* **11:** 610-616

Sievers F and Higgins DG (2014) Clustal Omega, accurate alignment of very large numbers of sequences. In *Multiple Sequence Alignment Methods*. Springer, pp. 105-116

Singh M and O'Hagan DT (2003) Recent advances in veterinary vaccine adjuvants. *International Journal for Parasitology* **33:** 469-478

Singh R, Gourinath S, Sharma S, Roy I, Gupta M, Betzel C, Srinivasan A and Singh T (2001) Enhancement of enzyme activity through three-phase partitioning: crystal structure of a modified serine proteinase at 1.5 Å resolution. *Protein engineering* **14:** 307-313

Singh V, Chaudhari S, Kumar S and Chhabra M (1995) Polyclonal antibody-based antigen-detection immunoassay for diagnosis of *Trypanosoma evansi* in buffaloes and horses. *Veterinary parasitology* **56:** 261-267

Smith GP (1985) Filamentous fusion phage: novel expression vectors that display cloned antigens on the virion surface. *Science* **228:** 1315–1317

Smith GP (1993) Surface display and peptide libraries - articles occasioned by a meeting at the Banbury-Center, Cold Spring Harbor laboratory, April 4-7, 1992 - preface. *Gene* **128:** 1-2

Smith SM and Gottesman MM (1989) Activity and deletion analysis of recombinant human cathepsin L expressed in *Escherichia coli*. *Journal of Biological Chemistry* **264:** 20487-20495

Smooker PM, Jayaraj R, Pike RN and Spithill TW (2010) Cathepsin B proteases of flukes: the key to facilitating parasite control? *Trends in parasitology* **26:** 506-514

Stuart K, Allen TE, Kable ML and Lawson S (1997) Kinetoplastid RNA editing: complexes and catalysts. *Current opinion in chemical biology* **1:** 340-346

Sullivan L, Wall SJ, Carrington M and Ferguson MA (2013) Proteomic selection of immunodiagnostic antigens for human African trypanosomiasis and generation of a prototype lateral flow immunodiagnostic device. *PLoS neglected tropical diseases* **7:** 1-14

Sun L, Lu X, Li C, Wang M, Liu Q, Li Z, Hu X, Li J, Liu F and Li Q (2009) Generation, characterization and epitope mapping of two neutralizing and protective human recombinant antibodies against influenza A H5N1 viruses. *PLoS One* **4:** 1-11

Sutherland IA, Moloo SK, Holmes PH and Peregrine AS (1991) Therapeutic and prophylactic activity of isometamidium chloride against a tsetse-transmitted drug-resistant clone of *Trypanosoma congolense* in Boran cattle. *Acta Tropica* **1:** 57-64

Svendsen L, Crowley A, Ostergaard LH, Stodulski G and Hau J (1995) Development and comparison of purification strategies for chicken antibodies from egg yolk. *Laboratory animal science* **45:** 89-93

Swallow BM (2000) Impacts of Trypanosomiasis on African Agriculture. *PAAT Technical and Scientific Series* **21**

Takle GB and Snary D (1993) *South American trypanosomiasis (Chagas' disease).* Blackwell Scientific publishers. 213-236.

Terpe K (2006) Overview of bacterial expression systems for heterologous protein production: from molecular and biochemical fundamentals to commercial systems. *Applied microbiology and biotechnology* **72:** 211-222

Terrestrial O (2013) Trypanosomosis (tsetse-transmitted). 1-11

Thuy NT, Goto Y, Lun Z-R, Kawazu S-I and Inoue N (2012) Tandem repeat protein as potential diagnostic antigen for *Trypanosoma evansi* infection. *Parasitology research* **110:** 733-739

Tini M, Jewell U, Camenisch G, Chilov D and Gassmann M (2002) Generation and application of chicken egg-yolk antibodies. *Comparative biochemistry and physiology part A: molecular & integrative physiology* **131:** 569-574

Tonikian R, Zhang Y, Boone C and Sidhu SS (2007) Identifying specificity profiles for peptide recognition modules from phage-displayed peptide libraries. *Nature protocols* **2:** 1368-1386

Towbin H, Staehelin T and Gordon J (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications *Proceedings of the National Academy of Sciences of the United States of America* **76:** 4350-4354

Tran T, Büscher P, Vandenbussche G, Wyns L, Messens J and De Greve H (2008) Heterologous expression, purification and characterisation of the extracellular domain of trypanosome invariant surface glycoprotein ISG75. *Journal of biotechnology* **135:** 247-254

Tran T, Claes F, Verloo D, De Greve H and Büscher P (2009) Towards a new reference test for surra in camels. *Clinical and Vaccine Immunology* **16:** 999-1002

Tran T, Napier G, Rowan T, Cordel C, Labuschagne M, Delespaux V, Van Reet N, Erasmus H, Joubert A and Büscher P (2014) Development and evaluation of an ITS1 “Touchdown” PCR for assessment of drug efficacy against animal African trypanosomosis. *Veterinary Parasitology* **202:** 164-170

Troeberg L, Morty RE, Pike RN, Lonsdale-Eccles JD, Palmer JT, McKerrow JH and Coetzer THT (1999) Cysteine Proteinase Inhibitors Kill Cultured Bloodstream Forms of *Trypanosoma brucei brucei*. *Experimental Parasitology* **91:** 349-355

Troeberg L, Pike RN, Lonsdale-Eccles JD and Coetzer TH (1997) Production of anti-peptide antibodies against trypanopain-Tb from *Trypanosoma brucei brucei:* effects of antibodies on enzyme activity against Z-Phe-Arg-AMC. *Immunopharmacology* **36:** 295-303

Troeberg L, Pike RN, Morty RE, Berry RK, Coetzer THT and Lonsdale-Eccles JD (1996) Proteases from *Trypanosoma brucei brucei* Purification, characterisation and interactions with host regulatory molecules. *European Journal of Biochemistry* **238:** 728-736

Turk B, Turk V and Turk D (1997) Structural and functional aspects of papain-like cysteine proteinases and their protein inhibitors. *Biological Chemistry* **378:** 141-150

Turk D, Guncar G, Podobnik M and Turk B (1998) Revised definition of substrate binding sites of papain-like cysteine proteases. *Biological Chemistry* **379:** 137-147

Turk V, Stoka V, Vasiljeva O, Renko M, Sun T, Turk B and Turk D (2012) Cysteine cathepsins: From structure, function and regulation to new frontiers. *Biochimica et Biophysica Acta (BBA) - Proteins and Proteomics* **1824:** 68-88

Vale GA (1993) Development of baits for tsetse flies (Diptera: Glossinidae) in Zimbabwe. *Journal of medical entomology* **5:** 831-842

Van den Bossche P, Chigoma D and Shumba W (2000) The decline of anti-trypanosomal antibody levels in cattle after treatment with trypanocidal drugs and in the absence of tsetse challenge. *Acta Tropica* **77:** 263-270

Van den Bossche P and Delespaux V (2011) Options for the control of tsetse-transmitted livestock trypanosomosis. An epidemiological perspective. *Veterinary parasitology* **181:** 37-42

Van Wyngaardt W, Malatji T, Mashau C, Fehrsen J, Jordaan F, Miltiadou D and Du Plessis DH (2004) A large semi-synthetic single-chain Fv phage display library based on chicken immunoglobulin genes. *BMC Biotechnology* **4:** 1-14

van Wyngaardt W, Mashau C, Wright I and Fehrsen J (2013) Serotype-and serogroup-specific detection of African horsesickness virus using phage displayed chicken scFvs for indirect double antibody sandwich ELISAs. *Journal of veterinary science* **14:** 95-98

Vather P (2010) Vivapain: A cysteine peptidase from *Trypanosoma vivax*. MSc dissertation. University of KwaZulu-Natal (Pietermaritzburg campus).

Vaughan TJ, Williams AJ, Pritchard K, Osbourn JK, Pope AR, Earnshaw JC, McCafferty J, Hodits RA, Wilton J and Johnson KS (1996) Human antibodies with sub-nanomolar affinities isolated from a large non-immunized phage display library. *Nature Biotechnology* **14:** 309-314

Verma R, Boleti E and George A (1998) Antibody engineering: comparison of bacterial, yeast, insect and mammalian expression systems. *Journal of immunological methods* **216:** 165-181

Vernet T, Khouri HE, Laflamme P, Tessier DC, Musil R, Gour-Salinll BJ, Storern AC and Thomas DY (1991) Processing of the papain precursor. Purification of the zymogen and characterization of its mechanism of processing. *The Journal of Biological Chemistry* **266:** 21451-21457

Vernet T, Tessier DC, Richardson C, Laliberte´ F, Khouri HE, Bell AW, Storer AC and Thomas DY (1990) Secretion of functional papain precursor from insect cells. Requirement for N-glycosylation of the proregion. *Journal of Biological Chemistry* **25:** 16661-16666

Vickerman K (1985) Developmental cycles and Biology of pathogenic Trypanosomes. *British Medical Bulletin* **2:** 105-114

Volpe JM and Kepler TB (2008) Large-scale analysis of human heavy chain V(D)J recombination patterns. *Immunome research* **4:** 3

Vreysen MJB, Saleh KM, Ali MY, Abdulla AM, Zhu Z-R, Juma KG, Dyck VA, Msangi AR, Mkonyi PA and Feldmann HU (2000) Glossina austeni (Diptera: Glossinidae)

Eradicated on the Island of Unguja, Zanzibar, Using the Sterile Insect Technique. *Economic Entomology* **93:** 123-135

Vreysen MJB, Seck MT, Sall B and Bouyer J (2013) Tsetse flies: Their biology and control using area-wide integrated pest management approaches. *Journal of Invertebrate Pathology* **112, Supplement 1:** S15-S25

Wiederanders B (2000) The function of propeptide domains of cysteine proteinases. *Advances in experimental medicine and biology* **477:** 261-270

Willats WG (2002) Phage display: practicalities and prospects. *Plant molecular biology* **50:** 837-854

Winter G, Griffiths AD, Hawkins RE and Hoogenboom HR (1994) Making antibodies by phage display technology. *Annual Review of Immunology* **12:** 433-455

Wu S and Letchworth GJ (2004) High efficiency transformation by electroporation of *Pichia pastoris* pretreated with lithium acetate and dithiothreitol. *Biotechniques* **36:** 152-154

Yamamoto Y, Kurata M, Watabe S, Murakami R and Takahashi S (2002) Novel cysteine proteinase inhibitors homologous to the proregions of cysteine proteinases. *Current Protein and Peptide Science* **3:** 231-238

Zhou M, Suganuma K, Ruttayaporn N, Nguyen T, Yamasaki S, Igarashi I, Kawazu S, Suzuki Y and Inoue N (2014) Identification and Characterization of a *Trypanosoma congolense* 46 kDa Protein as a Candidate Serodiagnostic Antigen. *The Journal of veterinary medical science/the Japanese Society of Veterinary Science* **76:** 799-806