

**Recombinant expression optimisation and characterisation of peptidases for  
diagnosis of animal African trypanosomosis**

by

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**MSc degree**

in

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## PREFACE

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The experimental work described in this dissertation was carried out in the School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from January 2016 to January 2018, under the supervision of Professor THT Coetzer. The studies represent original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others, it has been duly acknowledged in the text.

27/03/2018

\_\_\_\_\_  
Mr. AA Marivate

As the candidate's supervisor I agree to the submission of this dissertation.

27/03/2018

\_\_\_\_\_  
Prof. THT Coetzer

# DECLARATION - PLAGIARISM

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I, Amukelani Andrew Marivate, declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research.
2. This dissertation has not been submitted for any degree or examination at any other university.
3. This dissertation does not contain other persons' data, pictures, graphs or other information, unless specifically acknowledged as being sourced from other persons.
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## ABSTRACT

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Antigenic variation by African trypanosomes, the causative agents of animal African trypanosomiasis, has blunted efforts to develop a vaccine candidate. Therefore, disease control requires surveillance, diagnosis and effective treatment schedules for the affected livestock. Because of ever-increasing occurrences of drug-resistance and the non-specific symptoms of the disease, there is a great need for the development of point of care diagnostic tests that are inexpensive to the end-user. One of the main bottlenecks to affordable serological diagnostics is the availability of highly immunogenic antigens that are sufficiently stable and maintain their antibody binding properties in the diagnostic tests. Parasite aminopeptidases and cysteine peptidases are involved in the pathogenesis of African trypanosomiasis and therefore have high potential as antigens for the development of new chemotherapeutics and diagnostics. This study aimed to optimise the recombinant production of three known pathogenic factors namely cathepsin L-like peptidase from *Trypanosoma vivax*, *TviCATL*, and aminopeptidase one and two from *T. congolense* *TcoAP1* and *TcoAP2*.

For *TviCATL*, the full-length active site mutant construct (FL *TviCATL*<sub>C25A</sub>) was sub-cloned into pET32-a and pGEX4T-1 and subsequently used for recombinant expression optimisation experiments to improve target protein yield and solubility. A full-factorial design was employed to test varying cultivation conditions for improved soluble recombinant expression. Results showed that even after varying conditions, most of the recombinant FL *TviCATL*<sub>C25A</sub> was expressed in inclusion bodies. Subsequently, the sarkosyl method was employed to recover the protein from inclusion bodies with adequate yield. The size of the recombinant protein was found to be ~67 kDa and the authenticity of the expressed protein was confirmed using anti-His<sub>6</sub> and anti-FL *TviCATL*<sub>C25A</sub> antibodies. The recombinantly expressed and purified antigen was then used to produce polyclonal antibodies in chickens (IgY) for the detection of native *TcoCATL* within *T. congolense* bloodstream form cell lysate. These results demonstrate that this antigen has immense diagnostic potential and further development may allow for its use in rapid diagnostic tests.

For the two M1 aminopeptidases of *T. congolense*, a different approach was used for recombinant expression optimisation, *i.e.* co-expression with molecular chaperones. This study showed that the molecular chaperones could help recover the misfolded and subsequently aggregated aminopeptidases in varying degrees when compared to expression without the molecular chaperones. A suitable protein-chaperone grouping was found by trial and error by comparing recombinant co-expression of the aminopeptidases with several chaperone teams namely: pGro7 (GroES-GroEL), pG-KJE8 (DnaK-DnaJ-GrpE + GroES-GroEL), pG-Tf2 (GroES-GroEL + trigger factor), pKJE7 (DnaK-DnaJ-GrpE) and pTf16 (trigger

factor). In the present study, it was found that co-expression with the pKJE7 chaperone team could recover the most recombinant aminopeptidases from inclusion bodies and led to a two-fold improvement in soluble recombinant production of the aminopeptidases relative to the sample without molecular chaperone co-expression. The pKJE7 chaperone team co-expression experiment produced 24% soluble proportion of the total recombinant aminopeptidase for both *TcoAP1* and *TcoAP2*.

In the present study, two different approaches to recombinant soluble protein expression optimisation were undertaken and in the instance of molecular chaperone co-expression, a two-fold improvement of soluble recombinant aminopeptidases expression was achieved. In the case of FL *TviCATL<sub>C25A</sub>*, although the design of experiments approach to FL *TviCATL<sub>C25A</sub>* expression optimisation did not improve soluble expression of the protein, the use of the non-chaotropic sarkosyl method for its resolubilisation led to much higher yields and more efficient downstream processing. These improvements in protein yield helps in the development of diagnostic tests which require adequate amounts of stable homogeneous antigen, furthermore these improvements can be applied in the development of an anti-disease vaccine.

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*I dedicate this dissertation to my Mom, who has had my back since birth.*

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## LIST OF ABBREVIATIONS

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|          |  |
|----------|--|
| AMC      | 7-amino-4-methylcoumarin                                       |
| AP       | aminopeptidase   |
| ATP      | adenosine triphosphate   |
| AAT      | animal African trypanosomosis                                  |
| AT       | African trypanosomosis   |
| BCA      | bicinchoninic acid   |
| Bis      | N,N'-methylenebisacrylamide                                    |
| BSA      | bovine serum albumin   |
| BSF      | bloodstream form   |
| CATT     | card agglutination test  |
| CATL     | cathepsin L  |
| CP       | cysteine peptidase   |
| DALYS    | disability-adjusted life years                                 |
| DFMO     | eflornithine   |
| DMSO     | dimethyl sulfoxide   |
| DNA      | deoxyribonucleic acid  |
| DTT      | dithiothreitol   |
| E-64     | L- <i>trans</i> -epoxysuccinyl-leucylamido(4-guanidino) butane |
| ECL      | enhanced chemiluminescence                                     |
| EDTA     | ethylenediaminetetraacetic acid                                |
| ELISA    | enzyme-linked immunosorbent assay                              |
| <i>g</i> | relative centrifugal force                                     |
| GLB      | gel loading buffer   |
| GPI      | glycosylphosphatidylinositol                                   |
| GPI-PLC  | GPI-specific phospholipase C                                   |
| HAT      | human African trypanosomosis                                   |
| HRPO     | horseradish peroxidase   |
| ITS1     | internal transcribed spacer 1                                  |
| IFAT     | indirect immunofluorescence antibody                           |
| IgG      | immunoglobulin G   |
| IgY      | immunoglobulin Y   |
| IMAC     | immobilised metal affinity chromatography                      |
| kDa      | kilo-Dalton  |
| LAMP     | loop-mediated isothermal amplification                         |
| LED      | Light-emitting diode   |

|       |   |
|-------|---|
| MCF   | metacyclic form                                 |
| OPB   | oligopeptidase                                  |
| PAGE  | polyacrylamide gel electrophoresis              |
| PARP  | procyclic acidic repetitive protein             |
| PBS   | phosphate buffered saline                       |
| PCF   | procyclic form                                  |
| PCR   | polymerase chain reaction                       |
| RNA   | ribonucleic acid                                |
| RT    | room temperature ( $22 \pm 2^{\circ}\text{C}$ ) |
| RDT   | rapid diagnostic test                           |
| SDS   | sodium dodecyl sulfate                          |
| SRA   | serum resistance-associated                     |
| TBS   | tris-buffered saline                            |
| TEMED | N,N,N',N'-tetramethyl ethylenediamine           |
| Tris  | 2-amino-2-(hydroxymethyl)-1,3-propanediol       |
| Trx   | thioredoxin                                     |
| VSG   | variant surface glycoprotein                    |
| VAT   | variant antigen type                            |
| WHO   | World Health Organisation                       |

# Chapter 1: LITERATURE REVIEW

---

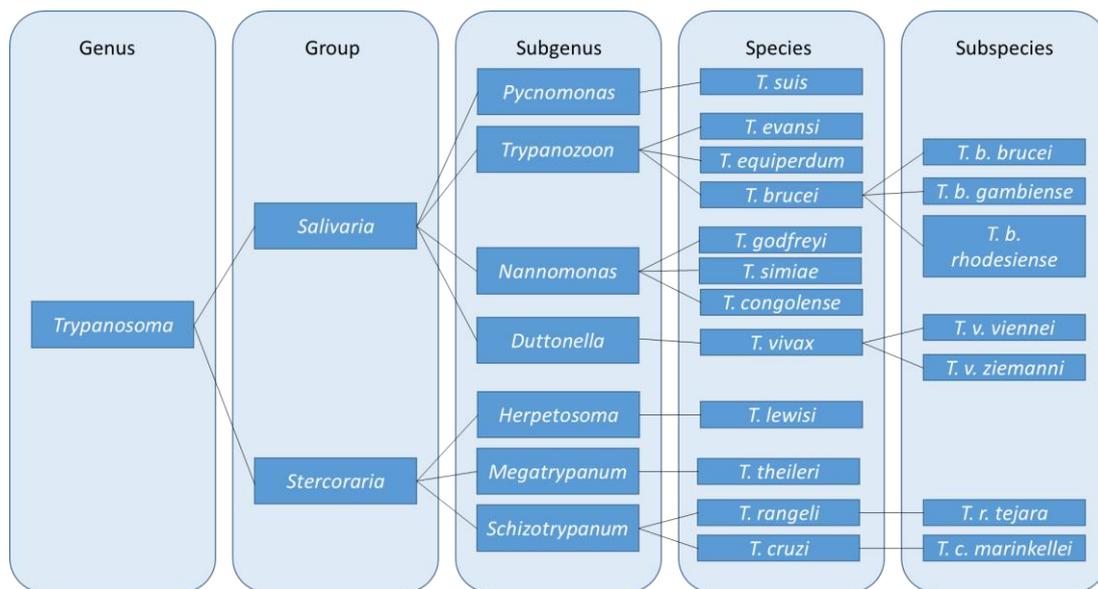
## 1.1 Trypanosomes

Trypanosomosis was initially studied by David Bruce in 1894 while investigating the death of cattle in what is now known as KwaZulu-Natal in South Africa (Duggan, 1977; Brown, 2008). Two African *Trypanosoma* species are pathogenic to humans, *i.e.* *T. b. rhodesiense* and *T. b. gambiense* are human African trypanosomosis causative agents in sub-Saharan Africa. *T. b. brucei*, *T. congolense*, *T. vivax* are all also transmitted by the tsetse fly (*Glossina spp.*) as is the case for the human infective forms, and *T. evansi* are the main causes of animal African trypanosomosis in sub-Saharan Africa (Steverding, 2017). In South America *T. vivax* infect ungulates, where it is transmitted non-cyclically by hematophagous flies rather than by tsetse flies since the parasite is confined to the insect vector's mouthparts (Osório *et al.*, 2008). Chagas disease in South America is caused by *T. cruzi*, an intracellular parasite spread by biting flies (Duggan, 1977; Rassi and Marin-Neto, 2010). The disease surra, which is prevalent in Africa, South America and Asia is caused by *T. evansi* and is transmitted by tabanids (Brun *et al.*, 1998). *T. equiperdum* has evolved a sexual mode of transmission and is transmitted during coitus in horses and causes dourine (Claes *et al.*, 2005).

### 1.1.1 Classification

African trypanosomes are flagellated haemoprotozoan parasites belonging to the order Kinetoplastida, which are characterised by the presence of a rod-shaped DNA-containing kinetoplast at the base of the flagellum, a single flagellum attached to the body as an undulating membrane, and a single highly branched mitochondrion (Uilenberg, 1998).

The species of trypanosomes infecting mammals have been divided into two groups that develop in different parts of the vector to the infective metacyclic stage (Enyaru *et al.*, 2010). In the group *Stercoraria*, transmission occurs through the posterior part of the vector, while in *Salivaria*, transmission occurs through the anterior part of the vector. Also, *Salivaria* are the only trypanosomes which exhibit antigenic variation. There are at present four subgenera of *salivarian* trypanosomes, *i.e.* *Duttonella*, *Nannomonas*, *Trypanozoon* and *Pycnomonas* (Haag *et al.*, 1998). Figure 1.1 shows the classification of trypanosomes of veterinary and medical importance.

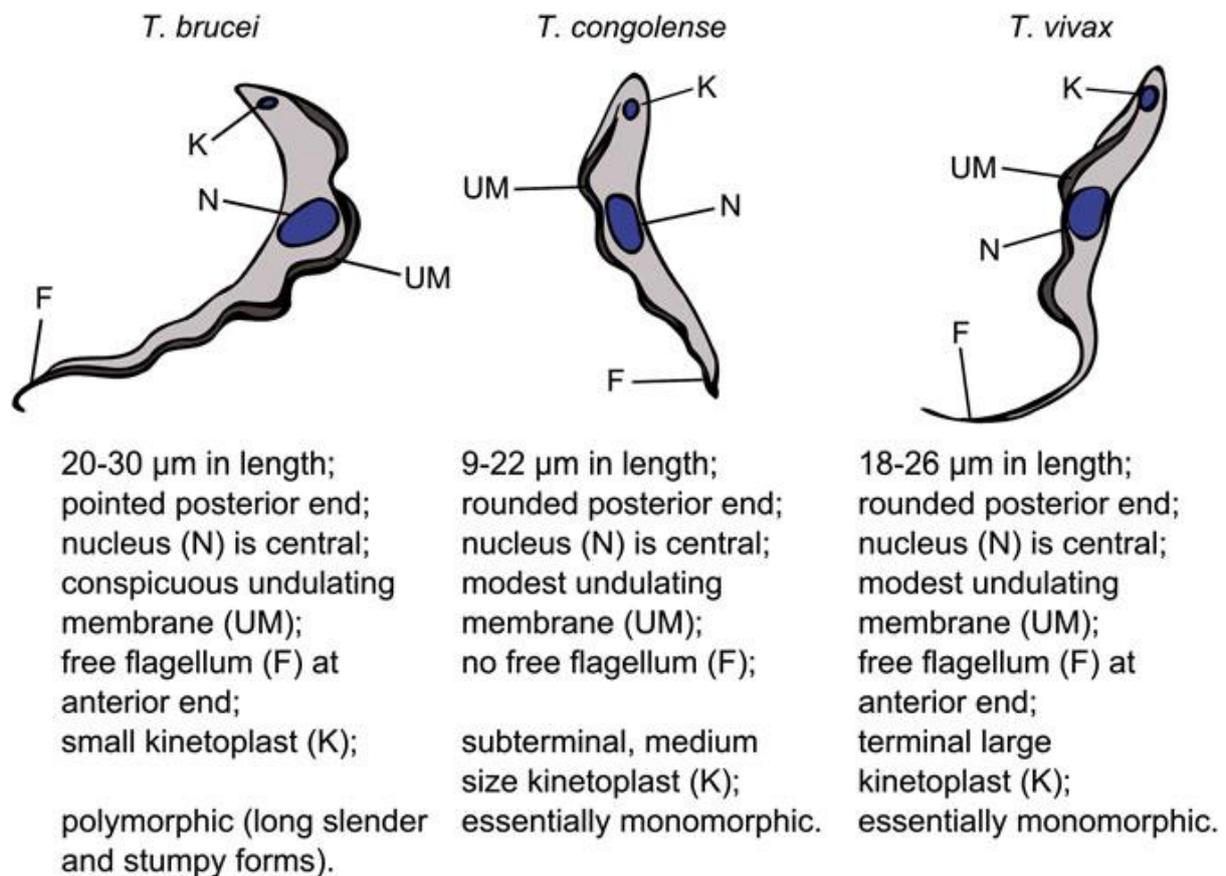


**Figure 1.1: Classification of trypanosomes of veterinary and medical importance.** (Stevens *et al.*, 2004).

## 1.1.2 Biology of African trypanosomes

### 1.1.2.1 Morphology

Trypanosomes are single-celled parasites that are elongated (ranging from 8-50  $\mu\text{m}$ ) and tapered at both ends (Figure 1.2). Since trypanosomes are eukaryotic, they contain typical features such as a nucleus and organelles including endoplasmic reticulum, Golgi apparatus, a mitochondrion and endocytosis and exocytosis systems (Bonhivers *et al.*, 2008). Trypanosomes have a flagellar pocket, flagellum and mitochondrial DNA. An invagination in the plasma membrane protects the flagellar pocket. Due to the presence of high a concentration of receptors responsible for endocytosis and immune evasion, the flagellar pocket is considered to be a site for protein trafficking, protein and lipid sorting, and a recycling compartment (Gadelha *et al.*, 2009). The flagellum which is responsible for parasite motility extends from the flagellar pocket. The basal body at which the flagellum is attached 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 (Peña-Díaz *et al.*, 2017). The mitochondrial DNA consists of a network of maxicircles (22 kb) and minicircles (1 kb). Maxicircles are responsible for encoding mitochondrial proteins while minicircles are responsible for extensive post-transcriptional modification or RNA editing of these regions using guide RNAs (Horn, 2014).



**Figure 1.2: Anatomical features of the bloodstream forms trypomastigotes of the three most important animal trypanosomes.** The trypomastigotes are the disease form and the target for therapy. (Giordani *et al.*, 2016)

### 1.1.2.2 The life cycle and distribution of African trypanosomes

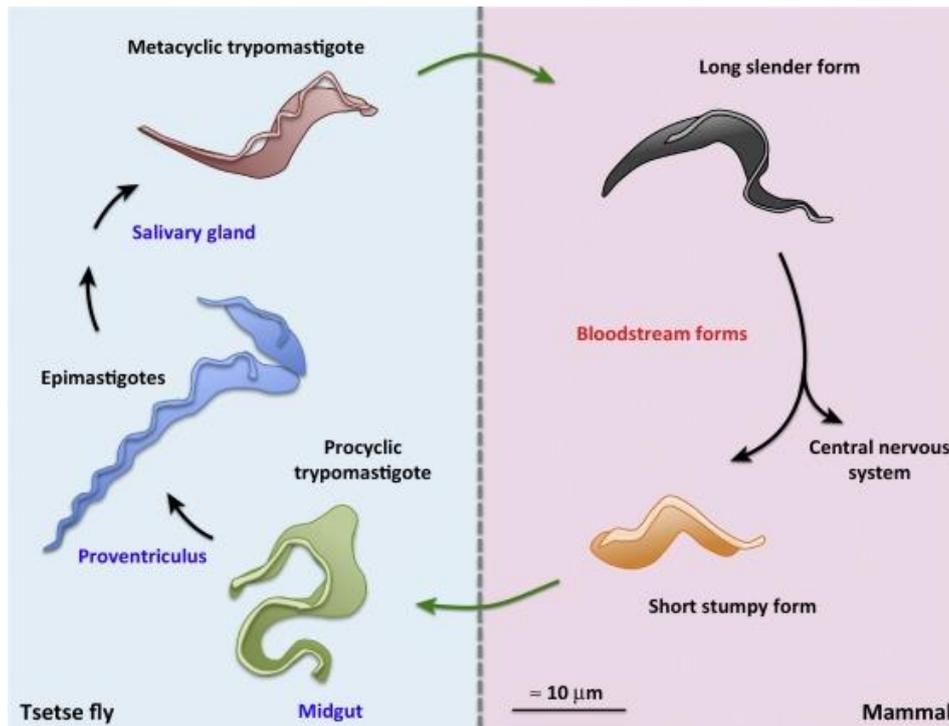
The presence of the trypanolytic factor in humans and some primate species renders them resistant to most trypanosomes, however *T. b. rhodesiense* and *T. b. gambiense* are themselves resistant to the factor and are therefore the only trypanosomes to infect humans (Poelvoorde *et al.*, 2004; Baral, 2010). *T. b. rhodesiense* expresses the serum resistance-associated (SRA) gene which renders it resistant to the trypanolytic factor in human sera, therefore, causing acute rather than chronic infections (De Greef *et al.*, 1992; Pays, 2006).

Trypanosomes have a dual host life cycle and therefore require special adaptations such as kinetoplast DNA, RNA editing, unique gene regulation, glycosomes and flagellum along with a flagellar pocket (Clayton *et al.*, 1995). The parasites require the various morphological and gene expression changes, which are involved in the various life cycle stages, to be effectively coordinated (Matthews, 1999) (Figure 1.3). Mammalian infection commences with the acquisition of a blood meal by an infected tsetse fly where the metacyclic form of parasites are injected into the host bloodstream. The metacyclic form parasites transform into proliferative bloodstream form trypomastigotes which are entirely coated with a continuous

layer of variant surface glycoprotein (VSG) homodimers (Vickerman and Luckins, 1969; Cross, 1975). The bloodstream form trypomastigotes are long and slender in appearance during the ascending phase of parasitaemia. At the peak of parasitaemia, the bloodstream form trypomastigotes, which are now short and stumpy, are no longer proliferative (Barry and McCulloch, 2001; Matthews *et al.*, 2004). The parasites then transform into insect vector infectious procyclic form parasites which can colonise the midgut of the insect vector and proliferate (McLintock *et al.*, 1993; Redpath *et al.*, 2000). The transformation from stumpy bloodstream form into procyclic form is accompanied by a change in metabolism, where ATP is generated by glycolysis to being generated by oxidative phosphorylation (Clayton, 1992; Vassella *et al.*, 2004; Matthews, 2005). The loss of the VSG surface coat, characterises the parasite's transformation into the proliferative procyclic form (Ziegelbauer and Overath, 1993; Gruszynski *et al.*, 2003).

The rapid loss of the VSG surface coat of the procyclic stumpy form requires the continued action of GPI-specific phospholipase C (GPI-PLC) and a surface zinc metallopeptidase (MSP-B) (Ziegelbauer and Overath, 1993; Gruszynski *et al.*, 2003). After the proliferation of procyclic form parasites within the midgut, migration towards the salivary glands occurs where they undergo asymmetric cell division to form stumpy and slender epimastigote form parasites (Van Den Abbeele *et al.*, 1999). Only the stumpy epimastigote parasites have a *brucei* alanine-rich protein (BARP) surface coat which replaces the procyclin coat. These stumpy epimastigote form parasites can attach, proliferate and mature into metacyclic form parasites within the salivary glands of the insect vector, where they re-obtain their VSG surface coat (Tetley *et al.*, 1987; Urwyler *et al.*, 2005; Urwyler *et al.*, 2007). Once colonised, the salivary glands are the generation site of the metacyclic form parasites which are infectious to mammals (Sbicego *et al.*, 1999). The metacyclic form parasites have a larger repertoire of VSG genes compared to that of the bloodstream form parasites (Barry and McCulloch, 2001).

The bloodstream form of the *T. b. brucei* parasites are pleomorphic, with two different physical forms being present concurrently (Vickerman, 1985). The slender forms replicate by asexual division and are susceptible to elimination by host peptidases (Sbicego *et al.*, 1999). The stumpy forms, which are non-proliferative, are resistant to elimination by host peptidases and can differentiate into procyclic form parasites which are then able to colonise the salivary glands of the insect vector (Clayton, 1992; Sbicego *et al.*, 1999). The non-proliferative character of the stumpy forms acts to prevent the uncontrolled growth of the slender forms which would result in the rapid killing of the infected mammalian host (Matthews and Gull, 1994).



**Figure 1.3: Life cycle of *T. brucei* showing the different morphological changes that occur during the different stages in the vector and mammalian host.**

[http://www.cell.com/trends/parasitology/fulltext/S1471-4922\(16\)30009-5](http://www.cell.com/trends/parasitology/fulltext/S1471-4922(16)30009-5) accessed on 16.05.2016.

### 1.1.2.3 Genomic organisation

Two genomes are present in African trypanosomes: one within the nucleus and one within the kinetoplast. The nuclear genome is of low complexity since 68% of the genome consists of single-copy sequences (Liu *et al.*, 2005). Although most of the nuclear genome is diploid, areas including the VSG genes, VSG expression sites, and mini-chromosomes are not. The nuclear genome is approximately  $3.5 \times 10^7$  bp in length, roughly the same as the genomes of parasites such as *Plasmodium falciparum*, *T. cruzi* and *Leishmania major* (Kooy *et al.*, 1989; El-Sayed *et al.*, 2000).

### 1.1.2.4 Antigenic variation

Trypanosomes have developed a process of antigenic variation to elude the host immune system. The outer surface of the bloodstream form trypanosomes are coated by the VSG, and each parasite in a population has the genetic capability of producing an antigenically distinct VSG (Vickerman, 1969; Taylor and Rudenko, 2006; Horn, 2014). The different VSG coats are termed variant antigen types (VATs). Antigenic variation is the term used to describe the varying expression of these VSG coats, resulting in differing populations of trypanosomes with a different VAT present in the blood over time. As a specific immune response develops against one VAT, the number of trypanosomes with that VAT decreases, the trypanosomes with a different VAT will survive and initiate the next wave of parasitaemia (Chung *et al.*, 2004).

The expression of different VSG coats is thought to occur when a VSG gene, often located in a non-transcribed region, is duplicated into a transcriptionally active region (Barry and Carrington, 2004).

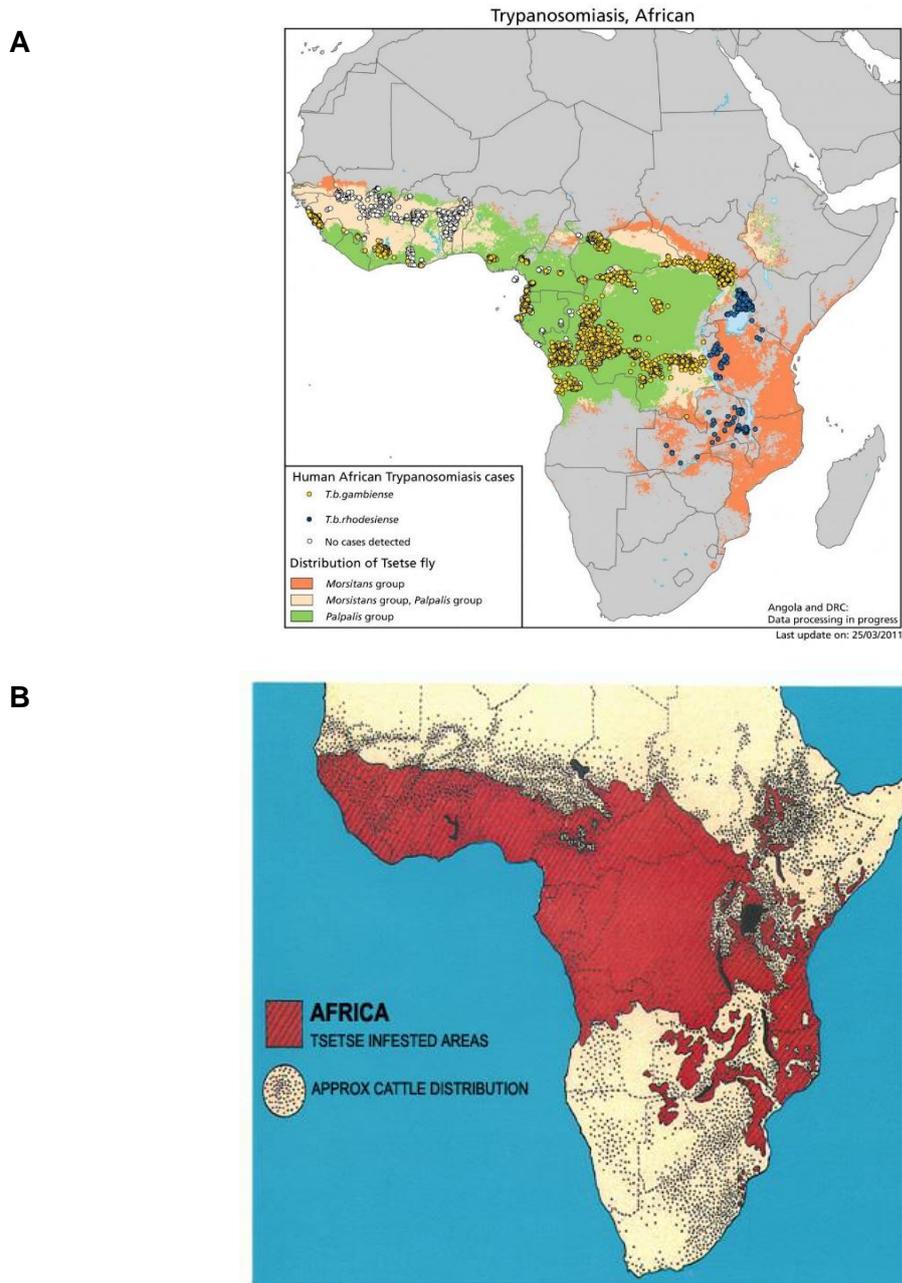
## 1.2 African trypanosomosis

In 2015, 2804 cases of HAT were reported to the WHO, with *gambiense* HAT comprising the bulk of cases (2733, a 90% reduction since 1999) and 71 cases were rhodesiense HAT (89% reduction). This includes cases diagnosed in both endemic and non-endemic countries (Büscher *et al.*, 2017). The number of cases reported does not reflect the true number of cases due to the limitation of diagnostic methods and the rural setting of disease incidence. The disease was estimated to have a health burden of approximately 1.6 million disability-adjusted life years (DALYS) in 2004 (Fevre *et al.*, 2008) which has a negative effect on economic development in central Africa. The impact of AT in Africa results in excess of \$4.5 billion in annual economic losses.

The disease affects mostly poverty-stricken communities living in remote rural areas of Africa and tends to be fatal if not treated. The geographic distribution of the foci of the disease is synonymous with the tsetse fly belt which stretches from north of the Kalahari desert to the south of the Sahara (Barrett *et al.*, 2003). Within this region, it is estimated that 42-62 million head of cattle and other domestic animals are at risk of animal African trypanosomosis (AAT) (Swallow, 2000).

Tsetse flies are distributed in approximately 10 billion km<sup>2</sup> of Africa (Figure 1.4, B) (Mattioli *et al.*, 2004). The *Palpalis* group of the tsetse fly are the insect vector for the human infective trypanosomes, whilst for the animal infective parasites it's the *Morsitans* group (Hargrove *et al.*, 2003) (Figure 1.4, A). Human infections by *T. b. gambiense* is known to occur in 24 countries across the west and central regions of Africa (Simarro *et al.*, 2008; Simarro *et al.*, 2012). This species is responsible for over 90% of reported HAT infections with the remainder of infections being caused by the *T. b. rhodesiense* species in east and southern Africa (Simarro *et al.*, 2008; Simarro *et al.*, 2011) (Figure. 1.4, A). The former causes chronic

infection, where death can occur within years while the latter causes acute infection with death occurring within months (Holt *et al.*, 2016).



**Figure 1.4: Distribution of tsetse flies in Africa and its effect on human and cattle population. (A)** Map showing human African trypanosomiasis cases, parasite species and tsetse fly distribution (Wertheim *et al.*, 2012). **(B)** Map Showing distribution of tsetse fly and cattle in Africa (IAEA, 2009).

Trypanosome infections in livestock are 100 to 150 times more likely to occur when compared to human infections (Alsan, 2015). Infections brought about by the *T. b. brucei* species are characterised by the onset of necrosis, oedema and numerous inflammatory disease symptoms (Eisler *et al.*, 2004). Infections by the *T. congolense* and *T. vivax* species cause extra-vascular haemolytic anaemia in infected cattle and is responsible for the development of chronic anaemia and results in decreased productivity and losses of livestock (Eisler *et al.*,

2004). N'dama cattle (*Bos taurus*) are able to limit parasitaemia, and the disease symptoms caused by *T. congolense* infection and are thus described as trypanotolerant (Authié, 1994), whereas African Zebu cattle (*Bos indicus*) are susceptible to the disease (Authié, 1994).

### 1.2.1 Pathogenesis

Pathogenic trypanosomes are responsible for three known pathological states in African livestock, which have been given the common names nagana (bovine trypanosomosis), *surra* (*T. evansi* infections) and dourine (*T. equiperdum*) (Table 1.1). Bovine trypanosomosis is caused mainly by the Salivarian group of African trypanosomes (Stevens *et al.*, 2004) (Table 2.1).

**Table 1.1 Overview of the pathogenic salivarian trypanosomes.** (Gibson, 2007).

| Subgenre    | Species                      | Disease                      | Distribution                                   | Potential mammalian hosts         |
|-------------|------------------------------|------------------------------|--|-----------------------------------|
| Trypanozoon | <i>T. brucei brucei</i>      | Nagana                       | Tropical Africa                                | Wild and domestic animals         |
|             | <i>T. brucei rhodesiense</i> | Rhodesian sleeping sickness  | East Africa                                    | Humans, wild and domestic animals |
|             | <i>T. brucei gambiense</i>   | Gambian sleeping sickness    | West and Central Africa                        |                                   |
|             | <i>T. evansi</i>             | Surra                        | North Africa, Middle East, Asia, South America | Wild and domestic animals         |
|             | <i>T. equiperdum</i>         | Dourine                      |  | Equines                           |
| Nannomonas  | <i>T. congolense</i>         | Nagana                       | Tropical Africa                                | Wild and domestic animals         |
|             | <i>T. simiae</i>             | Acute porcine trypanosomosis | Tropical Africa, South America                 |                                   |
| Duttonella  | <i>T. vivax</i>              | Nagana                       | Tropical Africa                                | Wild and domestic animals         |
| Pycnomonas  | <i>T. suis</i>               | Porcine trypanosomosis       | Tropical Africa                                | Suids                             |

These pathogenic trypanosomes can be separated into haematic trypanosomes, which include *T. congolense* and *T. vivax*, and humoral trypanosomes, which include *T. b. brucei*, *T. b. rhodesiense* and *T. b. gambiense*. Haematic trypanosomes are limited to the blood vessels of the host animal while humoral trypanosomes are present in plasma, intracellular tissues,

lymphatic system and are able to cross the blood-brain barrier (Uilenberg, 1998; Lonsdale-Eccles and Grab, 2002). Although *T. b. brucei* is capable of tissue invasion, it is generally less pathogenic than *T. congolense* and *T. vivax*. Anaemia is a symptom typical of both groups while infection by humoral trypanosomes may result in necrosis and inflammation (Losos and Ikede, 1970; Gibson, 2007).

The progress of AAT is dependent on the animal host, trypanosome species and strain. However, several clinical signs of the disease are shared between most domestic animals irrespective of the species of trypanosomes involved. Detection of parasitaemia and anaemia occur between 1-3 weeks after infection depending on the pathogenesis and infective dose of the trypanosome strain, and immune status of the host animal. The initial chancre is caused by the rapid multiplication of trypanosomes in the skin and the area surrounding the tsetse bite which results in the enlargement of the lymph nodes (Mattioli *et al.*, 1999). Once in the lymphatic system, the parasites have access to the host bloodstream and progress to the blood filtering organs such as the spleen and liver (Taylor and Authié, 2004).

Initial clinical signs of the acute phase of AAT include enlarged lymph nodes and spleen, weakness, lethargy, general loss of condition, reduced milk production and abortion. Infection with *T. congolense*, *T. vivax* or *T. b. brucei* may also result in damage to the bone marrow, heart, endocrine glands, and reproductive glands and in the case of *T. vivax*, the central nervous system. Death may occur in the first few weeks, or the animal may stabilise and begin a slow recovery. Animals are most likely to progress to the chronic phase of the disease characterised by intermittent parasitaemia, stunted growth, wasting and infertility (Bengaly *et al.*, 2002). Chronic trypanosomosis usually results in death by congestive heart failure (a result of prolonged anaemia), myocardial damage and increased vascular permeability (Taylor and Authié, 2004).

### **1.2.2 Control strategies**

A multi-faceted approach was adopted for the control of African trypanosomosis in animal and human populations. Vector control and the use of trypanocides are the historical control measures for bovine and other animal forms of African trypanosomosis (Van den Bossche, 2001; Baral, 2010). Vector control strategies include chemical control through the use of insecticides (Allsopp, 1984), the use of fly baits, traps and the adoption of the sterile insect technique (Vreysen *et al.*, 2000). For controlled cattle grazing areas, vector control can also be achieved by the expansive clearing of vegetation which sustains tsetse populations. Insecticide-treated cattle are protected from tsetse bites, and tsetse populations are also reduced due to the death of flies that take blood meals from insecticide-treated cattle. In *T. b. rhodesiense* endemic areas, where cattle serve as reservoirs for the disease in the absence

of wild animals, treatment of cattle with insecticides, could also help control the human disease. Vector control gets more costly upon scale-up and may not be sustainable, therefore, the use of trypanocides became the main control strategy in many rural and poor endemic areas (Chitanga *et al.*, 2011). Chemoprophylactic and chemotherapeutic control of the disease has two important effects namely, eliminating the reservoir of transmissible trypanosomes and reducing deaths caused by infection (Fevre *et al.*, 2008). Depending on the severity of symptoms exhibited, using trypanocide treatment in the chronic phase may take much longer for clinical recovery than effective treatment at the acute phase of the disease which usually leads to a prompt recovery (Giordani *et al.*, 2016). A few African cattle breeds such as Dahomey, N'Dama and Muturu developed resistance against trypanosome infection. This is known as trypanotolerance and is the ability to survive and be productive even when infected (Authié, 1994). Trypanotolerance allows the cattle to limit parasitaemia and anaemia enabling these animals to cope with infections. Raising trypanotolerant breeds is often seen as an important control strategy and has improved livestock productivity in multiple endemic areas (Naessens, 2006).

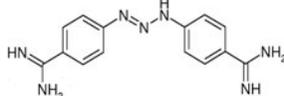
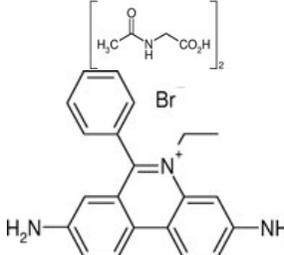
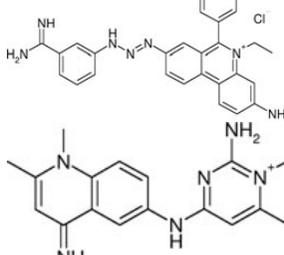
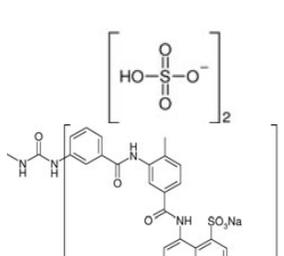
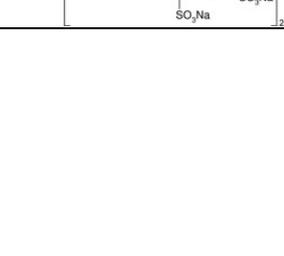
Treatment and prophylaxis of AAT relies mainly on five trypanocides (Table 1.2), most being actively used for the past 50 years (Munday *et al.*, 2015). Furthermore, several factors limit their usage, such as the trypanocides causing local irritation at the injection site and more importantly, past widespread usage has led to ever increasing trypanocide resistance, and their similarity in composition has promoted cross-resistance (Delespaux and de Koning, 2007; Giordani *et al.*, 2016). Many of the popular trypanocides have been shown to act against the parasite's kinetoplast causing the protozoan's death (Wilkinson and Kelly, 2009), however the actual biochemical mechanisms of action and resistance are yet to be elucidated. The different trypanocides have differing therapeutic effectiveness based on the pharmacokinetics that affect distribution and the specific potency against each trypanosome species which in turn have different biochemical physiology and host organ distribution between them (Giordani *et al.*, 2016).

The available trypanocides are mostly therapeutic while some are prophylactic (Stevenson *et al.*, 1995). The decision to administer prophylactic or therapeutic drugs is based on some observations such as drug availability and risk of infection (Diall *et al.*, 2017). In areas of high infection prevalence, prophylactic drugs should be applied across the whole herd as this approach will be more cost-effective, avoiding the loss of revenue associated with infections and significant reduction in morbidity and mortality, whereas therapeutic drugs are more suitable for low prevalence areas where infections of the affected animals have been confirmed (Gu *et al.*, 1999). Prophylactic and therapeutic drugs that are administered at single doses are preferred when compared to multi-dose regimens since this is not workable in

developing countries where animal care facilities are few and far between (Giordani *et al.*, 2016). As new trypanocides are unlikely to become available in the foreseeable future (optimistically it will take at least five years for the next most promising drug to go into market), efficient use of those already in market is paramount (Giordani *et al.*, 2016). However, trypanocide use in the field is hard to monitor let alone regulate. In high prevalence African countries, trypanocide administration is usually performed directly by farmers, that can buy them for less than US\$ 1 per treatment (Giordani *et al.*, 2016). Unfortunately, most farmers have little access to facilities that provide information regarding proper trypanocide usage and dosage and tools that allow for accurate diagnosis; usually farmers depend only on clinical symptoms that are not disease-specific, hence these factors lead to trypanocide misuse (Grace *et al.*, 2009). Furthermore, an unregulated market leads to the widespread use of poor quality or counterfeit trypanocides especially in Africa where documented product specifications are scarce (Tchamdja *et al.*, 2016). Two laboratories were established, one in Der Es Salaam and one in Dakar, under the watch of GALVmed-FAO (Global Alliance for Livestock Veterinary Medicines; Food and Agriculture Organization of the United Nations) to improve the standards of trypanocides and their usage (Sutcliffe *et al.*, 2014).

Vaccination is a viable alternative to chemotherapy in the control of African trypanosomosis. However, there are currently no vaccines available against trypanosomosis (Agbo *et al.*, 2003). Vaccine development has been challenging due to antigenic variation by the parasites (Lipsitch and O'Hagan, 2007). Consequently, a new vaccine approach involving the use of DNA vaccines is being explored. DNA vaccines have been shown to be successful in inducing immune protection against many protozoan-caused diseases (Handman *et al.*, 2000; Laddy and Weiner, 2006). Due to the limitations in anti-parasite vaccinations, it was necessary to introduce an alternative approach to vaccination. The anti-disease vaccine approach was thus developed, in which efforts are directed to targeting pathogenic factors rather than the parasite itself (Authié *et al.*, 2001). This approach led to the targeting of several peptidases, which play a role in the parasites' pathogenesis. Infections of trypanotolerant cattle with *T. congolense* resulted in an increased IgG response against *TcoCATL* antigen. The levels of anti-*TcoCATL* antibodies were directly proportional to levels of resistance in trypanotolerant cattle and correlated with a decrease in anaemia in cattle (Authié *et al.*, 2001).

**Table 1.2: Currently available trypanocides** (Giordani *et al.*, 2016).

| Name   | Trade name <sup>a</sup> | Structure   | Indication  |
|--|-------------------------|---|---|
| Diminazene aceturate   | Berenil                 |    | <i>T. vivax</i> and <i>T. congolense</i> but less potent on <i>T. evansi</i> and <i>T. b. brucei</i> infections             |
| Homidium bromide<br>Homidium chloride                                  | Ethidium novidium       |    | <i>T. congolense</i> and <i>T. vivax</i> but less potent against <i>T. b. brucei</i>  |
| Isometamidium chloride   | Trypamidium             |    | <i>T. vivax</i> and <i>T. congolense</i> but less potent against <i>T. b. brucei</i>  |
| Quinapyramine sulphate<br>Quinapyramine sulphate:chloride<br>(3:2 w/w) | Antrycide               |   | <i>T. congolense</i> , <i>T. vivax</i> , <i>T. evansi</i> , <i>T. b. brucei</i> , <i>T. equiperdum</i> and <i>T. simiae</i> |
| Suramin sodium   | Naganol                 |  | <i>T. equiperdum</i> , <i>T. b. brucei</i> and <i>T. evansi</i>   |

<sup>a</sup> Trade name list is not complete

### 1.2.3 Diagnosis

Successful control of trypanosomosis depends on reliable detection of the parasites, which is difficult in closely related sub-species (Agbo *et al.*, 2003). Point of care diagnostics is widely mooted as one of the best tools to combat AAT. Ideally, any point of care diagnostics up for consideration should meet the ASSURED criteria that is: it should be affordable, sensitive, specific, user-friendly, rapid, equipment-free and deliverable to end-users (Wild, 2013). *T. vivax*, *T. congolense* and *T. brucei* infection exhibit similar clinical symptoms. Therefore diagnosis of trypanosomosis 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.

#### 1.2.3.1 Parasitological diagnostics

Parasitological diagnosis aims at detection and identification of trypanosome parasites in the infected host blood circulation. Motile trypanosomes can be identified in thick or thin smears of blood on a microscope slide and through light microscopy. Experts are able to distinguish *T. congolense* and *T. vivax* based on their morphology but the sensitivity of the technique is not sufficient (Nantulya and Lindqvist, 1989). Introduction of haematocrit centrifugation, which concentrates the parasites in the buffy coat layer allowing simple detection of trypanosomes by light microscopy, improved the sensitivity of the method (Ancelle *et al.*, 1996; Matovu *et al.*, 2012). Blood parasite levels as low as  $5 \times 10^3$  *T. brucei*,  $2.5 \times 10^2$  *T. congolense* and  $5 \times 10^2$  *T. vivax* parasites per ml of blood can be detected using this method (Paris *et al.*, 1982). However, this technique depends on the availability of electricity, therefore, cannot readily 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.

#### 1.2.3.2 Molecular diagnostics

Effective identification of low-level parasite copy numbers was enhanced by the introduction of the polymerase chain reaction (PCR) that uses oligonucleotide primers that are highly specific for the detection of trypanosome DNA (Moser *et al.*, 1989). Since the use of several specific primers for PCR is time-consuming, a single molecular marker to identify several trypanosomes was attempted, namely the advanced single PCR utilised multi-copy internal transcribed spacer 1 (ITS1) within ribosomal RNA genes of trypanosomes. This method could distinguish a pool of trypanosomes in a single PCR run since each species of trypanosome contains 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 trypanosomosis since it is associated with improved species-specific assays but it requires substantially more complicated equipment and experienced personnel to conduct the test.

Although this method still shows low sensitivity for detection of *T. vivax* (Desquesnes *et al.*, 2001), a study conducted by Fikru and colleagues (2012), reported a five-fold higher detection efficiency for *T. vivax* as compared to the haematocrit centrifugation technique. The ITS1 PCR assay in a “touchdown” format was identified as a good diagnostic technique for evaluating drug efficacy against *T. congolense* infection in cattle (Tran *et al.*, 2014). Sequences from genes encoding for *Tvi*CATL from African and South American isolates were previously evaluated as suitable genetic markers. The use of these genetic markers for diagnosis showed immense potential as it used regions in the CATL coding sequences shared by all *T. vivax* genotypes but not those found in other trypanosomes making them perfect for a species-specific and sensitive PCR diagnostic assay (Cortez *et al.*, 2009).

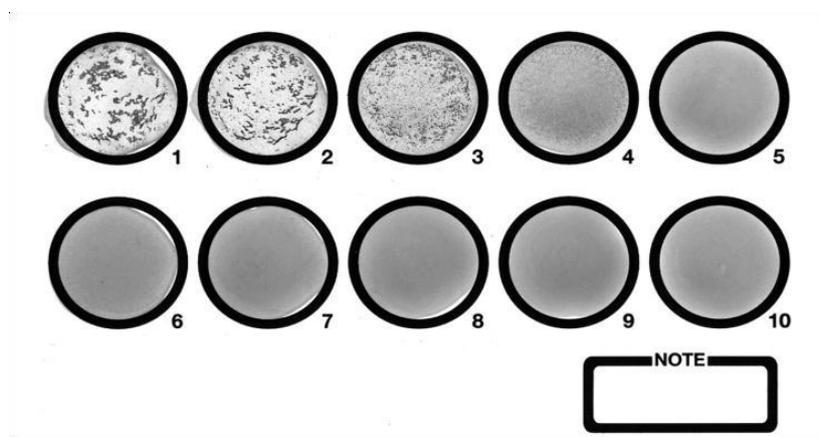
PCR-ELISA, which couples PCR for a specific species to ELISA was developed. In PCR-ELISA, nested PCR using specific primers is used for the amplification of DNA, after which the specificity of the PCR products is assessed by coating PCR products on ELISA plates. PCR-ELISA has been effective for the detection of *T. vivax* and *T. brucei* but not *T. congolense* in the peripheral blood of infected cattle. This technique is limited by its high cost, preventing its use in poorly resourced laboratories in African countries (Masake *et al.*, 2002; Cabrera *et al.*, 2009).

An alternative to PCR-based diagnostic techniques, the loop-mediated isothermal amplification (LAMP) method 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. Although this method has been used for the diagnosis of *T. congolense* infections in livestock, this method has mainly been used for the diagnosis of human-infecting *T. brucei* parasites (Mitashi *et al.*, 2012).

### **1.2.3.3 Serological diagnostics**

Serological diagnosis is a suite of immunodiagnostic techniques that involve antigen or antibody detection (Luckins, 1977; Nantulya and Lindqvist, 1989). Since trypanosome lysis relies on both complement and antibody, complement fixation tests (CFT) could be used for trypanosomosis diagnosis (Nantulya and Lindqvist, 1989). In the early stages of AAT serodiagnostics immunofluorescence assays (IFA) were the principal method for diagnosis and was shown to be sensitive, specific but not species-specific (Luckins, 1977). Serum or blood impregnated filter paper eluates can be used for IFA. Blood from mice infected with either *T. b. brucei* or *T. b. gambiense* is used as the antigen for IFA (WHO, 1998). The assay, however, is not quantitative, requires complicated equipment and the lack of a standardised antigen makes it a poor choice for active case finding in the field. Mass population screening

for the diagnosis of *T.b. gambiense* has advanced by the introduction of the card agglutination test (CATT)/*T. b. gambiense* (Büscher, 2014) (Figure. 1.5). The CATT agglutination assay is fast, simple and is useful for the diagnosis of *T. b. gambiense* infected patients by its ability to detect in sera or blood plasma, *T. b. gambiense*-specific antibodies (Magnus *et al.*, 1978; Büscher, 2014). The lysed *T.b. gambiense* bloodstream form parasites of the LiTat 1.3 VAT are used as the CATT antigen. Infections with trypanosome strains that lack the LiTat 1.3 gene may lead to false negative CATT results (Dukes *et al.*, 1992; Enyaru *et al.*, 1998). The efficiency of CATT relies on optimal fixing and stabilisation of the parasite using formaldehyde in order to detect the whole trypanosomes in the agglutination test (Nantulya and Lindqvist, 1989).



**Figure 1.5: A card agglutination test example using a 1:4 dilution of blood samples.** Sample 1 to 3 exhibit a strong positive reaction. Sample 4 exhibits a weak positive reaction while the remaining samples are negative for trypanosomal infection (Chappuis *et al.*, 2005).

The development of ELISA was a breakthrough since it can be used for large-scale testing. The major drawback of ELISA is that until recently the source of antigen for the test was either whole parasites or crude parasite lysate. ELISA was found to be a suitable diagnostic tool for the detection of bovine anti-trypanosome antibodies using both bloodstream 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 while 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-(*TcAGd*) and -(*TvAGd*) where plates were precoated with denatured antigen (AGd) from *T. congolense* (*TcAGd*) and *T. vivax* (*TvAGd*), 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). The use of whole parasite lysates as a source of antigen is a major limitation to

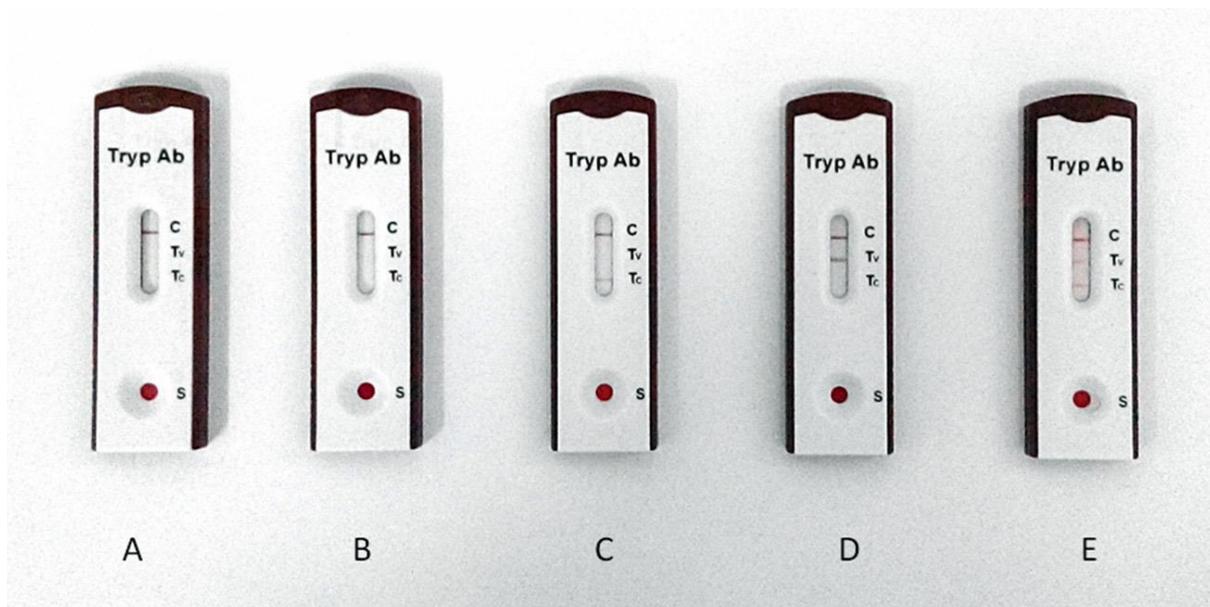
these techniques because it is difficult to standardise the preparation of trypanosome lysates. These lysates are prepared in different ways either from parasites propagated in rodents or grown in culture using different protocols. Additionally growing *T. vivax* in culture is particularly difficult (Boulangé *et al.*, 2017).

Standardised recombinant expression systems were used to produce antigens with diagnostic potential to avoid the use of whole parasites as antigens that are cultured *in vitro* or propagated *in vivo*. Targeting non-variant trypanosomal macromolecules, which are highly antigenic for example hsp70, showed improved specificity in 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 and Authié, 1994). In an inhibition ELISA format, recombinant hsp70/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 and Authié, 1994; Boulangé *et al.*, 2002; Bossard *et al.*, 2010). A proteomic approach was used for identifying trypanosomal antigens with diagnostic potential (Sullivan *et al.*, 2013). A flagellar-associated protein, TvGM6 was heterologously produced in an *E. coli* expression system and used for the detection of antibodies in sera from cattle with *T. vivax* infections via indirect ELISA (Pillay, Izotte, *et al.*, 2013). The protein TvY486\_0045500 and TvY486\_0019690 are invariant surface glycoproteins with diagnostic potential. These antigens were heterologously produced in *E. coli* and used in an indirect ELISA for the detection of *T. vivax* infections. High sensitivity and specificity were reported (Fleming *et al.*, 2014). When parasites are lysed, pathogenic factors such as cysteine peptidases are released within the host bloodstream, and these peptidases have diagnostic potential. The cathepsin-B of *T. congolense* TcoCB1 and the GM6 antigen of *T. vivax* TviGM6 are examples of pathogenic factors with diagnostic potential (Boulangé *et al.*, 2017).

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. To produce polyclonal antibodies, egg yolk immunoglobulin (IgY) technology represents a preferred method that offers advantages with respect to animal care, cost and antibody yield. Due to the phylogenetic distance between chickens and parasites, parasite antigens are highly immunogenic in chickens and antibody production in hens is readily stimulated. Recombinant trypanosomal antigens such as structural proteins; ISG65 and ISG75, and proteolytic enzymes; oligopeptidase B, pyroglutamyl peptidase and the cysteine peptidase TcoCATL from *T. congolense*, have been reported to be very

immunogenic in chickens as evidenced by antibodies with high signals in ELISA (Eysen, 2014). The chicken anti-trypanosomal antibodies produced in the study conducted by Eysen (2014) were used 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 immunodiagnosics is through identification of single chain variable fragments (scFv) of antibodies that recognise the diagnostic antigen of interest. Phage display technology is used to this end (Barbas, 2001).

Several components of an antigen ELISA can be packaged into a convenient, rapid diagnostic test (RDT) that in turn uses the process of chromatography that is especially suited to simple and rapid diagnosis (Figure 1.6) (Nguyen *et al.*, 2014).



**Figure 1.6: Example of a rapid diagnostic test for point-of-care use.** (A) Blood serum from an uninfected cow. No signal on Tv indicator and Tc indicator, but valid because of control line validation. (B) Blood serum from *T. theiliera* infected cow. No signal Tv indicator and Tc indicator (C) Blood serum from a *T. congolense*-infected cow. The positive signal on Tc line and no for Tv line. (D) Blood serum from *T. vivax*-infected cow. A positive signal from Tv indicator and no Tc indicator. (E) Blood serum from a cow with a mixed-infection of *T. congolense* and *T. vivax*. Positive on both Tc and Tv indicators. (Boulangé *et al.*, 2017).

The RDT is made-up of a lateral test flow strip encased within a plastic cassette. The strip itself is made-up of several components namely, a polyester conjugate pad, a whole blood separator sample pad, a nitrocellulose membrane and a paper adsorbent pad. In terms of the RDT shown in Figure 1.6, the recombinant antigens *TviGM6* (Tv test line, 1.2 mg/ml) and *TcoCB1* (Tc test line 0.65 mg/ml) and polyclonal anti-rabbit goat IgG (control line) were coated onto the nitrocellulose membrane. The gold conjugate used for detection was manufactured by adsorbing the recombinant antigens *TviGM6* and *TcoCB1* together with the rabbit IgG onto

gold nanoparticles. The nanoparticles were then coated onto the conjugate pad part of the lateral flow strip. This design allows for the testing of either blood or serum samples (Boulangé *et al.*, 2017). A test sample can then be applied to the RDT sample well with a drop of PBS buffer. The result develops within 10 min of incubation, if only the control line is made visible while the other test lines remain unchanged then the result is negative. If a line appeared for either the Tc (*TcoCB1*) or Tv (*TviGM6*) region in addition to the test line then the result was positive. If the control line wasn't visible after a test, then the test was invalid (Boulangé *et al.*, 2017).

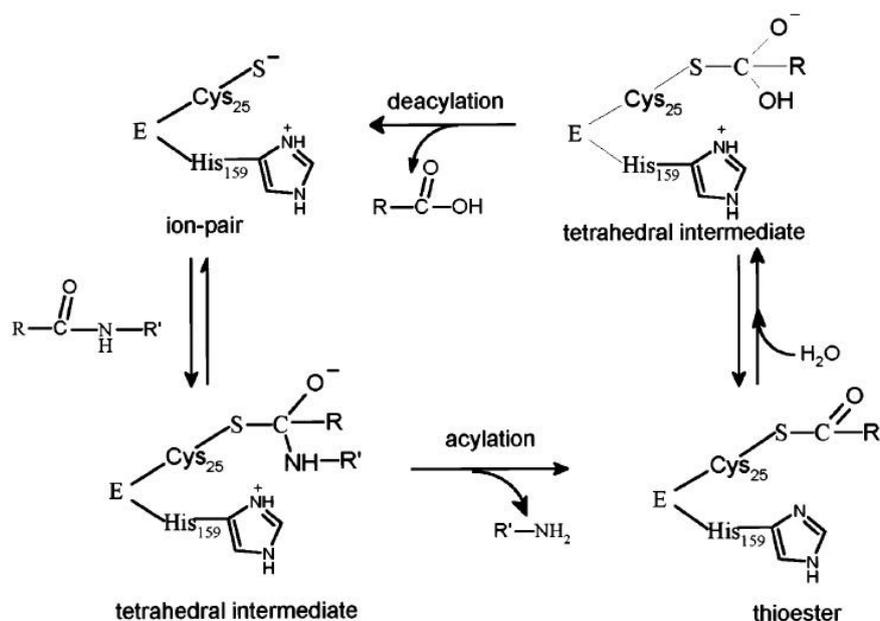
### **1.3 Peptidases**

Peptidases are enzymes that cleave peptide bonds in polypeptides (Barrett *et al.*, 2012) and either have amino or carboxy peptidase or endopeptidase activity (McKerrow *et al.*, 2006). Seven classes of peptidases have been discovered namely metallo, cysteine, aspartic, serine, threonine, glutamate and asparagine catalytic types (Barrett *et al.*, 2012). The clans, within these classes, usually have similar tertiary structures based on their evolutionary relationships (Caffrey and Steverding, 2009).

#### **1.3.1 Cysteine Peptidases**

##### **1.3.1.1 Classification of cysteine peptidases**

Cysteine peptidases are classified into clans, which are then differentiated into families based on sequence similarity, possession of inserted peptide loops and biochemical specificity to peptide substrates (Rawlings *et al.*, 2009). Cysteine peptidases contain a thiol group on the catalytic cysteine residue which is usually deprotonated by the histidine side-chain. The anionic sulfur atom is then capable of a nucleophilic attack on the carbonyl carbon of the peptide bond, forming a tetrahedral intermediate. The tetrahedral intermediate then undergoes acylation forming an enzyme-substrate thiol ester and releasing the C-terminal portion of the substrate. The enzyme-substrate thiol-ester is hydrolysed by water to form a second tetrahedral intermediate which undergoes deacylation releasing the N-terminal part of the substrate from the enzyme (Figure 1.7) (Rawlings *et al.*, 2009).

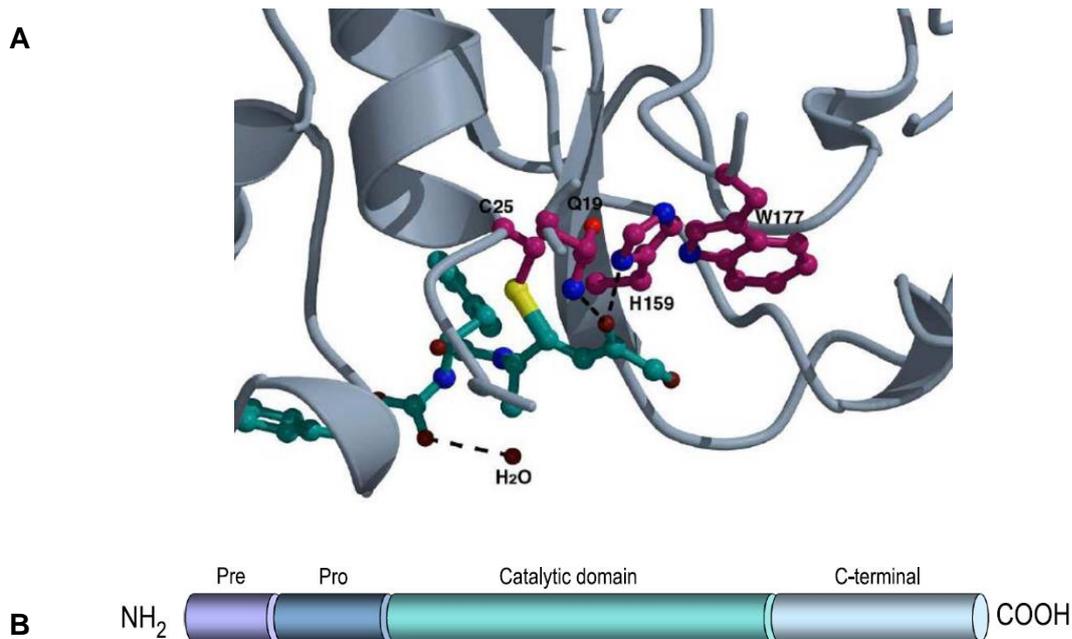


**Figure 1.7: Proposed reaction mechanism for papain-like cysteine peptidases.** These peptidases contain a nucleophilic cysteine thiol in their catalytic triad (Lecaille *et al.*, 2002).

Clan CA is characterised by a catalytic triad containing Cys<sup>25</sup>, His<sup>159</sup> and Asn<sup>175</sup> (papain numbering) (Barrett *et al.*, 2012). All clan CA peptidases are inhibited by trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64) and have a substrate specificity determined by the S<sub>2</sub> pocket (Barrett and Kirschke, 1981; Barrett *et al.*, 1982). Clan CA peptidases are either targeted to intracellular vesicular compartments or are secreted (Barrett and Kirschke, 1981). Family C1 of Clan CA contains papain family cysteine peptidases, including mammalian cathepsins B and L, and parasite peptidases: falcipains from *P. falciparum*, cysteine peptidase A, cysteine peptidase B and cysteine peptidase C from *Leishmania mexicana*, TcoCATL from *T. congolense* and TviCATL from *T. vivax* as well as TcrCATL from *T. cruzi* (Authié *et al.*, 1992; Cazzulo *et al.*, 2001; Grams *et al.*, 2001; Zadeh-Vakili *et al.*, 2004; Kumar *et al.*, 2007; Beckham *et al.*, 2009; Rawlings *et al.*, 2009). Family C2 of Clan CA contain the calpain-like cysteine peptidases which are calcium-dependent cytosolic cysteine peptidases which lack a signal sequence but possess a calcium binding domain (McKerrow *et al.*, 2006).

### 1.3.1.2 Cathepsin L-like cysteine peptidases

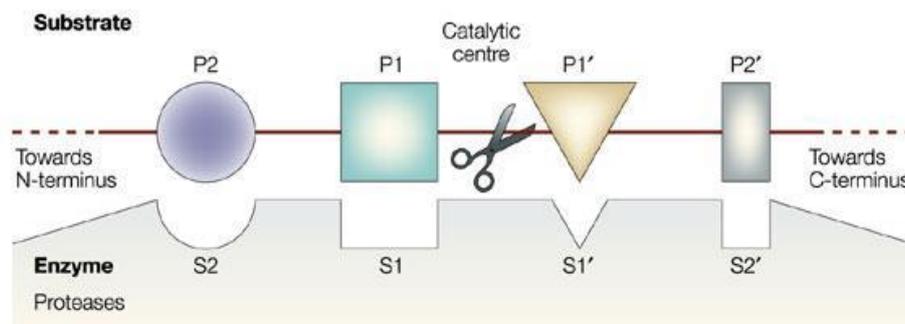
Cathepsin L-like peptidases (CATL) have structural features similar to most papain-like peptidases (Barrett *et al.*, 2012). The structure of CATL peptidases, including TcrCATL and TcoCATL homologues fold into two domains, an  $\alpha$ -helical L-domain and an R-domain rich in antiparallel sheets forming a  $\beta$ -barrel-like motif (Figure 1.8, A) (Lecaille, Cotton, *et al.*, 2001).



**Figure 1.8: Illustrations of *TcrCATL* interaction with an inhibitor. (A)** Amino acid side-chains involved in binding are highlighted in magenta. Hydrogen bonds are indicated by dashed lines (Choe *et al.*, 2005). **(B)** Schematic representation of the CATL peptidase from N to C terminus. The catalytic domain is flanked by the pro-region and the C-terminal domain, thus forming a zymogen (Hoelz *et al.*, 2016).

CATL peptidases are synthesised as inactive zymogens containing the pro-region of the pro-peptide (Figure 1.8, B). The zymogen is activated to the mature peptidase by cleavage of the pro-peptide in the acidic milieu of the endosome. The synthesis of cysteine peptidases as inactive precursors has many benefits. These include pro-regions acting as intramolecular chaperones to enhance protein folding, regulate peptidase activity by blocking the active site, and as a signal that directs peptidases to appropriate intracellular compartments (Lalmanach *et al.*, 1998). The propeptide contains a highly conserved helical ERFNIN motif found in all cathepsin L-like cysteine peptidases. This motif is thought to be crucial for trafficking into lysosomes and maturation of cysteine peptidases (Karrer *et al.*, 1993). The trypanosomal cysteine peptidases such as *TcrCATL* and *TcoCATL* contain a C-terminal extension which is not present in mammalian CATL peptidases (Åslund *et al.*, 1991; Rosenthal, 1999). The C-terminal extension of *TcoCATL* is linked to a catalytic domain by a proline-rich hinge region while that of *TcrCATL* is linked by a threonine-rich hinge region (Lalmanach *et al.*, 1998). The presence of the C-terminal extension in most trypanosomal CATLs confers relatively high antigenicity and immunogenicity (Cazzulo and Frasch, 1992). The C-terminal extension of plant cysteine peptidases was shown to play a fundamental role in zymogen activation, catalytic activity, folding and stability of the peptidase (Dutta *et al.*, 2011).

The substrate specifically binds to the active site cleft which contains subsites (S) each limited to bind only one specific amino acid residue of the peptide substrate (P). The peptidase active N-terminal (...S<sub>3</sub>-S<sub>2</sub>-S<sub>1</sub>) and primed C-terminal subsites (S<sub>1</sub>', S<sub>2</sub>', S<sub>3</sub>') is complementary to peptide substrate amino acid residues (...P<sub>3</sub>-P<sub>2</sub>-P<sub>1</sub>) and primed peptide substrate residues (P<sub>1</sub>', P<sub>2</sub>', P<sub>3</sub>'), respectively (Figure 1.9) (Schechter and Berger, 1967). The P<sub>2</sub> residue which is complementary to the active site S<sub>2</sub> pocket exhibits a very pronounced interaction with both L and R domains of cathepsins (Sajid and McKerrow, 2002; Turk *et al.*, 2012). Therefore, the substrate specificity of most papain-like peptidases is determined by the S<sub>2</sub> pocket. The trypanosomal peptidases including *TcrCATL* and *TcoCATL* prefer bulky hydrophobic amino acids such as phenylalanine in P<sub>2</sub> (Eakin *et al.*, 1992; Elaine *et al.*, 1997; Pillay *et al.*, 2010). *TcrCATL* exhibits a dual specificity for both basic (Arg) and hydrophobic residues (Phe) at the P<sub>2</sub> position. This is attributed to the presence of Glu<sup>205</sup> at S<sub>2</sub> of *TcrCATL* which is responsible for adjusting the conformation of the S<sub>2</sub> pocket in such a way that it is flexible to accommodate both hydrophobic and basic residues (Gillmor *et al.*, 1997). Conversely, *TcoCATL* exhibits restricted specificity for Phe residues at P<sub>2</sub>. This is because, at the bottom of the S<sub>2</sub> pocket of *TcoCATL*, there is Leu<sup>205</sup> instead of Glu<sup>205</sup>, which restricts the binding capacity of the S<sub>2</sub> pocket (Chagas *et al.*, 1997; Gillmor *et al.*, 1997). It has also been reported that the presence of conformationally constrained substrate analogues results in incorrect binding into the S<sub>2</sub> subsite of *TcoCATL* and this suggests that the hydrophobic Phe group is crucial for efficient binding and hydrolysis of the substrate (Lecaille, Authié, *et al.*, 2001).



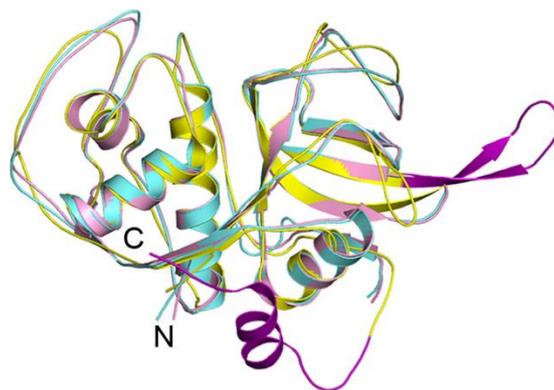
**Figure 1.9: The Schechter and Berger schematic representation of the interaction of the peptide substrate with the active site of cysteine peptidase.** Peptide (P) substrate with its N- and C-terminus indicated, interacts with peptidase sub-sites (S). The peptidase substrates are complementary to the sub-sites of the peptidases, and this allows correct sub-site fitting. The residue in the P<sub>2</sub> position is strictly bulky hydrophobic in the case *TcrCATL*. Sulfhydryl (SH) of the active site cysteine attacks the peptide bond during hydrolysis. [http://www.nature.com/horizon/peptases/background/figs/perspective\\_f1.html](http://www.nature.com/horizon/peptases/background/figs/perspective_f1.html) (Accessed 27 October 2016).

The cathepsin L-like peptidases *TbbCATL* (Mackey *et al.*, 2004), *TcoCATL* (Lalmanach *et al.*, 2002) and *TbrCATL* (Kerr *et al.*, 2010) are expressed throughout each of the parasites' four life cycle stages, but most significantly in the bloodstream form stage where it is localised in

the lysosomes (Mbawa *et al.*, 1991; Scory *et al.*, 1999; Caffrey *et al.*, 2001). The antigens used for serodiagnosis need to be expressed in the bloodstream form, and therefore the CATL antigens are potential diagnostic antigens.

Many isoforms of *Tco*CATL exist (Pillay *et al.*, 2010) and several isoforms are also observed in the CATL peptidases from *L. mexicana* and *T. cruzi* (Mottram *et al.*, 1997; Lima *et al.*, 2001). *Tcr*CATL is the major CATL lysosomal peptidase present in the *T. cruzi* parasite and is involved in the invasion process of host smooth muscle and epithelial cells (Aparicio *et al.*, 2004; Yoshida, 2006). It was suggested that *Tcr*CATL might be involved in immune system evasion by digesting the hinge region of human IgG subclasses (Berasain *et al.*, 2003). It was further suggested that *Tcr*CATL, *Tcr*OPB and metacyclic surface glycoprotein (gp82) play a vital role in the mobilisation of  $Ca^{2+}$  from intracellular stores, leading to host cell invasion by *T. cruzi* parasites (Burleigh *et al.*, 1997; Caler *et al.*, 1998).

The 3D structures of only two kinetoplastid CATLs have been solved to date in the presence of different substrates and inhibitors, namely those from *T. cruzi* and *T. b. rhodesiense* (Figure 1.10).



**Figure 1.10: The structures of *Tcr*CATL, *Tbr*CATL and *Pf*CATL3.** Ribbon representation and superimposition of *Tcr*CATL (pink), *Tbr*CATL (aquamarine) and *Pf*CATL3 (yellow) are shown. Insertions in *Pf*CATL3 are coloured purple (Kerr *et al.*, 2010).

The Tropical Disease Research (TDR) targets database (<http://tdrtargets.org/>) lists *Tcr*CATL and *Tbr*CATL as druggable targets, and these peptidases are also thought to be potential diagnostic antigens (Boulangé *et al.*, 2017). *Tbr*CATL, the CATL from *T. b. rhodesiense* was thought to have diagnostic potential due to the fact that *Tcr*CATL was a diagnostic antigen and that it was highly expressed in bloodstream form parasites but was not recognised in infected human sera (Manful *et al.*, 2010).

*Tvi*CATL, (CCD21670.1), *Tco*CATL, (CAA81061.1) and *Tcr*CATL (P25779.1) share a 78%, 72% and 71% sequence identity to *Tbr*CATL, (CAC67416.1) respectively (Figure 1.11) and thus should possess similar diagnostic and chemotherapeutic potential.

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TcoCATL ----MTRL--RFSVGLLVAACFPVALGVLHAEQSLQQQFAAFKQKYSRSYKDATEE 53
TviCATL -----MHA--HALVTLAAAVSVAPAAMAVLRADGPVEPLFAAFKQKYGSRYSYGTAAEE 51
TbrCATL MPRTMVRV--RFPVLLAMAACLASVALGSLHVEESLEMRFAAFKQKYGKVKYKDAKEE 58
TcrCATL ----MSGWARALLAAVLVVMACLVPAATASLHABETLTSQFAEFKQKHGRVYESAABE 55
      .:* . . . . . * . * . : : * * * : . : * * * *
TcoCATL AFRFRVFKQNERAKEEAAAANPYATFGVTRFSDMSPEEFRATYHNGAEYAAALKRPRKV 113
TviCATL AFRLRVFEDNMRRSRMYAAANPHATFGVTPFSDLTPEEFRTRYHNGERHFEAARGRVRTL 111
TbrCATL AFRRAFEENMEQAKIQAAANPYATFGVTPFSDMTREEFRARYRNGASYFAAAQKRLRKT 118
TcrCATL AFRLSVFRLENFLARLHAAANPHATFGVTPFSDLTREEFRSRYHNGAAHFPAQAQERARV 115
      * * : . * : : : * * * : * * * * * : * * * : * * * *
TcoCATL VNVSTGKAPPAVDNRKKGAVTPVKDQGCAGSCWAFSAIGNIEGQWVAGHELTSLSQML 179
TviCATL VQVPPGKAPAAVDWRRKGAFTPVKDGTCGSCWFSFAIGNIEGQWAAAGNPLTSLSEQML 177
TbrCATL VNVITGRAPAAVDWREKGAFTPVKDGQCGSCWAFSTIGNIEGQWVAGNPLVSLSEQML 184
TcrCATL VKVEVVGAPAAVDWRARGAVTAVKDGQCGSCWAFSAIGNVEQWFLAGHPLTNLSEQML 181
      * : * * * * * : * * * * * * * * * * * * * * * * * * * * * *
TcoCATL VSCDTTDYGCRGGLMDKSLQWIVSSNKGNVFTAQSYFYASGGKMPPCNKSGKVVGAKIS 233
TviCATL VSCDTKNGCGGGLMDNAFEWIVKENSQKVTYKSYFYVSGGEEPCKPRGHKVGATIT 231
TbrCATL VSCDTIDFGCGGGLMDNAFNWIVNSNGGNVFEASYFYVSGNGEQPQCQMGHEIGAAIT 238
TcrCATL VSCDKTDSGCGGLMNAFEWIVQENNGAVYTEDSYFYASGEGISPPCTTSGHTVGATIT 235
      * * * * * * * * * * * * * * * * * * * * * * * * * * * *
TcoCATL GHINLPKDENAIAEWLAKNGPVAIAVDATSFYLYGKGVLTSCISKGLDHDVLLVGYDDTS 293
TviCATL GHVDIPHDEDAIAKYLADNGPVAIVAVDATTMSYSGGVVTSCTSEALNHGVLLVGYNDSS 291
TbrCATL DHVDLPQDEDAIAAYLAENGLAIAVDATSFMDYNGGILTSCTSEQLDHGVLLVGYNDSS 298
TcrCATL GHVELPQDEAQIAAWLAVNGPVAIVAVDASSWMTYTGGMVTSQVSEQLDHGVLLVGYNDSA 295
      * : : * * * * * * * * * * * * * * * * * * * * * * * * * *
TcoCATL KPPYWIINKNSWSKGWGEEGYIRIEKGTNQLMKNYARSAVVSGPP---PPPP----- 339
TviCATL KPPYWIINKNSWSSWGEKGYIRIEKGTNQLVAQLASSAVVGGPPTPTPTPT----- 337
TbrCATL NPPYWIINKNSWSNMWGEDGYIRIEKGTNQLMNQAVSSAVVGGPTPPPPPP----- 347
TcrCATL AVPYWIINKNSWTTQWGEEGYIRIAKGSNQLVKEEASSAVVGGPGPTPEPTTTTTSAPG 344
      * * * * * * * * * * * * * * * * * * * * * * * * * * * *
TcoCATL PPASTPTQEFCEGAEQSGCTKATFPTGKCVQFGGAGSVIASCGSNLTIQIVYPLSSSCS 402
TviCATL TTNNNNNND-----RTWPIVKLHEDA-----LQR----- 368
TbrCATL PPSATPTQDFCEGKCTKGC SHATFPTGECVQTTGVSVIATCGASNLTIQIYPLSRSCS 410
TcrCATL PPSYFVQMSCTDAACIVGCENVLPTGQCLITTSQVSAIVTCGAETLTEEVFLTSTHCS 415
      : * * : * .
TcoCATL GFSIPLTVPLDKCLPIVVGSMYECGKAPTESARLVRHE----- 442
TviCATL -----
TbrCATL GLSVPITVPLDKCIPILIGSVEYHCSTNPPTKAARLVPHQ----- 450
TcrCATL EFSVRSSVPLNKCNRLLRGSVEFFCGSSSSGRLADVDRQRHQFYHSRHRRI 467

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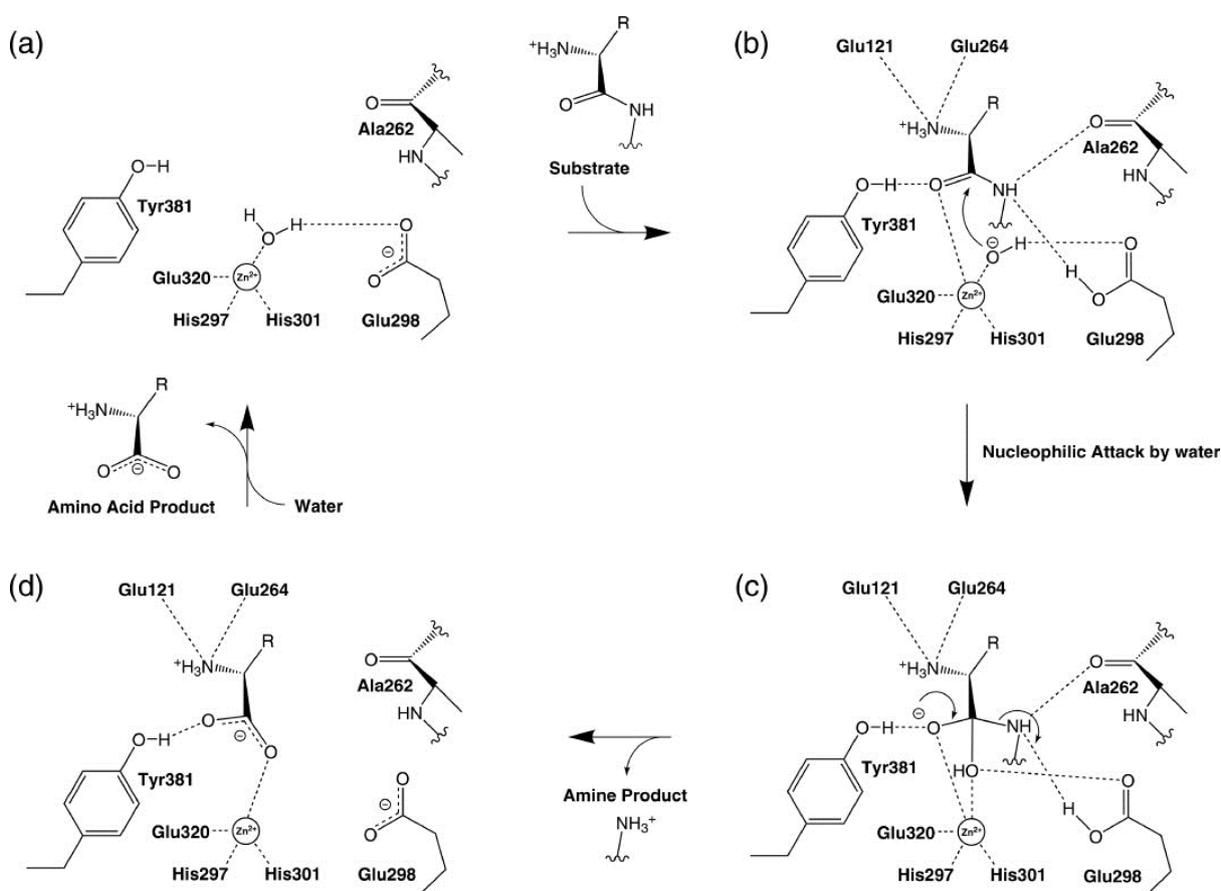
**Figure 1.11: Sequence alignment of the cathepsin-L like peptidases from *T. congolense*, *T. b. rhodesiense*, *T. vivax* and *T. cruzi*.** Multiple sequence alignment of the CA clan, C1 family of cysteine peptidases generated using ClustalΩ (Sievers *et al.*, 2011). Protein sequences were obtained from UniProtKB (<http://www.uniprot.org/help/uniprotkb>, accessed 15/10/2016): *TcoCATL* from *T. congolense*, Q26895, *TbrCATL* from *T. b. rhodesiense*, Q95PM0, *TviCATL* from *T. vivax*, F9WRA9, and *TcrCATL* from *T. cruzi*, P25779. The amino acid residues involved in the signal peptide are highlighted in yellow, those in propeptide in pink, the catalytic domain in blue, the catalytic site I grey, the C-terminal extension in green, and the 18-mer *TcoCATL* N-terminal peptide in red and yellow. Alignment characters are annotated as follows: conserved residues (\*), strongly similar properties (:), and weakly similar properties (.)

### 1.3.2 Metallopeptidases

Metallopeptidases are hydrolases that perform nucleophilic attack of peptide bonds with the help of a water molecule (Barrett *et al.*, 2012). These enzymes need a divalent metal cation, such as manganese, copper or zinc, to activate the water molecule. The metal ion is positioned by three amino acid ligands, namely, Glu<sup>320</sup>, His<sup>297</sup> and His<sup>301</sup> (Barrett *et al.*, 2012).

#### 1.3.2.1 Family M1 reaction mechanism

The best characterised family M1 metallopeptidase is *E. coli* aminopeptidase N (McCaman and Villarejo, 1982; Ito *et al.*, 2006). The reaction mechanism for the exopeptidase activity of this peptidase was proposed by Ito *et al.* (2006), as shown in Figure 1.12.



**Figure 1.12: Proposed reaction mechanism for *E. coli* aminopeptidase N.** It can be inferred that all Family M1 aminopeptidases have a similar mechanism for catalysis (Ito *et al.*, 2006). (a) Free enzyme; (b) Michaelis complex; (c) tetrahedral intermediate; (d) enzyme complexed with the amino acid product.

The residues necessary for catalysis are the Glu<sup>320</sup> of the “HEXXH” motif and an additional Tyr<sup>381</sup>, while the function of the two histidine residues, His<sup>297</sup> and His<sup>301</sup>, is to bind the divalent cation (Zn<sup>2+</sup>). As depicted in Figure 1.12 (a), the first step of catalysis is the hydrogen bonding of Glu<sup>298</sup> to a water molecule. The effect of this bond and the proximity of the zinc ion makes the water molecule strongly nucleophilic. The substrate is brought into the active site forming

a Michaelis complex (Figure 1.12, b) by the interaction of the carbonyl oxygen of the substrate with the hydroxyl group of Tyr<sup>381</sup> and Zn<sup>2+</sup>. At this point, the amine group of the substrate interacts with the hydroxyl group Glu<sup>298</sup>, allowing the formation of the enzyme-substrate complex.

Hydrolysis is initiated by the nucleophilic attack of the water molecule on the carbonyl carbon of the substrate (Figure 1.12, c). The hydroxyl group of Tyr<sup>381</sup> is thought to stabilise this reaction intermediate. Following the nucleophilic attack by the water molecule, the amine group linked to the rest of the peptide substrate is released, leaving the N-terminal amino acid residue linked to the enzyme via the Zn<sup>2+</sup> (Figure 1.12, d). This amino acid product is released when it is replaced by a second water molecule (Ito *et al.*, 2006; Mucha *et al.*, 2010).

### 1.3.2.2 Aminopeptidases

Aminopeptidases constitute a diverse set of exopeptidases with essential roles in a wide range of cellular functions. These enzymes catalyse the removal of amino acids from the N-terminus of polypeptides; the specific residues for which they show an affinity, as well as their structure, determine their subdivision into families (Drinkwater *et al.*, 2017).

Aminopeptidases belong to the clan MA which includes metallopeptidases that bind divalent cations (Figure 1.12). Within clan MA, aminopeptidase is found in families M1 and M17 (Rawlings *et al.*, 2009). Aminopeptidases all possess the conserved exopeptidase motif "GXMEN" which distinguishes them from the closely related leukotriene A4 hydrolases, which are also found in family M1 (Barrett and Rawlings, 2007). The most pertinent difference between the two families is the conserved metallopeptidase motif "HEXXH", absent in family M17 aminopeptidases, but present in family M1 aminopeptidases (Mucha *et al.*, 2010). The Glu of the motif and a Tyr residue are thought to be necessary for catalysis (Hooper, 1994). Also, aminopeptidases belonging to family M1 (Figure 1.13), bind a single divalent cation, while M17 aminopeptidases bind two. M17 aminopeptidases are only active as homo-hexamers whereas family M1 aminopeptidases are active as monomers (Matsui *et al.*, 2006). Sub-classification of aminopeptidases is based primarily on the number of amino acids cleaved from the N-terminus of substrates. Aminopeptidases cleave a single amino acid from the N-terminus while diaminopeptidases or aminodipeptidases remove a dipeptide from the N-terminus (Barrett *et al.*, 2012).

The classification of aminopeptidases is also dependent on the relative efficiency by which the enzyme removes different amino acids from the N-terminus. To date, leucine aminopeptidases, belonging primarily to family M17, have been prominently discussed, but argininy-, methionyl-, aspartyl-, alanyl- (mostly family M1), glutamyl-, prolyl-, and cysteinyl-aminopeptidases have also been described (Rawlings *et al.*, 2009). However, nomenclature

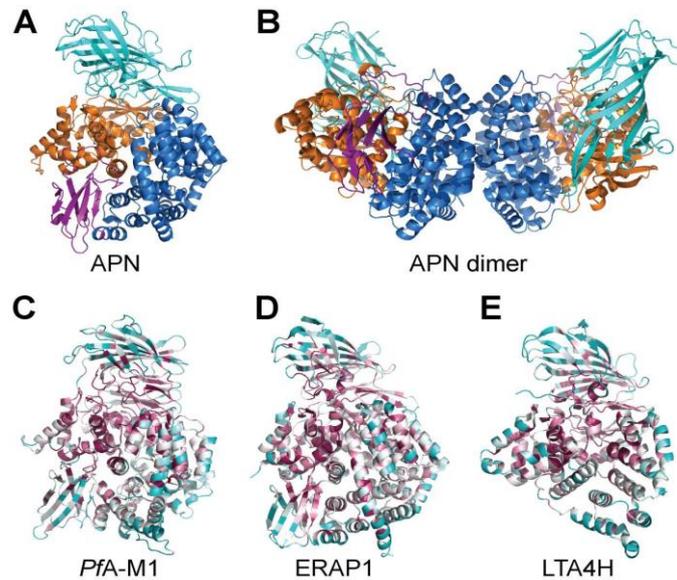
such as “leucine aminopeptidase” may be misleading since the specificity of the enzyme *in vitro* is usually determined using single amino acid synthetic substrates (e.g. H-Leu-AMC) which differ from more physiologically relevant substrates, such as peptide hormones including angiotensins (Taylor, 1993; Matsui *et al.*, 2006; Rawlings *et al.*, 2009). Also, although the metal ion used in activity assays appears to affect substrate preferences, different protocols use a variety of divalent cations, all of which have different effects on the catalytic activity of aminopeptidases (Taylor, 1993).

Aminopeptidases may also be classified based on the location of the enzyme within the cell. Some aminopeptidases are secreted, but most tend to either be membrane-bound (usually family M1) or cytosolic enzymes (usually family M17). Also, aminopeptidases may be localised to a single type of organelle, such as lysosomes, nuclei or mitochondria (Barrett *et al.*, 2012). Membrane-bound aminopeptidases and methionine specific aminopeptidases are both redundantly referred to as aminopeptidase M. Therefore; it is possible for structurally unrelated enzymes to have the same name and be located in different locales of the cell (Barrett *et al.*, 2012). Further distinctions between aminopeptidases may be made on the basis of their relative inhibition by bestatin or puromycin; metal ion co-factor and pH of optimal activity (Taylor, 1993). Aminopeptidases are inhibited by bestatin, amastatin and metal-chelating agents such as EDTA and 1,10 phenanthroline (Mucha *et al.*, 2010). Puromycin specifically inhibits M1 aminopeptidases and has been used to tell between cytosolic aminopeptidases and membrane-bound aminopeptidases which both belong to the family M1 (Gros *et al.*, 1985).

The large family of M1 aminopeptidases (clan MA, family M1) are zinc-dependent enzymes characterised by the presence of two conserved, catalytic sequence motifs; a consensus zinc-binding motif (HEXXH-(X<sub>18</sub>)-E) and the ‘GXMEN’ exopeptidase motif (Barrett *et al.*, 2012). Excluding viruses, M1 aminopeptidases are distributed throughout all phyla and have been implicated in a wide range of functions including cell maintenance, growth and development and defence. Their wide range of essential functions in combination with their presence in such a wide distribution of organisms shows the importance of the family and has led many research groups to assess their utility as therapeutic targets for both infectious and chronic diseases of humans and other animals.

Many parasite aminopeptidases have been investigated, including the aminopeptidases of the protozoan parasites *Plasmodium falciparum* (Mistry *et al.*, 2014), *P. vivax* (Kang *et al.*, 2015), *T. b. brucei* (Timm *et al.*, 2017), *T. cruzi* (Cadavid-Restrepo *et al.*, 2011), the parasitic flukes *Schistosoma mansoni* (Maggioli *et al.*, 2017), and *Fasciola hepatica* (Maggioli *et al.*, 2011);

the nematodes *Heterodera glycines* (Masler, 2004). A summary of the recently investigated parasite aminopeptidases is shown in Table 3.1.



**Figure 1.13: Structure and conservation of M1 aminopeptidases.** (A) X-ray crystal structure of human aminopeptidase N coloured based on domains with domain I in teal, domain II in orange, domain III in magenta, and domain IV in blue. (B) Crystallographically determined dimer of aminopeptidase N (APN) which is said to occur on the cell surface via its attachment to the domain I (teal). (C-E) Characteristic structures of M1 aminopeptidases coloured according to sequence conservation (high conservation is purple, the average is white and low is cyan). Sequence alignments and conservation calculations performed using ConSurf <http://consurf.tau.ac.il/2016/credits.php>: (C) the M1 aminopeptidase from *P. falciparum* (PfA-M1), (D) human endoplasmic reticulum aminopeptidase 1 (ERAP1), (E) human leukotriene A4 hydrolase (LTA4H). (Drinkwater *et al.*, 2017).

The main role proposed for these aminopeptidases in *P. falciparum* and *S. mansoni* is the proteolysis of ingested haemoglobin into single amino acids, which are subsequently used for necessary metabolic processes of the parasite (Stack *et al.*, 2007; Song *et al.*, 2008). Given that these enzymes seem to play an essential role in parasite development, aminopeptidase inhibitors seem to be effective in decreasing the growth of various intracellular parasites *in vitro*, most notably *Leishmania spp.*, and *P. falciparum* as well as the extracellular *T. b. brucei* (Knowles, 1993; Flipo *et al.*, 2007). The most promising of these studies resulted in the identification of a compound based on a malonic hydroxamic template, which has proven to be a selective inhibitor of PfA-M1, the family M1 aminopeptidase of *P. falciparum*. The inhibitor has been shown to reduce the growth of parasites *in vitro* and has good physicochemical and pharmino-kinetic properties (Flipo *et al.*, 2007; Skinner-Adams *et al.*, 2010).

There have been several attempts to use aminopeptidases as vaccines against parasitic diseases, most notably for fascioliasis and haemonchosis (Piacenza *et al.*, 1999; Reszka *et al.*, 2007; Acosta *et al.*, 2008). Interestingly, the fascioliasis vaccination included two cathepsin L-like cysteine peptidases together with the leucyl aminopeptidase of *F. hepatica*. The group

of animals receiving the mix of all three enzymes was significantly protected (78%) while the best level of protection (89%) was achieved by vaccination with the leucyl aminopeptidase alone (Piacenza *et al.*, 1999). In addition, the leucyl aminopeptidase of *F. hepatica* has been shown to be an immunodominant antigen and could potentially be used for the serological diagnosis of fascioliasis in humans (Marcilla *et al.*, 2008).

**Table 1.3: Summary of several characterised parasite aminopeptidases.**

| Species                       | Family | Substrate specificities | References                              |
|-------------------------------|--------|-------------------------|---|
| <i>Babesia bovis</i>          | MAP    | H-Met-AMC               | (Munkhjargal <i>et al.</i> , 2016)      |
| <i>Clonorchis sinensis</i>    | M17    | H-Leu-AMC               | (Kang <i>et al.</i> , 2012)             |
| <i>Cryptosporidium parvum</i> | M17    | H-Leu-AMC               | (Kang <i>et al.</i> , 2011)             |
| <i>Eimeria tenella</i>        | M1     | H-Ala-AMC<br>H-Arg-AMC  | (Gras <i>et al.</i> , 2014)             |
| <i>Plasmodium falciparum</i>  | M1     | H-Ala-AMC<br>H-Met-AMC  | (Poreba <i>et al.</i> , 2012)           |
| <i>Trypanosoma cruzi</i>      | M17    | H-Leu-AMC               | (Cadavid-Restrepo <i>et al.</i> , 2011) |

## 1.4 Recombinant Protein Expression

In recent years, much research and resources have been put into the development and improvement of large-scale recombinant protein production in biotechnology. Diagnostic agents, vaccines, growth factors and hormones depend on recombinant technology for their large-scale production (Mogk *et al.*, 2002). Much effort is invested in developing methods to produce the maximum yield of a properly folded and active protein. However, the recombinant overproduction of proteins in various heterologous expression systems often results in recombinant protein misfolding and aggregation (Kiefhaber *et al.*, 1991; Stegemann *et al.*, 2005). Prokaryotic systems, such as *E. coli*, are the principal hosts for recombinant expression since they are well understood compared to yeast and mammalian cell systems and therefore more easily genetically engineered and have the potential to produce large quantities of recombinant protein inexpensively (Mogk *et al.*, 2002).

### 1.4.1 *E. coli* host expression system

The advantages of the *E. coli* expression system include: easy cell growth, using relatively simple and widely available laboratory equipment; the genotypes and phenotypes of *E. coli* are well-known; it is the cheapest option; recombinant gene expression can be achieved from

cloning to pure protein product in as little as two weeks and the resources for optimising expression such as vectors and host strains are easily accessible (Fakruddin *et al.*, 2012).

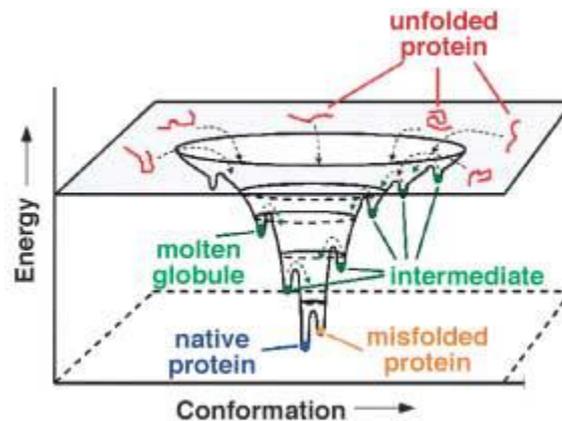
The main disadvantage of using *E. coli* as an expression host is the lack of protein biosynthesis machinery for the expression of eukaryotic proteins since *E. coli* is a prokaryote. Consequently, post-translational modifications such as glycosylation and eukaryote-specific codon sequences which are critical for proper folding of eukaryote-sourced proteins are missing in *E. coli* (Brown, 1995).

#### **1.4.1.1 Protein folding inside the *E. coli* cell**

Initially protein folding was thought to go through specific folding pathways to be transformed into the native structure speedily. However, proteins have been observed to achieve the correct conformation from various unfolded states. The mechanism of protein folding without a catalyst is presented in Figure 1.14. The native protein form represents the desired least-energy state form of the protein at the bottom of the funnel. Every other non-native form of a protein is at a higher energy state. Protein folding can therefore be imagined as protein striving to reach the lowest energy state as it “rolls” down the folding funnel. The folding funnel represents the various protein folding pathways a protein can go through from an unfolded state to the native minimum energy state which can progress fast or slow depending on the pathway followed (Schultz, 2000). Protein folding is an error-prone process, especially for large proteins. Over time incompletely folded protein intermediates with their exposed hydrophobic amino acid side chains may accumulate and thereby promote aggregation. The higher the concentration of these protein intermediates the higher the likelihood of protein aggregation occurring. Protein intermediates with incorrect disulfide bonds or prolyl peptide bonds are also prone to aggregation. This is circumvented by the expression of disulfide isomerase and peptidyl prolyl isomerase, responsible for the respective bond formation in the correct conformation, by the cell where folding is occurring (Schiene and Fischer, 2000).

Protein folding within cells is much more complex than in *in vitro* folding experiments. The biosynthesis of proteins occurs at the ribosomes from the N to the C-terminus in a vectoral manner. In *in vitro* experiments, protein folding is performed within diluted protein solutions and complete polypeptide chains, whereas *in vivo* the nascent polypeptides produced by the ribosome do not contain all the information necessary for folding (Mogk *et al.*, 2002). The highly dense environment of the cytosol increases the probability of nascent polypeptides and protein intermediates inappropriately interacting by hydrophobic interaction leading to misfolding and aggregation. Therefore, nascent polypeptides are required to fold as soon as possible, while proteins destined for other localisations should be translocated in an unfolded state. Due to ceaseless thermal atomic vibrations, native conformed proteins could

spontaneously unfold and lose activity, this is due to the conformational flexibility that native proteins have that is essential for protein function (Mogk *et al.*, 2002). Environmental stress conditions, for example a sudden temperature increase, can cause protein unfolding, degradation or aggregation of proteins. Therefore, to optimise cellular protein folding, protective systems have evolved over time consisting of families of highly conserved proteins named molecular chaperones (Mogk *et al.*, 2002).



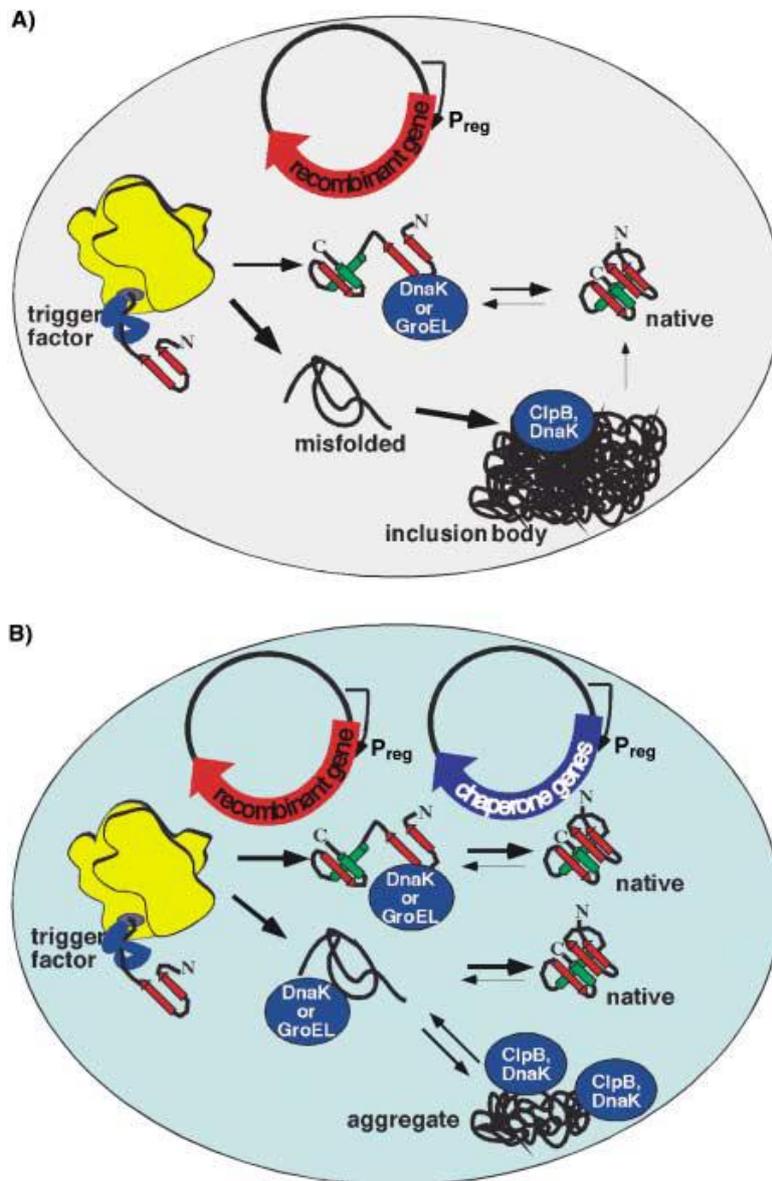
**Figure 1.14: Protein folding energy funnel.** In this representation, the “funnel” contains many local energy minima, in which a protein can settle during the folding process. Some minima are folding intermediates with native like structures whereas others are more misfolded intermediates (Schultz, 2000).

#### 1.4.1.2 *E. coli* molecular chaperone co-overexpression

Recombinant protein misfolding can be attributed to the limited availability of molecular chaperones of the *E. coli* host. The use of molecular chaperones is one of the standard approaches to improving the soluble recombinant expression of proteins in *E. coli* (Shuo-shuo *et al.*, 2011). Molecular chaperones are vital in the correct folding of newly synthesised proteins to their active state and also maintain the three dimensional structure of proteins by refolding misfolded and aggregated proteins, as shown in Figure 1.15 (de Marco *et al.*, 2007). The Hsp70 system (DnaK with co-chaperones DnaJ and GrpE), Hsp60 system (GroEL with GroES co-chaperone) and the ribosome-associated trigger factor are proposed to be the most vital chaperones for protein folding in *E. coli* (Hartl and Hayer-Hartl, 2002). Before molecular chaperone studies were common, it was thought that protein aggregation was an unsurmountable problem in recombinant expression systems. However, it has been demonstrated several times that protein aggregates can be disaggregated and refolded to their native state (Mogk *et al.*, 1999).

In one comprehensive study of molecular chaperone co-expression, the co-overproduction of the main cytosolic molecular chaperones in *E. coli* cells combined with disrupting ongoing *de*

*novo* protein synthesis in a two-step procedure resulted in improved solubility of 45 out of the 64 recombinant proteins that were assayed (de Marco *et al.*, 2007).



**Figure 1.15: Recombinant expression of recombinant protein (A) with and (B) without recombinant molecular chaperone co-expression.** (A) With insufficient amounts of molecular chaperones the probability of inclusion body formation is high whereas (B) recombinant co-overexpression of molecular chaperones together with recombinant protein increases levels of correctly folded recombinant protein (Mogk *et al.*, 2002).

A myriad of recombinant proteins has been co-expressed with recombinantly over-expressed molecular chaperones in the hopes of improving solubility and correct folding of the desired recombinant protein (Table 1.4).

**Table 1.4: Summary of several recombinant molecular chaperone co-expression studies.** Adapted from Betiku (2006)/

| Molecular chaperone            | Protein substrate                                   | Effect on solubility   | References                       |
|--------------------------------|---|--|----------------------------------|
| DnaK-DnaJ-GrpE                 | Human growth factor                                 | Co-expression inhibits inclusion body formation and improves recombinant protein solubility from 5% to >85% of total recombinant protein | (Blum <i>et al.</i> , 1992)      |
| GroEL-GroES                    | Protein-tyrosine kinase P <sup>50csk</sup>          | More than 50% of the total recombinant protein is soluble following co-expression  | (Amrein <i>et al.</i> , 1995)    |
| Trigger factor                 | Porcine epidemic diarrhoea virus spike glycoprotein | Soluble recombinant fragment of up to 7.5 mg/l was produced upon co-expression   | (Piao <i>et al.</i> , 2016)      |
| GroEL-GroES and trigger factor | Oxygen-regulated protein (ORP) 150                  | Approximately 86% of total recombinant ORP150 is soluble   | (Nishihara <i>et al.</i> , 1998) |
|                                | CD137 ligand  | A yield of 3 mg/L of pure soluble recombinant protein was obtained after co-expression   | (Wang <i>et al.</i> , 2012)      |
| GroEL-GroES and DnaK-DnaJ-GrpE | Lipase-948  | Co-expression resulted in soluble recombinant protein of 19.8% of total  | (Shuo-shuo <i>et al.</i> , 2011) |

### **1.4.2 Statistical approaches to soluble recombinant protein expression**

The standard approach to protein expression optimisation is usually performed by measuring one factor at a time to the experiments response while the other factors are kept constant. This approach is known as one factor at a time (OFAT). This approach has a major shortcoming in that it does not allow for the analysis of the interacting effects of the factors being measured. Consequently, factor interactions are undetected which makes this approach time-consuming (Noguère *et al.*, 2012).

A more systematic approach to testing factors has gained popularity in recombinant protein production namely the factorial approach. This approach is defined by the combinations of all states of the experimental factors. The bouquet of techniques that comprise the factorial approach is known as statistical design of experiment and has the advantage of allowing the identification of the crucial factors of any process such as recombinant protein expression (Papaneophytou and Kontopidis, 2014).

Protein expression experiments are often started with limited information on which factors are important for each individual protein. Subsequently, it is advisable to test as many factors as possible. This can be done efficiently by using a two-level full factorial design resulting in a design matrix. The important trait of this design is that the results of each experiment generate information about the interaction and effects of all the factors examined (Papaneophytou and Kontopidis, 2014).

## **1.5 Aims and objectives of current study**

### **1.5.1 General objective**

Three trypanosomal antigens have been identified as potential diagnostic markers for AAT namely, the full-length inactive mutant *T. vivax* cysteine peptidase FL*Tvi*CATL<sub>C25A</sub> and the two *T. congolense* family M1 aminopeptidases, *Tco*AP1 and *Tco*AP2. The overall objective of this study was to optimise standardised protocols for the soluble recombinant production of these antigens in an *E. coli* expression system for their eventual use in the development of point of care serodiagnostic assays that are affordable to end-users.

### **1.5.2 Aims**

1. To investigate the effect of several factors on the soluble recombinant expression of FL*Tvi*CATL<sub>C25A</sub> in the *E. coli* expression system using a statistical approach.
2. To produce antibodies against the FL*Tvi*CATL<sub>C25A</sub> antigen for examination of antigen detection within parasite lysates as a measure of diagnostic potential.

3. To investigate the effect of recombinant co-expression of several molecular chaperone teams with *TcoAP1* and *TcoAP2* on soluble recombinant expression of the *T. congolense* antigens *i.e.* for functional characterisation.

# Chapter 2: RECOMBINANT EXPRESSION OPTIMISATION OF THE *TRYPANOSOMA VIVAX* ANTIGEN FL *TviCATL*<sub>C25A</sub>

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## 2.1 Introduction

*Trypanosoma vivax* is a principal causative agent of trypanosomosis in African livestock (Lalmanach *et al.*, 2002). Control and therapeutic methods for this disease are required due to the negative impact it has on the economy of sub-Saharan Africa (Antoine-Moussiaux *et al.*, 2009). The diagnosis of trypanosomosis is a process that can provide sufficient and accurate information on the disease to allow for appropriate therapeutic measures to be taken (Giordani *et al.*, 2016). A range of diagnostic tests that are used include serological, parasitological, molecular and clinical tests. Diagnostic techniques used should be appropriate for use in the field and for the level of technology available in developing countries (Büscher, 2014).

Clinical diagnosis is dependent on the physical examination of cattle. The major problem with this technique is that the clinical signs are not specific for trypanosomosis (Büscher, 2014). Parasitological techniques involve viewing of wet blood films and Giemsa-stained thick and thin fixed blood films under a light microscope. This technique varies in sensitivity due to similarities in the morphology of trypanosome species (Holmes, 2013). For this reason, a haematocrit centrifuge can be used to increase the sensitivity by separating the buffy coat, containing the trypanosomes, from the blood cells and plasma. The disadvantage of using this technique, especially in the field, is that fresh blood, a haematocrit centrifuge and a microscope are needed (Büscher, 2014). Various PCR assays exist for the diagnosis of AAT and species identification, but none has been validated for diagnostic purposes. In theory, a PCR assay which targets repetitive sequences is more sensitive than those that target single copy or low copy sequences such as those developed for the distinction between *T. b. gambiense* and *T. b. rhodiense*. High specificities have been reported by most PCR studies. However, comparisons of their diagnostic abilities to those of commonly used diagnostic tests were not made (Tiberti *et al.*, 2013). An effective PCR diagnostic assay which used *TviCATL* gene sequences as genetic markers were found to be species-specific when evaluated against other trypanosome species. The problem with performing this technique in the field is that it is costly since it involves the use of specialised equipment by an experienced technician.

The most promising techniques for field use, especially for diagnosing *T. vivax* infections, are serological techniques, such as the enzyme-linked immunosorbent assay (ELISA) (Osório *et al.*, 2008). The advantage of the ELISA technique is that it can be adapted for field use, into

simple to use lateral flow devices which are RDTs. This technique also allows for the processing of a large number of samples due to this adaptation (Büscher, 2014).

There are two types of ELISAs that can be used for diagnosis namely, the antibody-detection ELISA (Boulangé *et al.*, 2017) and the antigen-detection ELISA (Nantulya and Lindqvist, 1989). The low availability of standardised recombinant trypanosome antigens for the detection and production of anti-trypanosome antibodies is a significant problem for both the antibody-detection ELISA and the antigen-detection ELISA. Up until now, ELISAs for both antibody and antigen detection has relied on the crude preparation of whole parasite lysate and native antigens acquired from infected rats; therefore, it is difficult to optimise and standardise the tests, and a large volume of cultured trypanosomes is required (Magez and Radwanska, 2014). Due to this problem, research has increased on the identification and recombinant expression of antigens that show promise for the detection of *T. brucei* (Tran *et al.*, 2008), *T. congolense* (Pillay *et al.*, 2010) and *T. evansi* (Tran *et al.*, 2009) infections in the sera of infected animal species.

There is currently no suitable antigen for the specific diagnosis of *T. vivax* infections (Boulangé *et al.*, 2017). For this reason, this study focussed on optimising the production of a recombinant antigen that could potentially be used to detect *T. vivax* infections. The antigen of concern was based on the *T. vivax* cathepsin L-like peptidase, *Tv*CATL. The single putative full length cathepsin L-like gene (UniProtKB ID: F9WRA9) identified in *T. vivax* strain Y486 (*Tv*CATL) codes for a protein of 454 amino acids in length with an expected molecular weight of 48 kDa and a theoretical pI of 7.51 as predicted by the Compute pI/MW tool on the ExPASy® server (Gasteiger *et al.*, 2005). FL*Tv*CATL<sub>C25A</sub> is the C25A mutation of *Tv*CATL and is also fused to the Trx Tag™ (19 kDa) giving the recombinant protein a total molecular weight of 67 kDa. Previously the inactive full-length form of *Tco*CATL showed relatively improved expression when compared to the active form of the peptidase (Authié, 1994; Pillay *et al.*, 2010; Boulangé *et al.*, 2011). Furthermore, in previous studies, only the catalytic domain of *Tco*CATL was expressed because this too showed better recombinant expression when compared to the full-length active construct of the peptidase (Pillay *et al.*, 2010).

The specificity of antibody detection assays is completely dependent on the homogeneity of the diagnostic antigen. Common antigen preparation protocols used in complement fixation tests (CFT), western blot or ELISA are made up of crude lysates cultured *in vitro* or *in vivo* from the trypanosome that is subject to investigation. Consequently, it is usual for cross-reactivity to occur from other African trypanosome infections and even with non-trypanosome parasites (Büscher, 2014). Due to high cross-reactivity observed between the trypanosome species in ELISA, it is vital to test infected sera using species-specific antigens (Büscher,

2014). Therefore, the production of FL*Tvi*CATL<sub>C25A</sub>, which has the potential to be specific for *T. vivax* infection detection and stable enough to fulfil the ASSURED criteria of lateral flow tests (Vather, 2010), could potentially be used in conjunction with the existing recombinant antigens, such as the full-length inactive mutant of *Tco*CATL, to increase the sensitivity and robustness of the immunoassays.

## 2.2 Materials and methods

### 2.2.1 Materials

**Molecular Biology:** EcoRI, NotI [for nomenclature see (Roberts *et al.*, 2003)], Shrimp alkaline phosphatase (SAP), T4 DNA ligase, O'GeneRuler™ 1 kb DNA Ladder, 10 mM dNTPs, high fidelity PCR enzyme mix, GeneJet™ Plasmid Miniprep Kit, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (IPTG) and the pTZ57R/T vector were obtained from Thermo Scientific (Waltham, MA, USA). The pET32-a expression vector was purchased from Novagen (Darmstadt, Germany) and the pGEX4T-1 expression vector from GE Healthcare (Uppsala, Sweden). FIREpol® Taq polymerase, 10 x PCR reaction buffer and 25 mM MgCl<sub>2</sub> were from Solis Biodyne (Tartu, Estonia). Bacteriological agar, tryptone and yeast extract were purchased from Merck Biolab (Darmstadt, Germany). Crystal violet and ethidium bromide were purchased from Sigma (St. Louis, MO, USA). The ZymoResearch Clean and Concentrator™ kit was obtained from Zymo Research (Orange, CA, USA) and the E.Z.N.A.® gel extraction kit from PEQlab (Erlangen, Germany). Seakem® LE agarose was purchased from Lonza (Rockland, ME, USA) and ampicillin sodium salt from Amresco (Solon, OH, USA).

***E. coli* cells:** Competent *E. coli* cells, JM 109 and BL21 (DE3) strains, were purchased from New England Biolabs (Ipswich, MA, USA). The JM109 *E. coli* permits blue/white screening for transformants in the presence of IPTG and X-gal. The BL21 (DE3) *E. coli* are deficient in both Lon and OmpT protease expression (Miroux and Walker, 1996).

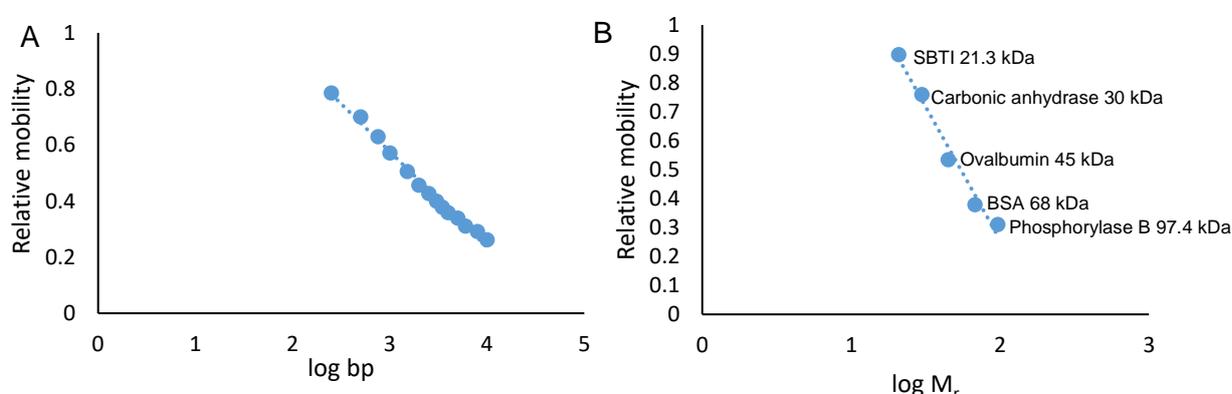
**Purification and quantification of recombinant proteins:** His-select® nickel affinity resin, dialysis tubing (10 kDa size cut-off), polyethylene glycol (PEG) Mr 20 000, 4-chloro-1-naphthol, phosphorylase B (97.4 kDa), bovine serum albumin (BSA, 68 kDa) ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (SBTI, 21.5 kDa), myoglobin (14 kDa), lysozyme (14 kDa) and the semi dry blotter were obtained from Sigma (St. Louis, MO, USA). The PageRuler™ prestained protein ladder was purchased from Pierce (Rockford, IL, USA) the Amicon® centrifugal concentrators from Merck Millipore (Billerica, MA, USA) and the BioTrace™ nitrocellulose from PALL Corp (Anne Arbor, USA).

**Immunochemical techniques:** Freund's complete and incomplete adjuvants, rabbit anti-chicken IgG HRPO conjugate and bovine serum albumin (BSA) were purchased from Sigma

(St. Louis, MO, USA), polyethylene glycol (PEG)  $M_r$  6 000 was purchased from Merck (Darmstadt, Germany) and Nunc-Immuno™ Maxisorp 96-well plates from Nunc Intermed (Roskilde, Denmark). The 2, 2'-azinobis [3-ethyl-3, dihydrobenzothiazole-6-sulfonate] (ABTS) ELISA chromogen was purchased from Roche (Mannheim, Germany) and the BIOTEK® ELx50™ Microplate washer from Biotek Instruments Inc. (USA).

## 2.2.2 Calibration curves for DNA fragment and protein size determination

Standard curves were drawn for molecular weight markers separated on a 1% (w/v) agarose gel (Figure 2.1, A) and on a 12.5% reducing SDS-PAGE gel (Section 2.2.7). The relative mobility of each DNA fragment or protein marker was plotted against the log of the number of base pairs (bp; Figure 2.1, A) or the respective relative molecular mass ( $M_r$ ; Figure 2.1, B).

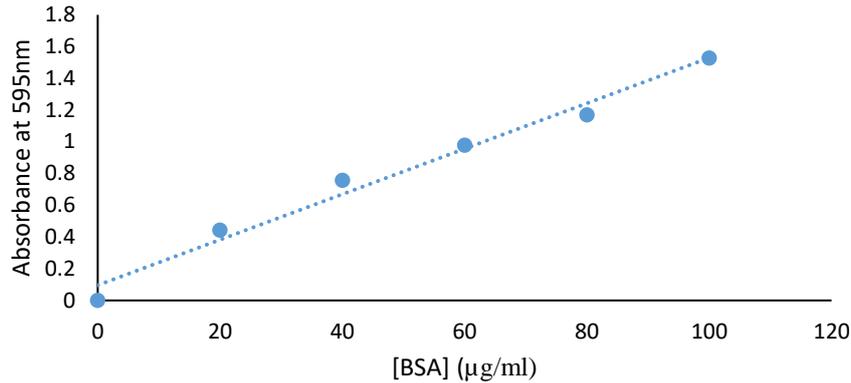


**Figure 2.1: Standard curves relating relative mobility to the log of the base pairs and molecular weight markers used in agarose and 12.5% reducing SDS-PAGE gels. (A)** O'GeneRuler™ 1 kb DNA ladder was used for agarose electrophoresis. The equation of the trend line is given by  $y = -0.3406x + 1.5982$ , with a correlation coefficient of 0.991. **(B)** The molecular weight marker used for reducing SDS-PAGE consisted of standard proteins of known size as indicated. The equation of the trend line is given by  $y = -0.9216x + 2.1001$ , with a correlation coefficient of 0.98.

## 2.2.3 Protein quantitation

### 2.2.3.1 Bradford protein assay

The Bradford (1976) assay was performed to determine the protein concentration. To obtain a standard curve (Figure 2.2), a 1 mg/ml BSA stock was used to prepare dilutions (20  $\mu$ g/ml - 100  $\mu$ g/ml) in triplicate. Protein standards (100  $\mu$ l) were mixed with 900  $\mu$ l of Bradford reagent [0.12 % (w/v) Coomassie brilliant blue G-250 dissolved in a 2 % (v/v) perchloric acid solution]. The absorbance at 595 nm was recorded after 15 min of incubation.



**Figure 2.2 Standard curve for the Bradford protein assay.** Bovine serum albumin standards ranging from 20 to 100 µg/ml were added to the Bradford reagent and the resulting absorbance values measured at 595 nm after incubation at 37°C for 30 min. The equation of the trend line is  $y = 0.0143x + 0.0959$  with a  $R^2$  value of 0.982. The absorbance readings at 595 nm represent the average of quintuplicate experiments after 15 min development.

### 2.2.3.2 Monitoring soluble recombinant protein expression level by western blot analysis

The amount of soluble recombinant FL*Tvi*CATL<sub>C25A</sub> upon expression was estimated by western blot. The intensity of protein bands on the nitrocellulose membrane was measured by densitometry using the G-Box system (GeneSys®) and GelAnalyzer® 2010a software (Lazar and Lazar, 2010). The yield of FL*Tvi*CATL<sub>C25A</sub> was calculated in arbitrary units, comparing the amount of FL*Tvi*CATL<sub>C25A</sub> in the soluble fraction of samples to the amount of FL*Tvi*CATL<sub>C25A</sub> in the whole cell lysate.

### 2.2.3.3 Statistical analysis

Design-Expert 10 software (Stat-Ease Inc) was used for the full factorial analysis of the experimental data. The quality of the fit to the non-linear model equation was expressed by the coefficient of correlation  $R^2$  and the significance of regression coefficient was tested by analysis of variance (ANOVA).

### 2.2.4 Sub-cloning of the FL*Tvi*CATL<sub>C25A</sub> gene into the bacterial pET32-a expression vector

FL*Tvi*CATL<sub>C25A</sub> had been previously cloned into the pT57R cloning vector and transformed into JM109 *E. coli* cells in our laboratory. Recombinant FL*Tvi*CATL<sub>C25A</sub>-pT57R plasmids (20 µl) were subjected to restriction enzyme digestion with NotI in buffer O at RT for 16 h with subsequent heat deactivation at 80°C for 20 min. The resultant reaction mixture was incubated with EcoRI in its unique buffer at RT and incubated overnight and thereafter inactivated at 65°C for 15 min. The restriction mixture (50 µl) was added to 1/6 volume of gel loading buffer and electrophoresed on a 1% (w/v) agarose gel in TAE buffer, both containing crystal violet

(10 µg/ml). Crystal violet was used instead of ethidium bromide to visualise bands before excision (Rand, 1996). Using ethidium bromide at this point in the cloning process would require visualisation with UV light which damages the DNA and would result in a less efficient cloning process. The gel was subsequently viewed on a lightbox, the 1300 bp product was excised and subsequently purified using the E.Z.N. A<sup>®</sup> gel extraction kit according to the manufacturer's instructions.

The pET32-a (Figure 2.3) and pGEX4T-1 expression vectors were each subjected to restriction enzyme digestion (20 µl) with NotI in buffer O at RT for 16 h with subsequent heat deactivation at 65°C for 15 min. The resulting reaction mixture was incubated with EcoRI in its unique buffer at RT for 16h and thereafter heat inactivated at 65°C for 15 min. Subsequently, 1 U of SAP was added and incubated at 37°C for 1 h to dephosphorylate the vector. A 3:1 ratio of vector to gene insert was incubated with 6 U of T4 DNA ligase at 37°C for 1 h followed by a further incubation step at RT for 16 h.



**Figure 2.3: Map of the pET-32a expression vector showing the multiple cloning sites (Novagen technical manual).**

The ligation mixture was transformed into competent *E. coli* BL21 (DE3) cells by heat shock transformation (Cohen *et al.*, 1972; Sambrook and Russell, 2001). Briefly, 10 ml of 2 x YT 1.6% (w/v) Tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl] liquid medium was inoculated with a single *E. coli* BL21 (DE3) colony and subsequently grown at 37°C for 16 h with agitation. Thereafter 1 ml of the overnight culture was diluted 1:100 with 99 ml of fresh 2 x YT liquid medium and grown at 37°C with agitation until an OD<sub>600</sub> of 0.3 to 0.4 was reached. The cultures were transferred into ice-cold, sterile centrifuge tubes and incubated on ice for 10 min. The cells were pelleted (4 500 g, 10 min, 4°C) and resuspended in ice-cold, sterile CaCl<sub>2</sub> solution [60 mM CaCl<sub>2</sub>, 10 mM HEPES, pH 7.0 (40 ml)]. The cell solution was pelleted again (4 500 g,

10 min, 4°C) and resuspended in ice-cold, sterile CaCl<sub>2</sub> solution (2 ml). The competent cells (20 µl) were incubated with the ligation mixture (1 µl) on ice for 30 min. Thereafter, cells were heat shocked at 42°C for 90 s and incubated on ice immediately for 2 min. The cells were added to pre-warmed super optimal cataboliser with catabolite repression (SOC) medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose (80 µl)] and incubated at 37°C for 1 h with gentle agitation. The resulting cell mixture (100µl) was plated onto two pre-warmed 2 x YT plates (containing 50 µg/ml ampicillin) and incubated at 37°C for 16 h.

Recombinant FL*Tvi*CATL<sub>C25A</sub>-pET32a and FL*Tvi*CATL<sub>C25A</sub>-pGEX4T-1 colonies were grown in 10 ml of 2 x YT liquid medium (containing 50 µg/ml ampicillin) and grown at 37°C for 16 h with agitation. The plasmid DNA was isolated using the GeneJet™ Plasmid Miniprep Kit, according to manufacturer's instructions and was subjected to a small scale restriction digestion (5 µl) with NotI and EcoRI simultaneously in buffer O at 37°C for 4 h. Restriction digestion products (10 µl), resulting from the NotI and EcoRI restriction enzyme digestions, were added to 1/6 volume of GLB and analysed on a 1% (w/v) agarose gel, containing ethidium bromide (0.5 µg/ml), in TAE buffer to test for positive clones. The plasmid DNA of the positive clones were then used as the positive template for colony PCR. The master mix for the PCR reaction contained FL*Tvi*CATL Fw and Rv primers (0.25 µM for each; Table 2.1), 1x High Fidelity PCR enzyme mix buffer, 2.5 mM MgCl<sub>2</sub>, 0.25 U FIREPol® Taq polymerase and 0.5 mM dNTPs in a total reaction volume of 25 µl. The PCR was carried out by denaturing the plasmid DNA at 95°C for 5 min, followed by 30 cycles of denaturing the DNA at 95°C for 1 min, primers annealed at 60°C, 64.3°C or 67°C for 1 min, elongation at 72°C for 2 min and finally an elongation at 72°C for 7 min. The colony PCR products were added to 6x GLB and electrophoresed on a 1% (w/v) agarose gel, containing ethidium bromide (0.5 µg/ml), in TAE buffer.

**Table 2.1: Primers used for the screening of recombinant colonies.** The restriction sites for EcoRI and NotI are underlined.

| Primer                              | Sequence   | T <sub>m</sub> (°C) |
|-------------------------------------|--|---------------------|
| Full length (FL) <i>Tvi</i> CATL Fw | 5'-AAA <u>GAA TTC</u> TAC ATG GCG GTG CTG CGC<br>GCG GAG-3'                | 67                  |
| Full length (FL) <i>Tvi</i> CATL Rv | 5'-AAA <u>GCG GCC GCC</u> CTA CCC TCG ATG TTG<br>TGC ATC GCA GGG CGG CG-3' | 78                  |

## 2.2.5 Factors and experimental design

Three factors of the recombinant protein expression process were chosen to be evaluated by the experimental design: temperature, induction duration and IPTG (inducer) concentration (Table 2.2).

**Table 2.2: Factors and levels assayed for recombinant expression optimisation**

|                         | Lowest level/Category A | Highest level/Category B |
|-------------------------|-------------------------|--------------------------|
| Temperature (°C)        | 30                      | 37                       |
| Induction duration (h)  | 4                       | 16                       |
| IPTG concentration (mM) | 0                       | 1                        |

A full two-level factorial design  $2^3$  was chosen to analyse the statistical effects of the factors on the soluble expression of FL*Tv*/CATL<sub>C25A</sub>. This design allows for main effects of the factors to be determined, but not their interactions. Due to the considerable variability inherent to bioprocesses, the factors were considered significant with p-values lower than 10% ( $p < 0.1$ ) using 90% confidence in the statistical analysis (Design-Expert 10 / Stat-Ease Inc.).

Single colonies of positively identified recombinant FL*Tv*/CATL<sub>C25A</sub>-pET32a were used to inoculate 10 ml of 2 x YT liquid medium containing ampicillin (50 µg/ml). After incubation at 37°C for 16 h with agitation, 1 ml of the 2 x YT culture was diluted 1:100 with 99 ml of fresh 2 x YT liquid medium (containing 50 µg/ml ampicillin) and grown at 37°C with agitation until an OD<sub>600</sub> of approximately 0.7 was reached. Expression was induced with IPTG (0.1 M) at a final concentration of 1 mM at 37°C for 4 h with agitation. Ampicillin (50 µg/ml) was added at the start of induction as well as after 2 h. The cells were then pelleted by centrifugation (5 000 g, 10 min, 4°C), the pellets were resuspended in 1% (v/v) Triton X-100-PBS (2 ml), lysozyme added (1 mg/ml final concentration) and incubated at 37°C for 30 min. The cell suspension was frozen at 70°C for 1 h and subsequently thawed at RT. The cell suspension was then sonicated four times for 30 s each, and the cellular debris was subsequently pelleted from the soluble protein lysate by centrifugation (5 000 g, 10 min 4°C). The protein lysate was then filtered through Whatman No. 1 filter paper and stored at -20°C.

Samples of the supernatant and the pellet were electrophoresed on two 12.5% reducing SDS-PAGE gels (Laemmli, 1970) (Section 2.2.7) with one stained with Coomassie blue R-250 and the other transferred onto nitrocellulose for western blot analysis (Section 2.2.8).

## 2.2.6 12.5% reducing SDS-PAGE analysis

Protein samples were analysed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) adapted from 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 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 tank buffer (250 mM Tris-HCl buffer, pH 8.3, 192 mM glycine, 0.1% SDS) using the BioRad® Mini protein III electrophoresis apparatus (BioRad, CA, USA). After electrophoresis, gels were stained for at least 4 h or for 16 h with Coomassie Blue staining solution [0.125% Coomassie Blue R-250, 50% (w/v) methanol, 10% (v/v) acetic acid], destained in destaining solution I [50% (v/v) methanol, 10% (v/v) acetic acid] followed by complete destaining and gel swelling to their original sizes in several changes of distilled water. To determine the molecular weight of proteins of unknown size, a set of standard proteins of known sizes were separated using reducing SDS-PAGE. The measurement of the relative migration distance ( $R_f$ ) of individual standard proteins, was used to construct a calibration curve to determine the molecular weight of proteins of unknown size (Section 2.2.2).

A sensitive silver staining method was used to visualise small amounts of protein on SDS-PAGE gels (Blum *et al.*, 1987). Following reducing SDS-PAGE, the gel was placed in a thoroughly cleaned glass container and incubated with fixing solution [50% (v/v) methanol, 12% (v/v) glacial acetic acid, 0.5% (v/v) formaldehyde] for 1 h or 16 h at RT 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)  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$ ] 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.

### **2.2.7 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 (20 V) and at maximum current setting for 55 min. Upon completion of protein transfer to nitrocellulose, the effectiveness of protein transfer to the membrane was checked by transiently staining it with Ponceau S solution [0.1 % (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. 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) fat-free powder milk in (TBS, 20 mM Tris-HCl buffer, pH 7.4, 200 mM NaCl)] for 1 h, after which the membrane was washed in TBS (3 x 5 min) and subsequently probed for 16 h at 4°C with either anti-6xHis IgY primary antibody [1:10 000 diluted in 0.5% (w/v) BSA-PBS] or anti-FLTvCATL<sub>C25A</sub> IgY [100 µg/ml diluted in 0.5% (w/v) BSA-PBS]. The membrane was washed in TBS (3 x 5 min) and incubated

in rabbit anti-chicken IgG horseradish peroxidase (HRPO) conjugate [1:15 000 diluted in 0.5% (w/v) BSA-PBS] at RT for 1h. Following washing in TBS (3 x 5 min), the membrane was placed in substrate solution [(0.06% (w/v) 4-chloro-1naphthol, 0.1% (w/v) methanol, 0.0015% (v/v) H<sub>2</sub>O<sub>2</sub> in TBS] until bands were visible.

### **2.2.8 Solubilisation, renaturation and purification of recombinant FL TviCATL<sub>C25A</sub>**

Recombinant proteins are usually the predominant protein when expressed in inclusion bodies and are protected against degradation by the host cell's proteolytic enzymes (Burgess, 2009). Renaturation of a protein generally involves the solubilisation of the inclusion bodies using chaotropic agents such as guanidine hydrochloride and urea or non-chaotropic agents such as SDS and *N*-lauroylsarcosine sodium salt (sarkosyl) (Burgess, 2009). The solubilised proteins are then renatured by removing excess denaturant in an optimised renaturation buffer by dilution, dialysis or chromatographic methods (Clark, 1998). The use of immobilised metal ion affinity chromatography (IMAC), in this case, a nickel chelate column, allows for the gradual removal of the denaturant and the subsequent renaturation of the immobilised recombinant protein (Petty, 1996). There are a plethora of commercial screens that help to identify suitable renaturation buffers. In the process of renaturing, the protein must undergo re-oxidation in order to allow for the formation of disulfide bonds in the correct conformation and is achieved by either the addition of 0.1 to 1 mM DTT in the inclusion body buffer or within the renaturation buffers (Burgess, 2009).

Sarkosyl solubilisation was performed (Schlager *et al.*, 2012) after 4 h of IPTG (1 mM) induced expression at 37°C. The cells were pelleted by centrifugation (5 000 *g*, 10 min, 4°C), and to the pellet, lysis buffer [8 mM Na<sub>2</sub>HPO<sub>4</sub>, 286 mM NaCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 2.6 mM KCl, 1% (w/v) SDS, pH 7.4, 1 mM DTT (5 ml for a 50 ml culture)] was added. The resulting solution was sonicated (2 x 2 min) and subsequently incubated on ice for 30 min. The solubilised proteins were separated from the insoluble debris by centrifugation (10 000 *g*, 20 min, 4°C). Samples of the supernatant and the pellet were electrophoresed on a 12.5% reducing SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie blue R-250.

An IMAC column was setup by stacking 1 ml of His-select<sup>®</sup> nickel affinity resin in a 10 ml column container. The column was first washed with 2 column volumes of dH<sub>2</sub>O then washed with 5 column volumes of wash buffer [2.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 286 mM NaCl, 8 mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1% (w/v) sarkosyl, pH 7.4]. Solubilised protein from the *E. coli* lysate was added to the column and mixed using an end-over-end rotator for 3 h at 4°C. Unbound protein fractions were collected, then the column was washed with wash buffer until the 280 nm absorbance reached a baseline value of 0.02. The bound proteins were eluted in 1 ml fractions with 10 ml elution buffer [50 mM imidazole, 2.6 mM KCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 286 mM NaCl, 8

mM Na<sub>2</sub>HPO<sub>4</sub>, 0.1% (w/v) Sarkosyl, pH 7.4]. The column was then regenerated using 2 column volumes of dH<sub>2</sub>O and 3 column volumes of equilibration buffer (10 mM imidazole, 500 mM NaCl, 50 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 6.8) and stored in 30% (v/v) ethanol at 4°C. Samples of the unbound and the eluted fractions were electrophoresed on a 12.5% reducing SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie blue R-250.

The fractions containing the target protein were pooled and concentrated against polyethylene glycol (PEG) M<sub>r</sub> 20 000 until there was a 5-fold decrease in volume. Protein concentration was determined by performing a Bradford assay (Bradford, 1976). Purified samples (10 µl) were electrophoresed on a 12.5% reducing SDS-PAGE gel (Laemmli, 1970) and visualised using silver stain (Blum *et al.*, 1987).

## **2.2.9 Antibody preparation and ELISA evaluation**

### **2.2.9.1 Preparation of immunogen, the immunisation of chickens and IgY isolation**

Chickens were used to raise antibodies against the purified recombinant FL*Tvi*CATL<sub>C25A</sub> protein with the use of these chickens approved by the University of KwaZulu-Natal animal research ethics committee (approval number 053/15/Animal). Two chickens were immunised intramuscularly on either side of the breastbone. The recombinant FL*Tvi*CATL<sub>C25A</sub> protein (50 µg/ml, 1.5 ml) was added to an equal volume of Freund's complete adjuvant and triturated to form a stable water-in-oil emulsion prior to immunisation. Booster injections, using Freund's incomplete adjuvant, were given at weeks 2, 4 and 6. Eggs collected prior to immunisation served as the pre-immune control, and those collected throughout the immunisation schedule were stored at 4°C.

Chicken immunoglobulin (IgY) was isolated from the yolks of the eggs collected at each week as described by Goldring and Coetzer (2003). Briefly, the yolks were separated from the egg whites and rinsed under running tap water. The yolk sac was punctured, the yolks collected, and the volume determined. The yolk was mixed with two yolk volumes of IgY buffer [100 mM NaH<sub>2</sub>PO<sub>4</sub>, pH 7.6, 0.02% (w/v) NaN<sub>3</sub>] and 3.5% (w/v) PEG 6 000 being subsequently added and dissolved with stirring. The resulting solution was centrifuged (4 420 g, 30 min, RT), and the supernatant filtered through adsorbent cotton wool. The final PEG concentration was increased to 12% (w/v) by the addition of 8.5% (w/v) PEG 6 000 and was once again dissolved by stirring. The mixture was pelleted by centrifugation (12 000 g, 10 min, RT), then the pellet was dissolved in IgY buffer equal to the yolk volume. Next, 12% (w/v) PEG 6 000 was stirred into the solution until it dissolved. The mixture was once again pelleted by centrifugation (12 000 g, 10 min, RT) and then the pellet was dissolved in a final IgY buffer [100 mM NaH<sub>2</sub>PO<sub>4</sub>, 0.1% (w/v) NaN<sub>3</sub>, pH 7.6] equal to sixth of the yolk volume then stored at 4°C. The

isolated IgY concentration was spectrophotometrically determined at 280 nm using the  $E_{280\text{ nm}}^{1\text{ mg/ml}} = 1.25$  extinction coefficient (Goldring *et al.*, 2005).

### 2.2.9.2 ELISA evaluation of antibody production

The progress of IgY antibody production by chickens during the immunisation period against FL*Tvi*CATL<sub>C25A</sub> recombinant protein was monitored by ELISA. The Nunc-Immuno™ Maxisorp ELISA plate wells were coated with 150 µl of rFL*Tvi*CATL<sub>C25A</sub>-PBS solution (1 µg/ml rFL*Tvi*CATL<sub>C25A</sub>, 100 mM NaCl, 100 mM NaH<sub>2</sub>PO<sub>4</sub>, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) per well for 16 h at 4°C. The coating solution was discarded, then 200 µl blocking buffer [0.5% (w/v) BSA-PBS, 0.1% (v/v) Tween-20] was added to each well to prevent non-specific binding of antibodies, then incubated at 37°C for 1 h. The wells were washed three times with 0.1% (v/v) Tween-20-PBS using the BIOTEK® ELx50™ microplate washer and were then incubated with 100 µl per well of 100 µg/ml of anti-FL*Tvi*CATL<sub>C25A</sub> IgY primary antibody diluted in blocking buffer for 2 h at 37°C. The wells were washed again as before then 100 µl of rabbit anti-chicken IgY HRPO conjugate diluted 1:15 000 in blocking buffer was added to each well and incubated at 37°C for 1 h. The wells were washed as before and 145 µl of ABTS solution [0.15 M citrate-phosphate buffer, 0.05% (w/v) ABTS, 0.0015% (v/v) H<sub>2</sub>O<sub>2</sub>, pH 5.0] was subsequently added to each well. The plate was incubated in the dark for 15 min prior to reading the absorbance at 405 nm using the VersaMax plate reader in 15 ml intervals until absorbance values of above 1.0 were obtained.

### 2.2.9.3 Culture and western blot of bloodstream form *Trypanosoma congolense* (strain IL 3000) parasites

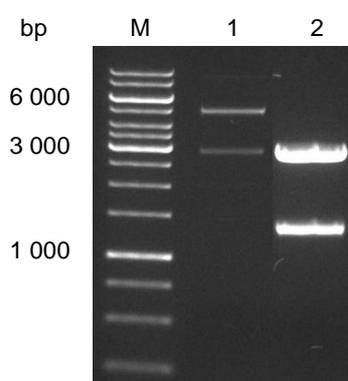
The *in vitro* culture of both *T. congolense* and *T. b. brucei* bloodstream form parasites were performed according to Eyford *et al.* (2011). Briefly, 10 ml Eagle's minimum essential medium [EMEM base powder, 2 mM glutamine, 10 mM proline, 20% (v/v) heat-inactivated foetal bovine serum] was used to culture both *T. congolense* and *T. b. brucei* bloodstream form parasites at 27°C and 5% (v/v) CO<sub>2</sub> until the culture became slightly overgrown.

Cultured bloodstream form parasites were pelleted by centrifugation (2 000 *g*, 10 min, RT), washed twice with PBS (2.7 mM KCl, 2 mM KH<sub>2</sub>PO<sub>4</sub>, 137 NaCl, 100 mM Na<sub>2</sub>HPO<sub>4</sub>, pH 7.4) and were subsequently resuspended in 50 µl of lysis buffer [10 µM, E64, 10 mM Na<sub>2</sub>EDTA, 1% (v/v) Triton X-100, 20 mM Tris-Cl buffer, pH 7.2]. An equal volume of reducing treatment buffer was added to the parasites before boiling for 10 min. A total of approximately 1 x 10<sup>5</sup> parasites were added to each lane (20 µl) and electrophoresed on a 12.5% reducing SDS-PAGE gel (Laemmli, 1970). The trypanosomal lysate from the SDS-PAGE gel was transferred onto a nitrocellulose membrane using the semi-dry blotter (Sigma) and the western blot performed as per Section 2.2.8.

## 2.3 Results

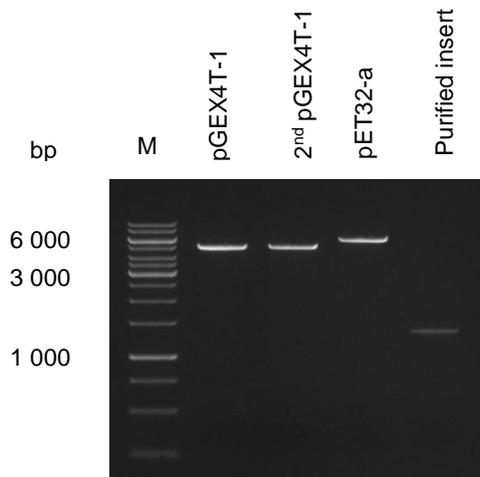
### 2.3.1 Sub-cloning of the *FL*Tvi*CATL<sub>C25A</sub>* gene into the bacterial pET32-a expression vector

The isolated plasmid DNA deriving from the *FL*Tvi*CATL<sub>C25A</sub>*-pTZ57R JM109 glycerol stocks were subjected to a small-scale restriction enzyme digest with *Eco*RI and *Not*I to obtain the gene insert for subsequent ligation into an expression vector. As shown in Figure 2.4 after restriction digestion two bands were produced at  $\pm 1\ 300$  bp and  $\pm 3\ 000$  bp which corresponds to the *FL*Tvi*CATL<sub>C25A</sub>* insert and the pTZ57R cloning vector respectively. The restriction digestion mixture was subsequently electrophoresed on a crystal violet containing agarose gel, and the 1 300 bp *FL*Tvi*CATL<sub>C25A</sub>* insert was excised and purified.



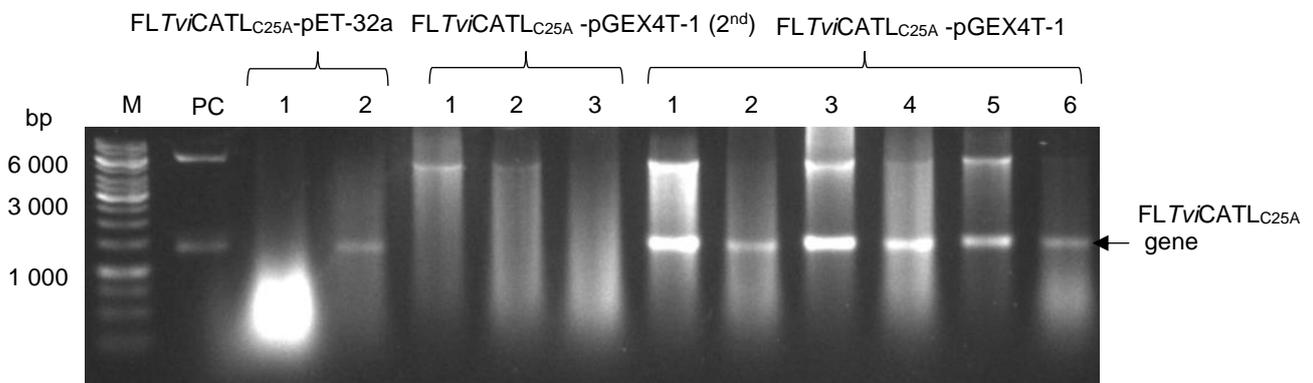
**Figure 2.4: Analysis of the *FL*Tvi*CATL<sub>C25A</sub>* insert after restriction digestion with *Eco*RI and *Not*I of the recombinant *FL*Tvi*CATL<sub>C25A</sub>*-pTZ57R clones.** Lane 1, undigested *FL*Tvi*CATL<sub>C25A</sub>*-pTZ57R cloning vector. Lane 2, double digested *FL*Tvi*CATL<sub>C25A</sub>*-pTZ57R. Samples were electrophoresed on a 1% (w/v) agarose gel containing 0.5  $\mu$ g/ml ethidium bromide. M: O'GeneRuler.

The purity, size and relative concentrations of the isolated *FL*Tvi*CATL<sub>C25A</sub>* insert along with the previously digested and de-phosphorylated pET32-a, pGEX4T-1 and the 2<sup>nd</sup> stock of pGEX4T-1 (purchased separately) expression vectors were determined from Figure 2.5. Ligation mixtures of the *FL*Tvi*CATL<sub>C25A</sub>* insert with either pET32-a or pGEX4T-1 expression vectors were prepared which included expression vector re-ligation controls and were subsequently transformed into *E. coli* BL21 (DE3) cells by heat shock transformation.



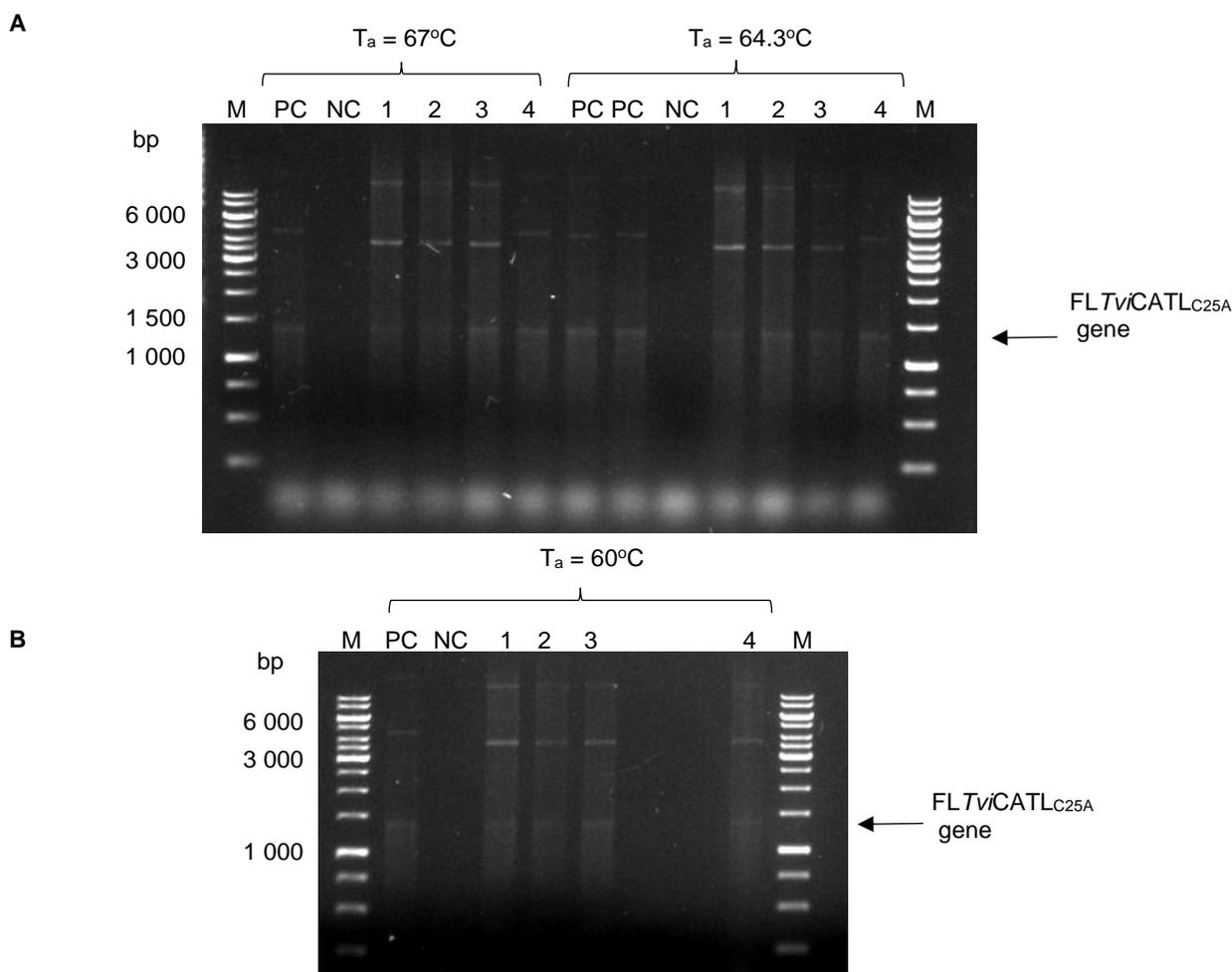
**Figure 2.5: Gel extraction of the *FLTviCATL<sub>C25A</sub>* insert after restriction digestion with *EcoRI* and *NotI*.** The *FLTviCATL<sub>C25A</sub>* insert was excised from the 1% (w/v) agarose gel and purified using the E.Z.N.A<sup>®</sup> gel extraction kit. Samples were electrophoresed on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. M: O'GeneRuler.

The plasmid DNA of the resulting *FLTviCATL<sub>C25A</sub>*-pGEX4T-1 and *FLTviCATL<sub>C25A</sub>*-pET32a clones were isolated and first screened by performing small-scale restriction enzyme digestion with *EcoRI* and *NotI* (Figure 2.6). One of the two *FLTviCATL<sub>C25A</sub>*-pET32a colonies that were screened contained the ±1 300 bp insert whereas all six of the screened *FLTviCATL<sub>C25A</sub>*-pGEX4T-1 colonies contained the ±1 300 bp insert.



**Figure 2.6: Restriction digest screening for recombinant *FLTviCATL<sub>C25A</sub>* clones ligated into pGEX4T-1 and pET32-a expression vectors.** Following the transformation of *E. coli* BL21 (DE3) with *FLTviCATL<sub>C25A</sub>*-pGEX4T-1 and *FLTviCATL<sub>C25A</sub>*-pET-32a ligation mixtures and the selection of colonies for screening, the isolated plasmid from each clone was subjected to small-scale restriction enzyme digestion using *EcoRI* and *NotI*. Samples were electrophoresed on a 1% (w/v) agarose gel and visualised with 0.5 µg/ml ethidium bromide. M: O'GeneRuler<sup>™</sup>, PC: *FLTviCATL<sub>C25A</sub>*-pTZ57R.

Plasmid DNA was isolated from recombinant *FLTviCATL<sub>C25A</sub>*-pGEX4T-1 and *FLTviCATL<sub>C25A</sub>*-pET32a and used as the template for colony PCR to amplify the 1 300 bp *FLTviCATL<sub>C25A</sub>* gene using the *FLTviCATL<sub>C25A</sub>* forward and reverse gene primers (Figure 2.7). An amplicon of ± 1 300 bp was observed in each case. Different annealing temperatures were compared to determine which led to better amplification of the gene.



**Figure 2.7: Colony PCR screening for recombinant FLTviCATL<sub>C25A</sub> clones ligated into the pGEX4T-1 and pET32-a expression vectors.** Following transformation of the FLTviCATL<sub>C25A</sub>-pGEX4T-1 and FLTviCATL<sub>C25A</sub>-pET32a ligation mixtures into *E. coli* BL21 (DE3) cells and selection of colonies, the isolated plasmid DNA from each clone was subjected to PCR amplification using the FLTviCATL<sub>C25A</sub> forward and reverse primers. Lanes labelled 1-3 contain FLTviCATL<sub>C25A</sub>-pGEX4T-1 plasmids while the lanes labelled 4 contain FLTviCATL<sub>C25A</sub>-pET-32a plasmids. Samples were electrophoresed on a 1% (w/v) agarose gel containing 0.5 µg/ml ethidium bromide. M: O'GeneRuler™. (A) Colony PCR with 64.3°C and 67°C annealing temperature. (B) Colony PCR at the 60°C annealing temperature. M: O'GeneRuler™, PC: FLTviCATL<sub>C25A</sub>-pTZ57R, NC: No template DNA.

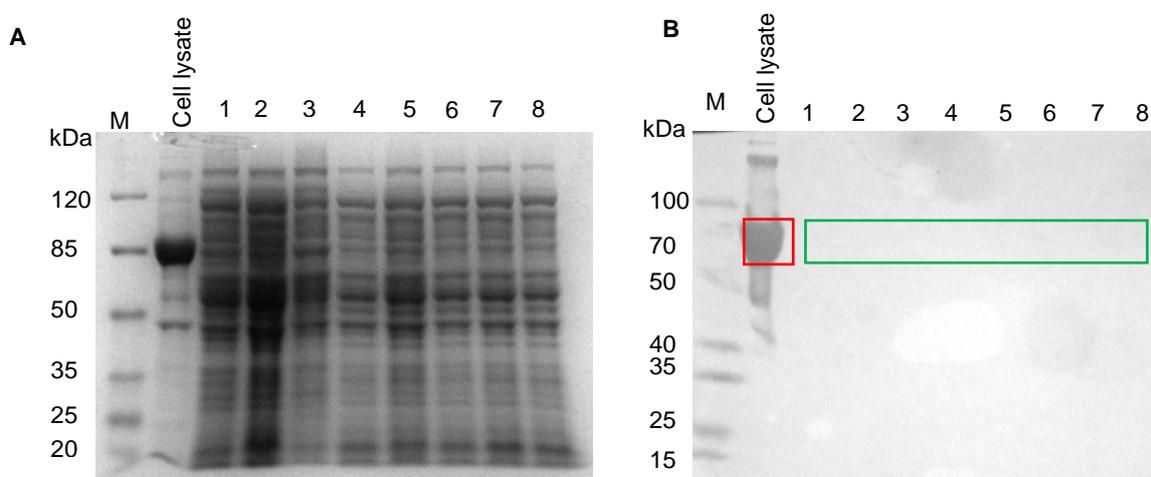
The plasmid DNA of FLTviCATL<sub>C25A</sub>-pGEX4T-1 clones 3 and FLTviCATL<sub>C25A</sub>-pET-32a clone 2 were sequenced using the FLTviCATL<sub>C25A</sub> forward and reverse primers for both clones as well as the T7 promoter and terminator primers for FLTviCATL<sub>C25A</sub>-pET32a and pGEX forward and reverse primers for FLTviCATL<sub>C25A</sub>-pGEX4T-1. The sequencing results showed that the recombinant clones all possessed the FLTviCATL<sub>C25A</sub> gene, identical to the sequence retrieved from GenBank® (CAEX01004850.1) apart from the intentionally introduced mutation (Figure A1).

## 2.3.2 Recombinant expression, solubilisation, refolding and purification of FL*Tvi*CATL<sub>C25A</sub>

### 2.3.2.1 Multi-factor analysis

The effects of three factors, *i.e.* expression temperature, induction duration and IPTG concentration were assessed based on soluble protein yield as the response measured to define a process with high yields and reduced time for expression of the recombinant FL*Tvi*CATL<sub>C25A</sub> protein in its soluble form. Soluble protein yield was measured by comparing the intensity of bands corresponding to FL*Tvi*CATL<sub>C25A</sub> after western blot analysis (Figure 2.8, B) using gel analysis software (GelAnalyzer<sup>®</sup>). To this effect, a statistical strategy based on a two-level full factorial screening design was performed, with two levels for each of the three factors (2<sup>3</sup>). A sequence of eight different experimental conditions was conducted to gather information about these factors that may affect the soluble expression of FL*Tvi*CATL<sub>C25A</sub>. Results are presented in Table 2.3.

Following expression with IPTG induction, a prominent protein band at approximately 70 kDa was observed for the cell lysate whereas small bands were observed at the expected size for the soluble fractions of the samples after staining with Coomassie blue R-250 (Figure 2.8, A).



**Figure 2.8: 12.5% Reducing SDS-PAGE and western blot analysis of multi-factor expression experiments of recombinant FL*Tvi*CATL<sub>C25A</sub> expression in pET32-a.** Samples of the soluble fractions and a reference insoluble fraction sample of the multi-factor analysis of recombinant FL*Tvi*CATL<sub>C25A</sub> expression were electrophoresed on 12.5% reducing SDS-PAGE gels and **(A)** stained with Coomassie blue R-250 while **(B)** was transferred onto nitrocellulose and incubated with chicken anti-6xHis HRPO conjugate [1:4000 in 0.5% (w/v) BSA-PBS]. Samples are labelled according to the experimental set-up shown in Table 2.3. The intensity of protein bands outlined in the green boxes was measured using GelAnalyzer 2010a (Lazar and Lazar, 2010). M Spectra BR prestained marker.

The results presented in the western blot analysis (Figure 2.8, B) show that the various changes in expression conditions didn't lead to any meaningful production of the recombinant FL*Tvi*CATL<sub>C25A</sub> in the soluble form since there were no bands detected in the soluble fractions for any set of the tested expression conditions.

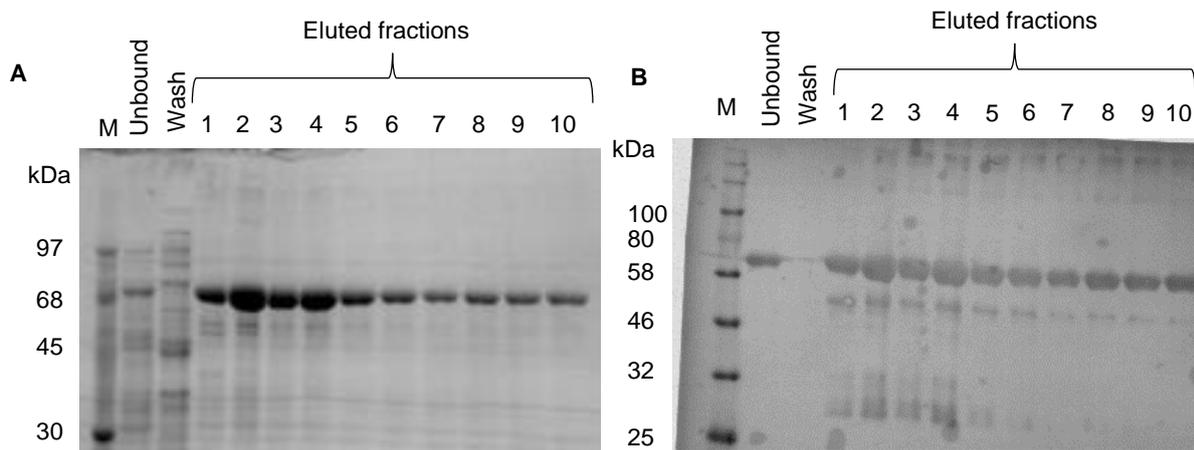
**Table 2.3: Two-level full factorial screening design and response**

| Run | Expression temperature (°C) | [IPTG] (mM) | Induction duration (h) | Relative soluble FL <i>Tvi</i> CATL <sub>C25A</sub> (%) |
|-----|-----------------------------|-------------|------------------------|---|
| 1   | 37                          | 0.1         | 16                     | 0   |
| 2   | 25                          | 0.1         | 16                     | 0   |
| 3   | 37                          | 1           | 16                     | 0   |
| 4   | 25                          | 0.1         | 4                      | 0   |
| 5   | 25                          | 1           | 16                     | 0   |
| 6   | 37                          | 0.1         | 4                      | 0   |
| 7   | 25                          | 0.1         | 4                      | 0   |
| 8   | 37                          | 1           | 4                      | 0   |

After this screening process and observing that most, if not all, the protein was expressed in the insoluble fraction, it was decided that resolubilising and refolding the protein from the inclusion bodies would yield more protein than continuing with the optimisation experiments.

### 2.3.2.2 Solubilisation, refolding and purification of recombinantly expressed FL*Tvi*CATL<sub>C25A</sub>

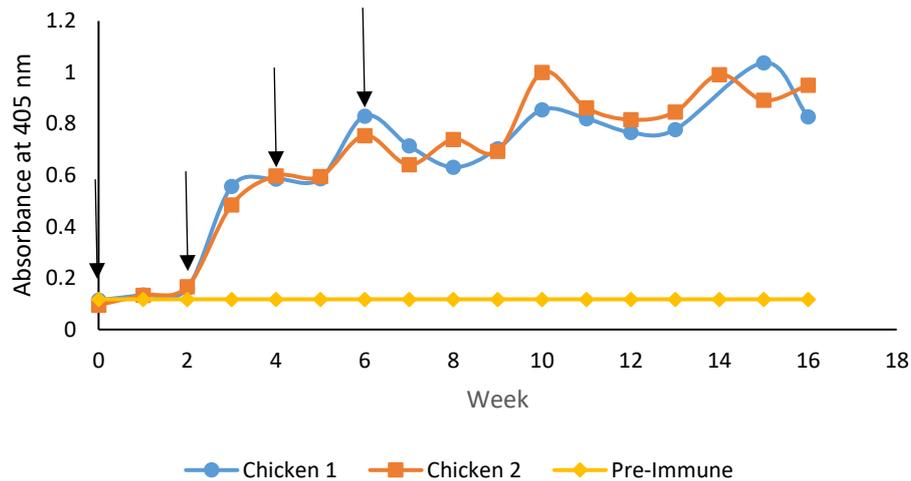
Solubilisation of the inclusion bodies using the sarkosyl method was performed and the recombinantly expressed His<sub>6</sub>-tagged FL*Tvi*CATL<sub>C25A</sub> was subsequently refolded and purified on a nickel chelate column. Solubilisation and on-column refolding using sarkosyl resulted in significant amounts of homogeneous recombinant FL*Tvi*CATL<sub>C25A</sub> protein being eluted (Figure 2.9). The purified antigen was then prepared for immunisation of chickens for antibody production.



**Figure 2.9: Nickel affinity on-column refolding and purification of solubilised recombinant FL*Tvi*CATL<sub>C25A</sub> using the sarkosyl method.** Samples from the sarkosyl method of on-column refolding and nickel affinity purification was analysed on **(A)** 12.5% reducing SDS-PAGE stained with Coomassie blue R-250 and **(B)** western blot probed with chicken anti-His<sub>6</sub> IgY [1:10 000]. M: PageRuler™ prestained marker.

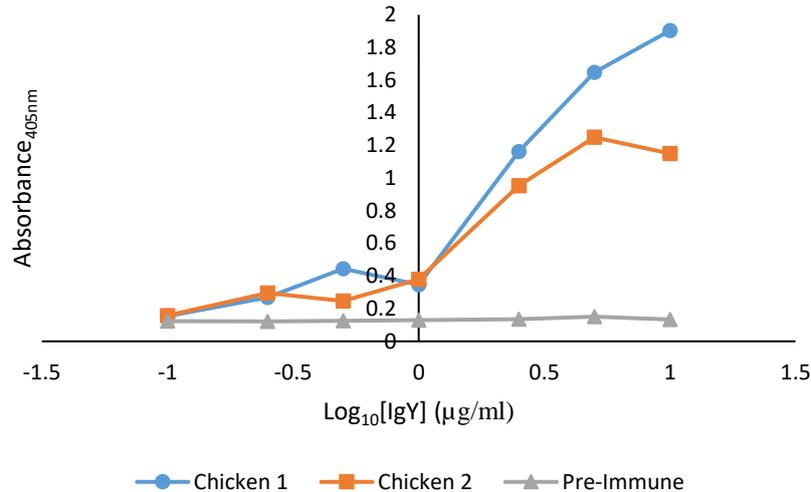
### 2.3.3 Evaluation of anti-FL*Tvi*CATL<sub>C25A</sub> antibody production by ELISA

The anti-FL*Tvi*CATL<sub>C25A</sub> IgY antibodies isolated from a single egg from each week during immunisation period were used for an initial ELISA to determine when the production of anti-FL*Tvi*CATL<sub>C25A</sub> IgY antibodies peaked (Figure 2.10). Both chickens produced high amounts of antigen-specific IgY at weeks 6 and 10. These levels of antibody were maintained for the remainder of the 16 weeks during which eggs were collected.



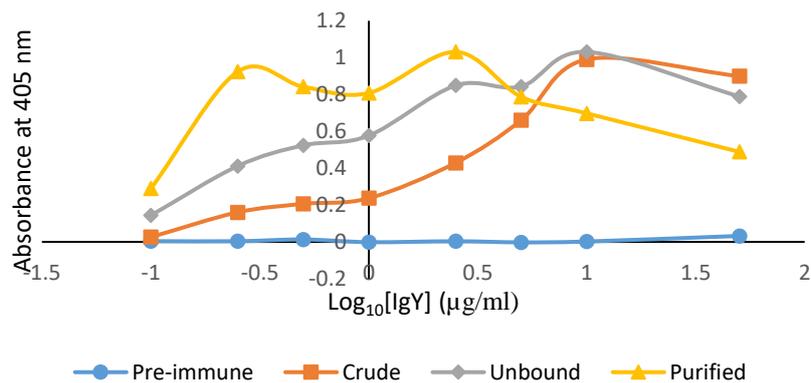
**Figure 2.10: ELISA of anti-FL*Tvi*CATL<sub>C25A</sub> IgY antibodies isolated from egg yolks of immunised chickens.** ELISA plates were coated with FL*Tvi*CATL<sub>C25A</sub> for evaluation of antibody production by the two immunised chickens over the 16-week period of egg collection. Antibodies (100 µg/ml) from each chicken were incubated and detected with rabbit anti-chicken IgG HRPO secondary antibody. The absorbance readings at 405 nm represent the average of duplicate experiments after 15 min development. Arrows indicate booster injection weeks.

An evaluation of the titres of anti-FL*Tvi*CATL<sub>C25A</sub> antibodies (IgY purified from eggs collected from weeks 9-11 pooled from each chicken) showed that slightly higher titres were obtained for antibodies produced by chicken one compared to chicken two (Figure 2.11). Both chickens produced antibodies with higher titres than the pre-immune control IgY sample.



**Figure 2.11: ELISA assessing anti-FLTvCATL<sub>C25A</sub> IgY response to recombinant FLTvCATL<sub>C25A</sub>.** ELISA plates were coated with FLTvCATL<sub>C25A</sub> and incubated with pooled anti-FLTvCATL<sub>C25A</sub> IgY from each chicken at 0.1, 0.25, 0.5, 1, 2.5, 5, 10 μg/ml). Rabbit anti-chicken IgG HRPO secondary antibody and ABTS/H<sub>2</sub>O<sub>2</sub> were used as the detection system. The absorbance readings at 405 nm represent the average of triplicate experiments after 15 min development.

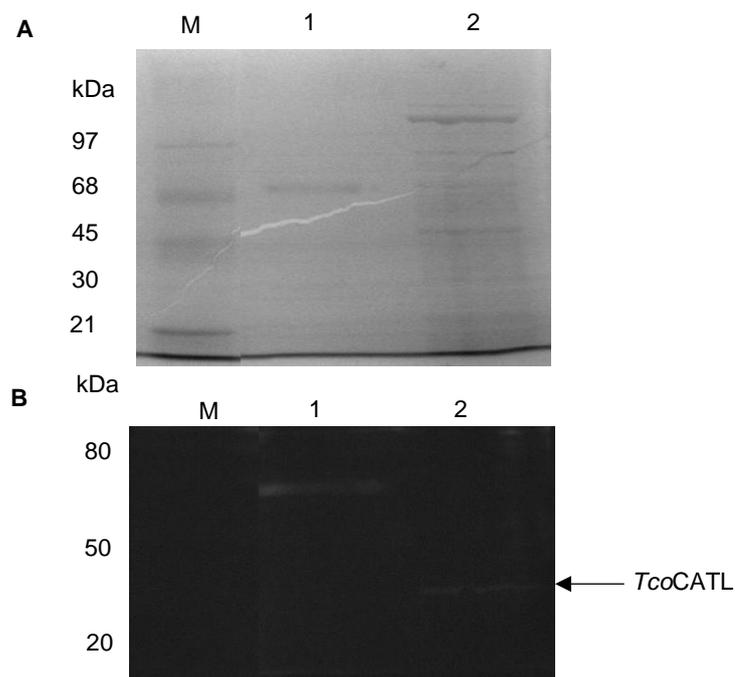
The ability of the affinity purified anti-FLTvCATL<sub>C25A</sub> IgY to recognise the corresponding recombinant antigen was assessed using ELISA. The affinity purified anti-FLTvCATL<sub>C25A</sub> antibodies showed higher absorbance values suggesting higher titres than the other antibody preparations: pre-immune, crude (pre-purification) and unbound (Figure 2.12). The affinity purified anti-FLTvCATL<sub>C25A</sub> antibodies from chicken two were then used in subsequent experiments to show immunological recognition of TcoCATL in *T. congolense* parasite lysate.



**Figure 2.12: Analysis of anti-FLTvCATL<sub>C25A</sub> antibodies from chicken two before and after affinity purification.** ELISA plates were coated with FLTvCATL<sub>C25A</sub> (1 μg/ml in PBS, pH 7.4). Affinity purified (▲), crude (■), unbound (◆), and pre-immune (●) antibodies were diluted (0.1 – 20 μg/ml). Rabbit anti-chicken IgG HRPO secondary antibody (1:15 000) and ABTS H<sub>2</sub>O<sub>2</sub> were used as the detection system. The absorbance readings at 405 nm represent the average of duplicate experiments after 30 min development.

### 2.3.4 Western blot analysis of *T. congolense* (IL3000) parasite lysate with anti-FL*Tvi*CATL<sub>C25A</sub> antibodies

A western blot was performed using *T. congolense* (IL 3000) to determine whether the anti-FL*Tvi*CATL<sub>C25A</sub> IgY antibodies recognise the native major CATL antigen in the bloodstream form of the parasite (Figure 2.13, B). The antibodies (100 µg/ml) detected the recombinant FL*Tvi*CATL<sub>C25A</sub> at the expected size of 70 kDa while a band corresponding to a size of approximately 33 kDa was detected within the parasite lysate which corresponds to that of native *Tco*CATL.



**Figure 2.13: 12.5% reducing SDS-PAGE and Western blot analysis of bloodstream form parasite lysate probed by purified anti-FL*Tvi*CATL<sub>C25A</sub> IgY.** The blot was incubated with anti-FL*Tvi*CATL<sub>C25A</sub> from chicken 2, weeks 9-11 (100 µg/ml) followed by rabbit anti-chicken IgY HRPO (1:15 000). The bands were developed with Pierce™ ECL western blotting substrate. M: prestained protein marker. Lane 1, recombinant FL*Tvi*CATL<sub>C25A</sub>, lane 2, *T. congolense* bloodstream form lysate. (A) 12.5% reducing SDS-PAGE stained with Coomassie Blue R-250, (B) luminescent image of the western blot.

## 2.4 Discussion

The diagnosis of animal African trypanosomiasis is a critical aspect in the fight against the disease since it gives critical information that allows for the correct use of chemotherapeutics. New targets for chemotherapies and diagnostics need to be identified due to the process of antigenic variation displayed by trypanosomal parasites which hinder any progress towards a vaccine (Barry and McCulloch, 2001). Numerous protozoan peptidases have been identified as pathogenic factors and are thus targets for such studies (Mottram *et al.*, 2004). This study was performed to produce a recombinant antigen that will be useful in the field diagnosis of *T. vivax* using the sera of the infected animals. The most promising technique is the serological ELISA technique. There are two types of serological ELISA techniques that can be used; the antigen-detection ELISA and the antibody-detection ELISA. For the first method, the recombinant antigen is used to produce antibodies that is used to detect the antigen in infected sera and the second would use the antigen directly to detect antibodies in infected animals.

The expressions optimisation of the full-length inactive mutant of *TviCATL* (FL*TviCATL*<sub>C25A</sub>) was undertaken for possible use in diagnostics for *T. vivax*. The reason why an inactive form of the protein was used was that in a previous study of the homologous protein of *T. Congolese*, very poor yield was obtained, and the protein was unstable. Therefore, for this study attempts were made to improve the recombinant expression of soluble FL*TviCATL*<sub>C25A</sub> in the *E. coli* expression system.

The 1.3 kb FL*TviCATL*<sub>C25A</sub> gene has an alanine coding sequence substituted for a cysteine coding sequence within the catalytic triad containing gene sequence, thus coding for an inactive site Ala<sup>25</sup>, His<sup>159</sup>, and Asn<sup>175</sup> (papain numbering) as compared to active *TviCATL*, which has Cys<sup>25</sup>, His<sup>159</sup> and Asn<sup>175</sup> (papain numbering) in the active site (Urban *et al.*, 2003).

There is a wide variety of prokaryotic and eukaryotic expression systems available to produce recombinant proteins. Each system is associated with advantages and disadvantages; therefore, recombinant expression of proteins remains a matter of trial and error. Factors such as recombinant protein solubility, yields, stability, folding and posttranslational modifications, are considered when choosing an expression system for recombinant protein production (Rosano and Ceccarelli, 2014). The prokaryote is the first choice for recombinant expression of proteins (Baneyx and Mujacic, 2004).

*E. coli* remains the most popular cell host for recombinant protein production, mainly because when compared to other expression host organisms it offers many advantages such as fast growth in relatively cheap media and simple process scale-up (Rosano and Ceccarelli, 2014). However, for eukaryotic proteins, the lack of post-translational modifying machinery and the almost inevitable production of inactive protein due to the formation of inclusion bodies, offers

a significant challenge in this expression system (Sahdev *et al.*, 2008). Multiple approaches have been devised to counteract these issues, converting inactive protein, expressed in inclusion bodies, into soluble and active protein. Basically, these approaches can be divided into three categories namely; (I) influential expression factors are modified which control the cellular machinery; (II) insolubly expressed protein is resolubilised; (III) and the expression of recombinant protein with a fusion tag/protein or chaperone (Sahdev *et al.*, 2008).

The first papain-like peptidase to be expressed in an *E. coli* system containing a T7 promoter was a human cysteine peptidase, cathepsin L, for which expression levels were very low, and the protein was produced in inclusion bodies (Smith and Gottesman, 1989). An attempt to express *TcoCATL* in *E. coli* was unsuccessful as it yielded incorrectly folded and inactive enzyme (Authié *et al.*, 2001). However, expression of an active enzyme at marginal yields was achieved through a more expensive and complex Baculovirus expression system (Authié *et al.*, 2001). The inactive mutant of *TviCATL* was previously expressed in inclusion bodies by (Vather, 2010), furthermore, not enough protein was expressed to sufficiently produce antibodies through chicken immunisation.

The soluble recombinant expression optimisation of *FLTviCATL<sub>C25A</sub>* in the *E. coli* bacterial system was thus attempted for this project. There is evidence of successful expression of CATLs in bacteria such as; full-length *TcrCATL*, and to a lesser extent, the target protein of this project *FLTviCATL<sub>C25A</sub>* was previously expressed in *E. coli* (Vather, 2010). Subsequently, the *FLTviCATL<sub>C25A</sub>-pTZ57R* cloning vector used to obtain the *FLTviCATL<sub>C25A</sub>* gene was sourced from Vather's (2010) work. The *FLTviCATL<sub>C25A</sub>* DNA insert was sub-cloned into pET32-a and pGEX4T-1 expression vectors using the EcoRI and NotI restriction sites. The *FLTviCATL<sub>C25A</sub>-pET32-a* and *FLTviCATL<sub>C25A</sub>-pGEX4T-1* plasmid DNA was then transformed into chemically competent BL21 (DE3) cells before expression. The BL21 (DE3) strain was chosen due to it containing the T7 RNA polymerase (under the transcriptional control of a *lacUV5* promoter, IPTG inducible) and the DE3 lysogen that renders the strain peptidase deficient (pET Expression System Manual).

Recombinant expression analysis of soluble *FLTviCATL<sub>C25A</sub>* was undertaken using statistical experimental design methodology. The statistical experimental design methodology, where the response is evaluated by changing more than one factor at a time, allows for the determination of the factors that are statistically significant, considering the interactions between them. This multi-factor approach allows for a more thorough analysis compared to the widely used and traditional one factor at a time approach, where the response is evaluated by changing one factor at a time while fixing the others. The multi-factor analysis enables the characterisation of experimental error, to compare the effects of factors between themselves

when factors are normalised, and hence, to gather high-quality information with as few experiments as possible. All these advantages make this approach a powerful tool to optimise conditions for recombinant protein expression (Larentis *et al.*, 2011).

Three factors were chosen for the analysis of soluble protein expression, namely, induction temperature, and induction duration and inducer concentration. Besides the choice of the expression system itself, these factors generally have the most critical impact on the yield of the soluble protein upon recombinant expression. Earlier observations suggest that induction conditions are essential factors since they affect the ratio of soluble and insoluble protein forms upon recombinant expression (Rosano and Ceccarelli, 2014). Most likely the influence of induction conditions is related to modulation of the *E. coli* cells chaperone activity or other protein synthesis machinery involved in protein folding. Induction conditions also play a crucial role in protein production since low inducer concentration may be insufficient for full induction, whereas a high concentration of the inducer may affect the growth of the cell, especially in the case of production of toxic proteins (Vasina and Baneyx, 1997). Moreover, excessive induction time may cause noticeable degradation of the expressed recombinant protein. For a thorough exploration of factors of importance, it was decided that the application of a full factorial design should be applied. This approach is used when two or more factors are varied simultaneously and allows reducing the total number of experiments very considerably when compared to one factor at a time analysis (for the same output of data). Therefore, the three factors that were chosen were tested at two levels using the matrix generated by the interactive statistical software Design-Expert 10. This software helps to set up an optimal experimental design with a minimal number of tests required to explore almost all possible combinations of the factors and their levels. The different expression conditions and their results are presented in Table 2.1. The soluble protein expression was estimated by quantification of soluble protein detected in the western blot compared to the whole rFL *Tvi*CATL<sub>C25A</sub> *E. coli* cell lysate (Figure 2.8, B). The result presented in Figure 2.8 panel B and Table 2.3, show that the intracellular concentration of soluble FL *Tvi*CATL<sub>C25A</sub> remained negligible despite the varying conditions of expression.

The recombinantly expressed FL *Tvi*CATL<sub>C25A</sub> was successfully resolubilised and renatured using a non-chaotropic (sarkosyl) method during IMAC purification. Previous attempts at on-column refolding and purification using the chaotropic (urea) method failed (Vather, 2010). The sarkosyl method of resolubilisation and refolding was adapted from (Schlager *et al.*, 2012). In this method, inclusion bodies were solubilised by SDS, the excess of which was precipitated using cloud point extraction by cooling and was removed from the solubilised protein by centrifugation. Cloud point extraction is a conventional technique used in the purification of membrane proteins (Arnold and Linke, 2007). According to Burgess (2009), it

has been seen that most proteins refold at a pH between 8 and 8.5, but the pH at which protein is refolded well is at one pH unit above or below the pI of the protein. The buffers used in the sarkosyl method all had a pH of 7.4 which is within one unit of FL*Tvi*CATL<sub>C25A</sub>'s pI which is 7.5 as suggested by Burgess (2009). The remaining wash and elution buffers contained a low concentration of sarkosyl, 0.1% (w/v), to maintain the protein's solubility during the refolding procedure using IMAC. The elution of refolded and purified FL*Tvi*CATL<sub>C25A</sub> in the sarkosyl method was performed using 50 mM imidazole concentration (Figure 2.9). This method was recently used to renature another cysteine peptidase, namely *Tco*MCA5 (Eyssen, 2014). The use of the sarkosyl method of solubilisation, refolding and purification resulted in the high yield of purified FL*Tvi*CATL<sub>C25A</sub>.

Production of polyclonal anti-FL*Tvi*CATL<sub>C25A</sub> was achieved using simple and efficient methods that have developed for chicken IgY isolation and purification Pauly *et al.* (2011). The anti-FL*Tvi*CATL<sub>C25A</sub> polyclonal IgY was purified on an AminoLink<sup>®</sup> resin matrixed with recombinant FL*Tvi*CATL<sub>C25A</sub> antigen. Large quantities of the anti-FL*Tvi*CATL<sub>C25A</sub> IgY were produced and isolated with the purified antibodies showing increased specificity at lower concentration when compared to the unbound and crude antibody samples (Figure 2.12). Dependent on the immunogenicity of the antigen, high antibody-titres of up to 8.37 mg/ml can be achieved after only 3 – 4 boost immunisations (Amro *et al.*, 2017). Due to the IgY antibodies' ability to recognise and bind multiple epitopes, they are often used in immunohistochemistry and immunofluorescence studies, furthermore, they are known to stay stable for over ten years in preservative without losing titre (De Meulenaer and Huyghebaert, 2001). Polyclonal antibodies have the advantage of being produced against multiple epitopes of the antigen, increasing the chances of protein detection but cross-reactivity is also likely (Trier *et al.*, 2012). Affinity purified anti-FL*Tvi*CATL<sub>C25A</sub> IgY identified native CATL from *T. congolense* bloodstream form lysates in a western blot. The expected size of 33 kDa, as shown in Boulange *et al.* (2011) study was detected on the nitrocellulose membrane using ECL as the detection system.

An ideal diagnostic antigen should be expressed in the mammalian infective bloodstream form *T. vivax*. Due to lack of *T. vivax* bloodstream-form parasite stocks, *T. congolense* bloodstream form parasites were used to confirm the antibodies ability to detect native CATL. The diagnostic potential of FL*Tvi*CATL<sub>C25A</sub> still needs to be verified by making use of *T. vivax* lysate, and *T. vivax* infected and non-infected bovine serum samples.

# Chapter 3: MOLECULAR CHAPERONE CO-EXPRESSION OF *TRYPANOSOMA CONGOLENSE* M1 AMINOPEPTIDASES

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## 3.1 Introduction

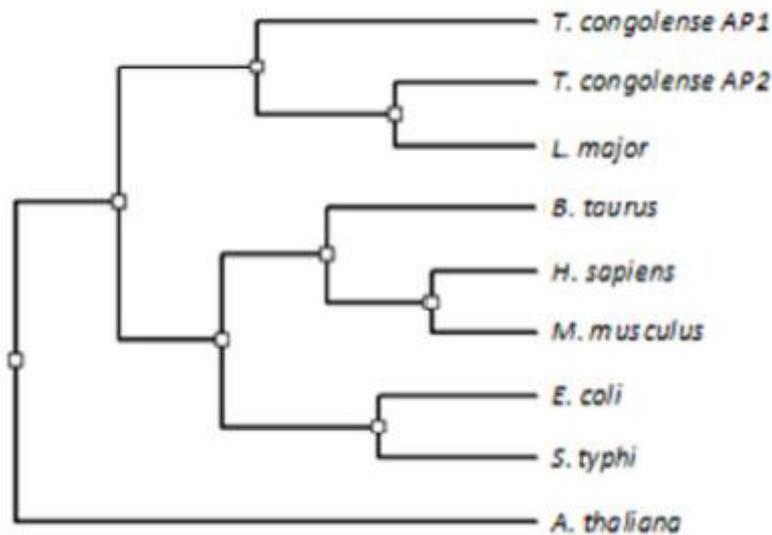
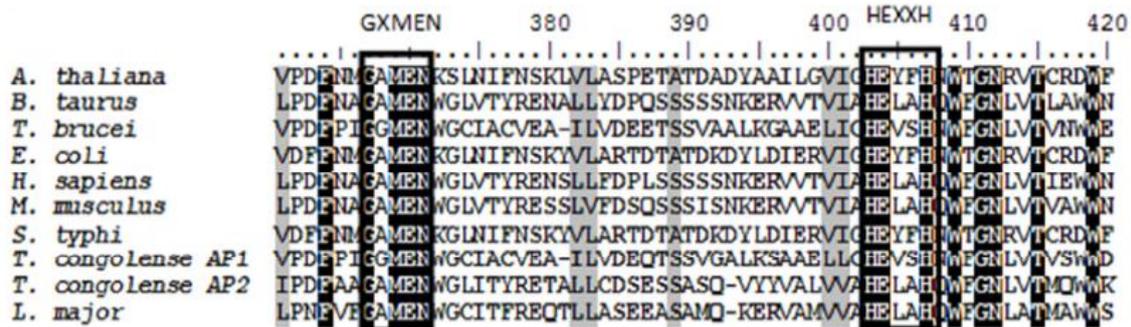
Tsetse-transmitted trypanosomiasis has been termed a notifiable disease, a disease that when detected should be reported to the World Organisation for Animal Health (OIE). Since the discovery that African trypanosomes cause AAT, research and control efforts have been consistent and have even picked up in recent decades (Diall *et al.*, 2017). However, despite recent progress, the disease still remains a major hurdle for efficient and more cost-effective livestock farming in large parts of sub-Saharan Africa which still has large farming potential (Alsan, 2015).

Trypanosomes have developed elegant immune evasion processes that help the parasites escape entire host immune responses (Stijlemans *et al.*, 2017). These immune evasion processes include the expression of variant surface glycoproteins that periodically switch to different variant antigen types to protect themselves from the host's immune system, a process known as antigenic variation (Radwanska *et al.*, 2002). Due to antigenic variation, the possibility of an AAT vaccine is very remote (Pays, 2006). Thus an anti-disease strategy is being considered in which parasite pathogenic factors are targeted (Antoine-Moussiaux *et al.*, 2009). Pathogenic factors are proteins which are essential to the parasite's survival within the host and are identified by studying the interaction between the parasite and mammalian host (Holzmuller *et al.*, 2008). Pathogeno-proteomics have facilitated the identification of new potential therapeutic agents as well as diagnostic targets (Holzmuller *et al.*, 2008). A number of metallopeptidases have been identified as pathogenic factors as they are responsible for a variety of cellular processes such as evasion of the host immune systems (Ferreira and Andricopulo, 2017).

The peptidases of protozoan parasites have drawn attention as potential drug targets, pathogenic factors and vaccine candidates (McKerrow *et al.*, 2006; Steverding, 2013). The cathepsin L-like (Boulangé *et al.*, 2011) and cathepsin B-like peptidases (Mendoza-Palomares *et al.*, 2008) of *T. congolense* have been studied. Interestingly, both these groups of peptidases are encoded by multiple genes with slight variations in sequences resulting in peptidases with different catalytic domains. Furthermore, both cathepsin B and L-like peptidases are expressed only in the bloodstream form of the parasites, making them attractive targets for therapeutic drugs.

Aminopeptidases constitute a diverse set of peptidases with essential roles in a wide range of cellular functions (Drinkwater *et al.*, 2017). The enzymes catalyse the removal of amino acids from the N-terminus of polypeptides; the specific residues of which they show an affinity, as well as their structure, determine their subdivision into families. The large family of M1 aminopeptidases (clan MA, family M1) are zinc-dependent enzymes characterised by the presence of two conserved, catalytic sequence motifs; a consensus zinc-binding motif (HEXXH-(X<sub>18</sub>)-E) and the “GXMEN” exopeptidase motif (Figure 3.1) (Osório *et al.*, 2008; Barrett *et al.*, 2012).

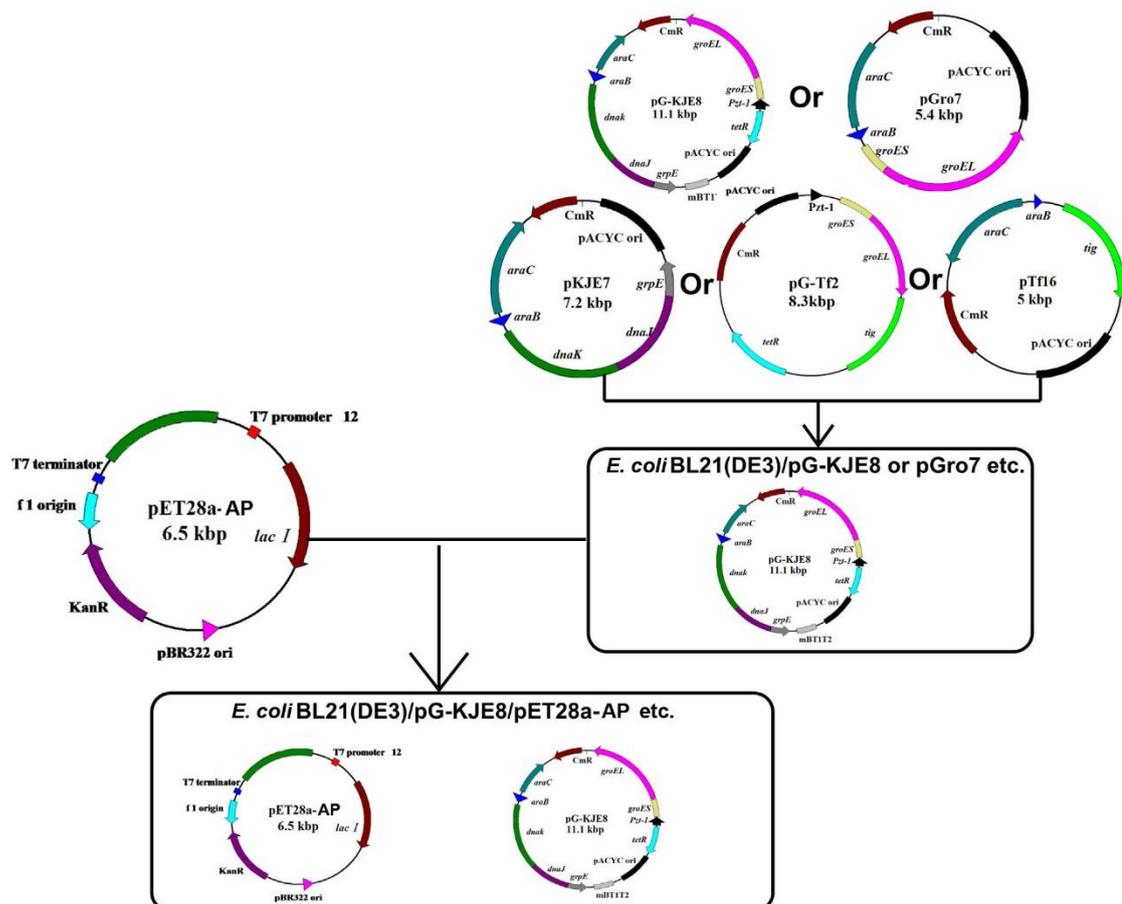
Aminopeptidases have been implicated in the development and pathogenesis of parasites (Drinkwater *et al.*, 2017), and they have been examined as potential anti-disease vaccines or chemotherapeutics for *Schistosoma mansoni* (Maggioli *et al.*, 2017), *T. b. brucei* (Knowles, 1993) and *T. cruzi* (Cadavid-Restrepo *et al.*, 2011). However, only a small selection of M1 aminopeptidases have been examined. The M1 aminopeptidases from *Toxoplasma gondii* (Zheng *et al.*, 2015) and the cattle pathogen *T. congolense* (Pillay, Boulangé, *et al.*, 2013) have both been characterised as immunogenic, but their potential as drug targets are yet to be fully elucidated. The only parasite M1 aminopeptidase that has been extensively evaluated as a potential drug target is PfA-M1 from *P. falciparum*, a causative agent of malaria (Harbut *et al.*, 2011; Mistry *et al.*, 2014).



**Figure 3.1: The phylogenetic relationships between the two *Trypanosoma congolense* family M1 aminopeptidases and other family M1 aminopeptidases. (A)** Multiple sequence alignment of a section of the family M1 aminopeptidases catalytic domains. The GXMEN and HEXXH cation-binding motifs, specific to the M1 aminopeptidases, are boxed. Sequences were obtained from Genbank® (<http://www.ncbi.nlm.nih.gov/genbank>, accessed 5-8-2016): *A. thaliana* (AAX59049.1), *B. Taurus* (NP001068612.1), *T. brucei* (AAS67871.1), *E. coli* (89107782), *H. sapiens* (P5144), *M. musculus* (31077182), *S. typhi* (CAD08183.1), *L. major* (CA5045941). **(B)** The phylogenetic tree is indicating the relationships between full protein sequences of several M1 aminopeptidases (Pillay, Boulangé, *et al.*, 2013).

Due to the promising future of parasite aminopeptidase research, producing it efficiently at a sufficient scale is of immense importance for meeting the needs of research and diagnostics. Batch cultivation has been proved to be an effective means of maximising biomass and target protein production (Rosano and Ceccarelli, 2014). Unfortunately, insoluble aminopeptidases are frequently formed due to recombinant over-expression in *E. coli* (Drinkwater *et al.*, 2017). It is well known that the over-expression of exogenous proteins usually results in substantial metabolic burden to cell and insoluble inclusion body due to protein misfolding (Sahdev *et al.*, 2008). Molecular chaperones are a highly conserved and ubiquitous group of proteins that are essential in the correct folding of protein during protein biosynthesis and cell stress response

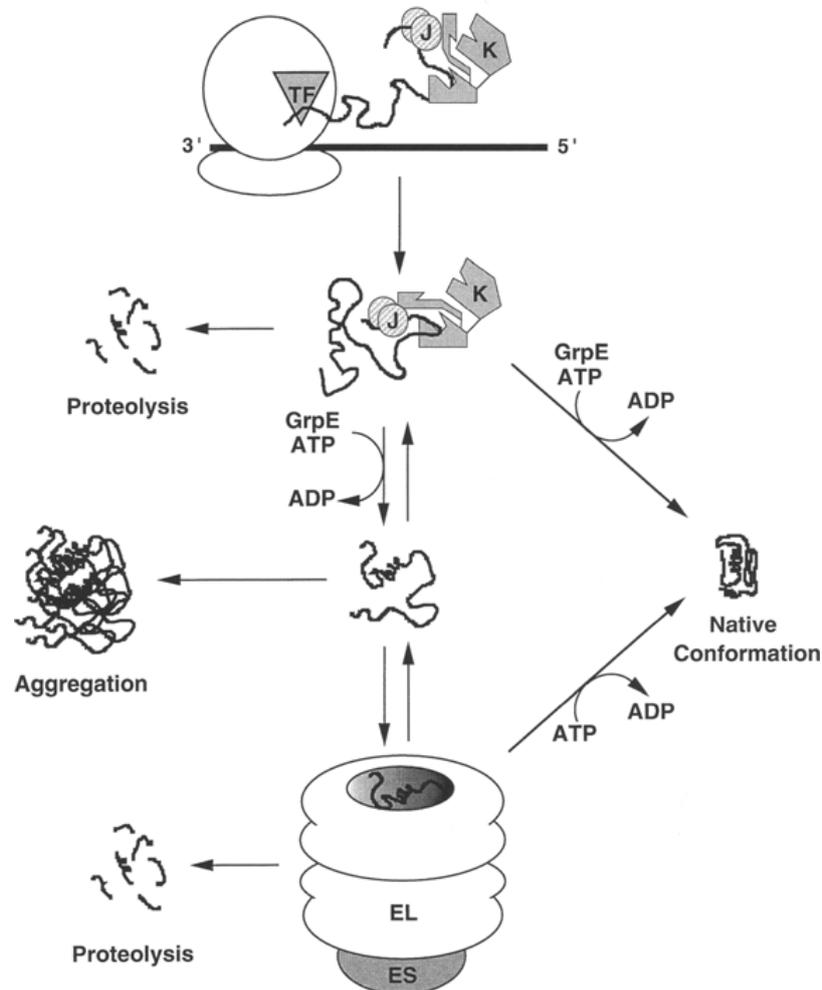
(Figure 3.2) (Nishihara *et al.*, 2000). Cells naturally increase the induction of molecular chaperone expression when under stressful conditions to help limit the presence of misfolded proteins during such events (Scatena *et al.*, 2010). Recombinant expression in general is usually recognised as stressful to the host cell, therefore it's likely that misfolding of the recombinant protein will occur (Rosano and Ceccarelli, 2014). Subsequently, the strategy of recombinantly over-expressing molecular chaperones with the target recombinant protein has been implemented to help reduce the misfolding of the desired protein (Figure 3.2).



**Figure 3.2: Molecular chaperone plasmid transformation flow chart.** Heat-shock transformation of molecular chaperone plasmid carrying chemically competent *E. coli* BL21 (DE3) with recombinant aminopeptidase plasmids. Adapted from Tong *et al.* (2016).

Hsp70, Hsp60 and the ribosome-associated trigger factor are suggested to be the most abundant and vital molecular chaperones for *de novo* folding of *E. coli* proteins (Durante and Colucci, 2010). To facilitate the application of these chaperone systems in *E. coli* host recombinant protein expression, commercial plasmids such as those offered by Takara Bio Inc were designed to allow efficient overexpression of these molecular chaperone teams. These commercially available chaperone teams have been implemented successfully for the soluble co-expression of several recombinant protein such as, *Psychrobacter sp.* G cold-

active lipase Lip-948 (Shuo-shuo *et al.*, 2011), *Thermomicrobium roseum* sarcosine oxide (Tong *et al.*, 2016) and human CD137 ligand (Wang *et al.*, 2012).



**Figure 3.3: Molecular chaperone protein folding model in *E. coli*.** The competition between chaperone interaction, folding, proteolysis and aggregation of a nascent polypeptide. Both DnaK (K) and GroEL (EL) process misfolded protein to either proteolysis or native conformation in an ATP-dependent manner that is managed by the DnaJ (J)-GrpE or GroES (ES) co-chaperones, respectively. Trigger factor (TF) is suggested to act as a general co-translational folding catalyst (Thomas *et al.*, 1997).

In the present study, two family M1 aminopeptidases of *T. congolense* were co-expressed with *E. coli* molecular chaperones, purified and enzymatically characterised. This information could contribute to further studies of these M1 type aminopeptidases as either vaccine candidates or drug targets.

## 3.2 Materials and Methods

### 3.2.1 Materials

**Molecular biology:** As outlined in section 2.2.1. M1 aminopeptidase 1 and 2 from *T. congolense* both in pET-28a sourced from Pillay's (2013) work previously done in our laboratory. The chaperone co-expression vectors (pTf16, pGro7, pG-KJE8, pKJE7 and pG-Tf2) were obtained from Takara (Kyoto, Japan). *E. coli* BL21 (DE3)/pTf16 and BL21 (DE3)/pG-Tf2 strains produces trigger factor (TF) and GroEL-GroES/TF molecular chaperone teams. On the other hand, *E. coli* BL21 (DE3)/pGro7, BL21 (DE3)/pG-KJE7 and BL21 (DE3)/pG-KJE8 produced GroEL-GroES, DnaK-DnaJ-GrpE and DnaK-DnaJ-GrpE/GroEL-GroES molecular chaperone teams, respectively.

**Table 3.1 Expression vector and chaperone plasmid set used for the recombinant chaperone co-expression of *T. congolense* aminopeptidases**

| Plasmid | Molecular chaperones       | Promoter | Inducer      | Resistance Marker |
|---------|----------------------------|----------|--------------|-------------------|
| pET-28a | -                          | lacUV5   | IPTG         | Kanamycin         |
| pGro7   | GroES-GroEL                | araB     | L-Arabinose  | Chloramphenicol   |
| pKJE7   | DnaK-DnaJ-GrpE             | araB     | L-Arabinose  | Chloramphenicol   |
| pG-Tf2  | GroES-GroEL-TF             | Pzt1     | Tetracycline | Chloramphenicol   |
| pTf16   | Trigger factor (TF)        | araB     | L-Arabinose  | Chloramphenicol   |
| pG-KJE8 | DnaK-DnaJ-GrpE GroES-GroEL | Pzt1     | Tetracycline | Chloramphenicol   |

### 3.2.2 Heat shock transformation of chaperone expression vectors into competent aminopeptidase expressing BL21 (DE3) *E. coli*

The heat shock competent BL21 (DE3)/*TcoAP1*-pET-28a and BL21 (DE3)/*TcoAP2*-pET-28a *E. coli* cells (20  $\mu$ l) were each thawed on ice to which 10 ng of either pG-KJE8, pKJE7, pGro7, pTf16 or pG-Tf2 plasmid was added to the cells. The cells were then incubated on ice for 30 min and heat shocked for 90 s at 42°C before being incubated on ice for 2 min. Pre-warmed SOC medium [2% (w/v) tryptone, 0.5% (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl<sub>2</sub>, 10 mM MgSO<sub>4</sub>, 20 mM glucose (80  $\mu$ l)] was added to the cells and incubated at 37°C for 1 h, with agitation (200 RPM). The transformed cells were spread plated onto 2 x YT agar

plates (1% agar w/v in 2 x YT medium with 34 µg/ml kanamycin and 20 µg/ml chloramphenicol) and incubated overnight, at 37°C.

### **3.2.3 Recombinant co-expression of aminopeptidases with molecular chaperones in *E. coli***

Single colonies of positively identified recombinant *E. coli* BL21 (DE3) were used to inoculate 10 ml of 2 x YT liquid medium containing kanamycin (34 µg/ml) and chloramphenicol (20 µg/ml). After incubation at 37°C for 16 h with agitation, 1 ml of the 2 x YT culture was diluted 1:100 with 99 ml of fresh 2 x YT liquid medium (containing 34 µg/ml kanamycin and 20 µg/ml chloramphenicol), then L-arabinose (0.5 mg/ml) and/or tetracycline (10 ng/ml) were added for molecular chaperone over-expression induction and grown at 37°C with 200 RPM agitation until an OD<sub>600</sub> of approximately 0.7 was reached. Recombinant aminopeptidase expression was induced with IPTG (0.1 M) at a final concentration of 1 mM at 37°C for 4 h with agitation. Ampicillin (50 µg/ml) was added at the start of induction as well as after 2 h. The cells were then pelleted by centrifugation (5 000 g, 10 min, 4°C), the pellets were resuspended in 1% (v/v) Triton X-100-PBS (2 ml), lysozyme added (1 mg/ml final concentration) and incubated at 37°C for 30 min. The cell suspension was frozen at -70°C for 1 h and subsequently thawed at RT. The cell suspension was then sonicated four times for 30 s each, and the cellular debris was subsequently pelleted from the soluble protein lysate by centrifugation (5 000 g, 10 min 4°C). The protein lysate was then filtered through Whatman No. 1 filter paper and stored at -20°C.

Samples of the supernatant and the pellet were analysed by a 12.5% reducing SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie blue R-250 (as per section 2.2.6).

### **3.2.4 Refolding and purification of *TcoAP1* and *TcoAP2***

Sarkosyl solubilisation was performed after 4 h of induced expression at 37°C with IPTG (1mM). The cells were pelleted by centrifugation (5 000 g, 10 min, 4°C), and to the pellet, lysis buffer (PCL) [8 mM Na<sub>2</sub>HPO<sub>4</sub>, 286 mM NaCl, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 2.6 mM KCl, 1% (w/v) SDS, pH 7.4, 1 mM DTT] was added. The resulting solution was sonicated (2 x 2 min) and subsequently incubated on ice for 30 min. The solubilised proteins were separated from the insoluble debris by centrifugation (10 000 g, 20 min, 4°C). Samples of the supernatant and the pellet were electrophoresed on a 12.5% reducing SDS-PAGE gel (Laemmli, 1970) and stained with Coomassie blue R-250.

After solubilisation of both aminopeptidases by sarkosyl, the denaturant was removed using the HiTrap desalting Sephadex G-25 resin (5 ml) on the ÄKTApurifier® system. Removal of the denaturant on the G-25 resin is also a form of purification as the resin not only removes the sarkosyl but any other low molecular weight (< 5000 Da) impurities as well. The resin was

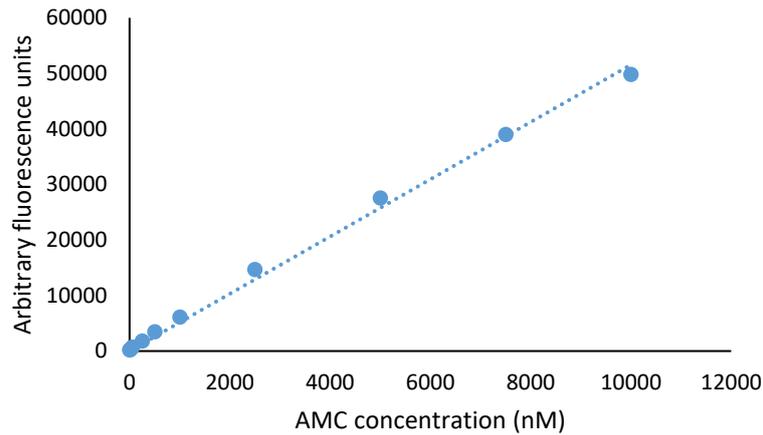
washed with 2 column volumes of MilliQ™ purified dH<sub>2</sub>O and equilibrated with 5 column volumes of wash buffer (manual run, 5 ml/min, 0.3 MPa, RT), the solubilised aminopeptidases (1.5 ml) were filtered through 0.45 μM filter and injected into the system. The desalting procedure was performed using ÄKTApurifier® system template method “HiTrap Desalting”. After the run, the resin was washed with 2 column volumes 0.2 M NaOH, followed by 5 column MilliQ™ purified dH<sub>2</sub>O and finally, 5 column volumes 20% ethanol. All reagents were prepared with dH<sub>2</sub>O, filtered and degassed using a vacuum degasser before use.

Refolded protein was purified on a 5 ml HisTrap® HP column using the ÄKTApurifier® and a gradient of 20 mM to 500 mM imidazole elution buffer (20 mM Tris-Cl, 0,5 M NaCl, pH 8.0). Purified protein containing fractions were identified by SDS-PAGE analysis and synthetic substrate fluorometric assay. The positively validated fractions were then pooled and stored in 20% glycerol, ready for further investigation.

### **3.2.5 Enzyme assay of *TcoAP1* and *TcoAP2***

An AMC calibration curve was constructed to quantify the hydrolysis of a fluorogenic peptide substrate by the purified recombinant *TcoAP1* and *TcoAP2*. The AMC standards ranging from 5 to 10 000 nM (50 μl) were incubated with 50 μl aminopeptidase assay buffer (50 mM Tris-Cl buffer, pH 8.0, 5 mM CaCl<sub>2</sub>) at 37°C for 10 min and the fluorescence (Ex<sub>360nm</sub> and Em<sub>460nm</sub>) measured using a FLUORStar Optima Spectrophotometer from BMG Labtech (Offenburg, Germany) (Figure 3.4). The slope of the calibration curve was used to determine the enzyme units. Activity was measured in arbitrary fluorescence units by subtracting any background activity of the purified samples against a negative control.

Samples of the eluted fractions from the on-column refolding and purification from the sarkosyl refolding method (50 μl) were incubated with aminopeptidase assay buffer containing 20 μM H-Gly-AMC substrate (50 μl) at 37°C for 10 min. Thereafter, the fluorescence (Ex<sub>360nm</sub> and Em<sub>460nm</sub>) was measured using a FLUORStar Optima spectrophotometer.

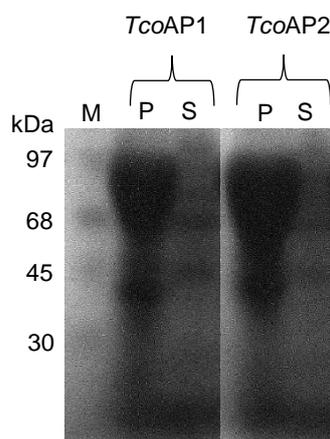


**Figure 3.4: AMC standard curve showing the relationship between fluorescence and AMC concentration.** AMC dilutions were incubated with aminopeptidase assay buffer at 37°C and the fluorescence measured (Ex<sub>360nm</sub> and Em<sub>460nm</sub>). The equation of the trendline is given by  $y = 5.1536x$  with a correlation coefficient of 0.996. The fluorescence readings represent the average of triplicate experiments after 10 min development.

### 3.3 Results

#### 3.3.1 Recombinant expression of *TcoAP1* and *TcoAP2* in *E. coli*

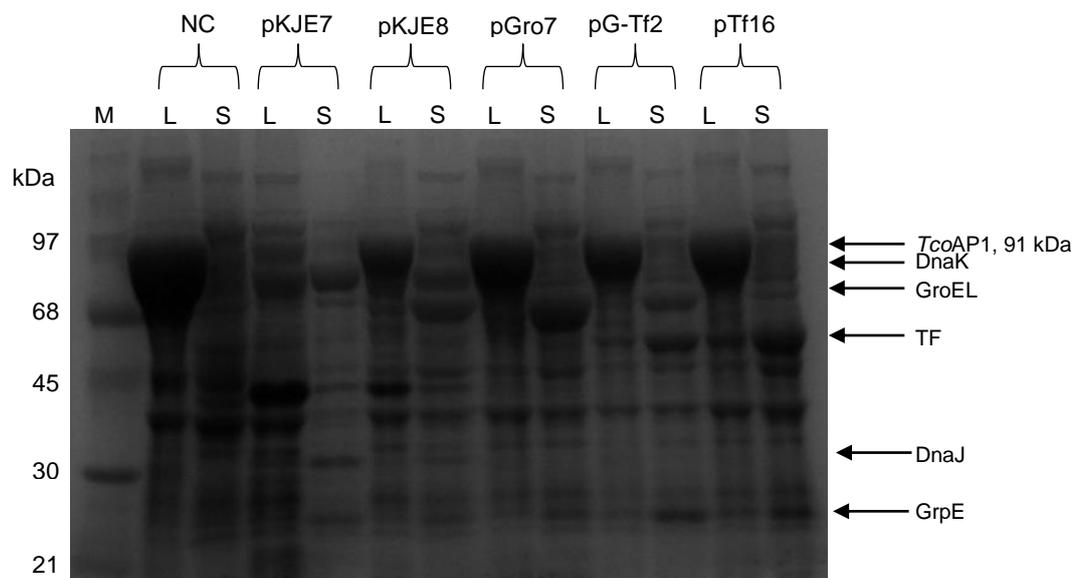
Expression of *TcoAP1* and *TcoAP2* mediated by pET28-a gave similar expression patterns in *E. coli* BL21 (DE3). Figure 3.5 shows the 91 kDa bands of *TcoAP1* and *TcoAP2* expressed with 1 mM IPTG. The expressed proteins were found almost exclusively in the insoluble fraction of the cell lysate.



**Figure 3.5: 12.5% reducing SDS-PAGE gel showing the expression of *TcoAP1* and *TcoAP2* in *E. coli* BL21 (DE3).** P: pellet (insoluble fraction) S: soluble fraction. Expression induced by addition of 1 mM IPTG. M: protein marker.

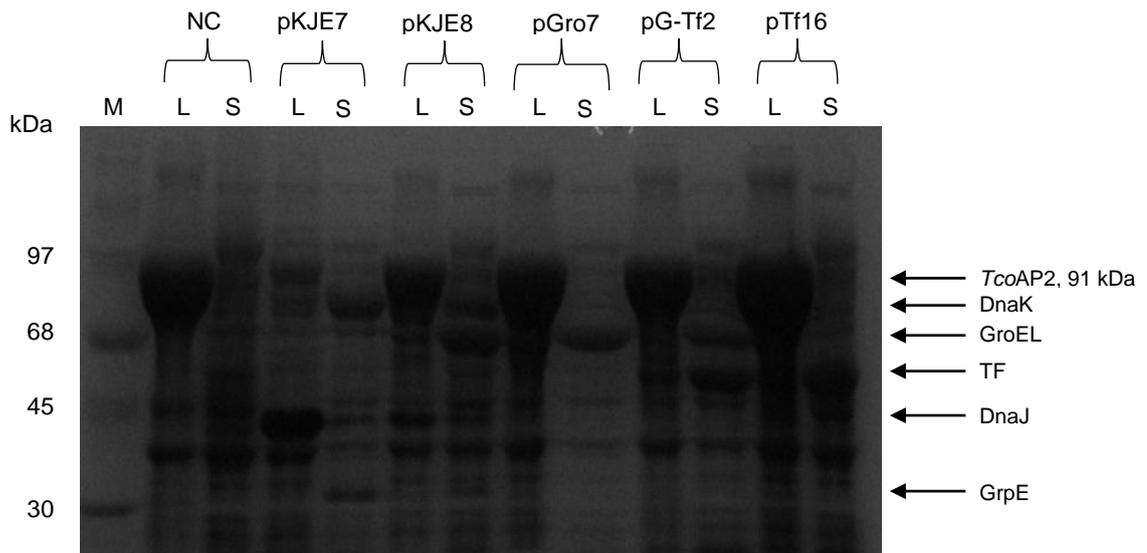
### 3.3.2 Molecular chaperone co-expression

Five different molecular chaperones were introduced for each target protein and co-expressed to improve the recombinant production of soluble aminopeptidases. The 12.5% reducing SDS-PAGE analysis of the molecular chaperone expressing *E. coli* cells showed bands in the soluble fractions corresponding to the molecular chaperones at the expected sizes *i. e.* DnaK at ~70 kDa, GroEL at ~60 kDa, TF at ~56 kDa, DnaJ at ~40 kDa, and GrpE at ~22 kDa which were not in the control samples (NC) that contained *TcoAP1* or *TcoAP2* expressing cells alone in Figure 3.6 and 3.7, respectively.



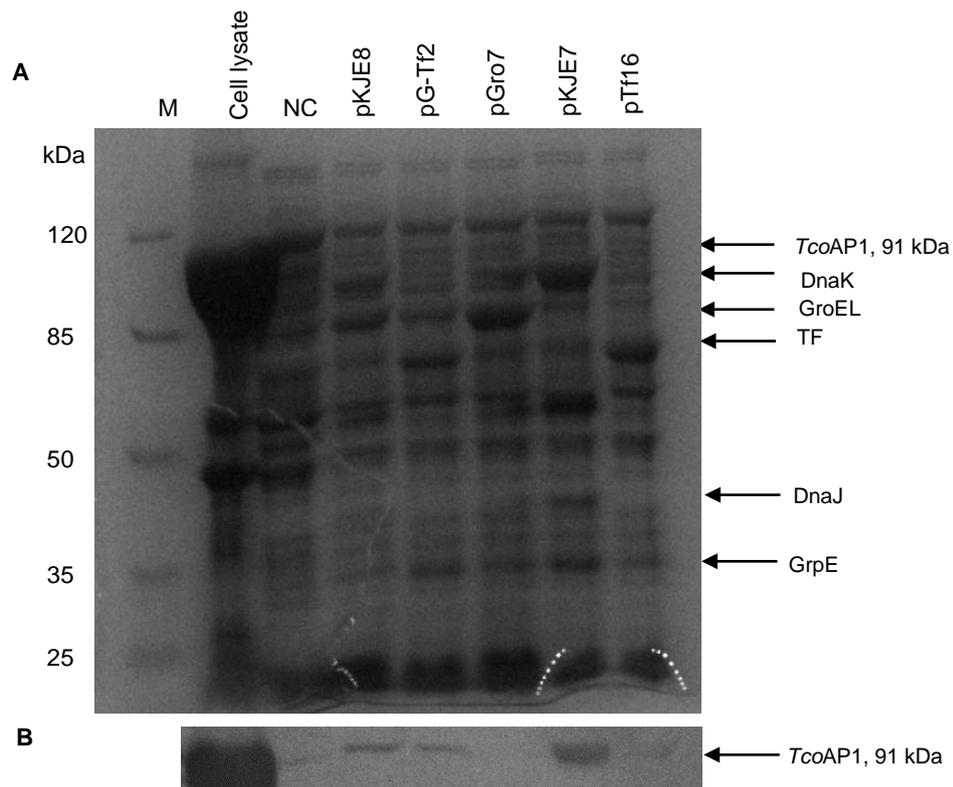
**Figure 3.6: *TcoAP1* and chaperone co-expression 12.5% reducing SDS-PAGE analysis.** *TcoAP1* was co-expressed with several molecular chaperones then samples from the cell lysate (L) and soluble fraction (S) were analysed by 12.5% reducing SDS-PAGE stained with Coomassie blue R-250. M: Pre-stained protein marker, NC: No chaperones.

Co-expression of *TcoAP1* with the molecular chaperones was analysed using 12.5% reducing SDS-PAGE, as shown in Figure 3.6, even with the presence of significant amounts of overexpressed molecular chaperones, most of *TcoAP1* was found within the insoluble fraction. A similar co-expression profile was seen for *TcoAP2*, were most of the protein was still found in the insoluble fraction (Figure 3.7).

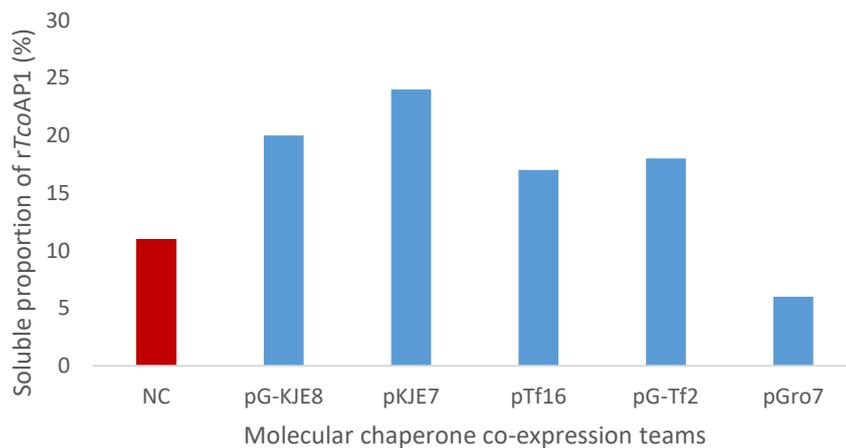


**Figure 3.7: *TcoAP2* and chaperone co-expression 12.5% reducing SDS-PAGE analysis.** *TcoAP1* was co-expressed with several molecular chaperones then samples from the cell lysate (L) and soluble fraction (S) were analysed by 12.5% reducing SDS-PAGE stained with Coomassie blue R-250. M: Pre-stained protein marker, NC: No chaperones.

In Figure 3.8, A, another 12.5% reducing SDS-PAGE analysis of the co-expression experiments was performed, this time only soluble fractions of the co-expression samples were loaded as well as the cell lysate of *TcoAP1* recombinant expression without chaperones. Furthermore, western blot analysis was performed to detect only the His<sub>6</sub>-tagged recombinant protein (Figure 3.8, B), for a more sensitive and specific analysis. In Figure 3.8, A, the production of soluble *TcoAP1* in conjunction with the molecular chaperones was compared with the whole cell lysate *TcoAP1* sample which was not co-expressed with the chaperones. The production of soluble *TcoAP1* seems almost negligible when compared to the total *TcoAP1* found in the cell lysate when viewing the 12.5% reducing SDS-PAGE gel. However, western blot analysis of the soluble *TcoAP1* co-expression samples (Figure 3.8, B) showed a visible increase in soluble *TcoAP1* protein expression compared to the soluble fraction of the *TcoAP1* expression without molecular chaperones (NC). Furthermore, co-expression of *TcoAP1* with the pKJE7 chaperones (DnaK-DnaJ-GrpE) led to the biggest improvement in soluble *TcoAP1* expression (Figure 3.9).



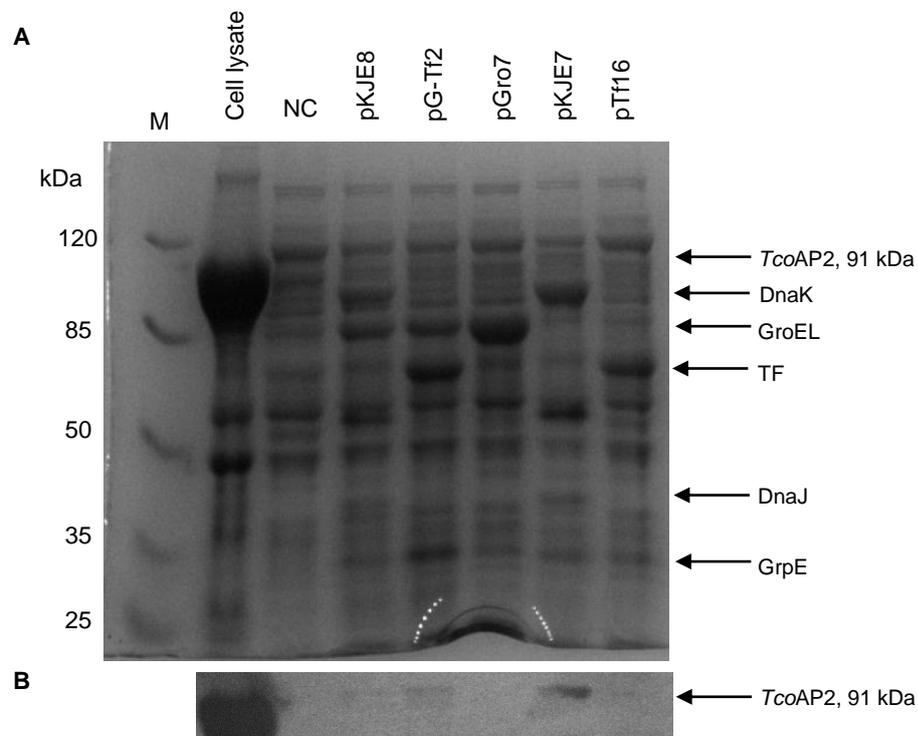
**Figure 3.8: 12.5% reducing SDS-PAGE and western blot analysis of soluble recombinant *TcoAP1* after molecular chaperone co-expression.** Samples of the soluble fractions and a reference cell lysate sample of recombinant *TcoAP1* co-expression with five different molecular chaperone teams were electrophoresed on 12.5% reducing SDS-PAGE gels and **(A)** stained with Coomassie blue R-250 while **(B)** was transferred onto nitrocellulose and incubated with chicken anti-His<sub>6</sub> HRPO conjugate [1:4000 in 0.5% (w/v) BSA-PBS]. M: Pre-stained protein marker. NC: No chaperones.



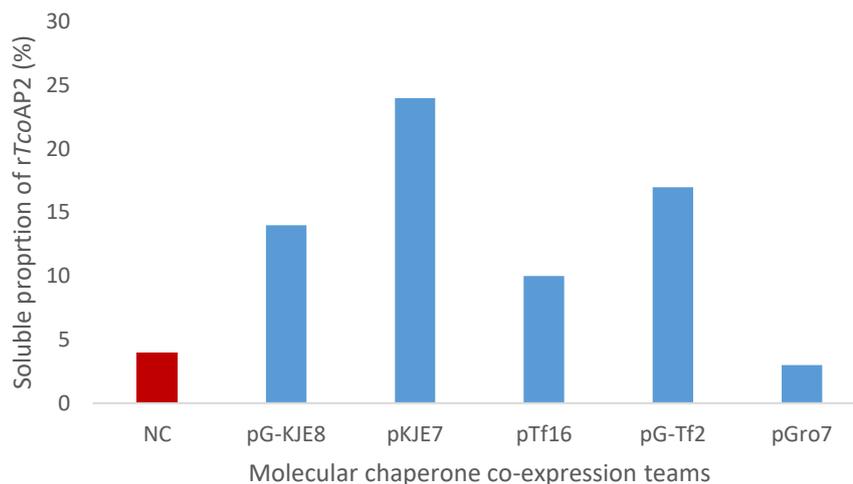
**Figure 3.9: Comparison of recombinant *TcoAP1* solubility after molecular chaperone co-expression.** The amount of soluble recombinant *TcoAP1* were compared according to band intensities of the western blot using GelAnalyzer<sup>®</sup>. NC: No chaperones.

In Figure 3.10, Panel A, another 12.5% reducing SDS-PAGE analysis of the co-expression performed is shown, again with only the soluble fractions to analyse soluble recombinant

*TcoAP2* expression, western blot analysis was also performed to detect only the His<sub>6</sub>-tagged recombinant protein (Figure 3.10, B). The production of soluble *TcoAP2* in conjunction with the molecular chaperones was compared with the whole cell lysate *TcoAP2* sample which was not co-expressed with the chaperones. The production of soluble *TcoAP2* also seemed negligible when compared to the total *TcoAP2* found in the cell lysate when viewing the 12.5% reducing SDS-PAGE gel (Figure 3.10, A). However, western blot analysis of the soluble *TcoAP2* co-expression samples (Figure 3.10, B) showed, as in the case of *TcoAP1*, a visible increase in soluble *TcoAP2* protein expression compared to the soluble fraction of the *TcoAP2* expression without molecular chaperones (NC). Again, co-expression of *TcoAP2* with the pKJE7 chaperones (DnaK-DnaJ-GrpE) led to the biggest improvement in soluble *TcoAP2* expression (Figure 3.11).



**Figure 3.10: 12.5% reducing SDS-PAGE and western blot analysis of soluble recombinant *TcoAP2* after molecular chaperone co-expression.** Samples of the soluble fractions and a reference cell lysate sample of recombinant *TcoAP1* co-expression with five different molecular chaperone teams were electrophoresed on 12.5% reducing SDS-PAGE gels and **(A)** stained with Coomassie blue R-250 while **(B)** was transferred onto nitrocellulose and incubated with chicken anti-His<sub>6</sub> HRPO conjugate [1:4000 in 0.5% (w/v) BSA-PBS]. M: Pre-stained protein marker. NC: No chaperones.

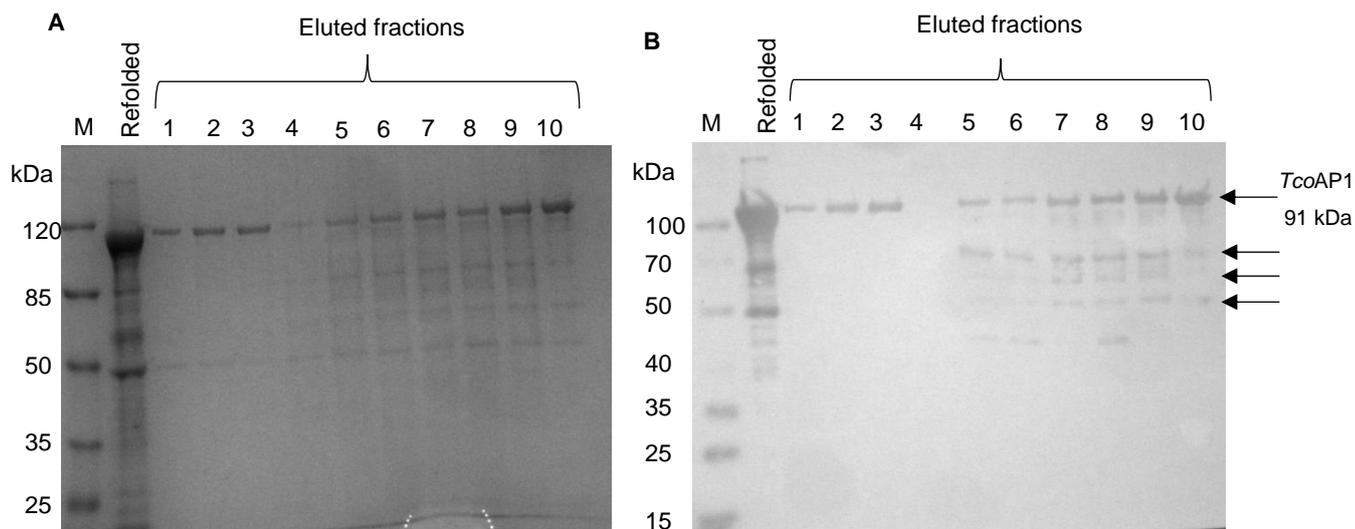


**Figure 3.11: Comparison of recombinant *TcoAP2* solubility after molecular chaperone co-expression.** The amount of soluble recombinant *TcoAP2* were compared according to band intensities of the western blot using GelAnalyzer®. NC: No chaperones.

### 3.3.3 Solubilisation, refolding and purification of recombinant *TcoAP1* and *TcoAP2*

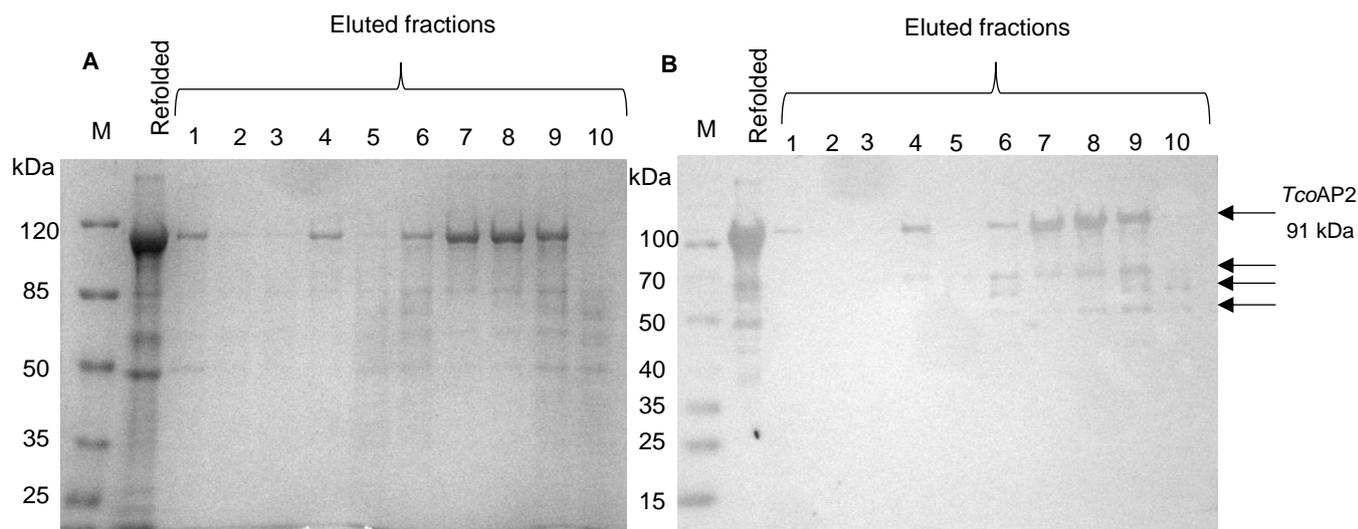
Despite improvements in soluble expression of both *TcoAP1* and *TcoAP2* upon co-expression of molecular chaperones, most of the target proteins were still found in the insoluble fraction. Therefore for downstream processes, it was decided that solubilising the vast amount of insoluble protein was the best option.

Solubilisation of the inclusion bodies using the sarkosyl method was performed, and the recombinantly expressed aminopeptidases were subsequently purified on a nickel chelate column then refolded on a G25 HiTrap desalting column. Reducing SDS-PAGE analysis of the refolding and purification of *TcoAP1* using the sarkosyl method is shown in Figure 3.12, panel A, where the eluted proteins were relatively pure being eluted in fractions 1 to 10. Western blot analysis confirms that the eluted protein was indeed the recombinantly expressed *TcoAP1*. In addition to the 91 kDa recombinant *TcoAP1*, there were three prominent lower molecular weight degradation products at ~70 kDa, ~60 kDa and ~50 kDa that were detected by anti-His<sub>6</sub> IgY antibodies in the eluted fractions 5-10 (Figure 3.12, B).



**Figure 3.12: Nickel affinity on-column refolding and purification of solubilised recombinant *TcoAP1* using the sarkosyl method.** Samples after refolding and from the eluted fractions after affinity purification were electrophoresed on two 12.5% reducing SDS-PAGE gel with one (A) stained with Coomassie blue R-250 and the other (B) transferred onto nitrocellulose, blocked with 5% (w/v) low fat milk-TBS and incubated with chicken anti-His<sub>6</sub> IgY HRPO conjugate [1:5000 in 0.5% (w/v) BSA-PBS] and 4-chloro-1-naphthol-H<sub>2</sub>O<sub>2</sub> were used as the detection system. M: Spectra BR pre-stained marker.

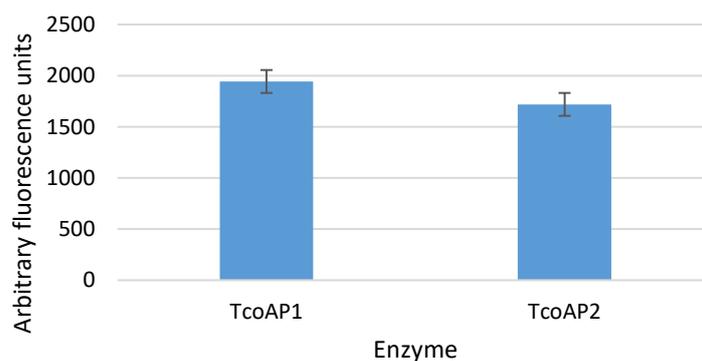
Reducing SDS PAGE analysis of the refolding and purification of *TcoAP2* using the sarkosyl method is shown in Figure 3.13, A, where the eluted proteins were relatively purified and eluted in fractions 1 to 10. Western blot analysis confirmed that the eluted protein was indeed the recombinantly expressed *TcoAP2*. Three other lower molecular weight bands at ~70 kDa, ~60 kDa and 50 kDa were detected, similar to the recombinant *TcoAP1* preparation, with the anti-His<sub>6</sub> IgY antibodies in the eluted fractions 5-10 shown in Figure 3.13, panel B.



**Figure 3.13: Nickel affinity on-column refolding and purification of solubilised recombinant *TcoAP2* using the sarkosyl method.** Samples from the refolded fraction and from eluted fractions after affinity purification were electrophoresed on two 12.5% reducing SDS-PAGE gel with one (A) stained with Coomassie blue R-250 and the other (B) transferred onto nitrocellulose, blocked with 5% (w/v) low fat milk-TBS and incubated with chicken anti-His<sub>6</sub> IgY HRPO conjugate [1:5000 in 0.5% (w/v) BSA-PBS] and 4-chloro-1-naphthol H<sub>2</sub>O<sub>2</sub> were used as the detection system. M: Spectra BR pre-stained marker.

### 3.3.4 Enzyme assay of *TcoAP1* and *TcoAP2*

To test if the sarkosyl method renatured recombinant aminopeptidases were indeed in their active form after refolding and purification, an H-Gly-AMC substrate activity assay was performed. As shown in Figure 3.14, both *TcoAP1* and *TcoAP2* showed activity against the substrate.



**Figure 3.14: Sarkosyl solubilised, refolded and purified recombinant *TcoAP1* and *TcoAP2* hydrolysis of H-Gly-AMC.** Equal amounts of the on-column refolded and purified *TcoAP1* and *TcoAP2* eluted after sarkosyl renaturation were incubated with aminopeptidase assay buffer containing the fluorogenic substrate (20  $\mu$ M) at 37°C for 10 min and the resulting fluorescence was measured at EX<sub>360nm</sub> and EM<sub>460nm</sub>. The fluorescence readings represent the average of triplicate experiments and are plotted as arbitrary fluorescence units.

### 3.4 Discussion

The need for co-ordinated actions against AAT have been recognised and therefore, in the last decade there has been renewed enthusiasm by governments, charities and philanthropists in funding research to tackle this huge challenge (Holt *et al.*, 2016). Effective treatment of AAT requires a reliable and inexpensive diagnosis of the disease. Diagnosis is complicated due to trypanosomiasis being caused by several species of trypanosomes that can infect several different host organisms leading to various parasite-host relationships. This results in most diagnostic tests having their own unique limitations that define their efficiency for a certain trypanosome-specific disease and therefore may need to be used in combination with each other in a rather scatter gun approach to diagnosis (Büscher, 2014).

*T. congolense* is a vector-transmitted parasite and is present in a vast reservoir of wildlife animals, control of the geographic spread of the disease in sub-Saharan Africa requires intense surveillance and treatment programs. Control of nagana relies on accurate diagnosis followed by chemotherapy. A main factor that hinders the control of the disease is the lack of easy-to-use field appropriate diagnostics that can be used followed by rapid response for infection treatment. Immunoassays based on the detection of trypanosome-specific antibodies have relied on the use of whole trypanosome lysates as antigens, which leads to problems of antigen stability, test reproducibility, and lack of specificity due to the persistence of antibody following treatment (Büscher, 2014).

Antigenic variation is a hurdle to the development of a standard vaccine for AAT. Consequently, the concept of an anti-disease vaccine, in which the pathogenic factors of the parasite are targeted rather than the parasite itself, has been planned (Antoine-Moussiaux *et al.*, 2009). The M1 aminopeptidases of *T. congolense* have been identified as possible pathogenic factors (section 3.2.2.2), that is, parasite antigens responsible for the development of disease in the host. Although several aminopeptidases from parasites such as *Plasmodium falciparum* (Mistry *et al.*, 2014) and *Schistosoma mansoni* (Maggioli *et al.*, 2017) have been evaluated as potential vaccines or chemotherapeutics, aminopeptidases of any trypanosome species have only previously been investigated once by Pillay (2013). This study led to the insufficient yield of the recombinant *T. congolense* M1 aminopeptidases required for efficient downstream processes. For these reasons, this study was conducted to improve the soluble recombinant expression of the two M1 type aminopeptidases *TcoAP1* and *TcoAP2* from *T. congolense*.

In Pillay's (2013) study, *TcoAP1* and *TcoAP2* were cloned, sequenced and expressed as inclusion bodies in *E. coli*. Originally, recombinant expression of *TcoAP1* and *TcoAP2* were attempted in the eukaryotic system, *Pichia pastoris*, because both aminopeptidases have a

high number of predicted disulfide bridges and were likely to undergo post-translational modifications similar to that of the native proteins resulting in soluble expressed functional proteins (Cereghino *et al.*, 2002). However, even after investigating several expression conditions using *P. pastoris*, no recombinant protein was produced. Subsequently, recombinant expression was conducted in *E. coli* BL21 (DE3), and as predicted, the recombinant proteins were expressed in inclusion bodies even when fused with solubility enhancing protein fusion tags such as GST and Trx. *E. coli* mutants such as BL21 (DE3) that are deficient in ATP- dependent proteases, *i.e.* Lon and OmpT deficient, are regularly used to improve the expression of unstable recombinant proteins such as aminopeptidases. However, the use of these types of *E. coli* often results in misfolding and aggregation of the non-native proteins after escaping degradation. This is partly due to the accumulation of misfolded recombinant protein saturating the limited amount of molecular chaperones normally available for correct protein folding (Nishihara *et al.*, 1998).

The number of aminopeptidase genes which were cloned recombinantly expressed and biochemically characterised is progressively increasing (Drinkwater *et al.*, 2017). However, high-level recombinant expression of aminopeptidases in *E. coli* typically results in misfolding and insoluble aggregation (Munkhjargal *et al.*, 2016; Maggioli *et al.*, 2017). The recombinant plasmids *TcoAP1*-pET-28a and *TcoAP2*-pET-28a were obtained from the lab. In this study, both 12.5% reducing SDS-PAGE and western blot analysis showed that after recombinant expression induction for 4 h at 37°C, both *TcoAP1* and *TcoAP2* were almost entirely present in inclusion bodies (Figure 3.5). In this study, these antigens were expressed under various conditions, but they were persistently produced in an insoluble form (data not shown). Aggregation of protein in inclusion bodies is caused by misfolding of overexpressed proteins which is a common occurrence in protein characterisation research (Rosano and Ceccarelli, 2014). An increasingly popular approach for the prevention of insoluble aggregation of proteins is co-expression with molecular chaperones; this was employed to produce soluble aminopeptidases in this study. Several investigations have elucidated the mechanisms of *in vivo* protein folding and it has been shown that molecular chaperones play crucial roles in this process (Mogk *et al.*, 2002). The *E. coli* cytoplasm is known to have three prominent molecular chaperone systems: Hsp60, Hsp70 and trigger factor (Baneyx and Mujacic, 2004). Upon leaving the exit tunnel of the ribosome newly biosynthesised polypeptides in *E. coli* initially associate with the chaperone trigger factor to prepare the nascent polypeptide to either spontaneously fold to its native conformation or for further folding assistance by other downstream chaperones (Hoffmann *et al.*, 2010). DnaK-DnaJ-GrpE disrupts inclusion body build-up by binding the misfolded protein intermediates for refolding or proteolysis, thus reducing aggregation, while GroES-GroEL encloses misfolded protein, preventing

aggregation and folding the protein to native state if possible or else it promotes proteolysis (Kerner *et al.*, 2005).

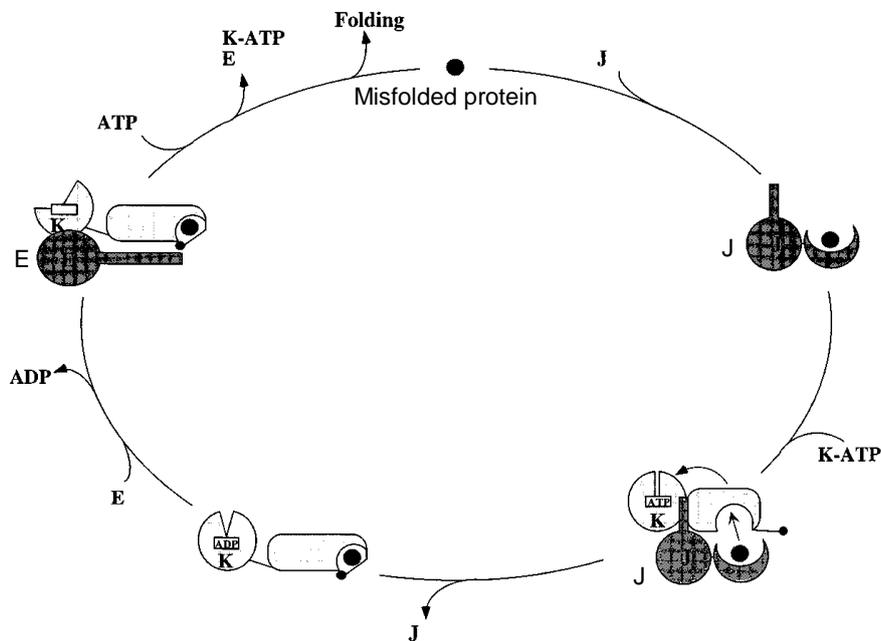
The molecular chaperones used in this study were sourced from Takara Bio Inc (Kyoto, Japan). The product consists of a set of five plasmids that encode for the overexpression of five molecular chaperone teams respectively (Table 3.1). The plasmids were designed to allow efficient recombinant expression of these chaperones which are recognised to work together in the protein folding process (Nishihara *et al.*, 1998). In a previous recombinant molecular chaperone co-expression study, 26 of the 50 various recombinant proteins of differing characteristics were shown to have improved solubility when co-expressed with differing combinations of the two major molecular chaperone teams, namely, GroEL-GroES and DnaK-DnaJ-GrpE (de Marco *et al.*, 2007). Normally these proteins form inclusion bodies when overproduced recombinantly in *E. coli* host (Dümmler *et al.*, 2005).

In this study, the soluble expression levels of recombinant *TcoAP1* were increased when co-expressed with the pKJE7 (DnaK-DnaJ-GrpE), pG-KJE8 (DnaK-DnaJ-GrpE + GroES-GroEL), pTf16 (trigger factor), and pG-Tf2 (GroES-GroEL + trigger factor) chaperone teams while a decrease in solubility was observed when co-expressed with pGro7 (Figure 3.8, B). For *TcoAP2* a similar pattern was observed with solubility of the recombinant protein increasing when co-expressed with the pG-KJE8, pKJE7, pTf16, pG-Tf2 chaperone teams while once more a decrease in solubility was observed upon co-expression with pGro7 (Figure 3.10, B).

The general improvement in *TcoAP1* and *TcoAP2* recombinant soluble expression when co-expressed with molecular chaperones could be attributed to the ability of the overexpressed molecular chaperone systems to refold, *in vivo*, large amounts of the overexpressed and misfolded antigens that occurred during co-expression. It could be inferred that normal expression induction of the molecular chaperones were insufficient to reduce the amount of misfolded overexpressed recombinant aminopeptidases as compared to co-expression with the overexpressed variant of the chaperone systems. The DnaK-DnaJ-GrpE molecular chaperone team's better performance relative to the other molecular chaperone combinations without DnaK-DnaJ-GrpE, could be attributed to the molecular chaperones' preference to bind to protein substrates larger than 60 kDa (Deuerling *et al.*, 2003) whereas GroEL-GroES preferentially binds to protein substrates within the size range of 10-55 kDa due to the assembled GroEL-GroES complex cavity being approximately 57 kDa (Sakikawa *et al.*, 1999). Furthermore, the observed decrease in soluble levels of the recombinant aminopeptidases when co-expressed with GroEL-GroES can be attributed to the chaperones involvement in promoting proteolysis. GroEL-GroES has been shown to be involved in promoting proteolysis during heat shock of endogenous protein aggregates in *E. coli* (Kędzierska *et al.*, 1999). The

trigger factor molecular chaperones' effect on the recombinant aminopeptidases solubility wasn't as marked as DnaK-DnaJ-GrpE probably because trigger factor is known to be a cold shock chaperone and performs better at low temperatures (Piao *et al.*, 2016), while, the co-expression experiments in this study were done at 37°C. In de Marco *et al.* (2007) study, recombinant sucrose synthases Susy and Mash from *Zea mays* that were of similar size to the *TcoAP*'s showed at least three-fold improvement in solubility when co-expressed with molecular chaperone combinations containing DnaK-DnaJ-GrpE.

In this study, recombinant *TcoAP1* and *TcoAP2* soluble expression was improved by at least two-fold when co-expressed with DnaK-DnaJ-GrpE (pKJE7), this was the largest improvement observed between the different co-expression experiments. The proposed model of this molecular chaperones' protein folding process is shown in Figure 3.15.



**Figure 3.15: Model of the protein folding process of the DnaK-DnaJ-GrpE system (Hsp70).** The misfolded protein (closed dot) associates with DnaJ (J), then the protein is passed to the ATP form of DnaK (K). This pass-over is facilitated by ATP hydrolysis. After protein transfer, DnaJ is released from the complex, and GrpE (E) binds to the complex triggering ADP dissociation from DnaK. This facilitates the binding of ATP which results in the dissociation of GrpE and folded protein from DnaK. (Bukau and Horwich, 1998).

Although co-expression with the various molecular chaperone teams led to the improved soluble expression of the aminopeptidases, when testing these co-expressed aminopeptidases for correct folding via enzymatic activity assays none of them showed activity after 30 min incubation with known synthetic fluorometric substrate H-Gly-AMC (data not shown). Furthermore, most of the recombinant antigens were still found in the insoluble fractions of the analysed samples. Although DnaK-DnaJ-GrpE improved the solubility of both recombinant aminopeptidases, the recombinant antigens' quality was probably compromised

since this molecular chaperone team is known to increase soluble aggregation of partially folded intermediates (Haacke *et al.*, 2009). Hence the observation of no enzyme activity even after improved soluble expression levels after co-expression. The occurrence of such side effects in molecular chaperone co-expression indicates that strategies need to be developed considering that both protein solubility and conformational quality are not necessarily linked (Martínez-Alonso *et al.*, 2010). Therefore, moving forward, the resolubilisation, refolding and purification of insoluble protein samples were performed.

It is possible for inclusion bodies to contain protein that is close enough to native conformation and therefore easy to solubilise using non-ionic detergents or low concentrations of sodium chloride (Burgess, 2009). However, this is rarely the case as the difficulty of solubilising recombinant *TcoAP1* and *TcoAP2* inclusion bodies in guanidine was demonstrated in Pillay *et al.*'s (2013) study. Therefore, in this study, a different non-ionic detergent, namely sarkosyl, was used for this purpose. Given the high number of cysteine residues in both APs, a reducing agent (DTT) was added to the inclusion body solubilisation buffer (but excluded from the refolding buffer) in order to minimise the incorrect formation of disulfide bonds as recommended by Burgess (2009). Following solubilisation of the inclusion bodies, on-column refolding was performed using the G25 HiTrap desalting column. The wash and elution buffers contained low concentrations of sarkosyl, 0.1% (w/v), to maintain the antigens' solubility during the refolding procedure using both IMAC and the desalting column. The use of the sarkosyl method resulted in a moderately improved yield of pure aminopeptidases when compared to the only previous study of them. Enzyme activity assays using the H-Gly-AMC synthetic substrate were performed with the purified and refolded aminopeptidases, and correct folding was confirmed (Figure 3.14).

Both molecular chaperone co-expression and sarkosyl resolubilisation methods were performed to mediate the recovery of soluble recombinant aminopeptidases from insoluble aggregates and showed relative improvement in recombinant antigen solubility in this study when compared to the only previous recombinant expression study of the *T. congolense* aminopeptidases.

## Chapter 4: GENERAL DISCUSSION

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Animal African trypanosomosis (AAT), a neglected tropical disease, is endemic to approximately 70% of the countries on the African continent. The disease is estimated to have a yearly financial impact of between US \$ 1.5 to 5 billion (Leigh *et al.*, 2015). The debilitating disease caused by trypanosome parasites is mainly spread by the infected tsetse fly (Matthews *et al.*, 2015). These parasites' ability to vary their surface coats through an activity known as antigenic variation hugely deters the development of a vaccine (Morrison *et al.*, 2009). Consequently, the popular remedial approaches for AAT are targeting the tsetse-fly or using trypanocidal drugs. The establishment of diagnostic tests for use in cattle-herding areas is critical for successful treatment by allowing the proper administration of the available trypanocides (Giordani *et al.*, 2016).

Trypanosomosis diagnostics are developed to either detect the whole parasite or its components (mainly protein antigens, DNA or induced antibodies). Antigen detection allows for diagnosis of current infections, whereas antibody detection generally cannot determine if an infection is current or was recently cured since antibodies can persist in the bloodstream after cure. The gold standard microhaematocrit centrifugation is a parasite detection method that is simple to use. However, sensitivity is low (Masake *et al.*, 1995) in comparison to molecular techniques such as loop mediated isothermal amplification (LAMP) or PCR techniques that need relatively complex equipment and expertise (Mitashi *et al.*, 2012). Sandwich ELISA, also known as capture or antigen ELISA, is applicable for high-throughput screening and uses relatively inexpensive equipment when compared to PCR, but currently, no eligible antigen targets have been identified, despite several investigations (OIE, 2013). Antibody detection was originally done by indirect fluorescence antibody test (IFAT) or parasites fixed on a slide (Katende *et al.*, 1987). Other forms of antibody detection techniques include the use of indirect ELISA that is most popular for antibody detection in sera diagnosis. The use of whole parasite lysates as a source of antigen is a major limitation to these techniques because it is difficult to standardise trypanosome lysates. These lysates are prepared in different ways either from parasites propagated in rodents or grown in culture using different protocols. Additionally growing *T. vivax* in culture is particularly difficult (Boulangé *et al.*, 2017). Furthermore, because of the high-level homology between the *salivaria* group of trypanosomes, species-specific diagnosis remains elusive, although not impossible, with this issue marginally resolved by analysing cross-reactions between species (Hopkins *et al.*, 1998).

These hurdles can be overcome by using purified recombinant antigens previously identified as pathogenic factors, instead of whole parasite lysates, because antigen production can be

standardised resulting in improved species-specific diagnosis. However, this approach requires relatively expensive and complex equipment, and expertise which reserves this approach for extensive epidemiological studies rather than point-of-care decisions in the field performed by the farmers (Boulangé *et al.*, 2017). An optimised ELISA test can be converted to an immunochromatographic or rapid diagnostic test (RDT) or “dipstick” format for use under field conditions, perfect for end-users. The Surra Sero K-SeT antibody RDT based on recombinant variant surface glycoprotein rVSG RoTat 1.2 was developed for *T. evansi* diagnosis and is commercially available (Birhanu *et al.*, 2015). Further RDTs based on *T. congolense* cathepsin B and *T. vivax* GM6 antigen, a flagellar tandem repeat protein, showed much promise for field diagnosis (Boulangé *et al.*, 2017).

Over time more antigens with diagnostic potential for trypanosomosis have been identified and produced, using optimisable and standardised recombinant technology, to avoid the use of unstable native parasite antigens for diagnosis. The ideal trypanosome antigens with diagnostic potential are immunodominant antigens found in the bloodstream development stage of the parasite (Thuy *et al.*, 2012). Trypanosome cysteine peptidases are considered as immunodominant antigens with potential for the development of AAT serodiagnostic tools (Lalmanach *et al.*, 2002). In addition to the cysteine peptidases, the family M1 aminopeptidases were also identified as potential pathogenic factors (Pillay, Boulangé, *et al.*, 2013). However, a recurring theme that is revealed in recombinant expression studies of these antigens is the production of the desired antigen within inclusion bodies due to misfolding and aggregation. Soluble recombinant expression of these antigens, in high enough yields and correct conformation, is required for downstream processes such as antibody production and functional characterisation.

The aim of this research project was to optimise the soluble recombinant expression of the previously studied and potential diagnostic targets, full-length mutant *Tv*CATL from *T. vivax* FL*Tv*CATL<sub>C25A</sub> (Vather, 2010) and the two *T. congolense* family M1 aminopeptidases *Tco*AP1 and *Tco*AP2 (Pillay, Boulangé, *et al.*, 2013). Recombinant protein expression in *E. coli* has developed over the years to produce high amounts of soluble target when compared to other methods of obtaining target protein (Rosano and Ceccarelli, 2014).

The first papain-like peptidase to be expressed in an *E. coli* expression system containing a T7 promoter was human cathepsin L. The recombinant expression levels of protein obtained in that study were very low and the resultant protein was mostly expressed in an inactive form within inclusion bodies (Smith and Gottesman, 1989). Early attempts to express *Tco*CATL from *T. congolense* in *E. coli* was unsuccessful as this yielded misfolded, inactive protein (Boulangé *et al.*, 2001). However, a later expression study produced a marginal increase in

yields and the production of active enzyme using the much more expensive and complicated baculovirus expression system (Boulangé *et al.*, 2001). A CATL-like peptidase from *Leishmania braziliensis* was also expressed in *E. coli* mostly in inclusion bodies where resolubilisation and on-column refolding were used to recover an active enzyme (Lanfranco *et al.*, 2008).

The FL *Tvi*CATL<sub>C25A</sub> construct used in the present study is an inactive mutant of *Tvi*CATL and was investigated for its potential as a diagnostic target (Vather, 2010). *Tvi*CATL belongs to Clan CA of the CP family and has a classical papain-like catalytic triad of Cys<sup>25</sup>, His<sup>159</sup> and Asn<sup>175</sup> (papain numbering), however, this mutant construct had Cys<sup>25</sup> replaced by Ala<sup>25</sup> by Vather *et al.* (2010). The mutant construct contains a pro-peptide domain (120 residues), which is auto-catalytically cleaved at acidic pH in the case of active constructs (Jackson, 2011), a catalytic domain connected to a C-terminal domain by a poly-proline hinge region and Trx fusion tag from the pET32-a expression vector which reportedly improves solubility of recombinant proteins (Young *et al.*, 2012). The C-terminal region is unique to parasite cysteine peptidases and is completely absent in the mammalian homologues (Mottram *et al.*, 2004). The function of the C-terminal region is currently speculative, however, it has been hypothesised that it may play a role in targeting the enzyme to the lysosome or glycosome, as is the case for the phosphoglycerate kinase of *Crithidia fasciculata* (Swinkels *et al.*, 1988). Moreover, the presence of an N-glycosylation site in this region could potentially allow attachment of a lysosome targeting marker.

The recombinant expression of FL *Tvi*CATL<sub>C25A</sub>, *Tco*AP1 and *Tco*AP2 using the standard cultivation conditions established in our laboratory led to the recombinant antigens being produced almost entirely in inclusion bodies. Inclusion bodies are composed predominantly of the recombinant protein with contaminating proteins representing less than 15% of the inclusion body (Ramón *et al.*, 2014). Various factors may contribute to inclusion body formation including: very high concentration of the recombinant protein within the cytosol leading to insoluble protein aggregation, inadequate amounts of endogenous molecular chaperones resulting in promotion of hydrophobic interactions between partially folded polypeptides, inhibition of disulfide linkage formation since the *E. coli* cytosol is in a reducing state and a lack of the post-translational modifying cellular machinery thus producing less stable eukaryotic recombinant protein (Walsh, 2002). Recombinant proteins within inclusion bodies are predominantly composed of partially folded protein intermediates that are consequently biologically inactive (Walsh, 2002). Hence, to obtain these recombinant antigens for effective downstream processing, inclusion body formation must be avoided. If this is not possible then the soluble recombinant antigens must be recovered from the inclusion bodies.

For the optimisation of FL*Tvi*CATL<sub>C25A</sub> soluble recombinant protein expression, the BL21 (DE3) *E. coli* strain was chosen. This strain of *E. coli* is deficient in the Lon and OmpT proteases with the former responsible for degrading foreign proteins while the latter is an outer membrane protease that degrades extracellular proteins (Daegelen *et al.*, 2009). These modifications help prevent the digestion of recombinant protein after cell lysis. Cultivation of *E. coli* for recombinant protein expression is usually done with a set of techniques that are in combination known as batch cultivation (Sørensen and Mortensen, 2005). Batch cultivation is a relatively easy and inexpensive approach to achieving high-density *E. coli* cultures. In various protein expression experiments, achieving an OD<sub>600nm</sub> of 0.8 (approximately 6.4 x 10<sup>8</sup> cells/ml) within 4 hours is common when using “standard” cultivation conditions *i.e* 2 x YT complex medium, 37°C cultivation temperature and 200 RPM agitation. *Escherichia coli* reaches mid exponential growth phase, the preferred phase for recombinant gene expression (Khow and Suntrarachun, 2012), at approximately 4 h when cultivating at 37°C. The 2 x YT medium was used instead of the more popular LB broth because it has consistently shown to improve cell densities during recombinant expression when comparisons were done (Atlas, 2015). Optimising the batch cultivation conditions is regarded as the simplest approach to improving soluble recombinant protein expression.

In this study, a factorial design was implemented for optimising cultivation conditions to improve the soluble expression of recombinant FL*Tvi*CATL<sub>C25A</sub>. Three factors were initially chosen for screening, namely, cultivation temperature, inducer (IPTG) concentration and induction time. These factors were chosen because they are generic factors that can be applied to most soluble protein production studies and because these factors are known to have an impact on recombinant protein solubility during recombinant expression in *E. coli* host due to them primarily affecting the rate of protein biosynthesis. Generally reducing the agitation rate should decrease the formation of inclusion bodies, however, this will inevitably decrease cell growth and total protein production (Papanephytou and Kontopidis, 2014). High cultivation temperature promotes cell growth; however, high temperature can have a negative impact on soluble protein expression since higher growth rates lead to a higher likelihood of plasmid loss, increased errors during the gene expression process and inevitably leads to an increase in improperly synthesised recombinant protein that become misfolded (Mosrati *et al.*, 1993). This is a vital issue when it comes to gene expression from expression vectors. In general, protein aggregation is favoured at high temperatures because hydrophobic interactions between protein macromolecules are promoted as the temperature increases (Kiefhaber *et al.*, 1991). Taking the effects of high temperature in consideration, it is evident that decreasing temperature usually has a beneficial impact on improving solubility of recombinant protein. Decreased temperature, coupled with increased induction duration

greatly benefited the soluble expression of many recombinant proteins, but arriving at the best combination of these two factors is generally a trial-and-error exercise (Papaneophytou and Kontopidis, 2014). In terms of inducer concentration, this factor has been found to affect recombinant protein yields. A low concentration results in inefficient protein expression induction, whereas excess inducer concentration can be toxic to the *E. coli* host, causing reduced cell growth and recombinant protein concentration (Ramirez *et al.*, 1994). It has been previously shown that IPTG concentrations between 0 and 1 mM does not affect *E. coli* growth rate or maximum cell density, however, this depends on the combination of cell host, expression vector and recombinant protein used in a study.

To screen the above-mentioned factors for their influence on recombinant FL*Tvi*CATL<sub>C25A</sub> solubility, a design of experiments approach called full factorial design was employed. Initially little information was available to determine the influence of various factors on soluble recombinant expression of FL*Tvi*CATL<sub>C25A</sub>, therefore examining multiple factors simultaneously was advisable and this approach allowed for that. Full factorial design was performed by using a two-level full factorial, meaning that two levels were chosen for each factor, namely a lower (-) and higher (+) level. For induction temperature the lower level was 30°C and the higher level was 37°C, for induction duration 4 h and 16 h were the respective levels while for the inducer concentration 0 and 1 mM were tested. The three factors and their levels were then combined to develop a design matrix (using Design Expert® software) on which the factor screening experiments were based. After performing all the experiments based on this matrix, no observable improvement on soluble expression of rFL*Tvi*CATL<sub>C25A</sub> was achieved. This consequently led to the discontinuation of attempts to optimise the cultivation conditions for soluble expression improvement and initiated the resolubilisation process in attempts to recover the recombinantly expressed protein from inclusion bodies.

Polyclonal anti-FL*Tvi*CATL<sub>C25A</sub> antibodies were produced in chickens due to the efficiency and simplicity of chicken IgY isolation and purification methods (Pauly *et al.*, 2011). The anti-FL*Tvi*CATL<sub>C25A</sub> polyclonal IgY was purified on affinity resins prepared by immobilising recombinant FL*Tvi*CATL<sub>C25A</sub> on the AminoLink® coupling matrix. Large quantities of the anti-FL*Tvi*CATL<sub>C25A</sub> IgY were produced and isolated with the purified antibodies showing high specificity at relatively low concentrations. Parasite CATL's are localised in lysosomes and are released into the host bloodstream upon parasite death and lysis (Caffrey and Steverding, 2009), therefore the generated polyclonal antibodies were tested against the bloodstream form of a parasite for antigen detection. The affinity purified anti-FL*Tvi*CATL<sub>C25A</sub> IgY recognised native 33 kDa CATL in *T. congolense* bloodstream form lysates in a western blot.

For future work, it will be necessary to conduct antibody detection inhibition ELISA in parallel with an indirect antibody detection ELISA to compare the specificity, sensitivity and reproducibility of the diagnostic procedures. In this analysis it will be necessary to include *Kinetoplastidae* other than trypanosomes present in areas where AAT is endemic to use as negative controls. To further improve serodiagnosis accuracy, it would be ideal to develop an antigen detection ELISA (Eisler *et al.*, 2004). The antibodies that were produced in this study may be the starting point for such a development, in which the detection of *Tvi*CATL antigen from *T. vivax* infected sera can be investigated. The steps for the proposed antigen detection ELISA are: coat with chicken anti-FL*Tvi*CATL<sub>C25A</sub> antibodies, probe with *T. vivax* infected sera from cattle and use a rabbit anti-IgY-HRPO conjugate with the appropriate chromogen for detection.

The family M1 aminopeptidases are metallopeptidases requiring a divalent metal co-factor for the catalysis of single amino acid residues from the N-terminal end of peptides. Aminopeptidases have only been previously studied in *T. congolense* (Pillay, Boulangé, *et al.*, 2013). The leucyl aminopeptidases of *Fasciola hepatica* and *Haemonchus contortus* were evaluated for their protective potential in conventional vaccines with some success (Acosta *et al.*, 2008). Interestingly, the leucyl aminopeptidase of *F. hepatica* was used in conjunction with the primary cysteine peptidase as a cocktail vaccine. However, the best protection (89%) was achieved using leucyl aminopeptidase alone (Piacenza *et al.*, 1999). Furthermore, it has been shown that inhibition of the M1 aminopeptidases of *P. falciparum* results in growth inhibition *in vitro* (Skinner-Adams *et al.*, 2010).

In Pillay *et al.* (2013) study, *Tco*AP1 and *Tco*AP2 were expressed in inclusion bodies in *E. coli*. Initially, recombinant expression of both aminopeptidases was attempted in the eukaryotic system, *Pichia pastoris*, since the aminopeptidases had a high number of predicted disulfide bridges and would be likely to undergo post-translational modifications similar to those of the native proteins resulting in soluble expressed functional proteins (Cereghino *et al.*, 2002). However, even after testing several expression conditions using *P. pastoris*, no expression was achieved. Subsequently, within the same study, recombinant expression was conducted in *E. coli* BL21 (DE3), and as predicted, the recombinant proteins were expressed in inclusion bodies using the pGEX-4T-1 (GST fusion tag), pET28-a and pET32-a (Trx fusion tag) vectors (Pillay, Boulangé, *et al.*, 2013). Several similar aminopeptidases have previously been expressed as soluble active proteins in *E. coli* including the M1 aminopeptidase of *P. falciparum*, PfAM1, the leucyl aminopeptidases of *F. hepatica* and those of three Leishmania species, all of which had fused polyhistidine tags to aid purification (Morty and Morehead, 2002; Acosta *et al.*, 2008; González-Bacerio *et al.*, 2014). Similarly, the methionine aminopeptidase of *P. falciparum*, leucyl aminopeptidases of the tick *Haemaphysalis*

*longicornis*, the apicomplexan parasite *Toxoplasma gondii* and the pathogenic bacterium *Salmonella enterica*, were all expressed as soluble active enzymes using a GST fusion protein tag in an *E. coli* expression system (Mathew *et al.*, 2000; Hatta *et al.*, 2006; Jia *et al.*, 2010). The leucyl aminopeptidases of the flukes *Schistosoma mansoni* and *S. japonicum*, were expressed in a different expression system namely the eukaryotic Baculovirus expression system (McCarthy *et al.*, 2004). It is speculative as to why the expression of both *TcoAP1* and *TcoAP2* failed in *P. pastoris* (Pillay *et al.*, 2013), but it was suggested that the active form of the *T. congolense* aminopeptidases may be toxic to the yeast, as was the case for the serine peptidase trypsin (Hanquier *et al.*, 2003). Processing of the zymogen during the recombinant expression resulted in very low yields of the active trypsin being recovered. However, it was found that by preventing maturation using a single point mutation resulted in a large amount of the zymogen being recombinantly expressed in *P. pastoris* (Hanquier *et al.*, 2003).

In the present study, both reducing SDS-PAGE and western blot analysis showed that after recombinant expression induction under standard conditions (4 h incubation at 37°C), both *TcoAP1* and *TcoAP2* were almost entirely present in inclusion bodies. In this and the previous (Pillay, Boulangé, *et al.*, 2013) study, these proteins were expressed under various conditions but were persistently produced in an insoluble form. Aggregation of protein in inclusion bodies is caused by misfolding of overexpressed proteins which is a common occurrence in protein characterisation research (Rosano and Ceccarelli, 2014). An increasingly popular approach for the prevention or mitigation of insoluble aggregation of proteins is co-expression with molecular chaperones. This approach was subsequently employed in the present study to produce soluble aminopeptidases. Molecular chaperones are involved in the crucial stages of *de novo* protein folding, and several studies have shed more light on the exact mechanisms of this process (Mogk *et al.*, 2002). The *E. coli* cytoplasm is known to have three prominent chaperone systems, namely, Hsp60, Hsp70 and trigger factor (Baneyx and Mujacic, 2004). Upon leaving the ribosome's exit channel, newly biosynthesised polypeptides in *E. coli* initially covalently interact with the chaperone trigger factor to prepare the nascent polypeptide to either spontaneously fold to its native conformation, or for further folding assistance by other downstream chaperones (Hoffmann *et al.*, 2010). DnaK-DnaJ-GrpE (Hsp70 chaperone family) decreases inclusion body formation by degrading misfolded proteins, while GroEL-GroES (Hsp60 chaperone family) participates in the transition of protein from insoluble to soluble fractions and promotes disaggregation, thus limiting the formation of inclusion bodies (Kerner *et al.*, 2005). The soluble expression levels of recombinant *TcoAP1* were increased when co-expressed with the pKJE7 (DnaK-DnaJ-GrpE), pG-KJE8 (DnaK-DnaJ-GrpE + GroES-GroEL), pTf16 (trigger factor), and pG-Tf2 (GroES-GroEL + trigger factor) chaperone teams while a decrease in solubility was observed when co-expressed with pGro7. For *TcoAP2* a similar

pattern was observed with solubility of the recombinant protein increasing when co-expressed with the pG-KJE8, pKJE7, pTf16, pG-Tf2 chaperone teams while once more a decrease in solubility was observed upon co-expression with pGro7.

The pKJE7 (DnaK-DnaJ-GrpE) chaperone team gave the largest improvement of recombinant soluble levels for both *TcoAP1* and *TcoAP2*. The DnaK-DnaJ-GrpE molecular chaperone team performs three different tasks when it comes to interacting with protein substrates: it plays a part in *de novo* folding of protein, it refolds or degrades denatured protein and more importantly in the context of this study, it is involved in reversing insoluble aggregation of protein (Betiku, 2006). It has been suggested that it disaggregates protein by performing an ATP-dependent unfolding process that allows for lengthy binding of thermolabile protein substrate (Goloubinoff *et al.*, 1989), therefore preventing aggregation and stabilising the protein substrate for refolding (Buchberger *et al.*, 1996). The DnaK-DnaJ-GrpE molecular chaperone team performed better than the other chaperone teams probably because it preferentially binds to newly synthesised polypeptides that are larger than 60 kDa (Deuerling *et al.*, 2003) whereas the GroEL-GroES chaperone team prefers to bind newly synthesised polypeptides that are in the size range of between 10-55 kDa (Sakikawa *et al.*, 1999). Both *TcoAP1* and *TcoAP2* were recombinantly expressed as ~91 kDa proteins. Trigger factor is a cold shock molecular chaperone (Piao *et al.*, 2016), and therefore did not perform as well as the DnaK-DnaJ-GrpE molecular chaperone team probably because recombinant co-expression experiments were done at 37°C cultivation temperature.

Although the molecular chaperone co-expression experiments markedly improved the soluble expression of the recombinant aminopeptidases, when compared to their previous study (Pillay, Boulangé, *et al.*, 2013), most of the recombinant antigens were still found in inclusion bodies. As with FLTviCATL<sub>C25A</sub>, renaturation was performed on the recombinant aminopeptidases using the sarkosyl method adapted from Schlager *et al* (2012).

All three recombinantly expressed antigens were resolubilised and renatured using the non-chaotropic (sarkosyl) method during immobilised metal affinity chromatography (IMAC) purification. Previous attempts at on-column refolding and purification using the chaotropic (urea) method failed for FLTviCATL<sub>C25A</sub> Vather (2010), whereas Pillay *et al.* (2013) attempts at renaturing the recombinant aminopeptidases using the chaotropic agent guanidine was tedious and led to a large loss of the expressed antigens during this process. In the sarkosyl resolubilisation method, inclusion bodies were solubilised by SDS, the excess of which was precipitated using cloud point extraction by cooling and was removed from the solubilised protein by centrifugation. Cloud point extraction is a standard technique used in the purification of membrane proteins (Arnold and Linke, 2007). The remaining wash and elution buffers

contained a low concentration of sarkosyl [0.1% (w/v)] to maintain protein solubility during the on-column refolding procedure. This method was recently used to renature a cysteine peptidase, namely *TcoMCA5* (Eyssen, 2014). The use of the sarkosyl method of solubilisation, refolding and purification resulted in much higher yields for all three recombinantly expressed antigens when compared to previous recombinant expression studies (Vather, 2010; Pillay, Boulangé, *et al.*, 2013).

In the present study, the active-site mutant full-length CATL from *T. vivax*, FL*TviCATL*<sub>C25A</sub> and the family M1 aminopeptidases of *T. congolense*, *TcoAP1* and *TcoAP2* were expressed and renatured using an *E. coli* expression system in conjunction with the sarkosyl renaturation method to produce high yields of homogeneous recombinant antigens that were amenable to their use as antigens in diagnostic tests. For future studies, it is important to continue improving the yields of homogenous recombinant antigen for downstream processes such as protein crystallisation to obtain the three-dimensional structures of these therapeutically important antigens. Interestingly, the design of experiments that were used in this study is a versatile approach that can also be used to optimise the factors involved in crystallisation trials such as pH, protein and PEG concentrations. Previous crystallisation condition optimisation for *TcoCATL* were attempted by Jackson (2011) and was partially successful as needle-like crystals were obtained, however, it may be of interest to use the design of experiments approach to improve outcomes.

In conclusion, two different approaches to recombinant soluble protein expression optimisation were undertaken and in the instance of molecular chaperone co-expression, a two-fold improvement of soluble recombinant aminopeptidase expression was achieved when co-expressed with DnaK-DnaJ-GrpE. In the case of FL*TviCATL*<sub>C25A</sub>, although the design of experiments approach to FL*TviCATL*<sub>C25A</sub> expression optimisation did not improve soluble expression of the protein, the use of the non-chaotropic sarkosyl method for its resolubilisation led to much higher yields and more efficient downstream processing. This study developed optimised and standardised procedures for high-yield recombinant expression and purification of three potential diagnostic antigens, namely, the *T. vivax* recombinant antigen, FL*TviCATL*<sub>C25A</sub> and the *T. congolense* recombinant antigens *TcoAP1* and *TcoAP2*, using the inexpensive and readily obtainable *E. coli* BL21 (DE3) expression system.



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