RECOMBINANT EXPRESSION AND EVALUATION OF α- AND β-TUBULIN FROM *TRYPANOSOMA CONGOLENSE* AS VACCINE CANDIDATES FOR AFRICAN TRYPANOSOMIASIS

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PREFACE

The experimental work described in this dissertation was carried out in the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, from January 2008 to January 2010, under the supervision of Prof. Theresa H. T. Coetzer.

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

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Cara-Lesley Bartlett (candidate)

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Prof. Theresa H.T. Coetzer (supervisor)

DECLARATION - PLAGIARISM

I, Cara-Lesley Bartlett, declare that

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ABSTRACT

African trypanosomiasis is caused by protozoan parasites known as trypanosomes, which are transmitted by the tsetse fly, affecting both humans and animals. *Trypanosoma congolense* is one of the main trypanosome species affecting cattle and causes the disease known as nagana. Control of animal African trypanosomiasis currently relies on chemotherapy and vector control methods, neither of which has proven satisfactory. An effective vaccine against trypanosomiasis would be the most cost effective solution to control the disease; however, due to the phenomenon of antigenic variation, intrinsic to the parasite's outer coat of variable surface glycoprotein, this has not yet been achieved. Recent vaccine efforts have been centred on identification of invariant parasite antigens for use as vaccine candidates.

Trypanosome cytoskeleton components have in recent years been shown to be capable of providing a protective immune response against trypanosome infection. These include tubulin proteins, which form the main components of the cytoskeleton, as well as microtubule associated proteins (MAPs) and paraflagellar rod proteins.

In the present study α- and β-tubulin from *T. congolense* were recombinantly expressed and their immuno-protective potential in mice assessed. Amplification of both α- and β-tubulin ORFs from *T. congolense* genomic DNA was followed by cloning of the amplicons into the T-vector pTZ57R/T, and thereafter sub-cloning into the bacterial expression vector, pET238a and the yeast expression vector pPICZαA28. Only the α-tubulin amplicon was successfully sub-cloned into pICZAαA28; however, no protein expression was achieved upon transfection of the methylotrophic yeast, *Pichia pastoris,* with this construct. Sub-cloning of both α- and β-tubulin inserts into pET28a was successful. Expression of recombinant α- and β-tubulin as fusion proteins with a histidine tag, both at a size of 55 kDa, was achieved in *Escherichia coli* host BL21 (DE3).

Recombinant proteins were successfully purified using nickel chelate chromatography under denaturing conditions. Refolding was first attempted by dilution of purified denatured proteins in a refolding buffer followed by reconcentration, but was largely unsuccessful. A second, more successful refolding method was performed wherein denatured proteins were refolded by application of a decreasing gradient of urea, while bound to a nickel chelate column. Native tubulin from cultured *T. congolense* procyclics was successfully purified and renatured using a polymerisation/depolymerisation method for use as a control for immunisation.

Mice were immunised separately with refolded recombinant α- and β-tubulin, native tubulin or an irrelevant protein VP4AA expressed in the same way as the tubulins. ELISA analysis confirmed the production of antibodies against each protein. Parasitaemia developed in all mice following challenge with *T. congolense.* Only the group immunised with β-tubulin recorded no deaths during the monitoring period despite the presence of parasitaemia, with 60% of mice immunised with α-tubulin or VP4AA and the no antigen control and no mice from the native tubulin immunised group surviving. The results showed that partial protection against trypanosomiasis caused by *T. congolense* infection was achieved in the group immunised with β-tubulin and suggest that β-tubulin may have vaccine potential.

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ABBREVIATIONS

2XYT	2 x yeast tryptone medium
ABTS	2,2-azino-di-[3-ethylbenzthiazoline sulfonate]
AEBSF	4-(2-aminoethyl)benzenesulfonyl fluoride
BCA	bicinchoninic acid
β-ME	β-mercaptoethanol
BMGY	buffered glycerol complex
BMM	buffered minimal methanol
BSA	bovine serum albumin
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E-64	L-*trans*-epoxysuccinyl-leucylamido(5-guanidino)butane
EDTA	ethylenediaminetetra-acetic acid
EGTA	ethylene glycol-bis(beta-aminoethyl ether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
g	relative centrifugal force
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GPI	glycosylphosphatidylinositol
GST	glutathione-*S*-transferase
GTP	guanosine-5-triphosphate
GDP	guanosine diphosphate
h	hour(s)
HRPO	horse radish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-beta-D-thiogalactopyranoside
kDa	kiloDalton
MAP	microtubule associated protein
MES	r(-)-2-(4-morpholino)-ethane sulfonate
min	minute(s)

OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PCV	packed cell volume
PMSF	phenylmethylsulfonyl fluoride
PLC	phospholipase C
RT	room temperature
sec	second(s)
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TNF	tumour necrosis factor
VSG	variable surface glycoprotein
YPD	yeast extract peptone dextrose

CHAPTER ONE
LITERATURE REVIEW

1.1 INTRODUCTION

African trypanosomiasis is a disease caused by flagellated protozoan parasites of the genus *Trypanosoma* (Lumsden, 1977; Turner, 1985; Stevens and Brisse, 2004). Trypanosomes found in sub-Saharan Africa are generally transmitted by tsetse flies, a biting fly of the genus *Glossina*. Of great economic importance are the species infective to animals, most importantly livestock, which include *Trypanosoma congolense, T. vivax* and *T. brucei brucei.* These trypanosomes cause Nagana, the name given by the Zulu people to trypanosomiasis in cattle. Two species of trypanosome are known to parasitise humans, namely *T. b. rhodesiense* and *T. b. gambiense* – subspecies of *T. brucei*, resulting in a disease known as sleeping sickness. *T. b. rhodesiense* and *T. b. gambiense*, however, also cause disease in animals. Although human African trypanosomiasis was almost eradicated by the early 1960's, it has since re-emerged (Simarro *et al.*, 2008) and now along with animal African trypanosomiasis, results in severe health and social problems, as well as economic losses across a large extent of sub-Saharan Africa. Control of trypanosomiasis has been hampered due to the existence of only a handful of chemotherapeutic drugs, which are either toxic or lacking in efficacy, as well as the absence of a vaccine capable of providing efficient protection/immunoprotection against trypanosomiasis. The current review presents an overview of trypanosomiasis and the efforts underway towards development of a vaccine, as well focusing in on the structural proteins α- and β-tubulin, which will be investigated for their potential to provide immunoprotection against African trypanosomiasis.

1.2 Epidemiology and cost of trypanosomiasis

The distribution of trypanosomiasis throughout Africa depends primarily on the distribution of the tsetse fly vector. Tsetse flies are abundant throughout nearly a third of the continent (10 million km^2), with tsetse flies inhabiting areas in 36 countries (Kristjanson *et al.*, 1999;

Aksoy, 2003). Figure 1 shows the distribution of cattle and tsetse throughout Africa. The map shows tsetse ranging largely throughout the tropical regions of the continent, prohibiting the presence of cattle in many of these regions (Fig. 1). There are estimated to be 50 000 to 70 000 people currently infected with trypanosomiasis, with an estimate of 100 000 cases of sleeping sickness reported annually, with at least a 60 million people [(Aksoy, 2003); Programme Against African Trypanosomiasis (PAAT), 2009] and 50 million head of cattle (PAAT, 2009) as well as millions of other head of livestock (Kristjanson *et al.*, 1999; PAAT, 2009) at risk of infection throughout the continent.

Besides the detrimental effect of trypanosomiasis on human health, significant economic losses have resulted from the effect of trypanosomiasis on livestock farming. It is estimated that direct economic losses for livestock production are in the region of US$1340 million annually. More recent estimates from the United Nation's Programme Against African Trypanosomiasis (PAAT, 2009) put the figures for economic losses in cattle production in the range of US$ 1.0 - 1.2 billion, with approximately 3 million cattle deaths each year (PAAT, 2009). Ten countries in Africa are completely infested with tsetse flies, with the economic losses in these countries ranging from $192 - $960 million annually, due to the inability to carry out farming activities in these areas (Kristjanson *et al.*, 1999). In addition to the above losses, the costs of treating infected livestock amounts to roughly $35 million per year. Losses to the agricultural Gross Domestic Product are estimated to be in the region of US$ 4.5 billion for all the countries infested with tsetse flies (Antoine-Moussiaux *et al.*, 2009; PAAT, 2009). It is estimated that improved control of trypanosomiasis would result in economic gains of around $700 million in livestock production.

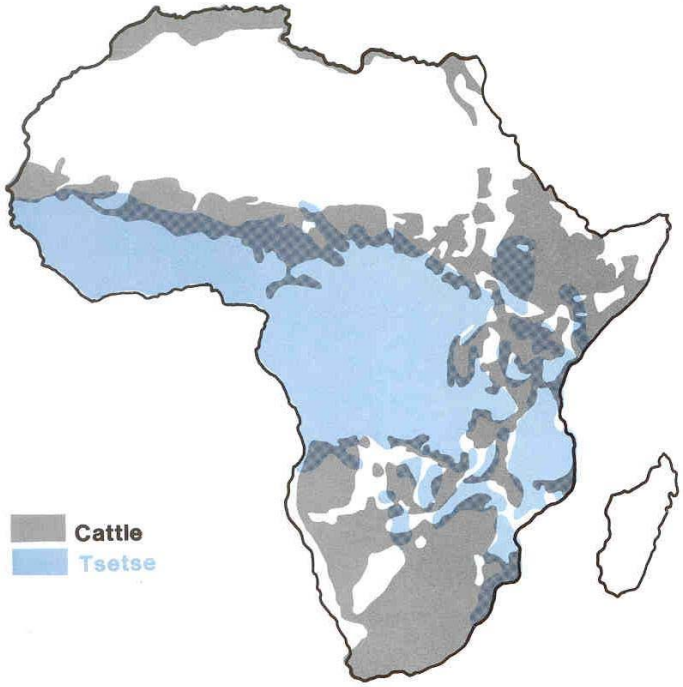

Figure 1: Distribution map of tsetse flies and cattle across Africa. Cattle are mainly absent from areas where tsetse are prevalent. Cattle are found to overlap with tsetse in areas bordering tsetse ranges (ILRI, 2009).

1.3 Control for trypanosomiasis

As a vaccine for trypanosomiasis has yet to be developed, the only strategies that have had any success to date in combating the disease are trypanocidal drugs, vector control methods and the rearing of trypanotolerant cattle in areas infested with tsetse flies. Current strategies for vaccine development will be discussed in Section 1.5.

1.3.1 Chemotherapy

There are only four drugs currently available for the treatment of human African trypanosomiasis (HAT), of which three were developed more than 50 years ago (Fairlamb, 2003). These drugs include suramin and pentamidine for treatment of early-stage infection as well as melarsoprol (an arsenical) and eflonithine for late-stage treatment of HAT

(Fairlamb, 2003). Similarly, only a few drugs are available for the treatment of trypanosomiasis in cattle, namely isometamidium chloride and homidium, which are mainly used for prophylaxis but also used therapeutically; as well as ethidium bromide and diminazene aceturate (berenil) which are mainly for therapeutic use (Delespaux *et al*., 2008 and (Geerts *et al.*, 2001). The efficacy and/or toxicity levels of all the drugs used to treat HAT are not satisfactory (Gutteridge, 1985; Fairlamb, 2003), whilst the main problems faced with the drugs used to treat animal trypanosomiasis are cost and drug resistance (Geerts *et al.*, 2001).

The development of drug resistance in trypanosomes is a concern, as this decreases the number of effective drugs available, and in turn decreases the productivity of, and increases the costs to, livestock farmers in Africa, (Anene *et al.*, 2001; Geerts *et al.*, 2001; Fairlamb, 2003; Holmes *et al.*, 2004; Delespaux *et al.*, 2008). The development of drug resistance amongst trypanosomes is not surprising, given that there are only a limited number of trypanosidal drugs available on the market, and these have been in use for over five decades (Geerts *et al.*, 2001). The initial discovery of drug resistance was made in trypanosomes during the early 20th century, despite this however, the mechanisms of drug resistance occurring in trypanosomes are only now slowly being elucidated (Mäser *et al.*, 2003; Delespaux *et al.*, 2008).

1.3.2 Vector control

Other efforts aimed at controlling trypanosomiasis in sub-Saharan Africa are focused on controlling the tsetse fly vector. This is accomplished by insecticides, traps designed to catch tsetse flies or release of sterile males. Vector control has had some success; for example tsetse flies were effectively eradicated from the island of Zanzibar in 1997, by using the sterile insect release strategy (Vreysen, 2001). This allowed for improved livestock production, which improved the socio-economic status of the communities in this region (Kabayo, 2002).

The widespread use of insecticides to control tsetse populations in infected areas has seen some slight development of vector resistance to insecticides (Aksoy *et al.*, 2003). Concerns over the effect of insecticides on the environment have been the main consideration raised. Thus, the use of insecticides in controlling trypanosomiasis is not seen as a permanent solution.

Tsetse traps have been effective in controlling insect numbers, especially when used in addition to insecticides. However, traps require effort to maintain, as they are susceptible to damage, and therefore require long-term community involvement (Kabayo, 2002). Recently, efforts to improve the use of tsetse traps have included treated traps that lure tsetse with odour baits in order to increase the number of flies caught, as well as treating cattle and other livestock with insecticide (Omolo *et al.*, 2009).

1.3.3 Trypanotolerant cattle

Certain breeds of cattle were found to be more tolerant to trypanosome infection than other breeds (Authie, 1994; Lalmanach *et al.*, 2002). These tolerant breeds, known as trypanotolerant cattle, once infected by trypanosomes, are able to limit the pathogenicity of the parasites and maintain a better state of health than other breeds. The breeds that are more susceptible to disease following trypanosome infection are termed trypanosusceptible. Trypanosusceptible breeds are not as able to cope with infection as the trypanotolerant breeds, and frequently succumb to the disease as a result of high levels of parasitaemia and anaemia (Murray *et al.*, 2004). The disadvantage of farming with the trypanotolerant breeds, such as the N'Dama, is that these are usually smaller, and in terms of milk production, less productive than the larger, trypanosusceptible breeds, such as the Zebu (Murray *et al.*, 2004). The genetic factors resulting in trypanotolerance have up to now been largely unknown, however, research is currently underway to investigate the phenomenon by the use of whole transcriptome analysis of both trypanotolerant and trypanosusceptible cattle (Berthier *et al.*, 2008).

1.4 The life cycle and structure of trypanosomes

The main species of African trypanosomes infective to humans and animals are transmitted via the saliva of tsetse flies when an infected fly feeds on the blood of a human or animal. For the above reason, tsetse fly-transmitted trypanosomes are known as *Salivaria* (Barrett *et al.*, 2003; Stevens and Brisse, 2004). Trypanosomes transmitted by the tsetse fly, *T. brucei*, *T. b. rhodesiense*, *T. b. gambiense*, *T. congolense* and *T. vivax*, must be adapted to spend part of their life cycle in the insect host. By contrast, *T. evansi*, is transmitted mechanically between infected camels (Stevens and Brisse, 2004; Li *et al.*, 2007) by biting flies such as horse flies (genus *Tabanus*) causing surra, affecting camels mainly. The need for parasite adaptation to an insect vector is therefore not required in *T. evansi*. *T. equiperdum* also does not require an insect vector, since it is transmitted between horses via copulation, causing dourine. The form of insect adapted African trypanosomes varies throughout the lifecycle as the parasite occupies different host environments. Figure 2 presents the life cycle of African trypanosomes. In the bloodstream of the mammalian host, trypanosomes exist as long, slender blood stream forms capable of proliferation. Some long slender forms differentiate into non-proliferative short stumpy forms, which when ingested by a tsetse fly during a blood meal, enter the midgut of the tsetse, where they develop into the procyclic stage. The procyclics proliferate in the midgut, and from there, migrate to the salivary glands of the tsetse where they develop into epimastigotes and attach to the cell surface tissues in the salivary gland. The epimastigotes continue to divide and also differentiate into the metacyclic stage, which ceases to proliferate, and are free to be injected into the mammalian host during the next blood meal. Once injected into the host, metacyclics develop into the long slender bloodstream form, completing the life cycle (El-Sayed *et al.*, 2000).

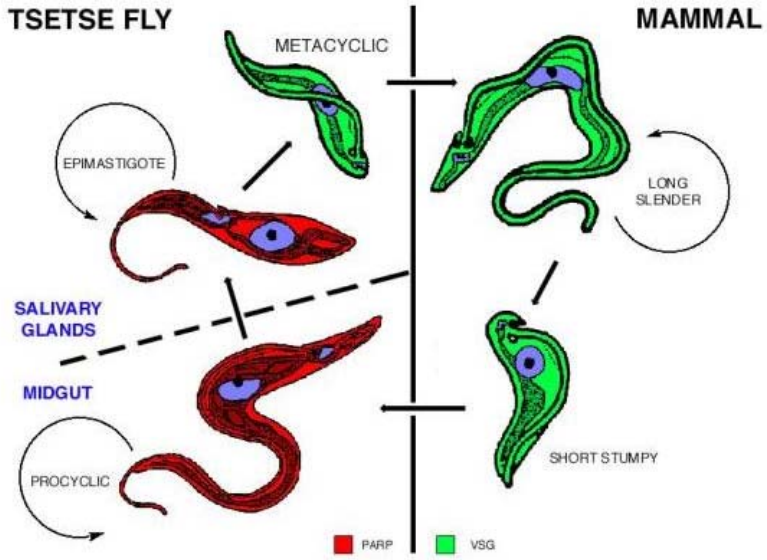

Figure 2: Life cycle of African trypanosomes. Trypanosomes in the metacyclic stage are delivered into the mammalian bloodstream via a tsetse fly bite, where they develop into long slender bloodstream form parasites. The long slender form divide in the bloodstream of the mammalian host, some of which differentiate into short stumpy form parasites. Short stumpy form parasites, when taken up by a tsetse fly during a blood meal, are capable of transforming into procyclic stage parasites in the tsetse fly midgut. After division in the midgut, the procyclic stage parasites migrate to the salivary glands of the tsetse fly host, where they differentiate into epimastigotes. Epimastigotes continue to proliferate and differentiate into the metacyclic stage, capable of infecting a mammalian host during the next tsetse bloodmeal. Of all the life cycle stages, only the metacyclic and short stumpy parasites are not capable of proliferation. Blood stream and metacyclic stage parasites exhibit an outer coat of variable surface glycoprotein (VSG) shown by the colour green, while the tsetse stage parasites have a coat of procyclic acidic repetitive proteins (PARPs), or procyclins, shown by the colour red (El-Sayed *et al*., 2000).

Upon injection of trypanosomes into the derma of the host, the parasites multiply at the site of infection, forming a chancre and from there migrate into the bloodstream of the host, where they remain extracellular. The trypanosomes being free in the bloodstream, are subjected to the host humoral immune response. A layer of closely packed proteins on the parasite surface, forms a protein "coat" around the parasite. This coat is composed mainly of the protein known as variable surface glycoprotein (VSG). In tsetse-transmitted parasites, VSG is formed on the parasite surface during the metacyclic stage of the life cycle, whilst the parasites are in the salivary glands of the tsetse fly, and remains present on the surface of both bloodstream stages. During the epimastigote and procyclic stages, the parasite surface is covered in procyclic acidic repetitive proteins (PARPs) which are also

known collectively as procyclins (El-Sayed *et al.*, 2000; Utz *et al.*, 2006). The trypanosome VSG coat changes or varies throughout the course of infection and is therefore responsible for the capacity for antigenic variation present in African trypanosomes (Donelson, 2003). Antigenic variation of the VSG coat is the primary reason for the difficulty involved in developing a vaccine against African trypanosomiasis. The VSG and the phenomenon of antigenic variation will be discussed in greater detail later.

1.4.1 The trypanosome cytoskeleton

All eukaryotic cells possess a cytoskeleton; however, the trypanosome cytoskeleton is unique amongst eukaryotes due to the simplicity of its structure. The trypanosome cytoskeleton has several functions. Maintenance of cell shape and motility are the main functions, as well as adherence to cell surfaces of the insect host. The cytoskeleton is therefore important as the shape of the parasite cell must change to adapt to life in both the mammalian and insect hosts during the various life cycle stages (Kohl and Gull, 1998).

The trypanosome cytoskeleton is much simpler than that found in most other eukaryotic cells, and consists mainly of microtubules (Schneider *et al.*, 1987). It has several components; these are the subpellicular microtubules, the axoneme, the basal body, the paraflagellar rod, the flagellum attachment zone and the filaments which allow for the parasite to attach to the tissues of the insect host (Kohl and Gull, 1998). Figure 3 illustrates the various components of trypanosome cellular structure, including the position of the mitochondrion, nucleus, kinetoplast, basal body, flagellar pocket and flagellum. Also shown are the positions of the major cytoskeletal components: the subpellicular microtubules and the axoneme of the flagellum (Fig. 3).

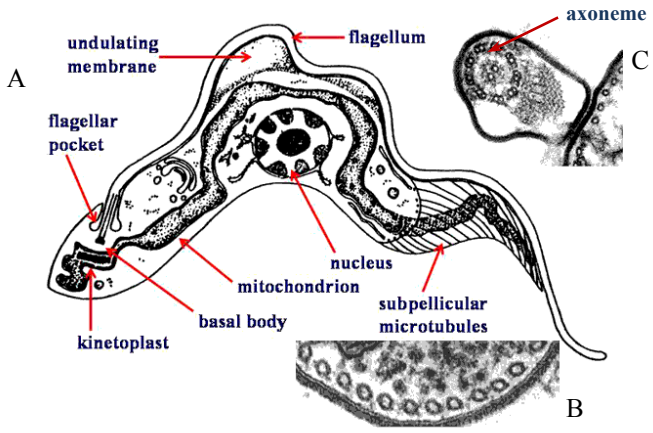

Figure 3: Illustration and electron micrographs of a trypanosome showing various cellular components. **A**: Illustration of trypanosome cellular structure, showing the nucleus, a single mitochondrion, the flagellum, flagellar pocket and subpellicular microtubules. **B**: Electron micrograph cross-section of a trypanosome cell showing the arrangement of the subpellicular microtubules along the internal surface of the cell membrane, forming the corset. **C**: Electron micrograph cross-section showing the attachment of the flagellum to the outer surface of the cell membrane as well as the axoneme (Wiser, 2007).

The subpellicular microtubules are found directly below the surface membrane, and form a corset or cage structure around the parasite (MacRae and Gull, 1990; Kohl and Gull, 1998; Jackson *et al.*, 2006). The sub-pellicular microtubule corset as well as the basal body, axoneme and the mitotic spindle of the parasite are primarily composed of microtubules. The sub-pellicular corset, which is unique to trypanosomes, is made up of more than 100 microtubules (Kohl and Gull, 1998). The corset microtubules are cross-linked by a unique set of microtubule associated proteins (MAPs), which aid in the polymerisation of the tubulin filaments into the stable microtubules of the sub-pellicular corset (Kohl and Gull, 1998). Recently discovered trypanosome MAPs include MAP p15 and MAP p52, so named because of their molecular weights of 15 and 52 kDa respectively.

1.4.1.1 Microtubule structure

Microtubules are highly necessary structural components of all eukaryotic cells, as they play vital roles in cell division, transport within the cell, as well as cellular architecture in the form of a cytoskeleton (Nogales *et al.*, 1998). Although several types of tubulin exist (ε, δ, η, ζ), microtubules are composed primarily of heterodimers of the proteins, α- and β-tubulin (Nogales *et al.*, 1998; Gull, 2001). Tubulins may be found in a soluble form in the

cell, or they may polymerise into microtubules (Schneider *et al.*, 1987; Kohl and Gull, 1998). α- and β-tubulin proteins bind together to form heterodimers, which assemble head-to-tail into linear structures known as protofilaments (Fig. 4). Tubulin heterodimers exhibit polarity; with the α-tubulin subunit having a negative charge and the β-tubulin subunit a positive charge. Consequently, protofilaments and microtubules will also have polarity (Fig. 4). A singlet microtubule is formed when ~13 protofilaments join together laterally to form a hollow tube of approximately 24 nm in diameter (Kohl and Gull, 1998; Dutcher, 2001). Singlet microtubules may also associate with one another to form doublet or triplet microtubules (Dutcher, 2001). Microtubules can reach lengths of up to 50 μm (Karsenti *et al.*, 2006), and have a characteristic known as dynamic instability: this is where microtubules will undergo continued periods of growth and shortening, known as rescues and catastrophes respectively (VanBuren *et al.*, 2005).

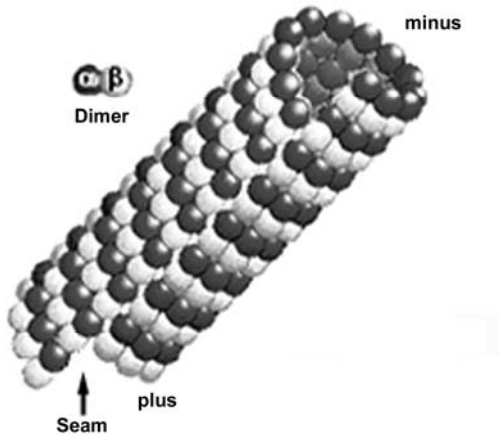

Figure 4: Microtubule protofilament structure. Protofilaments are assembled by the linear arrangemet of αβ tubulin heterodimers into tubular structures having an inbuilt polarity due to the polar nature of the heterodimers (Böhm, 2002).

α- and β-tubulin each have a molecular mass of 55 kDa (Gallo and Anderton, 1983; Voet and Voet, 1995). Despite having an amino acid sequence identity of around 40%, the tertiary structures of α- and β-tubulin are nearly identical. The basic structure of each monomer of α- and β-tubulin consists of a core of two β sheets surrounded by α-helices (Fig. 5). Both α- and β-tubulin have a domain structure that consists of an amino-terminal

domain containing the guanosine triphosphate (GTP) binding site and a site which binds taxotere (an analog of the microtubule stabilising compound, Taxol, also known as paclitaxel), and a carboxy-terminal domain (Nogales *et al.*, 1998).

In order for the assembly of α- and β-tubulin dimers to associate into microtubules, GTP is required. One GTP molecule is bound to a GTP binding site in the N-terminal domain of each of the α- and β-monomers, designated N and E respectively. The ribbon structure of the αβ heterodimer (Fig. 5) shows the orientation of the subunits to one another in the dimer and also the positions of the bound GTP molecules on the subunits. A molecule of taxotere, is shown bound to the taxol binding site of the β subunit. Taxol has been found to bind to only a single site on the heterodimer while it forms part of a protofilament (Nogales *et al.*, 1998). The GTP molecule bound to the α subunit is non-exchangeable; while the GTP bound to the β subunit is exchangeable (Nogales *et al.*, 1998). The GTP bound to the β subunit is hydrolysed to GDP at the same time as, or immediately after the αβ heterodimer is incorporated into a microtubule filament. If another αβ heterodimer is not added shortly after one of the GTPs is hydrolysed, the second GTP will be hydrolysed as well, causing the dimer to dissociate from the filament. This instability of microtubules allows them to be broken down and reassembled according to the needs of the cell (Voet and Voet, 1995; Karsenti *et al.*, 2006).

The amino acid sequences of both α- and β-tubulin are highly conserved across all eukaryotic species, as they have very similar functions in many different eukaryotic organisms (Erickson, 2007). Tubulins can also undergo a wide variety of enzymatic post-translational modifications including acetylation/deactylation, phosphorylation, polyglycylation or, polyglutamylation. Another modification of tubulin is the tyrosination/detyrosination cycle, which involves the removal of the C-terminal tyrosine residue present in most α-tubulin isoforms, occurring as a result of microtubule stabilisation. The removal of the glutamic acid residue adjacent to the C-terminal tyrosine prevents tyrosination, and results in the production of non-tyrosinatable α-tubulin (MacRae, 1997). In the majority of eukaryotic cells, genes for α- and β-tubulin consist of

several different genes coding for slightly different forms of these proteins, known as isoforms. The existence of different tubulin isoforms, together with the various post-translational modifications mentioned above, allows for production of slightly different microtubule populations able to perform different functions or bind different molecules within the cell (MacRae, 1997). It was suggested by Sasse and Gull in 1988, that in *T. brucei,* detyrosination of α-tubulin post-translationally may be a mechanism allowing the parasite to distinguish between old and new microtubules used in the construction of the cytoskeleton (Sasse and Gull, 1988).

The C-terminal domain of α- and β-tubulin contains binding sites for proteins such as microtubule associated proteins (MAPs), as well as a hypervariable region consisting of a short sequence of amino-acid residues at the C-terminus. Most of the variations between tubulin isoforms, and also between species, are due to this hypervariable region (Nogales *et al.*, 1998).

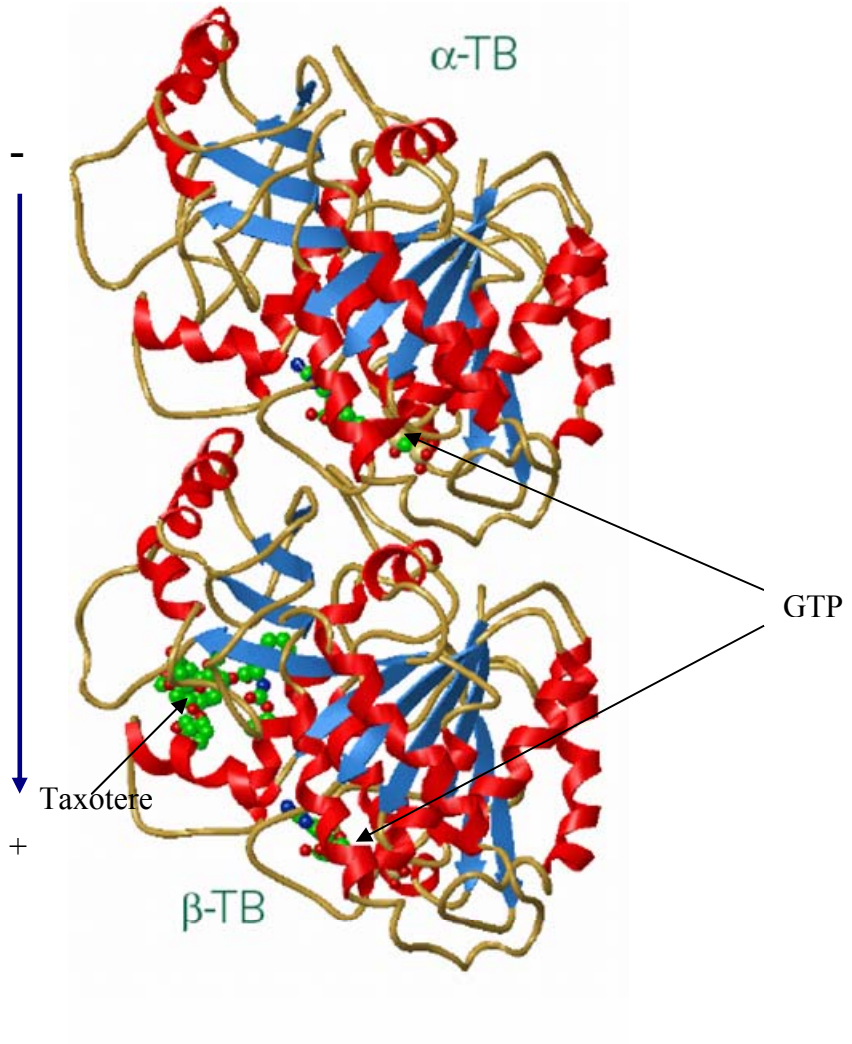

Figure 5: Ribbon diagram of a heterodimer of α- and β-tubulin. The position of the α- (α-TB) and β- (β-TB) subunits are shown as well as the bound GTP molecules and a molecule of taxotere, an analog of Taxol, which when bound stabilises microtubules and prevents depolymerisation. The polarity of the heterodimer is indicated by a minus sign at the α-subunit and a plus sign at the β-subunit (Nogales *et al.*, 1998).

1.4.1.2 Structure and function of variable surface glycoprotein (VSG)

The surface of each individual African trypanosome is covered with approximately 5.5 million molecules of the coat protein known as variable surface glycoprotein (VSG). VSG constitutes more than 95% of the total cell surface proteins (Barry and Carrington, 2004). Each VSG molecule is composed of approximately 450 amino acid residues, with a molecular weight of around 60 kDa (Turner, 1985). The VSG consists of a conserved C-terminal domain and a variable N-terminal domain, with a short signal sequence at the N-

terminus and a short glycosylphosphatidylinositol (GPI) signal sequence at the C-terminus (Fig. 6).

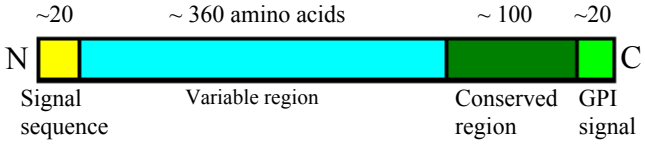

Figure 6: Schematic diagram of VSG protein. A signal sequence (~20 amino acid residues) is located at the N-terminus, followed by a variable region (~360 amino acid residues) and a shorter conserved region (~100 amino acid residues). Finally, a GPI signal sequence (~20 amino acid residues) is located at the C-terminus (After Wiser, 2007).

The VSG molecules form homodimers which are anchored to the cell surface membrane via a covalent linkage between the C-terminal domain and a GPI anchor present in the surface membrane of the parasite (Fig. 7) (Turner, 1985; Barry and Carrington, 2004; Taylor and Rudenko, 2006).

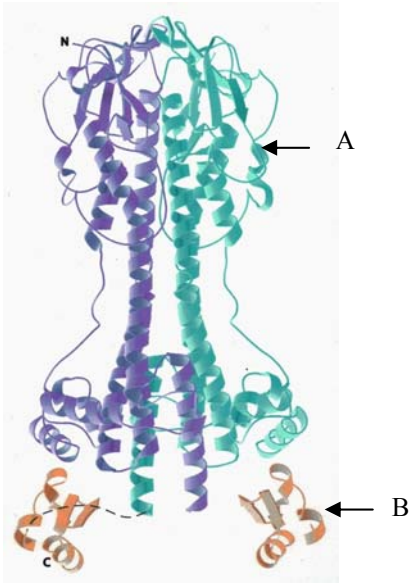

Figure 7: Ribbon model of VSG homodimers and GPI anchors. VSG is shown in its homodimer organisation (A), with the N-terminal region facing the extracellular environment. The C-terminal domain attaches to GPI anchors (B) present on the surface membrane of the parasite (Barry and Carrington, 2009).

The VSG coat forms the barrier between the parasite and the host. VSG has several functions, such as the ability for trypanosomes to resist lysis by alternative pathway activated complement proteins of the host (Vincendeau and Bouteille, 2006). VSG is highly immunogenic, and causes the host immune system to secrete cytokines, such as interleukin (IL) 2 and most importantly, tumour necrosis factor (TNF)-α (Taylor, 1998). VSG also stimulates the production of autoantibodies by molecularly mimicking host tissues. The most important function of VSG is the role it plays in antigenic variation, a phenomenon which allows the parasites to evade the specific immune response of the host (Vincendeau and Bouteille, 2006).

1.5 Vaccine Development

Drug therapy of infected mammals as well as vector control methods have had some success in curbing the spread of trypanosomiasis, as it has been noted that the human form of the disease was almost eliminated from Africa in the 1960's, but has since re-emerged (Kabayo, 2002). Despite these limited successes, all these currently employed strategies have shortcomings. A study conducted by Kristjanson *et al*. (1999) calculated a benefit/cost ratio of 34:1 for the development of a trypanosomiasis vaccine. When the shortcomings of the above control methods are considered, the above calculation makes development of a cost effective vaccine for humans and animals a very attractive solution to the problem of trypanosomiasis.

In the following sections the research that has been done toward developing a vaccine for trypanosomiasis will be reviewed and problems encountered discussed. The potential use of trypanosome α- and β-tubulin from *T. congolense* will also be put forward as a promising vaccine candidate

1.5.1 VSG and Antigenic Variation

The trypanosome genome contains a vast array of VSG genes and pseudogenes. For example, *T. brucei* has more than 1000 genes and pseudogenes coding for VSG, near the

telomeres of the chromosomes (Taylor and Rudenko, 2006). Each individual trypanosome expresses a single form of VSG on its surface at any particular time, known as a variable antigen type (VAT). During the course of infection, the majority of trypanosomes in the blood will be of a single VAT. However, one out of every 10^4 – 10^5 parasites will spontaneously switch off the gene for the particular VAT it currently expresses, and begin to express different VSG genes, antigenically different to the former VAT. The majority of the parasites will still be of the first VAT, and will eventually be killed by the host's antibody response mounted against that particular VSG. Those parasites that have switched VSG genes, are undetected by the current antibody population. The host then mounts an immune response to the new VAT, during which time another gene switch will have occurred. This results in successive waves of parasitaemia seen in the infected host (Barry, 1997; Taylor and Rudenko, 2006).

The infected human or animal will therefore contain parasites of several different VATs, all at different stages of proliferation. Antigenic variation benefits the parasite in that it allows the infection to be sustained for periods from several months to even years, without killing the host (Pays and Nolan, 1998).

1.5.2 Anti-parasite and anti-disease vaccines

The ability of the trypanosome VSG coat to undergo antigenic variation has made the development of a vaccine based on VSG impossible. For this reason, researchers have found it necessary to look for alternative ways to develop a vaccine against trypanosomiasis. Several attempts have been made over the years, with varying success. These approaches include the conventional vaccine approach, which aims to kill the disease-causing parasite in the host, and so rid the host of infection completely. Targets for a conventional vaccine have included tubulin proteins, which form the subpellicular microtubules, as well as the microtubule associated proteins (MAPs) (Lubega *et al.*, 2002a; Rasooly and Balaban, 2004; Li *et al.*, 2007). Antigens derived from the flagellar pocket region of the parasite have also been researched as possible vaccine candidates (Mkunza *et al.*, 1995). Another approach aims to limit the pathogenicity of the parasite, in order to

mitigate the harmful symptoms of infection, even though live parasites are present within the host; this is known as an anti-disease vaccine (Playfair *et al.*, 1991; Authie, 1994).

Multi-component vaccines are seen presently as being the most effective strategy in vaccine development. These modern vaccines consist of several components, which each have a separate function. Thus, a multi-component vaccine that contains several peptide epitopes, capable of activating both T- and B- lymphocytes, has been envisioned (Lozano and Patarroyo, 2007). A multi-component vaccine for trypanosomiasis may therefore consist of conventional anti-parasite as well as anti-disease vaccine components

1.5.2.1 Flagellar pocket antigens

The flagellar pocket is an invaginated region on the parasite surface, where endo- and exocytosis occur. The trypanosome flagellum arises from the cell at the flagellar pocket (Nolan *et al.*, 2004). Varying degrees of protection have been observed in mice and cattle immunised with antigens derived from the flagellar pocket region of *T. b. rhodesiense* (Mkunza *et al.*, 1995).

An antigen derived from the flagellar pocket region of *T. b. rhodesiense* was used to immunise cattle (Mkunza *et al.*, 1995). Out of a total of 90 experimental animals, 40 served as controls, 20 were treated with the trypanocidal drug samorin as a prophylactic and 30 were immunised with the flagellar pocket antigen. Experimental cattle were monitored for infection occurring via the bite of tsetse flies endemic to the region throughout one rainy season. At the end of the rainy season, the number of cattle that became infected with trypanosomes was 58% for the controls, 41% for the group treated with samorin and 26% for the group immunised with the flagellar pocket antigen. Only the flagellar pocket antigen gave statistically significant ($p < 0.001$) protection against trypanosome infection as compared to the control groups (Mkunza *et al.*, 1995). Similar results were achieved in two further experiments with cattle. The second experiment used 250 cattle, and the same experimental conditions for a period of 6 months, and found that the level of protection

against infection by the flagellar pocket antigen was again statistically significant ($p < 0.005$) with 9% of immunised cattle (150/250) becoming infected compared to 22% infection of the untreated controls (100/250). A third experiment involving 177 cattle (117 immunised and 60 controls) over a period of 15 months, with only a difference in the carrier protein used for immunisation, resulted in statistically significant protection ($p < 0.001$) with 13% infection in the control group and 0.9% infection in the immunised group. The breed of cattle used in the above experiments is not given; however, the experiments were performed in a manner that closely simulates the naturally occurring conditions, whereby cattle are infected by trypanosomes via tsetse bites. The trypanosome species were native to the area in Southeastern Kenya where the experiment was performed, and the mode of infection was the same as would occur naturally, adding to the significance of the results.

1.5.2.2 Microtubule associated proteins (MAPs)

Tubulin proteins and subpellicular microtubules are highly important for trypanosome viability. Immunisation of mice with tubulin proteins or MAPs, have elicited antibodies capable of protecting mice from trypanosomiasis, as well as limiting trypanosome growth *in vitro*. Tubulins as well as their associated proteins (MAPs), have been suggested as possible vaccine candidates for trypanosomiasis (Lubega *et al.*, 2002a; Rasooly and Balaban, 2004; Li *et al.*, 2007).

In a study done by Rasooly and Balaban (2004), the MAP p15 was described as a possible vaccine candidate. MAP p15 was chosen as a suitable antigen as it is invariant, and not present in mammalian cells. Also, success had previously been achieved using MAP p52 to provide immunoprotection in mice against *T. b. brucei* infection (Balaban *et al.*, 1995; Rasooly and Balaban, 2004). Immunisation with native MAP p52 from *T. b. brucei* as well as trypanosomal aldolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which were co-purified with MAP p52, were found to provide 100% protection to rats and mice when immunised three times over 3 weeks with the above three proteins, and

challenged with *T. b. brucei* (Balaban *et al.*, 1995). Mice were immunised with recombinant and native MAP p15 in separate experiments and challenged with *T. b. brucei*. All seven mice immunised with recombinant MAP p15 were immunoprotected with no evidence of parasitaemia. The same result was obtained with 7/8 mice immunised with native MAP p15. Mice immunised with a subpellicular fraction containing MAP p52 resulted in 4/4 mice surviving challenge with *T. b. brucei*, while 0/4 mice survived when immunised with buffer alone and similarly challenged (Rasooly and Balaban, 2004). It appears from this study that immunisation with MAP p15 specifically, is able to provide complete protection to mice against an otherwise lethal challenge with *T. b. brucei*. These results seem promising, however, the small sample sizes of seven (MAP p15) and four (MAP p52) mice immunised with the antigens, cast doubt on the validity of the conclusions that MAPs p15 and p52 are capable of providing highly efficient immunoprotection to mice against trypanosomiasis.

1.5.2.3 Tubulin

Lubega and colleagues found that tubulin isolated from *T. brucei* and renatured was able to provide protection to mice when challenged with either *T. brucei*, *T. congolense* and *T. b. rhodesiense* in separate experiments (Lubega *et al.*, 2002a; Lubega *et al.*, 2002b). Mice immunised with either synthetic tubulin peptides or denatured *T. brucei* tubulin and similarly challenged were not protected to the same degree as mice immunised with renatured *T. brucei* tubulin. Renatured rat brain tubulin did not provide any protective immunity when injected into mice that were subsequently challenged with the above mentioned trypanosome strains (Lubega *et al.*, 2002a).

A later study demonstrated that the renatured *T. brucei* tubulin injected into rabbits was able to induce the production of antibodies that were able to inhibit the growth and cause immunoagglutination of trypanosomes *in vitro* in a dose-dependent manner. Growth inhibition and immunoagglutination was also observed in the presence of denatured *T. brucei* tubulin as well as synthetic tubulin peptides, however, this was not as pronounced as

in the case where renatured *T. brucei* tubulin was used. Antibodies against renatured rat brain tubulin had no effect on trypanosome growth *in vitro* (Lubega *et al.*, 2002b).

The potential use of tubulin as a vaccine candidate, as demonstrated by Lubega *et al*. (2002a and b) was confirmed in a study by Li *et al*. (2007), which showed that recombinant β–tubulin from *T. evansi* was able to give protection to mice against disease when challenged with *T. evansi* (83.3%), *T. equiperdum* (70%) and *T. b. brucei* (76.6%). This study also found that serum from a rabbit immunised with β-tubulin inhibited the growth of these three trypanosome species *in vitro* (Li *et al.*, 2007).

The above results indicate that tubulin and their associated proteins (MAPs) provide a promising avenue for future research into the development of a vaccine against trypanosomiasis. These studies should focus on the potential of tubulin as a vaccine candidate in cattle and other livestock species, as it has been noted that trypanosomiasis disease progresses differently in mice and cattle (Mkunza *et al.*, 1995; Magez *et al.*, 2002; Naessens, 2006).

1.5.2.4 Anti-disease Vaccines

The concept of an anti-disease vaccine was first described with reference to malaria, and involves the production of a vaccine that protects the host from the pathogenic effects of the parasite, without necessarily killing the parasite itself (Playfair *et al.*, 1991). Thus, an anti-disease vaccine would enable the host to remain healthy, while parasites continue to survive in the host's body.

The anti-disease vaccine approach was considered as a possibility for the treatment of trypanosomiasis after it was noted that certain breeds of cattle were more tolerant to trypanosome infection than other breeds, as discussed in Section 1.3.3. Certain parasite factors are responsible for pathogenic effects in the host. Immune system antibody response has been noted to differ between trypanotolerant and susceptible cattle (Taylor, 1998).

Trypanotolerant cattle are able to resist or control the pathogenicity of the trypanosomes during infection; for this reason, efforts have been directed toward the identification of the factors responsible for the phenomenon of trypanotolerance. The identification of one such molecule involved in trypanotolerance is the trypanosome cysteine protease, congopain. It has been found that trypanotolerant cattle produce antibodies that inhibit the activity of congopain (Authié *et al.*, 2001; Lalmanach *et al.*, 2002). In recent years congopain has been studied as a drug target and also as an anti-disease vaccine candidate.

Congopain is a 33 kDa cysteine protease isolated from *T. congolense* and homologous to the mammalian lysosomal proteinase, cathepsin L, a member of the papain family of cysteine proteases (Authie, 1994). Unlike cathepsin L, trypanosome cysteine proteases possess a unique C-terminal extension linked to the central domain via a polyproline hinge region. As a result of its exclusivity to trypanosomes, the C-terminal extension is highly immunogenic in mammals (Lalmanach *et al.*, 2002). For this reason, trypanosome cysteine proteases (e.g. congopain) have been expressed as recombinant proteins without the C-terminal extension to direct the immune response to the catalytic domain with the potential of producing antibodies that inhibit the activity of congopain (Authié *et al.*, 2001; Boulangé *et al.*, 2001).

During trypanosome infection in cattle, congopain is released into the host bloodstream, where it has been observed to degrade host proteins and interfere with antigen processing and presentation. This contributes greatly to the pathogenic effect experienced by the host during trypanosome infection (Lalmanach *et al.*, 2002). In order to test the hypothesis that congopain plays a role in the pathogenicity of trypanosomiasis, trypanosusceptible Boran cattle were immunised with trypanosome cysteine proteinases (CP1 and CP2, where these have 90% sequence identity and CP2 is identified as native congopain) and challenged with *T. congolense*. After immunisation, antibodies against trypanosomal cysteine proteinases (congopain) were found, as well as a rapid IgG antibody response to VSG proteins after challenge (Authié *et al.*, 2001). The cattle immunised with trypanosome cysteine proteinases were found to be healthier following infection than the non-immunised cattle.

The presence of antibodies against congopain that were able to inhibit the activity of congopain in the immunised cattle, and that the immunised cattle were healthier than controls following challenge, lead the authors of the above study to conclude that congopain may be involved in the pathogenesis of trypanosomiasis in cattle. This is confirmed by the observation that the trypanotolerant cattle produce a greater IgG response to congopain as well as VSG and heat shock protein (hsp) 70/BiP, than the trypanosusceptible breeds (Taylor *et al.*, 1996). The identification of other pathogenic factors would allow researchers to identify molecules that could be used in an anti-disease vaccine for trypanosomiasis.

1.5.2.5 Glycosylphosphatidylinositol (GPI) anchor

The GPI molecules which anchor the VSG proteins to the surface membrane were first discovered in African trypanosomes, but have since been found to occur in many eukaryotic cells (Ferguson, 1999). GPI anchors have been suggested as possible targets for the development of an anti-disease vaccine for trypanosomiasis, as they do not exhibit antigenic variation (Magez *et al.*, 2002).

The enzyme phospholipase C (PLC), produced by the trypanosome, hydrolyses the VSG from the GPI anchor at a specific point on the GPI anchor when the trypanosome is under stress or is lysed. The hydrolysis releases a soluble form of VSG (sVSG) into the blood stream, carrying with it the glycosyl-inositol-phosphate moiety of the GPI and a specific epitope known as the cross-reacting determinant, which may be recognised by the host's antibodies. The dimyristoylglycerol compound of the GPI is left behind on the cell membrane (Webb *et al.*, 1997; Magez *et al.*, 2002). It has been shown that PLC from *T. brucei* (GPI-PLC), is not required for parasite survival in mice. Mice have shown a decreased level of pathogenicity when infected with a strain of *T. brucei* lacking the PLC enzyme I (Webb *et al.*, 1997; Magez *et al.*, 2002). The cleavage of the GPI-VSG moieties by PLC has been demonstrated to stimulate the production of host inflammatory cytokines, such as tumor necrosis factor (TNF) (Magez *et al.*, 2002). An anti-disease vaccine based on the GPI anchors has been suggested as an effective strategy after it was found that anti-GPI

antibodies isolated from sera of individuals infected with malaria, were able to block malaria toxin from inducing TNF production (Magez *et al.*, 2002). No success has yet been achieved using this strategy; however, vaccine trials are underway.

1.6 Assessment of α- and β-tubulin from *T. congolense* as vaccine candidates.

The studies of Lubega *et al*. (2002a and b) and of Li *et al*. (2007) have demonstrated that the tubulin proteins, and β-tubulin in particular, are capable of providing protective immunity to mice infected with a variety of trypanosome strains.

As previously discussed, trypanosomes have a unique cytoskeleton in that it is composed primarily of microtubules. Trypanosome tubulin is also significantly different to mammalian tubulin. This was demonstrated in the experiments by Lubega *et al*. (2002), where antibodies raised against trypanosome tubulin did not recognise renatured rat brain tubulin, and similarly, antibodies raised against renatured rat brain tubulin did not inhibit trypanosome growth *in vitro* (Lubega *et al*., 2002a and b).

The work of Li *et al*. (2007) demonstrated that recombinant β-tubulin alone from *T. evansi* could provide immunoprotection against infection with *T. evansi*, *T. equiperdum* and *T. b. brucei* in mice, whereas the work of Lubega *et al*. (2002a) did not identify the antigen in the tubulin preparations used that was able to provide protective immunity. *In vitro* studies showed that anti-tubulin antibodies are internalised by the trypanosomes, which inhibits their growth and kills them. The mechanism by which antibodies inhibit parasite growth is still not understood fully, although the flagellar pocket region is thought to play a role in the internalisation process (Li *et al.*, 2007). The results of Li *et al*. (2007) and Lubega *et al*. (2002a and b) indicate the possibility that antibodies against sub-surface antigens may be internalised and by the parasite and are able to bind to and inhibit the function of these sub-surface antigens within the cell. These observations raise questions as to how this internalisation may be achieved.

The β-tubulin amino acid sequences of *T. congolense* and *T. vivax* are nearly identical, which is significant as these are the main infective trypanosome strains in African cattle. It is therefore likely that a vaccine developed against β-tubulin from one of these strains will be effective against the other strains as well.

1.7 Objectives of the present study

The following study aims to investigate the immuno-protective potential of recombinant α- and β-tubulin from *T. congolense* in mice against challenge with *T. congolense,* using native tubulin isolated from *T. conglense* cultured procyclics as a control for immunisation. Chapter two presents the cloning of the *T. congolense* α- and β-tubulin ORFs into the bacterial expression vector pET28a and also of the α-tubulin ORF into the yeast expression vector, pPICZαA28. The high-level expression of α- and β-tubulin as fusion proteins with a histidine tag, using the pET28a expression system and verification by western blot with anti-tubulin monoclonal antibodies are also presented in Chapter two. Chapter three presents the purification of recombinant α- and β-tubulin by nickel chelate chromatography and refolding of these proteins by dilution and reconcentration and by application of a decreasing gradient of urea while bound to a nickel chelate resin. The isolation of native tubulin from *T. congolense* for use as a control for immunisation is also reported in Chapter three, as well as the results of the immunisation of mice with recombinant α-, β-, native tubulin and an irrelevant viral protein, VP4AA, expressed in the same way as α- and β-tubulin, followed by challenge with *T. congolense*.

CHAPTER TWO

CLONING AND EXPRESSION OF α- AND β-TUBULIN FROM *TRYPANOSOMA CONGOLENSE* FOR ANALYSIS AS VACCINE CANDIDATES AGAINST TRYPANOSOMIASIS

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ABSTRACT

Initial efforts towards development of a vaccine against animal African trypanosomiasis have been hampered by the phenomenon of antigenic variation inherent to the outer coat of variable surface glycoprotein surrounding the trypanosome parasite. For this reason, invariant parasite antigens have been sought as vaccine candidates. Parasite tubulin proteins have been identified as being capable of producing protective antibody responses. In the present study α- and β-tubulin from *Trypanosoma congolense* were cloned and expressed with the view to assess their immuno-protective potential. Following amplification from genomic DNA, the α- and β-tubulin amplicons, at sizes of 1356 and 1329 bp respectively were ligated into the T-vector pTZ57R/T and thereafter sub-cloned into the bacterial expression vector pET28a and the yeast expression vector, pPICZαA28. Only α-tubulin was successfully cloned into pPICZαA28, however, following transfection of the methylotrophic yeast *Pichia pastoris* X33 with this construct, no recombinant protein was found to be expressed. Recombinant α- and β-tubulin in pET28a were successfully expressed as fusion proteins with a histidine tag in *Escherichia coli* expression host BL21 (DE3) both at a size of 55 kDa. The identity of both recombinant α- and β-tubulin was confirmed by western blot with anti-α and anti-β-tubulin monoclonal antibodies.

2.1 INTRODUCTION

African trypanosomiasis is caused by parasitic protozoans known as African trypanosomes, affecting humans and animals, most importantly livestock which are parasitised mainly by the species *Trypanosoma congolense* and *T. vivax*, throughout large regions of sub-Saharan Africa and results in death unless treatment is given. African trypanosomes are transmitted by the biting tsetse fly, and have lifecycle stages adapted to this host. Farming activities in areas where tsetse are prevalent are greatly hindered, as cattle which supply meat and milk and are necessary to provide traction for farming activities are largely absent, resulting in extensive economic losses for the affected region (Kristjanson *et al.*, 1999; Taylor and Rudenko, 2006). The development of a vaccine is seen as being the most successful means of controlling this disease (Taylor and Authié, 2004).

Despite continued efforts, and a calculated cost:benefit ratio of 1:34 for the development of a vaccine against African trypanosomiasis, meaning that the benefits resulting from an effective vaccine outweigh the costs involved in its development by 34 to 1, or for every one US$ spent in vaccine research, gains of US$ 34 would result from the use of the vaccine, no effective vaccine has been forthcoming (Kristjanson *et al.,* 1999). The main hindrance to vaccine development has been the phenomenon of antigenic variation inherent to trypanosomes (Taylor and Rudenko, 2006). Therefore, it has been necessary to identify invariant parasite factors that may serve as appropriate vaccine candidates. Thus far, invariant antigens that have been investigated for vaccine potential have included trypanosomal cysteine proteases, flagellar pocket antigens, microtubule associated proteins (MAPs) and glycosyl-phosphatidylinositol (GPI) anchors (Mkunza *et al.*, 1995; Authié *et al.*, 2001; Lalmanach *et al.*, 2002; Magez *et al.*, 2002; Rasooly and Balaban, 2004).

Recently, both native and recombinant trypanosome tubulin have been identified as possible vaccine candidates (Lubega *et al.*, 1998; Lubega *et al.*, 2002a; Rasooly and Balaban, 2004; Li *et al.*, 2007). Tubulins are invariant structural proteins, and are present abundantly as the main components of the microtubules that make up the trypanosome cytoskeleton. Although several forms of tubulin have been identified, microtubules are

comprised of heterodimers of only two forms, namely α- and β-tubulin (Nogales and Wang, 2006). Native tubulin has been isolated directly from trypanosome cells as a mixture of α- and β-tubulin (Stieger *et al.*, 1984; MacRae and Gull, 1990; Lubega *et al.*, 2002a) as performed by Lubega *et al*. (2002a) in their study of the immuno-protection potential of native tubulin from *T. brucei*, while either α- or β-tubulin may be separately expressed as recombinant proteins, as was done by Li *et al*. (2007) in their evaluation of the immnuo-protection potential of recombinant β-tubulin from *T. evansi*.

Proteins identified as having potential as vaccine candidates must be available in sufficient quantity for immunisation trials and if proven to be effective, for large scale commercial preparation. Due to the greater cost and low yields typically obtained for the isolation of native eukaryotic proteins, it is more economical and time-saving to obtain the protein via recombinant expression (Marston, 1986). Recombinant proteins have the advantage of being routinely expressed in quantities above those achieved through purification of native protein, with greater ease and at lower cost (Singh and Panda, 2005). Recombinant proteins are used for purposes that include protein and enzyme characterisation studies, for example, recombinantly produced α- and β-tubulin from the pepper plant *Capsicum annuum*, was used as a control for screening of antimitotic drugs against plant pathogenic fungi, where isolation of native *C. annuum* tubulin resulted in low yields (Jang *et al.*, 2008). Recombinant protein is also used for production of vaccines and other commercial and medical applications, such as the production of commercially available enzymes for molecular biology applications (by companies such as Fermentas) and recombinantly produced insulin in the treatment of diabetes (Winter *et al.*, 2002).

Recombinant expression systems have included insect, mammalian, yeast and bacterial cells as expression hosts (Verma *et al.*, 1998). Yeast has the advantages of ease of purification of recombinant proteins, as recombinant proteins are secreted from the cell into the surrounding medium. For example, C2, the catalytic domain of the trypanosomal cysteine protease, congopain has been successfully expressed in the yeast *Pichia pastoris* for use in vaccine related studies (Bizaaré, 2009; Huson *et al.*, 2009; Ndlovu, 2009).

Because yeast is a eukaryotic expression system, the recombinant proteins expressed in yeast are also post-translationally modified in a similar fashion as in mammalian cells (Verma *et al.*, 1998; Daly and Hearn, 2005). Many different factors must be considered in order for heterologous protein expression in yeast to be successful. These factors include the yeast strain selected for expression and the choice of expression vectors and promoters used, as well as the techniques employed in the transformation and integration of the construct into the yeast genome (Daly and Hearn, 2005).

Bacterial expression systems, typically *Escherichia coli,* offer advantages over yeast in that they are able to express a larger variety of recombinant proteins in a much shorter space of time (Marston, 1986). While some bacterially expressed recombinant proteins are soluble, a major disadvantage of many proteins expressed in bacteria is their tendency to form inclusion bodies, i.e. densely packed and denatured insoluble masses of the recombinant protein formed inside the host cell, resulting from over-expression (Marston, 1986; Singh and Panda, 2005). Inclusion bodies must be purified, solubilised and refolded into their biologically active form, as would occur in native protein; tasks which are labour intensive and reduces the yield of recombinant protein (Rudolph and Lilie, 1996; Lilie *et al.*, 1998). A further disadvantage of *E. coli* expression systems is their inability to glycosylate proteins; for this reason, and to avoid the necessity of inclusion body purification and refolding, yeast expression systems are often more advantageous (Verma *et al.*, 1998).

Previous studies into protozoan tubulin have suggested it as a possible drug target for treatment of disease caused by trypanosomes and other related kinetoplastid parasites. This is due to differences noted in the properties and drug susceptibility between mammalian and protozoan tubulins (Stieger *et al.*, 1984; Werbovetz *et al.*, 1999; MacDonald *et al.*, 2003). For example, the observation that the tubulins of lower eukaryotes are only marginally affected by colchicine, a microtubule inhibitor of higher eukaryotes, and the ability of phenothiazines, a group of drugs having clinical uses, to kill trypanosomes by destruction of the pellicular microtubules (Stieger *et al.*, 1984; MacDonald *et al.*, 2003). A study by Rasooly and Balaban (2004) investigating the immuno-protection potential of a

microtubule associated protein (MAP), p15, as an anti-parasite vaccine yielded some success, as 100% protection was achieved in mice from an otherwise lethal challenge with *T. brucei*. The successes of Lubega *et al*. (2002a and b) and Li *et al*. (2007), with trypanosome tubulin have further strengthened the case for investigating trypanosome tubulins and other cytoskeletal proteins as potential vaccine candidates.

Structurally, α- and β-tubulin are identical; however, they share approximately 40% amino acid sequence identity (Nogales *et al.*, 1998). Due to the differences in sequence identity, it would be of interest to investigate the immunoprotective potential of recombinant α-tubulin as well as β-tubulin, based on the success of Li *et al*. (2007) with recombinant β-tubulin. Since α- and β-tubulin form heterodimers that polymerise into microtubules, they are in close association with each other and may have similar immuno-protection potential. Also considering that native tubulin isolated by Lubega *et al*. (2002a) was a mixture of α- and β-tubulin, it is not apparent from that study which of these proteins was responsible for the immuno-protection observed, or if both proteins are possibly involved.

Here we report on the amplification of the α- and β-tubulin ORFs from *T. congolense* (strain IL 1180) genomic DNA. This is followed by the cloning and expression of the α- and β-tubulin ORFs into the bacterial expression vector pET28a, and the yeast expression vector pPICZαA28, which is a pPICZαA vector containing the multiple cloning site of pET28a. The expression of α- and β-tubulin in the above two systems is analysed with a purpose of producing recombinant antigen for testing as a vaccine against trypanosomiasis.

2.2 METHODS AND MATERIALS

2.2.1 Materials

The cloning vector pTZ57R/T (InsTAclone PCR Cloning Kit, Fermentas, Vilhius) was used to clone the α- and β-tubulin ORFs after PCR amplification. The expression vectors pET28a and pPICZαA28 were used to express the protein in the *E. coli* strains BL21 (DE3) and JM 109, and the yeast *Pichia pastoris* strain X33, respectively.

Kits used included GeneJET plasmid mini-prep kit (Fermentas), TransformAid™ Bacterial Transformation Kit (Fermentas), peqGOLD Gel Extraction Kit (Peqlab Biotechnologie, Erlangen), InsTAclone PCR Cloning Kit (Fermentas) and DNA Clean and Concentrate Kit (Zymo Research). Chemicals were purchased from Roche (Mannheim) and Sigma (St. Louis, USA), while enzymes were purchased from Fermentas (Lithuania), New England BioLabs (Canada) and Solis-Biodyne (Tartu, Estonia). Specialised chemicals including Coomassie R-250, 4-chloro-1-naphthol and horse radish peroxidase (HRPO)-linked goat anti-mouse IgG from Sigma. L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) or Pefabloc, 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) and bovine serum albumin (BSA) were from Roche. Leupeptin was from Cambridge Research Biochemicals (Cambridge, England). Low range molecular weight markers were purchased from BioRad (United States of America).

2.2.2 Isolation of genomic DNA from *Trypanosoma congolense*

An estimated total number of 1 x 10^9 cells of *T. congolense* procyclics (strain IL 1180) cultured in MEM medium containing minimum essential medium (MEM) powder (Sigma) and fetal calf serum (FCS), were collected by centrifugation (400 x *g*, 10 min, RT). The cell pellet was resuspended in 2 ml of TE buffer (10 mM Tris-Cl, 10 mM EDTA, pH 7.4). SDS was added to bring the solution to a concentration of 0.5% (w/v) SDS. RNAse A (Roche), 1 µl, was added to the solution and incubated at 37° C for 15 minutes. Proteinase K (Roche) was added to a final concentration of 100 µg/ml. The solution was incubated at 50° C for 3 h and NaCl was added to a final concentration of 0.1 M. An equal volume of Tris-saturated phenol was added and mixed. The solution was centrifuged (2400 x *g*, 5 min, RT) in a Heraeus Pico 17 centrifuge. The upper aqueous phase was extracted with an equal volume of a 1:1 phenol:chloroform mixture. The solution was centrifuged as before. The upper phase was extracted once more with an equal volume of chloroform and centrifuged as before. The upper phase was extracted with an equal volume of diethyl ether and centrifuged as before. The bottom phase was retained and 2 volumes of cold ethanol were added in order to precipitate the DNA. The solution was centrifuged (2400 x *g*, 10 min, RT)

to pellet the DNA, which was washed twice with 70% (v/v) ethanol. The DNA pellet was dissolved in TE buffer (10 mM Tris-Cl, 1 mM EDTA, pH 7.4).

2.2.3 Identification of *T. congolense* α-tubulin ORF

The complete *T. congolense* α-tubulin ORF had not been identified and reported on any online database at the time of PCR primer design. For this reason, it was necessary to perform database searches using basic local alignment search tool (BLAST) using the existing α-tubulin ORF sequence of *T. brucei* (Tb927.1.2400) to identify the complete *T. congolense* ORF within the *T. congolense* genome database on the GeneDB website. The *T. brucei* α-tubulin sequence was used as the query sequence for a BLAST search against the *T. congolense* contigs latest assembly database in GeneDB. The search found an ORF within the contig_0001094 (see Appendix A) which closely matched the *T. brucei* α-tubulin ORF, and this was identified as the ORF of *T. congolense* α-tubulin (see Appendix B).

2.2.4 Polymerase chain reaction (PCR) for amplification of α- and β-tubulin ORFs

2.2.4.1 Oligonucleotide primer design

The ORFs for α- and β-tubulin (GeneDB: congo819f03.q1k3) were amplified by PCR using primers purchased from Proligo (Cergy, France) and the University of Cape Town (South Africa). Restriction enzymes cut along a DNA strand at a site specified by a short palindromic nucleotide sequence, which is specific for each particular enzyme. These short palindromic sequences are known as restriction sites, and are engineered into plasmid and insert sequences to allow for the inserts to be cloned into various plasmid vectors. The oligonucleotide primers were designed to include the restriction sites EcoR1 [nomenclature as in (Roberts *et al.*, 2003)] and Not1 for α-tubulin and EcoR1 and Nde1 for β-tubulin, to allow for the ligation of the insert into the various expression vectors (Table 2.1). The reverse primers, α-Tub-Rv, and β-Tub-Rv also code for a factor Xa cleavage site, which allows for the removal of the carrier protein and/or His-tags from the protein of interest by cleavage with Factor Xa.

Table 2.1: Nucleotide sequence of primers used for amplification of the α- and β-tubulin ORFs of *T. congolense*.

Primer name	Nucleotide sequence*
α-Tub-Fw	5' – AAAGAATTCCATATGCGCGAGGCTATCTGCATC – 3'
α-Tub-Rv	5' – AAAGCGGCCGCCTACCC↓TCGATGGTACTCCTCAACGTCCTCCTC – 3'.
β-Tub-Fw	5' – AAAGAATTCCATATGCGTGAGATCGTTTGTATTCAG – 3'
β-Tub-Rv	5' – AAAAGAATTCCTACCC↓TCGATGTACTGCTCCTCTTCATCAAACTCACC – 3'

* EcoR1, Nde1 and Not1 restriction sites and Factor Xa cleavage site coding sequence are shown in colours indicated. The position of the arrow (↓) indicates the position of cleavage by Factor Xa in the translated sequence.

2.2.4.2 PCR amplification of α- and β-tubulin ORF from trypanosome genomic DNA

The primer set α-Tub-Fw and α-Tub-Rv was used to amplify the α-tubulin ORF and the set β-Tub-Fw and β-Tub-RV was used to amplify the β-tubulin ORF. The PCR reaction was carried out in a volume of 50 µl, with 2.5 mM Mg^{2+}, 1 x reaction buffer, primers each at 20 pmol, dNTP's at 200 µM, 50 ng genomic DNA (Table 2.2). One unit of FirePol DNA polymerase (Solis-Biodyne) was added to the reaction tube after 1 min incubation at 94° C. After addition of DNA polymerase, a further 4 min incubation at 94° C was used, followed by 30 cycles consisting of incubation at 94° C for 30 sec, at 50° C for 1 min and at 72° C for 1.5 min. The final elongation step was carried out at 70° C for 10 minutes, where after DNA was stored at 4° C.

Table 2.2: PCR reaction components for amplification of α- and β-tubulin ORF from *T. congolense* genomic DNA.

PCR reaction components	Concentration	Volume (µl)
Template DNA (50 ng)	1 ng/µl	1
Forward primer (10 µM)	0.8 µM	4
Reverse primer (10 µM)	0.8 µM	4
10 x PCR reaction buffer	1 x	5
$MgCl^{2+}$ (25 mM)	2.5 mM	5
dNTP mixture	200 µM	2
Sterile dH_2O	-	28.5
Taq DNA polymerase	1 U	0.5
Total volume (µl)		50.0

2.2.5 Agarose gel electrophoresis analysis of DNA

DNA is analysed for size and purity by agarose gel electrophoresis. A 0.8% (w/v) agarose gel was used to analyse DNA samples produced from PCR or plasmid isolation. Preparation of the gel entailed adding 0.24 g agarose powder to 30 ml 1 x TAE buffer and heating the solution until the agarose dissolved. Once the solution was cooled to approximately 50° C, ethidium bromide was added to give a final concentration of 0.5 µg/ml, and the solution poured into a gel casting tray and allowed to set. Once the gel was set, it was submerged in an electrophoresis tank containing 1x TAE buffer. DNA samples were prepared by addition of loading dye containing bromophenol blue and 20% (v/v) glycerol, and loaded into the wells of the gel. The DNA was electrophoresed at 80 V for approximately 45 min in order to separate the DNA into bands of various sizes. After electrophoresis, the DNA was visualised by fluorescence of the ethidium bromide dye by UV light.

2.2.6 Gel extraction of PCR product

The α- and β-tubulin amplicons were purified by gel extraction in order to remove any contaminating DNA or nucleotides using the PeqGOLD E.Z.N.A Gel Extraction Kit. The DNA sample to be purified was run on a 0.8% (w/v) agarose gel at 50 V for approximately 45 min to fractionate the DNA. The gel was exposed to UV light briefly to identify the band of interest and this was excised from the gel and placed in a clean microfuge tube. The mass of the gel in the tube was determined and a volume of DNA Binding Buffer equal to the weight of the gel was added to the tube, assuming the density of the gel is roughly 1 g/ml. The gel was melted by incubation at 55 - 65°C for 7 min. The melted gel was then placed into a fresh HiBind® spin column within a collection tube and centrifuged (10 000 *g*, 1 min, RT) after which the flow-through was discarded. A further 300 µl of Binding Buffer was added to the spin column and centrifuged as before, and the flow-through discarded. The spin column was again washed twice by addition of 750 µl of SPW Wash Buffer and centrifugation as before. The spin column was then dried by centrifuging as before. The spin column was transferred to a clean microfuge tube and the DNA eluted by addition of 30 µl Elution Buffer followed by centrifugation as before. Purity of the DNA sample was analysed by agarose gel electrophoresis (Section 2.2.5).

2.2.7 Ligation of insert into T-vector

Ligation of the α- and β-tubulin ORFs into the T-vector pTZ57R/T (Fig. 2.1) was performed using the InsTAclone PCR Cloning Kit (Fermentas) at a ratio of 3:1 insert to vector concentration. A reaction volume of 15 µl was used which contained 4 µl insert, 1 µl pTZ57R/T, 3 µl 5x ligation buffer, 6.6 µl nuclease-free water, and 0.4 µl T4 DNA ligase (5 U). The ligation reaction was performed overnight at 4° C.

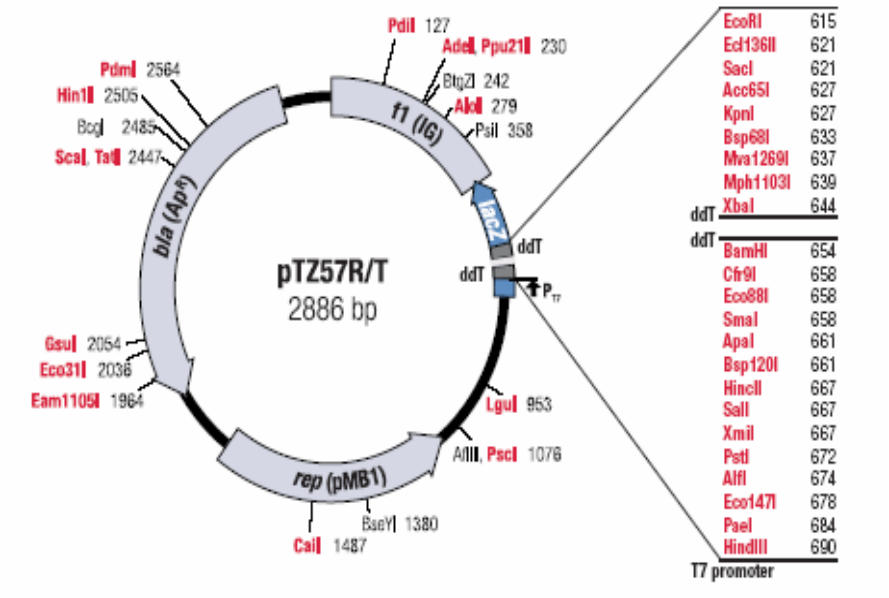

Figure 2.1: Map of T-vector pTZ57R/T (Fermentas).

2.2.8 Transformation of *E. coli* JM 109 with ligation mixture

The pTZ57R/T vector and α- or β-tubulin insert ligation mixture was transformed into *E. coli* JM 109 cells using the TransformAid™ Bacterial Transformation Kit (Fermentas). Non-recombinant *E. coli* JM 109 cells were streaked onto an antibiotic-free 2X YT agar plate and incubated overnight at 37° C. A small section (4 x 4 mm) of the 2X YT agar plate containing cultured *E. coli* cells was cut from the plate and transferred to 1.5 ml of pre-warmed C-medium and incubated at 37° C for 2 h with shaking. The cells were pelleted by centrifugation (17 000 *g*, 1 min, RT) and the supernatant discarded. The pelleted cells were resuspended in 300 µl of T-solution, prepared freshly using equal volumes of T-solutions A and B, and incubated on ice for 5 min. The cells were pelleted again by centrifugation as before, and the supernatant discarded. The cells were resuspended finally in a volume of 120 µl of T-solution and incubated on ice for 5 min. At the same time, 2.5 µl of the ligation mixture was transferred to a sterile microfuge tube and placed on ice for 2 min. Next, 50 µl of resuspended cells were added to the tube containing the ligation mixture and incubated on ice for 5 min. The resuspended cells were plated onto pre-warmed 2X YT agar plates

containing ampicillin (50 µg/ml), X-gal and IPTG to allow for blue and white colony selection, and incubated overnight at 37° C.

2.2.9 Colony PCR

After transformation of *E. coli* JM 109 with pTZ57R/T/α- and β-tubulin, 2X YT agar plates containing X-gal, IPTG and ampicillin were inspected for the presence of blue and white colonies. The resultant recombinant white colonies were screened by colony PCR to detect the presence of an insert at the expected size of 1300 bp. The specific primers for α- and β-tubulin were used to screen for the respective inserts. Template DNA was obtained from a sample of cells transferred from a colony by a toothpick. The PCR reaction was performed in a reaction volume of 25 µl with reaction conditions that included initial denaturation at 94° C for 15 min, followed by 30 cycles of denaturation at 94° C for 30 s, annealing at 55° C for 1 min, elongation for 1.5 min at 72° C. A final elongation step was carried out at 70° C for 10 min, and thereafter the reaction tubes were stored at 4° C. The reaction components and their concentration used in colony PCR are given in Table 2.3.

Table 2.3: Reaction components of colony PCR

Components of reaction mixture	Concentration	Volume (µl)
Forward primer (10 µM)	0.8 µM	2
Reverse primer (10 µM)	0.8 µM	2
10 x PCR reaction buffer	1 x	2.5
$MgCl^{2+}$ (25 mM)	2.5 mM	2.5
dNTP mixture	200 µM	1
Sterile dH_2O	-	14.7
Taq DNA polymerase	0.5 U	0.25
Total volume (µl)		25

2.2.10 Isolation of plasmid DNA by miniprep using GeneJET™ Plasmid Miniprep Kit

The technique commonly used to isolate plasmid DNA from bacterial (*E. coli*) cells is known as alkali lysis or the miniprep procedure. The GeneJET™ Plasmid Miniprep Kit (Fermentas) was used to purify recombinant plasmids from *E. coli* JM 109 and BL21 (DE3) according to the manufacturer's instructions. Cells containing recombinant plasmid were cultured overnight at 37° C in 2X YT media containing 34 μg/ml kanamycin (or 50 μg/ml ampicillin). Two ml of culture was centrifuged (17 000 *g*, 2 min, RT) to pellet cells, and the supernatant discarded. The cell pellet was resuspended in 250 μl of resuspension solution, and the cells lysed by addition of 250 μl of lysis solution. Cell membranes and chromosomal DNA were precipitated by addition 350 μl of neutralization solution, and pelleted by centrifugation (17 000 *g*, 5 min, RT). The supernatant was transferred to a GeneJET™ spin column, centrifuged (17 000 *g*, 1 min, RT), and the flow-through discarded. The column was washed with 500 μl wash solution and centrifuged (17 000 *g*, 1 min, RT) and the flow-through discarded. The wash step was repeated twice. The spin column was centrifuged again (17 000 *g*, 1 min, RT) to remove residual wash solution. Finally, 50 μl of elution buffer was added to the spin column, which was transferred to a clean microfuge tube and the column incubated at room temperature for two min. The spin column and microfuge tube were centrifuged (17 000 *g*, 2 min, RT) to elute the plasmid DNA.

2.2.11 Restriction digestion of insert and plasmid

The presence of the α- and β-tubulin inserts in the pTZ57R/T vector was assessed by restriction digestion of the plasmid with restriction enzymes EcoR1, Not1 and Nde1. Inserts were excised from the pTZ57R/T vector by digestion with the restriction enzymes EcoR1 and Not1 for pTZ57R/T/α-tubulin and EcoR1 and Nde1 for pTZ57R/T/β-tubulin, according to the manufacturers' instructions. The restriction mixture consisted of the DNA sample to be analysed, restriction enzyme buffer (10x), nuclease-free dH_2O, and the restriction enzyme. Digestion was carried out at 37° C for 2 h or overnight at room temperature (RT).

2.2.12 Sequencing of pTZ57R/T/α- and β-tubulin constructs

Clones that tested positive for the presence of an insert at the expected size of 1.3 kb and having the correct restriction enzyme sites were sent for sequencing. Sequencing of the inserts was performed by automated dideoxynucleotide termination method using standard primers T7 forward and M13 reverse, at the International Livestock Research Institute (ILRI) in Kenya. Sequencher 4.7 software (Genecode, Michigan) was used to analyse the sequences.

2.2.13 Sub-cloning of α- and β-tubulin insert into pET28a and pPICZαA28

Inserts sequenced and found to be free of mutations were used for sub-cloning into the bacterial expression vector pET28a and yeast expression vector pPICαA28 (Fig. 2.2). Inserts were excised from the T-vector pTZ57R/T mini-prep preparations by digestion with restriction enzymes as detailed in Section 2.3.10. Expression vectors pET28a and pPICZαA28 were similarly digested with EcoR1 and Not1 in preparation for ligation with α-tubulin and with EcoR1 and Nde1 for ligation with β-tubulin. The digested vectors pPICZαA28 and pET28a were digested with shrimp alkaline phosphatase (SAP) for 1 h at 37° C in order to dephosphorylate the cut ends of the vector, followed by deactivation of the SAP by incubation for 15 min at 65° C. The digested inserts and expression vectors were purified by gel extraction (Section 2.2.6) to remove any contaminating nucleotide sequences. The purified inserts and vector preparation were analysed by agarose gel electrophoresis (Section 2.2.5) to determine relative quantities necessary for ligation.

Ligation of inserts and expression vectors was carried out using the same protocol as ligation of PCR product with T-vector detailed in Section 2.2.7. Transformation of ligation mixture into *E. coli* JM 109 was performed using the TransformAid Bacterial Transformation Kit (Fermentas), as described in Section 2.2.8, with the modification that cells were plated onto 2X YT plates containing antibiotics; kanamycin for pET28a and Zeocin™ for pPICZαA28 to allow for selection of recombinant colonies. Recombinant colonies were screened by colony PCR (Section 2.2.9) and restriction digestion (Section 2.2.11) to ensure the presence of the correct insert before expression of recombinant

proteins was commenced. pET28a/α- and β-tubulin plasmids were transformed into the expression host *E. coli* BL21 (DE3) prior to expression, while the pPICZαA28/α-tubulin plasmid was transfected into yeast cells *P. pastoris* X33 for yeast expression (cloning of β-tubulin into pPICZαA28 was unsuccessful).

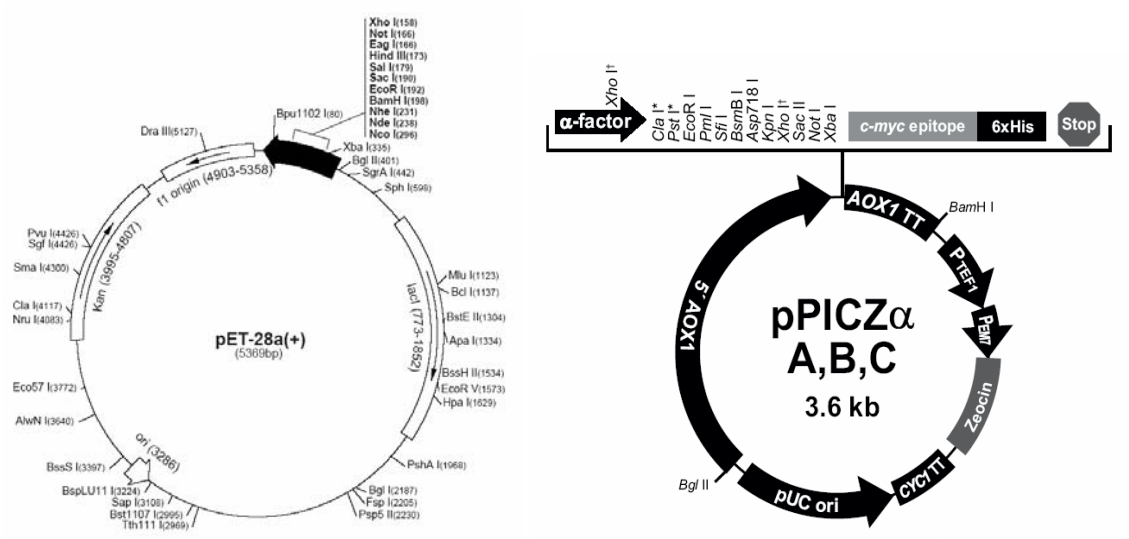

Figure 2.2: Maps of bacterial expression vector pET28a (Novagen) and yeast expression vector pPICZαA (Invitrogen).

2.2.14 Preparation of electrocompetent *P. pastoris*

A small-scale culture of *P. pastoris* X33 was grown in YPD medium until an O.D. at 600 nm of 1 – 2 was obtained. The number of cells obtained was estimated by the equation $O.D._{600}$ x 5 x 10^7. The number of transformations possible was then calculated by the equation: no. of cells/ 8 x 10^7 (Wu and Letchworth, 2004). The cells were pelleted by centrifugation (2000 *g*, 10 min, RT) and resuspended in a volume of lithium acetate solution (100 mM lithium acetate, 10 mM DTT, 0.6 M sorbitol, 10 mM Tris-HCl buffer, pH 7.5) equal to the number of transformations possible multiplied by 8. The cells were then incubated for 30 min at room temperature and pelleted by centrifugation (2000 *g*, 10 min, RT). The cells were resuspended in ice-cold 1 M sorbitol to a volume equal to the

number of transformations x 1.5 ml and again pelleted by centrifugation (2000 *g*, 10 min, RT). This was repeated a further 2 times. Finally, cells were resuspended in ice-cold 1 M sorbitol to a concentration of 10^{10} cells/ml and stored at -80° C until needed.

2.2.15 Transfection of pPICZαA28/α-tubulin into *P. pastoris* yeast cells

The pPICZαA28/α-tubulin mini-prep plasmid preparation was linearised by digestion with Pme1 (Fermentas) (Section 2.2.11) in order to allow the plasmid to integrate into the *P. pastoris* genome upon transfection. The linearised plasmid was purified by gel extraction (Section 2.2.6) prior to electroporation. Previously prepared electrocompetent *P. pastoris* X33 cells were thawed and placed on ice. The *P. pastoris* cells (200 µl) and linearised plasmid (5 µl) were transferred to sterile, ice-cold gap vials (BioRad), and electroporation was carried out at 1.5 kV, 25 µF and 186 Ω using a BioRad electroporator. Immediately after electroporation, 1 ml of ice-cold 1 M sorbitol was added to the cells. The cells were briefly centrifuged to pellet cells and plated onto YPD agar plates containing the antibiotic Zeocin™ and incubated at 30° C for 4 days.

2.2.16 Yeast expression of pPICZαA28/α-tubulin in *P. pastoris*

A single colony of *P. pastoris* X33 containing pPICZαA28/α-tubulin was inoculated into YPD medium (50 ml) containing Zeocin™ (100 µg/ml) and incubated for 3 days at 30° C with shaking. The culture was used to inoculate BMGY medium (500 ml) containing ampicillin (50 µg/ml) and incubated for 2 days at 30° C with shaking until an O.D. at 600 nm of 2 was reached. The cells were collected by centrifugation (2000 *g*, 10 min, 4° C) and resuspended in BMM medium (500 ml x 2), each flask covered with sterile gauze, and incubated at 30° C for 4 days with shaking along with the addition of 2.5 ml methanol daily to each flask. Cells were pelleted by centrifugation (2000 *g*, 10 min, 4° C), and the supernatant retained for analysis of protein expression by SDS-PAGE.

2.2.17 Three phase partioning (TPP) of yeast supernatant

TPP was carried out in order to concentrate by salting out any recombinant protein present in yeast culture supernatant after expression prior to analysis by SDS-PAGE (Dennison, 2003). A volume of 50 ml of yeast culture supernatant was used, to which 30% (v/v) t-butanol and 20% (w/v) $(NH_4)_2SO_4$ was added and allowed to dissolve, followed by centrifugation (5000 *g*, 10 min, RT). The supernatant was retained and made up to 40% (w/v) $(NH_4)_2SO_4$ which was dissolved and centrifuged as before. The resulting precipitate was retained and dissolved in 100 µl PBS for analysis by SDS-PAGE.

2.2.18 Bacterial expression (Terrific broth)

Expression of recombinant α- and β-tubulin in pET28a was carried out in the *E. coli* expression host BL21 (DE3). Recombinant colonies were inoculated into Terrific broth medium (500 ml) containing 34 µg/ml kanamycin and incubated overnight at 37 °C. Cells were harvested by centrifugation (5 000 *g*, 20 min, 20° C). The pelleted cells were resuspended in ice-cold 100 mM Tris-Cl buffer pH 7.4, 10 mM EDTA, and centrifuged (12 000 *g*, 30 min, 4° C). The cells were resuspended (10 mM Tris-Cl buffer pH 8.2, 1 mM EDTA, 100 mM NaCl, 1 µg/ml lysozyme), containing protease inhibitors E-64, leupeptin and 4-(2-aminoethyl)benzenesulfonyl fluoride (AEBSF) at 1 µM each and stored at -20° C.

2.2.19 SDS-PAGE analysis of recombinant α- and β-tubulin expression

Proteins were analysed using 10% SDS-PAGE (Laemmli, 1970). The BioRad mini-PROTEAN 3 gel system was used and electrophoresis conducted at 18 mA per gel. BioRad low molecular weight markers were used to determine the size of protein samples by construction of a standard curve relating the log molecular mass to the relative mobility of protein samples on an SDS-PAGE gel (Fig. 2.3). After completion of electrophoresis, proteins were stained with Coomassie blue R-250 or blotted onto nitrocellulose membrane for western blots. A VersaDoc imaging system together with Quantity One analysis software (BioRad) were used for imaging of SDS-PAGE gels and western blots.

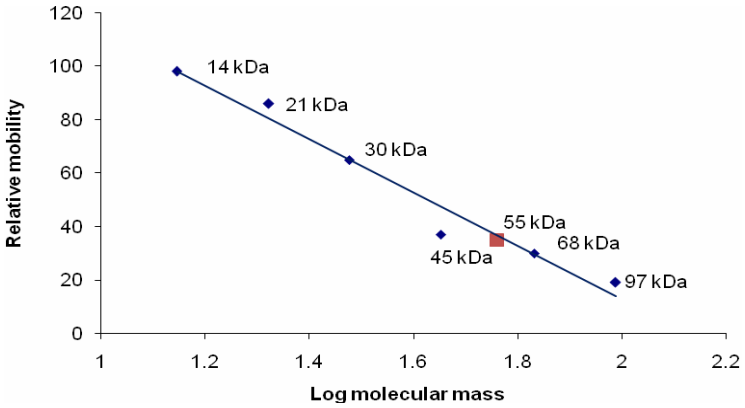

Figure 2.3 Standard curve relating the relative mobility of molecular weight marker proteins on an SDS-PAGE (10%) gel to their log molecular mass. The protein markers include phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), chicken egg ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa) and chicken egg white lysozyme (14 kDa). The equation of the trend line was y = -99.789x + 212.5, with a correlation coefficient of 0.968.

2.2.20 Western blot to detect the presence of a His-tag on recombinant α- and β-tubulin

Western blotting was performed according to Towbin *et al.* (1979). Proteins were transferred from an unstained SDS-PAGE gel to a nitrocellulose membrane using a dry blotter apparatus (model B 2529, Sigma), at 40 mA for 1.5 h. The nitrocellulose was transiently stained with 1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid, to reveal molecular weight marker band positions, and the bands marked with pencil. The nitrocellulose was washed with distilled water containing a drop of NaOH to remove the Ponceau S and blocked for 1 h in Tris-buffered saline (TBS) (20 mM Tris-Cl, 20 mM NaCl, pH 7.4) containing 5% (w/v) low fat milk. The nitrocellulose was washed with TBS and incubated with the primary antibody, anti-His monoclonal antibody (Sigma, USA), diluted in TBS containing 5% (w/v) low fat milk, for 2 h at room temperature or overnight at 4° C. The nitrocellulose was again washed with TBS and incubated with the detection antibody, HRPO-linked goat anti-mouse IgG (Sigma, USA), in TBS containing 5% (m/v) low fat milk for 1 h at room temperature. After a final wash with TBS, the nitrocellulose was incubated with the substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v) methanol, 0.0015% (v/v) H_2O_2 in TBS] in the dark until bands appeared on the membrane.

2.3 RESULTS

2.3.1 Genomic DNA Isolation from *T. congolense*

Genomic DNA was isolated from *T. congolense* procyclics (strain IL 1180) by phenol:chloroform extraction in order to obtain sufficient DNA to be used as a template for PCR amplification of the α- and β-tubulin ORFs. The result is shown in Fig. 2.4, where. *T. congolense* DNA was isolated from three separate preparations of cultured procyclics, designated A, B and C. These were run in duplicate on a 0.8% agarose gel (Fig. 2.4). The sizes of the bands of genomic DNA were larger than the largest molecular weight marker band (10 000 bp), which is not ideal but unfortunately was due to the unavailability of a higher range molecular weight marker.

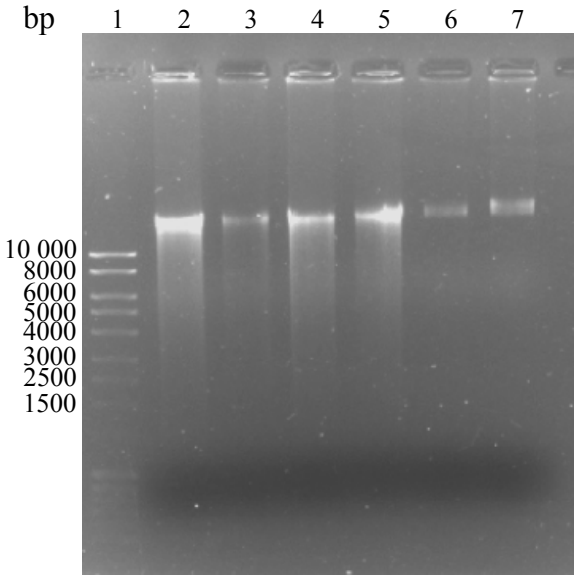

Figure 2.4: Isolation of genomic DNA from T. congolense strain IL 1180. Genomic DNA (1 µl) was electrophoresed through a 0.8% agarose gel. Lane 1, DNA MassRuler; lanes 2 and 3, culture A; lanes 4 and 5, culture B; lanes 6 and 7, culture C. DNA was visualised by ethidium bromide staining.

2.3.2 Amplification of *T. congolense* α- and β-tubulin ORF's from genomic DNA

PCR was used to amplify the ORF of both α-tubulin and β-tubulin from *T. congolense* (Fig. 2.5). The PCR products for both α- and β-tubulin were found to have an approximate size of 1300 bp when run on a 0.8% agarose gel. This size corresponds to the expected sizes of

1356 bp for α-tubulin (Section 2.2.3) and 1329 bp for β-tubulin (GeneDB: congo819f03.q1k3).

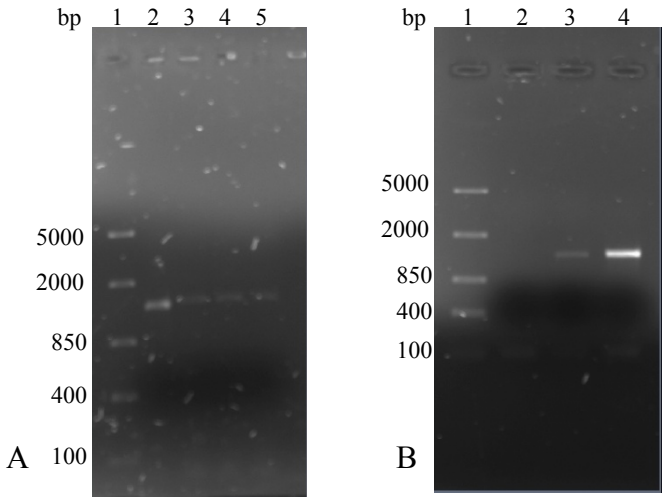

Figure 2.5: Amplification of α- and β-tubulin ORFs from *T. congolense* genomic DNA. Electrophoresis was carried out in a 0.8% agarose gel. Panel A: α-tubulin. Lane 1, molecular weight marker (Fermentas MiddleRange); lane 2, positive control (β-tubulin), lane 3, α-tubulin PCR sample 1; lane 4, α-tubulin PCR sample 2; lane 5, α-tubulin PCR sample 3. Panel B: β-tubulin. Lane 1, molecular weight marker (Fermentas MiddleRange); lane 2, no template control; lane 3, β-tubulin PCR sample 1; lane 4, β-tubulin PCR sample 2.

2.3.2 Cloning of β - and α -tubulin ORFs into T-vector pTZ57R/T

The β- and α-tubulin amplicons were cloned into the pTZ57R/T cloning vector prior to sequencing and sub-cloning into the expression vectors pET28a and pPICZαA28. Purified insert was ligated into pTZ57R/T and transformed into *E. coli* JM 109 cells. Recombinant colonies were screened by colony PCR to detect the presence of the insert at the expected size.

The results of the colony PCR in Fig. 2.6 indicate that all ten of the colonies screened were positive for the presence of the β-tubulin ORF, as a band at 1300 bp could be observed in all screened colonies. This band corresponds to the expected size of 1329 bp for β-tubulin. The positive control containing *T. congolense* genomic DNA as the template instead of a bacterial colony, showed a band at 1300 bp, while the negative control containing no template DNA showed no band.

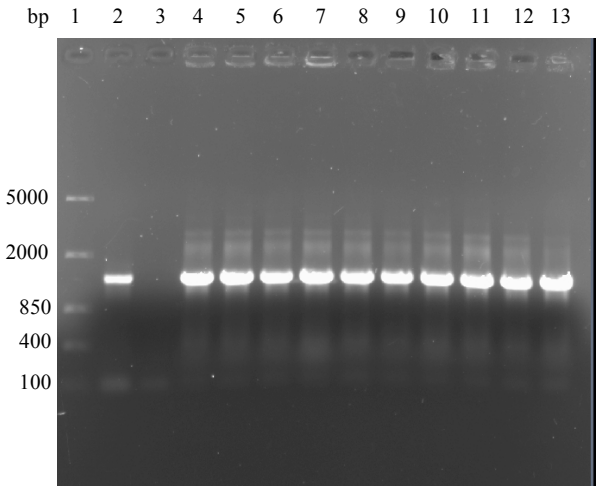

Figure 2.6: Agarose gel electrophoresis analysis of pTZ57R/T/β-tubulin by colony PCR. Lane 1, molecular weight marker (Fermentas MiddleRange); lane 2, positive control using *T. congolense* genomic DNA as template; lane 3, PCR control excluding template DNA; lanes 4 – 13 colonies selected for screening of recombinants.

The α-tubulin ORF amplified from *T. congolense* genomic DNA was ligated into the cloning vector pTZ57R/T. The ligated plasmid and insert were transformed into *E. coli* strain JM 109 cells and plated onto 2X YT plates containing X-gal, IPTG and ampicillin. The resulting colonies were screened by blue and white colony selection to detect recombinants. Five white colonies were selected and screened by colony PCR to ensure that the expected insert was present (Fig. 2.7, lanes 12 to 15 and 17). All five colonies screened were found to include the insert at 1300 bp, which corresponds to the expected size of 1356 bp for *T. congolense* α-tubulin. The positive control contained *T. congolense* genomic DNA as the template instead of a bacterial colony (Fig. 2.7, lane 18), while the negative control contained no template DNA (Fig. 2.7, lane 19).

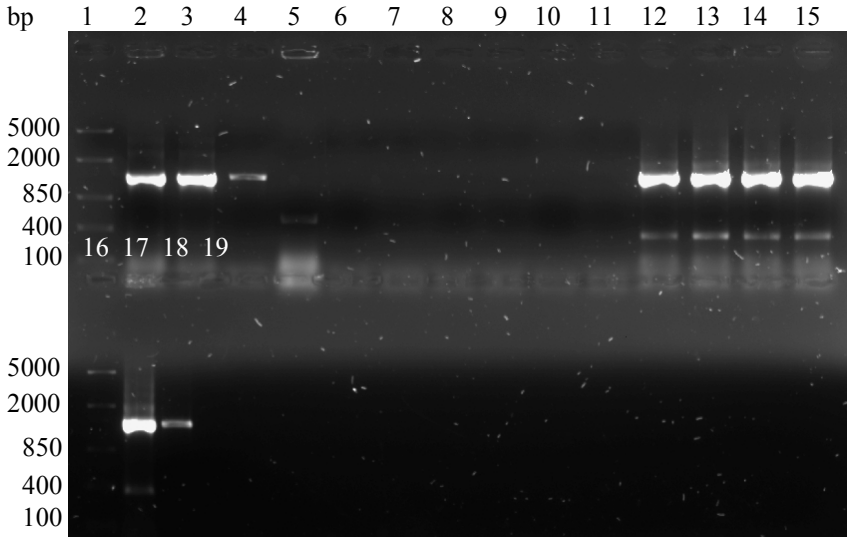

Figure 2.7: Agarose gel (0.8%) electrophoresis analysis of recombinants for pET28a/β-tubulin, pPICZαA28/β-tubulin and pTZ57R/T/α-tubulin by colony PCR after transformation into *E. coli* JM 109 cells. Lanes 1 and 16, molecular weight marker (Fermentas MiddleRange); lanes 2-4, PCR of individual colonies for pET28a/β-tubulin; lanes 5-11, PCR of individual colonies for pPICZαA28/β-tubulin; lanes 12-15 and 17, PCR of individual colonies for pTZ57R/T/α-tubulin; lane 18, positive PCR control; lane 19, PCR control excluding template DNA (negative control).

Five of the positive colonies for pTZ57R/T/β-tubulin were screened by digestion with the restriction enzymes EcoR1 and Nde1 to ensure that the insert was correctly in place. The analysis of the digestion is given in Fig. 2.8 and shows that three out of the five colonies screened contained plasmid with insert correctly ligated with expected restriction sites present. Colonies 1, 2 and 5 showed excision of the insert following digestion by EcoR1, linearisation of the plasmid with Nde1 and excision of the insert with double digestion by EcoR1 and Nde1, as expected. Colony 3 produced a linear DNA band when digested with EcoR1 (lane 6), instead of excising the insert, no digestion was observed with Nde1 (lane 16) and the plasmid was linearised when double-digested with EcoR1 and Nde1 (lane 27). The insert was not excised from colony 4 after digestion with EcoR1 (lane 8). Thus, colonies 1, 2 and 5 were used for sequencing.

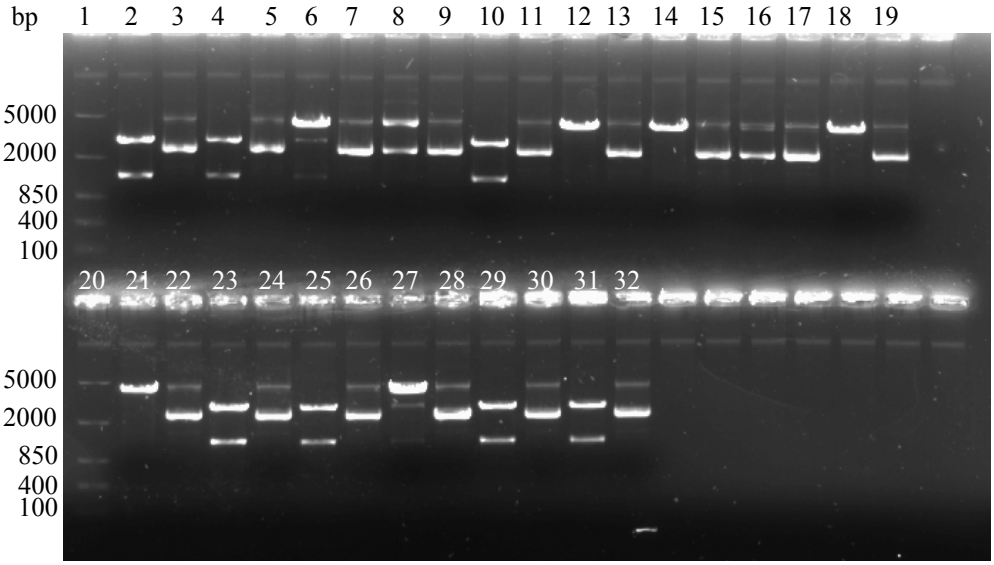

Figure 2.8: Analysis of pTZ57R/T/β-tubulin plasmid mini-prep from five recombinant *E. coli* colonies, digested by restriction enzymes EcoR1 and Nde1. Lane 1, molecular weight marker (Fermentas MiddleRange); lanes 2, 4, 6, 8 and 10, colonies 1-5 digested with EcoR1; lanes 3, 5, 7, 9, 11, undigested colonies 1-5; lanes 12, 14, 16, 18 and 21, colonies 1-5 digested with Nde1; lanes 13, 15, 17, 19 and 22, undigested colonies 1-5; lanes 23, 25, 27, 29 and 31, colonies 1-5 digested with EcoR1 and Nde1; lanes 24, 26, 28, 30 and 32, undigested colonies 1-5.

Recombinant plasmid from the five colonies, positive for the α-tubulin insert, was isolated by mini-prep for analysis by restriction digestion. Colony 3 (Fig. 2.7) did not yield a sufficient amount of plasmid DNA after mini-prep and was therefore excluded from further analysis. The remaining four colonies were digested by restriction enzymes EcoR1 and Not1 to determine the presence of the insert at the expected size and having correctly located restriction sites. The results of the restriction in Fig. 2.9 show excision of the insert with EcoR1, linearisation of the plasmid with digestion by Not1 and excision of the insert with double digestion by EcoR1 and Not1 in colonies 1-3, as expected. Colony 4 did not show excision of the insert with digestion by EcoR1 (Fig. 2.9, lane 15) and was therefore excluded from further analysis. Colonies 1-3 were used for sequencing.

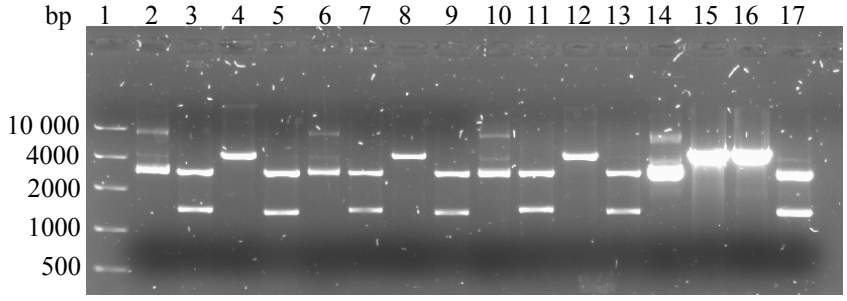

Figure 2.9: Agarose gel (0.8%) electrophoresis analysis of pTZ57R/T/α-tubulin mini-prep plasmid from recombinant *E. coli* colonies digested with EcoR1 and Not1. Lane 1, molecular weight marker (Fermentas MiddleRange); lanes 2-5, colony 1: undigested, digested with EcoR1, Not1 and EcoR1/Not1 respectively; lanes 6-9, colony 2; lanes 10-13, colony 3 and lanes 14-17, colony 4 in the same sequence as colony 1.

Plasmid from colonies 1-3 for pTZ57R/T/α-tubulin and colonies 1, 2 and 5 of pTZ57R/T/β-tubulin were sequenced to verify that the DNA sequence of each insert was free from mutations that may alter the correct expression of the protein. Analysis of the sequenced constructs showed that several point mutations were present, however no frame shift mutations were detected in the sequenced constructs.

2.3.3 Sub-cloning of α- and β-tubulin ORFs into pET28a and pPICZαA28

The α- and β-tubulin ORFs were ligated into suitable expression vectors for either yeast or bacterial expression. The bacterial expression vector pET28a and yeast expression vector pPICZαA28 were chosen for sub-cloning.

The pET28a and pPICZαA28 expression vectors were digested with EcoR1 and Not1 in preparation for ligation with the α-tubulin insert. Similarly, the α-tubulin insert was excised from the cloning vector pTZ57R/T by digestion with EcoR1 and Not1. Both vectors and insert were purified by agarose gel extraction and analysed for purity by agarose gel electrophoresis, which revealed a single band for each purified vector and the purified α-tubulin insert (Fig. 2.10).

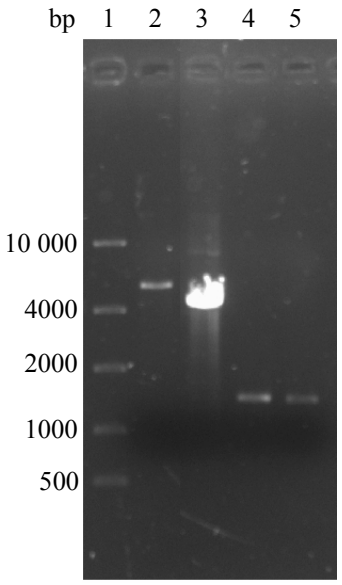

Figure 2.10: Agarose gel (0.8%) electrophoresis analysis of gel extraction of pET28a, pPICZαA28 and α-tubulin insert digested with restriction enzymes EcoR1 and Not1. Lane 1, molecular weight marker (Fermentas HighRange); lane 2, pET28a (1 µl); lane 3, pPICZαA28 (1 µl); lanes 4 and 5, α-tubulin (1 µl).

The pET28a vector was purified from culture and digested with restriction enzymes EcoR1 and Nde1 in preparation for ligation with the β-tubulin insert. The β-tubulin insert was excised from the pTZ57R/T cloning vector by double digestion with EcoR1 and Nde1. Both pET28a and insert were purified by gel extraction and analysed by agarose electrophoresis for purity and to determine the relative quantities of vector and insert needed for ligation, as shown in Fig. 2.11, showing a single pure band for both pET28a and β-tubulin insert.

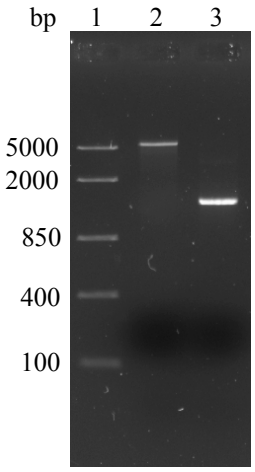

Figure 2.11: Agarose gel (0.8%) electrophoresis analysis of digestion of pET28a and β-tubulin insert with EcoR1 and Nde1. Lane 1, molecular weight marker (Fermentas MiddleRange); lane 2, pET28a (1 µl); lane 3, β-tubulin insert (1 µl).

The ligated pET28a/α-tubulin plasmid was transformed into *E. coli* JM 109 and plated onto 2X YT agar containing kanamycin. The resulting recombinant colonies were screened by colony PCR to detect the presence of the α-tubulin insert (Fig. 2.12). The results show that all eight colonies screened contained an insert at the expected size of 1300 bp for α-tubulin, indicating that the ligation was successful.

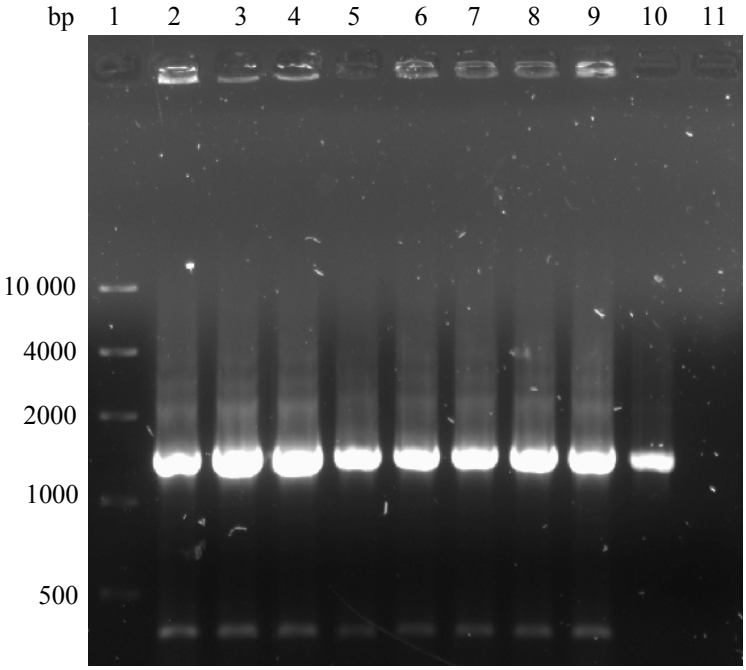

Figure 2.12: Agarose gel (0.8%) electrophoresis analysis of screening of recombinant pET28a/α-tubulin colonies after transformation into *E. coli* JM 109. Lane 1, molecular weight marker (Fermentas MiddleRange); lanes 2-9, PCR of individual colonies for pET28a/α-tubulin; lane 10, positive PCR control; lane 11, PCR control excluding template DNA (negative control).

The purified pET28a and β-tubulin insert were ligated and transformed into *E. coli* JM 109 and plated onto 2X YT agar containing kanamycin. Three colonies grew and were screened by colony PCR to detect the presence of the β-tubulin insert. The results of the PCR were analysed by agarose electrophoresis (Fig. 2.7) and showed that all three colonies were positive with a band present at the expected size of 1300 bp, indicating that the ligation was successful.

Plasmid from four of the pET28a/α-tubulin colonies screened (Fig. 2.12) were isolated by mini-prep and double digested with EcoR1 and Not1 to detect the presence of the α-tubulin insert. In Fig. 2.13 it can be seen that digestion with EcoR1 and Not1 produces a band at approximately 5500 bp for each colony screened, which corresponds to the expected size of 5369 bp for the pET28a vector, and a band at 1300 bp for each colony screened, confirming the presence of the α-tubulin insert.

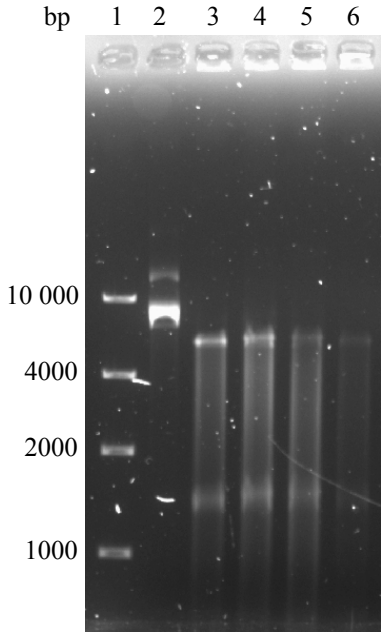

Figure 2.13: Agarose gel (0.8%) electrophoresis analysis of pET28a/α-tubulin digested with EcoR1 and Not1 to detect α-tubulin insert. Lane 1, molecular weight marker (Fermentas MiddleRange); lane 2 pET28a/α-tubulin undigested; lanes 3-6, pET28a/α-tubulin double digested with EcoR1 and Not1.

In order to assess the presence of the β-tubulin insert with the correct restriction sites, plasmid from two colonies positive for the insert were digested with restriction enzymes EcoR1 and Nde1 and analysed by agarose gel electrophoresis (Fig. 2.14). pET28a/β-tubulin from both colonies was linearised by digestion with EcoR1 and Nde1 and the β-tubulin insert excised by double digestion with EcoR1 and Nde1, as indicated by a band present at 1300 bp in lanes 5 and 9 in Fig. 2.14.

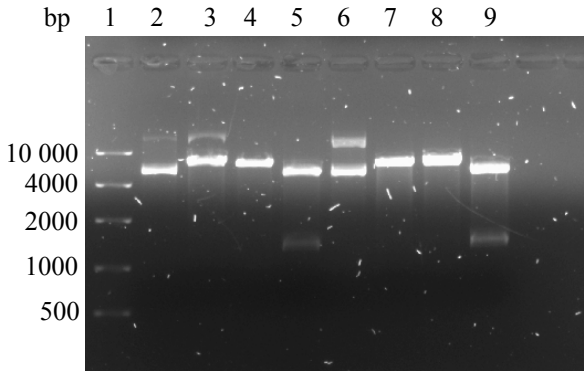

Figure 2.14: Agarose gel (0.8%) electrophoresis analysis of pET28a/β-tubulin digested with EcoR1 and Nde1 to detect β-tubulin insert. Lane 1, molecular weight marker (Fermentas HighRange); lanes 2-5, colony 1: undigested, digested with EcoR1, Nde1 and EcoR1/Nde1 respectively; lanes 6-9, colony 2: undigested, digested with EcoR1, Nde1 and EcoR1/Nde1 respectively.

The ligated pPICZαA28/α-tubulin plasmid was transformed into *E. coli* JM 109 plated onto 2X YT agar containing Zeocin™ and the resulting colonies screened by colony PCR to detect recombinants. Recombinant colony plasmid was isolated by mini-prep and linearised by digestion with restriction enzyme Pme1. The linearized plasmid was purified by gel extraction and transformed by electroporation into yeast strain *Pichia pastoris*. Colony PCR was used to screen for recombinant colonies. Fig. 2.15 shows a faint band at the expected size of 2000 bp following amplification using vector primers AOX-Fwd and AOX-Rv.

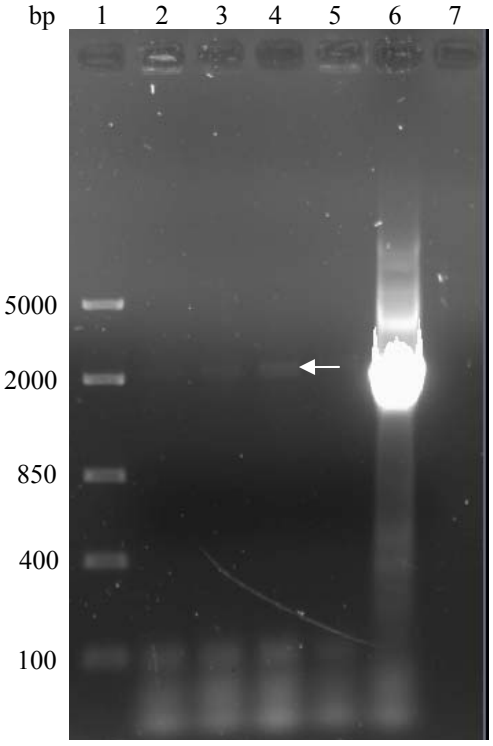

Figure 2.15: Agarose gel (0.8%) electrophoresis analysis for screening of recombinant pPICZαA28/α-tubulin colonies after transformation into *P. pastoris* (strain X33). Lane 1, molecular weight marker (Fermentas MiddleRange); lanes 2-5, PCR of individual colonies for pPICZαA28/α-tubulin; lane 6, positive PCR control; lane 7, control excluding template DNA (negative control).

2.3.4 Expression of recombinant α- and β-tubulin in yeast

P. pastoris strain X33 was transfected with pPICZαA28/α-tubulin and cultured with daily addition of methanol to induce the expression of recombinant α-tubulin. After culturing, supernatant was concentrated by TPP and analysed for the presence of α-tubulin expression by SDS-PAGE and western blot (Fig. 2.16). The results indicated that no expression occurred in yeast, as no bands in the expected range of 55 kDa for tubulin were visible. No bands were detected in the supernatant sample when a western blot was probed with anti-α-tubulin antibodies. The anti-α-tubulin monoclonal antibodies were produced against *T. brucei* cytoskeleton, are of antibody subclass IgG2b and are specific to α-tubulin. This was

compared to a control consisting of *T. congolense* cell lysate, which when probed, produced a band at 55 kDa corresponding to the expected size for tubulin (Fig. 2.16).

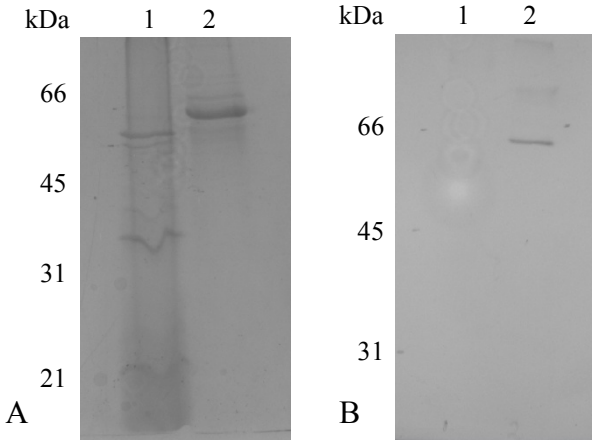

Figure 2.16: Analysis of expression of pPICZαA28/α-tubulin in *P. pastoris* X33 by SDS-PAGE and western blot. Panel A: SDS-PAGE (10%) gel**.** Lane 1, yeast culture supernatant (40% TPP, 5 µl); lane 2, *T. congolense* cell lysate (10 µl containing 1 x10^6 cells). Proteins stained with Coomassie R-250. Panel B: western blot. Lane 1, yeast culture supernatant (40% TPP, 5 µl); lane 2, *T. congolense* cell lysate (10 µl containing 1 x10^6 cells). Electroblotted proteins were incubated with mouse anti-α-tubulin monoclonal antibody (1:500 dilution) followed by goat anti-mouse IgG HRPO-conjugate (1:3000) The reaction was developed using 4-chloro-1-naphthol and H_2O_2.

2.3.5 Bacterial expression of α- and β-tubulin

The pET28a/α-tubulin and pET28a/β-tubulin constructs were transformed into the expression host, *E. coli* BL21 (DE3). After overnight expression of pET28a/α- and β-tubulin in Terrific broth in the presence of kanamycin, cells were lysed and the lysate separated into soluble and insoluble fractions to determine in which fraction the recombinant tubulins were present. As shown in Fig. 2.17, both α- and β-tubulin fusion proteins with a 6xHis-tag are expressed as insoluble inclusion bodies with a size determined as 55 kDa on reducing SDS-PAGE.

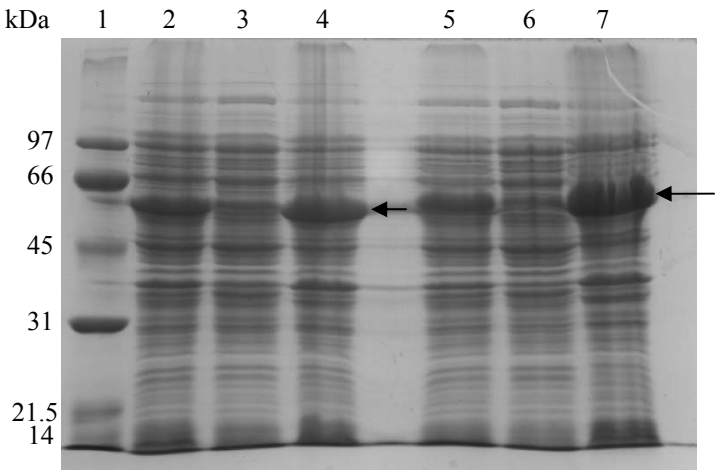

Figure 2.17: SDS-PAGE gel (10%) analysis of recombinant α- and β-tubulin expression in *E. coli* host BL21 (DE3). Lane 1, BioRad low molecular weight marker; lane 2, pET28a/α-tubulin total lysate; lane 3, pET28a/α-tubulin soluble fraction; lane 4, pET28a/α-tubulin insoluble fraction; lane 5, pET28a/β-tubulin total lysate; lane 6, pET28a/β-tubulin soluble fraction; lane 7, pET28a/β-tubulin insoluble fraction. Proteins stained with Coomassie blue R-250. Arrows indicate the position of α- and β-tubulin at 55 kDa.

To confirm the presence of a His-tag on the recombinant α- and β- tubulin proteins, both proteins were electro-blotted onto nitrocellulose and probed with antibody to the 6xHis-tag in a western blot (Fig. 2.18), which resulted in development of bands at the expected size of 55 kDa, thereby indicating the presence of the His-tag.

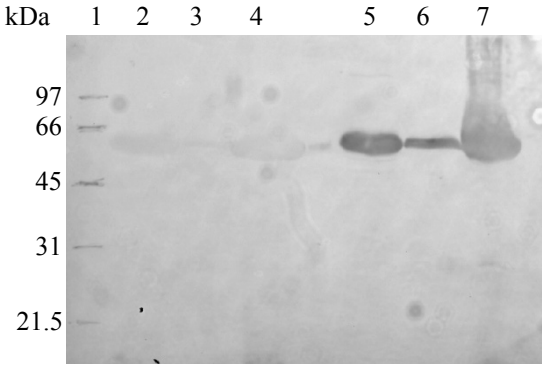

Figure 2.18: Western blot analysis to determine the recognition of the His-tag in recombinant pET28a/α- and β-tubulin. Lane 1, BioRad low molecular weight markers (5 µl), lane 2, α-tubulin total lysate (15 µl); lane 3, α-tubulin soluble fraction (15 µl); lane 4, α-tubulin insoluble fraction; lane 5, β-tubulin total lysate (15 µl); lane 6, β-tubulin soluble fraction (15 µl); lane 7, β-tubulin insoluble fraction (15 µl). Proteins were electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Transferred proteins were incubated with anti-His monoclonal antibody (1:1000 dilution) and secondary antibody goat anti-mouse IgG HRPO-conjugate (Sigma) (1:3000 dilution). The reaction was developed using 4-chloro-1-naphthol/H_2O_2 .

Due to the fact that both α- and β-tubulin have the same molecular weight as observed on SDS-PAGE, both α- and β-tubulin were probed with monoclonal anti-α-tubulin and anti-β-tubulin antibodies, in order to confirm the identity of both proteins and to detect cross-reactivity. The anti-β-tubulin monoclonal antibodies were produced against *Physarum polycephalum* amoebal tubulin, are of antibody subclass IgG2b and are specific to β-tubulin. As seen in Fig. 2.19, both α- and β-tubulin are detected by their respective antibodies and no cross-reactivity between α- and β-tubulin was observed.

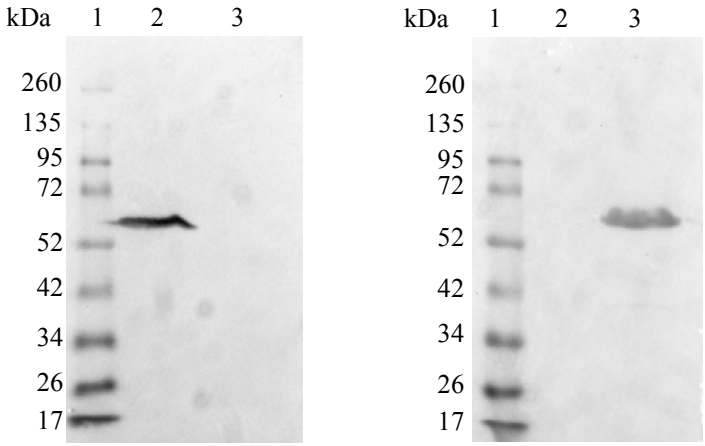

Figure 2.19: Western blot analysis of α- and β-tubulin with anti-α-tubulin and anti-β-tubulin monoclonal antibodies. Panel A: Blot incubated with anti-α-tubulin monoclonal antibody (dilution 1:500). Lane 1, Spectra™ Multicolor Broad Range Protein Ladder (Fermentas); lane 2, pET28a/α-tubulin total lysate (15 µl); lane 3, pET28a/β-tubulin total lysate (15 µl). Panel B: Blot incubated with anti-β-tubulin monoclonal antibody (dilution 1:250). Lane 1, Spectra Multicolor Broad Range Protein Ladder (Fermentas); lane 2, pET28a/α-tubulin total lysate (15 µl); lane 3, pET28a/β-tubulin total lysate (15 µl). Proteins were electrophoresed on a 10% SDS-PAGE gel and electroblotted onto a nitrocellulose membrane. Secondary antibody was goat anti-mouse IgG HRPO-conjugate (Sigma) (1:3000 dilution). The reaction was developed using 4-chloro-1-naphthol/H_2O_2.

2.4 DISCUSSION

The lack of suitable chemotherapeutic drugs against animal African trypanosomiasis, as well as vector control methods that are unsustainable over the vast area of sub-Saharan Africa where the tsetse fly occurs, have created the need for an effective vaccine against this disease. Trypanosome tubulin proteins have been identified as potential candidates for a conventional vaccine against trypanosomiasis, due to their invariant nature, and suitable sequence distance from mammalian tubulins (Lubega *et al*., 2002a and b; Li *et al*., 2007). Complete and partial protection against infection with *T. brucei*, *T. congolense* and *T. b. rhodesiense* was observed in mice after immunisation with renatured native tubulin, containing both α- and β-tubulin, isolated from *T. brucei* (Lubega *et al*., 2002a). A high

level of protection against infection with *T. evansi*, *T. equiperdum* and *T. b. brucei* was observed in mice that had been immunised with recombinant β-tubulin from *T. evansi*. Therefore, recombinant α- and β-tubulin from *T. congolense* was expressed in order to be tested as a vaccine in animals to protect against *T. congolense* infection. The rationale for expressing both α- and β-tubulin lies in the observation that protection was achieved using native tubulin which was a mixture of α- and β-tubulin, and it was hence not possible to distinguish which of these proteins conferred the immuno-protection, or whether both were involved (Lubega *et al*., 2002a).

Genomic DNA from *T. congolense* (strain IL 1180) was successfully isolated by phenol: chloroform extraction, and used as template DNA for the amplification of the α- and β-tubulin ORFs by PCR. Genomic DNA was used as, uniquely amongst eukaryotes, the trypanosome genome seems to contain no introns (Perelman and Boothroyd, 1990). Introns are non-coding stretches of DNA situated throughout the genomes of most eukaryotic species, and may be located within the transcribed region (exons) of genes. For this reason, in species containing introns the gene of interest must be amplified from transcribed mRNA by reverse transcriptase PCR (RT-PCR).

The purified α- and β-tubulin amplicons were ligated into the T-vector, pTZ57R/T using T4 DNA ligase. The pTZ57R/T vector is purchased from the manufacturer (Fermentas) as a linearised plasmid containing 3'-ddT overhangs. This prevents the plasmid from re-circularising and allows for the ligation of an insert containing 3'-dA overhangs, which are frequently added to PCR products by *Taq* DNA polymerase (Fermentas InsTAclone PCR Cloning Kit, www.fermentas.com). The ligated pTZ57R/T/α- and β-tubulin constructs were transformed into *E. coli* JM 109 and screened by colony PCR, which indicated that the ligation was successful due to the presence of an insert at 1300 bp for both α- and β-tubulin.

Restriction sites were engineered into the designed primer to amplify the α- and β-tubulin ORFs and allow for their excision from the T-vector in order for sub-cloning of the α- and β-tubulin inserts into the expression vectors pET28a and pPICZαA28. Restriction digestion

of the construct also confirms the identity of the insert contained therein and the correct restriction site sequence. Constructs were digested with restriction enzymes and analysed by agarose electrophoresis to identify those having the correctly placed restriction sites of Nde1, EcoR1 and Not1. Those constructs for which the correct restriction sites were confirmed were sequenced to verify the identity of the insert and to rule out the possibility of frame shift mutations in the sequence added by the *Taq* polymerase during the elongation step of the PCR reaction.

Frame shift mutation free inserts were excised from the T-vector and ligated into the expression vectors pPICZαA28 and pET28a. Both α- and β-tubulin inserts were successfully ligated into the pET28a vector, however only α-tubulin was successfully ligated into the pPICZαA28 vector. The reason for the failure of the β-tubulin insert to ligate into pPICZαA28 after several attempts may be due to several possibilities. The possibility that the insert was not correctly processed can be ruled out, as the β-tubulin insert, restricted with EcoR1 and Nde1 and used for ligation into the pPICZαA28 vector was successfully cloned into the pET28a vector using the same restriction sites. The pPICZαA28 vector was similarly restricted with EcoR1 and Nde1, and analysed by agarose electrophoresis, which revealed a linearised product as compared to the uncut vector. Colonies were observed after transformation of *E. coli* with the ligation mixture, however upon colony PCR screening, no PCR product was detected. The problem could therefore lie with the restriction of the vector, as the same vector when linearised with EcoR1 and Not1, was able to ligate with the α-tubulin insert. It may be possible that either the Nde1 site was not correctly restricted, or that it may not have been present; therefore pPICZαA28 would only have been restricted with EcoR1. Re-circularisation of the vector may therefore have occurred during the ligation reaction instead of ligation of the vector with the insert. This would have allowed colonies to form which are resistant to Zeocin™ but do not contain an insert.

The pPICZαA28/α-tubulin construct was linearised with Pme1 and transfected into *P. pastoris* strain X33 for expression. Expression in this system is induced by methanol, which

activates the AOX1 promoter and allows for gene expression. Expression of α-tubulin, however, was not successful, as no recombinant protein was identified on SDS-PAGE or by western blot. The presence of the construct in the *P. pastoris* genome was verified by colony PCR, which produced a PCR product of 2000 bp, corresponding to the AOX1 gene. The lack of expression of α-tubulin may be due to the possibility that growing/culture conditions may not have been optimal for the expression of recombinant α-tubulin. Linder *et al*. (1997) reported that *P. pastoris* required cotransformation with both α2- and β2-tubulin isoforms of *Reticulomyxa filosa* (freshwater amoeba) in order for detectable levels of expression of α2-tubulin to occur; while in *P. pastoris* cells transformed with only a single construct containing the α2-tubulin gene expression, levels were undetectable. Linder *et al*. (1997) also observed that as the *R. filosa* tubulins increased in level, a corresponding decrease in yeast tubulins was also observed. This observation led them to put forward that the *R. filosa* tubulins could be substituted for the yeast tubulins within the cell. Due to the high level of conservation of tubulins found to exist amongst eukaryotes, this is plausible (Erickson, 2007). It may be possible that similar regulation of tubulin expression occurred in *P. pastoris* transformed with *T. congolense* α-tubulin. It may also be possible that cotransformation of *P. pastoris* with both *T. congolense* α- and β-tubulin is necessary for expression in *P. pastoris* to occur.

In the present study, both α- and β-tubulin were successfully expressed using the pET28a expression system and *E. coli* host strain BL21 (DE3). The pET28 expression system regulates expression of the target gene by the bacteriophage T7 promoter located upstream of the target gene. The bacteriophage T7 RNA polymerase, present in the host cell *E. coli*, binds to the T7 promoter and allows for transcription of the target gene. Expression can be regulated by the addition of the lactose analog isopropyl β-D thiogalactoside (IPTG) to the media which induces the expression of the T7 RNA polymerase (pET system manual, Novagen).

Leaky expression was found for both pET28a/α- and β-tubulin constructs in the absence of IPTG in the *E. coli* BL21 (DE3) host. High levels of protein were obtainable without

induction, therefore expression was carried out overnight in Terrific broth without induction by IPTG, resulting in high yields of recombinant protein. The leaky expression observed can be explained by the observation that leaky expression occurs in pET system plasmids when using host strains containing DE3 lysogens (pET system manual, Novagen).

In the pET28a system, proteins are expressed as fusion proteins with a 6xHis-tag allowing for purification by nickel chelate chromatography. In order to confirm the presence of the His-tag on the expressed protein, both recombinant α- and β-tubulin were probed with anti-His-tag monoclonal antibodies by western blot. The results of the blot revealed the presence of a His-tag for both α- and β-tubulin, however a far stronger signal was observed in the case of β-tubulin compared to α-tubulin. The reason for the difference in signal intensity is not known, because although α- and β-tubulin share only 40% amino acid sequence identity, they are structurally the same (Nogales *et al*., 1998) (Chapter 1, Fig. 5) (see Appendix C). The protein used for western blot analysis however, is denatured and would not have the native structure, therefore it may be possible that the denatured α-tubulin may have assumed a non-native structure wherein the His-tag is not as fully displayed as on β-tubulin. This may have affected binding ability of the anti-His-tag antibody.

As further confirmation of the identity of the recombinant proteins, both α- and β-tubulin were probed with anti-α-tubulin (TAT-1) and anti-β-tubulin (KMX-1) monoclonal antibodies, kind gifts of Prof. Keith Gull (University of Oxford, UK). The results indicated that both α- and β-tubulin were recognised by their respective antibodies, and furthermore, no cross-reactivity was observed. Because both α- and β-tubulin have the same molecular mass, antibodies specific to each of these proteins are useful for their identification.

Both α- and β-tubulin have been successfully expressed as fusion proteins with a 6xHis-tag using the pET28a bacterial expression system, and *E. coli* host BL21 (DE3). The proteins are expressed as insoluble inclusion bodies, which will require purification, solubilisation and refolding prior to the investigation of the immuno-protection potential of α- and β-tubulin from *T. congolense* in mice.

CHAPTER THREE

PURIFICATION OF RECOMBINANT α- AND β-TUBULIN FROM TRYPANOSOMA CONGOLENSE AND ASSESSMENT AS VACCINE CANDIDATES IN MICE

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ABSTRACT

Trypanosomes possess a unique cytoskeleton composed primarily of microtubules. Microtubules themselves consist of heterodimers of α- and β-tubulin. Trypanosome tubulin proteins and other cytoskeletal components have been identified as antigens capable of eliciting a protective immune response against African trypanosomiasis. The following study presents the successful purification and refolding of recombinant 55 kDa α- and β-tubulin and native tubulin from *Trypanosoma congolense*, and their assessment as vaccine candidates in an immunisation and challenge experiment in mice. Recombinant α- and β-tubulin were expressed as fusion proteins with a histidine tag in a bacterial expression system and purified to a greater than 1 mg/ml concentration by nickel chelate chromatography. Refolding of the recombinant proteins was attempted via dilution of the protein in a refolding buffer followed by reconcentration of the refolded protein; however this method was not successful. A second refolding method where the proteins were refolded while bound to a nickel chelate resin via application of a decreasing gradient of urea was more successful in refolding both recombinant proteins. Mice were immunised separately with recombinant α- and β-tubulin, native tubulin or an irrelevant protein VP4AA, expressed in the same manner as the tubulins. Antibodies were produced against each protein as shown by ELISA. All mice developed parasitaemia after challenge with *T. congolense* (strain IL1180), with only the β-tubulin group recording no deaths during the monitoring period as compared to 60% survival in the α-tubulin, VP4AA and PBS

immunised groups, and 0% survival in the native tubulin group. The results obtained are consistent with the observation that β-tubulin has immuno-protective potential against trypanosomiasis.

3.1 INTRODUCTION

African trypanosomiasis is a devastating disease caused by parasitic kinetoplastid protozoans known as trypanosomes. African trypanosomes are transmitted to humans and animals, by the biting tsetse fly, which is endemic to vast regions of sub-Saharan Africa. African trypanosomes are free-living in the bloodstream of the mammalian host. In ruminant livestock, of which cattle are the most important, a severe wasting disease results. Symptoms include anaemia and weight loss, leading to decreased productivity of the animal and may eventually lead to death unless treatment is given (Lalmanach, *et al*., 2002). Although direct and indirect losses to the continent estimated at US$ 4.5 billion annually are due to trypanosomiasis, and coupled with the severe health effects of the disease on both humans and animals, only a handful of drugs are available for treatment and a vaccine has yet to be developed (Kristjanson *et al.*, 1999; Antoine-Moussiaux *et al.*, 2009). The underlying reason for the difficulty in vaccine development for African trypanosomiasis lies in the antigenic variation intrinsic to the outer protein coat of the parasite, consisting of variable surface glycoprotein (VSG). With over 1000 genes for VSG present in the genome of African trypanosomes, and the ability of the parasite to switch between any one of these genes, a vaccine targeting VSG is not viable (Donelson *et al.*, 1998; Taylor and Rudenko, 2006). For this reason, efforts aimed at a vaccine have been geared towards the identification of invariant antigens that could serve as vaccine candidates for African trypanosomiasis.

Two avenues currently being investigated for African trypanosomiasis vaccine development are conventional (anti-parasite) and anti-disease vaccines. The conventional method of vaccine development has been aimed at preventing the parasite from establishing infection in the host (Lalmanach *et al*., 2002). The aim of an anti-disease vaccine is amelioration of the pathogenic factors of the parasite without removing it entirely from the

host (Playfair *et al*., 1991; Authié *et al*., 2001). Anti-disease vaccine candidates have included trypanosome cysteine peptidases, which have been found to play a role in the pathogenesis of the disease (Authié *et al*., 2001; Lalmanach *et al*., 2002). Serine peptidases of trypanosomatids, such as oligopeptidase B, have also been identified as anti-disease vaccine candidates as well as targets for drug therapy, due to their pathogenicity to the host during infection (Morty *et al.*, 2001; de Matos Guedes *et al.*, 2007).

In recent years, the components of the trypanosome cytoskeleton have been identified as potential vaccine candidates, due to their abundance within the cell and invariant nature (Rasooly and Balaban, 2004). The trypanosome cytoskeleton is composed largely of microtubules, which form the subpellicular corset, and are also present in the flagellum. Microtubules are themselves comprised of heterodimers of the structural proteins, α- and β-tubulin, and stabilised by microtubule associated proteins (MAPs) (Kohl and Gull, 1998). MAPs have been found to be immuno-protective, as indicated by studies involving native and recombinant MAP p15 and native MAP p52, which resulted in high levels of protection against trypansome infection in mice (Rasooly and Balaban., 2004). The immuno-protection potential of trypanosome tubulins have also been investigated as anti-parasite vaccine candidates, as studies showed that protozoan tubulin has differing drug susceptibility compared to mammalian tubulin (Werbovetz *et al.*, 1999), which prompted investigation into its immunotherapeutic ability. Lubega *et al*. (2002a) found that mice were significantly protected from infection with *T. brucei*, *T. congolense* and *T. b. rhodesiense*, when immunised with native renatured tubulin from *T. brucei,* while denatured tubulin showed no protection. Serum from a rabbit immunised with renatured native *T. brucei* tubulin was also found to inhibit the growth and cause immunoagglutination of trypanosomes *in vitro* (Lubega *et al*., 2002b). In a related study by Li *et al*. (2007), recombinantly expressed β-tubulin from *T. evansi* was found to provide immuno-protection to mice against infection when challenged with *T. evansi* (83.3%), *T. equiperdum* (70%) and *T. b. brucei* (76.6%).

Due to the previous success of both native tubulin and recombinant β-tubulin in providing immuno-protection against trypanosome infection, it was decided to investigate the immuno-protection potential of recombinant α- and β-tubulin, as well as native tubulin from the more economically important trypanosome species, *T. congolense*, against challenge with *T. congolense* in mice, in an attempt to confirm previous research. Vaccine challenge experiments involving parasites are frequently performed using mice, as these animals are less costly and easier to maintain than larger mammals (Ahmed *et al.*, 2003; Foote *et al.*, 2005; Radwanska *et al.*, 2008). Typically, immunisation schedules involve multiple immunisations of the antigen with a suitable adjuvant. The amount of antigen and adjuvant administered varies. Three booster immunisations in total seem to be commonly used in mice, as performed by Ahmed *et al*. (2003). The presence of an adjuvant prevents the antigen from dispersing too rapidly from the site of immunisation, thus allowing for a sustained release of immunogen and a greater immune response (Wilson-Welder *et al.*, 2008). The titre of antibodies produced against the immunogen following immunisation is measured by enzyme-linked immunosorbent assay (ELISA) such as in immunisation experiments by Li *et al*. (2007), which allows for the antibody levels to be assessed over the course of the immunisation schedule. Challenge of experimental animals after immunisation by infection with live parasites can be performed by direct injection, or by insect bite, as done by Mkunza *et al*. (1995) and Authié (1994). Monitoring of the presence of parasites in the host is then performed to determine the success of the potential vaccine. In an anti-disease vaccine experiment for trypanosomiasis, the length of time after infection before parasites are detected, the number of parasites in the blood, as well as anaemia, measured by packed cell volume (PCV) are monitored over the course of infection (Authié *et al*., 2001). The parasite levels, PCV and general health of the animal indicate the effectiveness of the vaccine in reducing the disease symptoms. A conventional anti-parasite vaccine, however, is considered successful if infection does not result at all after challenge with parasites.

In the present study, immunisation of mice with purified refolded recombinant and native tubulin was performed using the adjuvants alum and saponin. After verifying the

production of anti-tubulin antibodies, mice were challenged with *T. congolense* (strain IL 1180) and monitored for infection.

3.2 MATERIALS AND METHODS

3.2.1 Materials

General reagents including buffer salts, of the highest grade, were purchased from Saarchem (South Africa), Merck (Germany), Fermentas (Lithuania), Roche Diagnostics (Germany), BDH (England) and ICN Biomedicals (USA).

Specialised chemicals including Coomassie R-250, 4-chloro-1-naphthol, horse radish peroxidase (HRPO)-linked goat anti-mouse IgG and His-Select® nickel affinity gel were from Sigma. 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) and bovine serum albumin (BSA) were from Roche. Low range molecular weight markers were purchased from BioRad. Recombinant VP4AA was supplied by Phillia Vukea (University of KwaZulu-Natal) and cultured *T. congolense* procyclics (strain IL 1180) supplied by Richard Kangethe (University of KwaZulu-Natal). Centriprep® centrifugal filter devices were from Amicon, Millipore, USA, Nunc-Immuno™ 96-well plates from Nunc Intermed (Denmark) and Amicon stirred ultrafiltration cell (model 202) from Millipore, USA. ELISA plate absorbance readings were measured using a FLUOStar OPTIMA Spectrophotometer (BMG Labtech, Germany).

Ethical approval was granted by the animal ethics committee of the University of KwaZulu-Natal (UKZN) for all experimentation and procedures performed using animals at UKZN (Reference number 002/07/Animal and 025/08/Animal).

3.2.2 Bacterial inclusion body purification and solubilisation

Bacterial inclusion body purification and solubilisation were carried out according to Sijwali *et al*. (2001). Cells resuspended in lysis buffer were thawed at room temperature, sonicated (VirSonic 60, Virtis, USA) and centrifuged (12 000 *g*, 30 min, 4° C), to give the

soluble and insoluble (containing inclusion bodies) fractions of the cell lysate. The insoluble fraction was washed twice (2.0 M urea, 20 mM Tris HCl buffer pH 8.0, 2.5% Triton X-100) and centrifuged (12 000 *g*, 30 min, 4° C) between washes. The insoluble fraction was again washed twice with 20% (w/v) sucrose, 20 mM Tris HCl buffer pH 8.0, and centrifuged (12 000 *g*, 30 min, 4° C), with the final centrifugation at 17 000 g for 30 min at 4° C. The purified inclusion bodies were solubilised in solubilisation buffer containing 6 M guanidine-HCl, 20 mM Tris HCl buffer pH 8.0, 500 mM NaCl, 10 mM imidazole, at room temperature for 60 min. The solubilised inclusion bodies were centrifuged (27 000 *g*, 30 min, 4° C) to pellet any insoluble material, and immediately bound to a nickel-affinity gel (His-Select™, Sigma) for affinity purification or on-column refolding.

3.2.3 Nickel-affinity chromatography (His-Select, Sigma)

Recombinant pET28a/α- and β-tubulin from solubilised inclusion bodies (Section 3.2.2) were purified by nickel affinity chromatography, using His-Select resin (Sigma). One ml of His-Select resin was loaded into a Poly-Prep Chromatography Column (BioRad), and equilibrated with 5 column volumes of solubilisation buffer (Section 3.2.2). The column was then loaded with 5 ml of solubilised inclusion bodies and incubated for 60 min at room temperature to allow protein binding. The supernatant was drained, and the column washed sequentially with 10 column volumes of solubilisation buffer, followed by 8 M urea, 20 mM Tris-Cl buffer pH 8.0, 500 mM NaCl, and lastly 8 M urea, 20 mM Tris-Cl buffer pH 8.0, 30 mM imidazole. Bound proteins were eluted with 10 ml of elution buffer A (8 M urea, 20 mM Tris-Cl buffer pH 8.0, 0.5 M imidazole). Eluted protein samples were stored at 4° C.

3.2.4 Refolding of recombinant tubulin by dilution and concentration

The following method was derived from Sijwali *et al*. (2001), and involves dilution of the denatured protein in a refolding buffer, containing reduced and oxidised glutathione, to effect refolding of the protein, followed by re-concentration. The denatured protein purified

by affinity chromatography (Section 3.2.3) was rapidly diluted to 20 μg/ml (approximately 100 x) in ice-cold refolding buffer (100 mM Tris-Cl buffer pH 8.0, 1 mM EDTA, 20% glycerol, 1 M KCl, 1 mM reduced glutathione, 0.5 mM oxidised gluthathione) and incubated for 20 h at 4° C without stirring. The protein was concentrated by ultrafiltration using a X-M 30 membrane and low pressure, to 10% of the starting volume, where after it was diluted 10x with sterile distilled water and concentrated to between 0.5 and 1 ml. DTT was added to a final concentration of 2.5 mM, and the protein incubated for 40 min at room temperature with gentle stirring. The refolded protein was stored on ice for immediate use or at -20° C for long term storage.

3.2.5 On-column refolding of recombinant tubulin

Proteins were refolded on-column by a decreasing gradient of urea, according to the method of Jang *et al*. (2008). Inclusion bodies were purified (Section 3.2.2) and solubilised in binding buffer [6 M guanidine HCl, 20 mM Tris-Cl buffer pH 8.0, 300 mM NaCl, 5 mM imidazole, and 1 mM β-mercaptoethanol (β-ME)] for 30 min at room temperature with stirring, and centrifuged (23 700 *g*, 10 min, 4° C) to pellet insoluble material. The supernatant was further clarified by filtration through a 0.45 μm Acrodisc® syringe filter (Pall Corporation). The supernatant was loaded onto a column containing 1 ml of His-Select nickel affinity resin (Sigma) pre-equilibrated with binding buffer, and incubated for 60 min at room temperature. The column was washed with binding buffer followed by washing buffer (6 M urea, 20 mM Tris-Cl buffer pH 8.0, 300 mM NaCl, 1 mM β-ME). The protein bound on the column was refolded with a decreasing gradient of urea (6-0 M) starting with washing buffer and finishing with refolding buffer (20 mM Tris-Cl buffer pH 8.0, 300 mM NaCl, 1 mM β-ME). The column was then washed with refolding buffer and proteins eluted by addition of elution buffer B (20 mM Tris-Cl buffer pH 8.0, 300 mM NaCl, 0.5 M imidazole, and 1 mM β-ME). Proteins were analysed by SDS-PAGE (Section 3.2.8).

3.2.6 Purification and renaturation of native tubulin from *T. congolense*

3.2.6.1 Harvesting of trypanosomes

The method of Lubega *et al*. (2002) was used for purification of native tubulin from cultured *T. congolense* procyclics (strain IL 1180). A total number of 3.57 x 10^9 cultured trypanosomes were pelleted by centrifugation (3000 *g*, 10 min, 4° C), washed twice by suspension in Pipes buffer (100 mM Pipes buffer pH 6.9, 1 mM EDTA, 1 mM $MgSO_4$, 1 mM PMSF), and stored in liquid nitrogen until needed.

3.2.6.2 Purification of native tubulin

Once trypanosomes were thawed on ice, cells were disrupted with liquid nitrogen and a mortar and pestle for 30 min, followed by resuspension in Pipes buffer and sonication (3 x 2 min at 0.04 Watt output power) using a VirSonic 60 sonicator (Virtis, USA). The homogenate was centrifuged (3 000 *g*, 10 min, 4° C) and the supernatant retained, while the pellet was sonicated again to disrupt the remaining cells and centrifuged as before. The homogenate fractions were pooled and centrifuged (10 000 g, 10 min, 4° C) whereafter the supernatant was centrifuged again (100 000 *g*, 1 h, 25° C) and filtered through a 0.22 µm syringe filter. The resulting supernatant was incubated in the presence of 2 µg/ml paclitaxel (Taxol) for 1 h at 37° C to promote tubulin polymerisation, and followed by centrifugation (100 000 *g*, 1 h, 25° C) to pellet the polymerised tubulin. After solubilisation of the pellet in 8 M urea for 1 h at 25° C, the solution was diluted 20x with alkaline buffer (50 mM KH_2PO_4, 0.1 mM PMSF, 1 mM EGTA, 50 mM NaCl, pH 10.7) and incubated for a further 30 min. The pH of the solution was adjusted to pH 8.0 with HCl, and concentrated to one third of the total volume by ultrafiltration using a YM10 Centriprep® centrifugal filter device (Amicon). The solution was re-diluted 3-fold with MES buffer (0.025 mM MES buffer pH 6.0, 1 mM EGTA, 0.5 mM $MgSO_4$, 1 mM GTP) and again concentrated to one third of the final volume. Dilution and concentration were repeated twice. The final volume was centrifuged (40 000 g, 2 h, 4° C) to remove any aggregated tubulin, and the supernatant containing renatured tubulin stored at -80° C until needed.

3.2.7 Protein concentration determination

The concentration of protein samples was determined using the Bicinchoninic Acid (BCA) Protein Assay (Pierce, Rockford, IL, USA) as well as by comparison to BSA samples of known concentration on SDS-PAGE (Section 3.2.8). The BCA assay involves the addition of a working reagent to a protein sample at a ratio of 20:1 in a microtitre plate. The plate is incubated for 30 min at 37° C and the absorbance read at 562 nm: a wavelength at which the BCA-protein complex absorbs highly. To determine the concentration of the protein, BSA samples of known concentration were included in the assay (125 to 2000 µg/ml). To determine the concentration of the samples, it is necessary to construct a standard curve (Fig. 3.1) using the BSA standards for each separate BCA assay, as the assay does not reach an end-point.

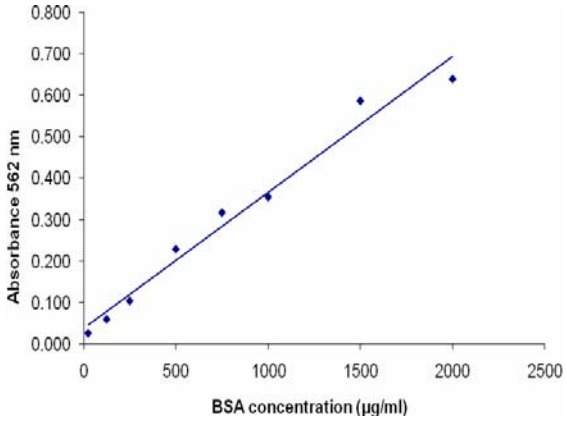

Figure 3.1: Standard curve for quantification of proteins using the BCA™ Protein Assay Kit using known concentrations of BSA. The absorbance of BSA at a range of 125 – 2000 µg/ml was measure at 562 nm. The equation of the trend line is y = 0.000x + 0.037, with a correlation coefficient of 0.975.

3.2.8 SDS-PAGE analysis of recombinant α- and β-tubulin

Proteins were analysed using 10% SDS-PAGE (Laemmli, 1970). The BioRad mini-PROTEAN 3 gel system was used and electrophoresis conducted at 18 mA per gel. BioRad low molecular weight markers were used to determine the size of protein samples by construction of a standard curve relating the log relative molecular mass (M_r) to the relative mobility of protein samples on an SDS-PAGE gel (Fig. 3.2). After completion of electrophoresis, proteins were stained with Coomassie blue R-250 or blotted onto

nitrocellulose membrane for western blots. A VersaDoc imaging system together with Quantity One analysis software (BioRad) were used for imaging of SDS-PAGE gels and western blots.

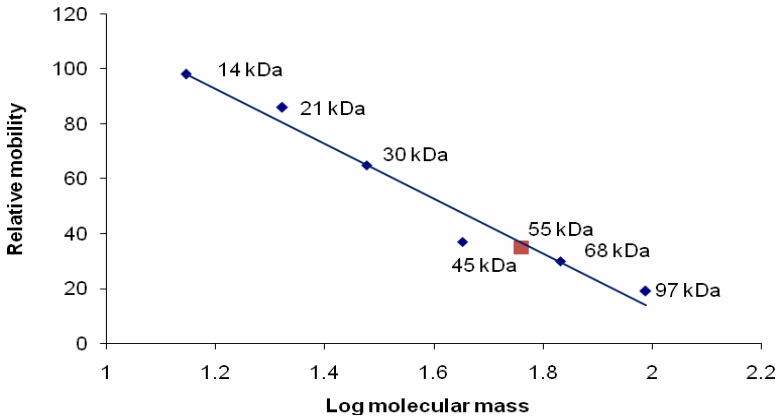

Figure 3.2: Standard curve relating the relative mobility of molecular weight marker proteins on an SDS-PAGE (10%) gel to their log relative molecular mass (Mr). The protein markers include phosphorylase B (97.4 kDa), bovine serum albumin (68 kDa), chicken egg ovalbumin (45 kDa), bovine erythrocyte carbonic anhydrase (30 kDa), soybean trypsin inhibitor (21 kDa) and chicken egg white lysozyme (14 kDa). The size of α- and β-tubulin is shown (55 kDa). The equation of the trend line was y = -99.789x + 212.5, with a correlation coefficient of 0.968.

3.2.9 Western blot to detect binding of monoclonal anti-tubulin antibodies to native tubulin

Western blotting was performed according to (Towbin *et al.*, 1979). Proteins were transferred from an unstained SDS-PAGE gel to a nitrocellulose membrane using a dry blotter apparatus (model B 2529, Sigma), at 40 mA for 1.5 h. The nitrocellulose was transiently stained with Ponceau S to reveal molecular weight marker band positions, and the bands marked with pencil. The nitrocellulose was washed with distilled water containing a drop of NaOH to remove the Ponceau S and blocked for 1 h in Tris-buffered saline (TBS) (20 mM Tris-Cl, 20 mM NaCl, pH 7.4) containing 5% (w/v) low fat milk. The nitrocellulose was washed with TBS and incubated with the primary antibody (mouse monoclonal anti-α- or anti-β-tubulin; gifts of Prof. Keith Gull, Oxford University, UK) diluted in TBS containing 5% (w/v) low fat milk, for 2 h at room temperature or overnight at 4° C. The nitrocellulose was again washed with TBS and incubated with the detection

antibody, HRPO-linked goat anti-mouse IgG, in TBS containing 5% (m/v) low fat milk for 1 h at room temperature. After a final wash with TBS, the nitrocellulose was incubated with the substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v) methanol, 0.0015% (v/v) H_2O_2 in TBS] in the dark until bands appeared on the membrane.

3.2.10 Immunisation of mice with recombinant α- and β-tubulin and challenge with *T. congolense* (strain IL 1180).

In order to asses the immuno-protective potential of recombinant α- and β-tubulin against challenge with *T. congolense*, mice were first immunised with refolded recombinant α- or β-tubulin in order to elicit the production of antibodies against these antigens.

Refolded α- and β-tubulin (Section 3.2.4) were dialysed against PBS to remove imidazole and renatured native tubulin was concentrated by ultrafiltration (Centriprep®, YM 10). Native tubulin (Section 3.2.6) was used as a control for immunisation as well as the irrelevant recombinant viral protein, VP4AA expressed in the same way as tubulin. The concentration of all antigens was determined by BCA assay (Pierce, Rockford, IL, USA) according to the manufacturer's instructions (Section 3.2.7).

A total of 25 female BALB/c mice were selected for experimentation, and were split into 5 groups of 5 mice each and immunised with either α-tubulin, β-tubulin, native tubulin, VP4AA or no antigen (replaced by PBS) along with adjuvants saponin (1 mg/ml in PBS) and alum (Brenntag) in an immunogen to adjuvant ratio of 10:1 and 1:1 respectively. Mice were injected biweekly, with 20 μg of protein per immunisation, into the intraperitoneal cavity. Blood was collected from the tail vein prior to initial immunisation and weekly thereafter. A total of 3 immunisations were given to each mouse and the serum pooled for each group.

Mice immunised with α- and β-tubulin were challenged with *T. congolense* in order to test the hypothesis that tubulin is capable of providing protection against trypanosome infection. Mice in all experimental groups were infected with 10^3 parasites per animal

contained in fresh blood from infected mice and animals monitored for the presence of parasites by microscopic examination of blood smears.

3.2.11 Enzyme-linked immunosorbent assay (ELISA) for monitoring antibody production

The antibody response of mice immunised with recombinant α- and β-tubulin as well as controls was assessed by ELISA using pooled sera from each group as the primary antibody. Microtitre plates (NUNC-Immuno™) were coated with the appropriate antigen (α-, β-tubulin, native tubulin of VP4AA) at 1 μg/ml in PBS overnight at 4° C (100 ng in 100 μl/well). Wells were blocked with 0.5% (w/v) BSA-PBS (200 μl/well for 1 h at 37° C) and washed 3 x with 0.1% (v/v) Tween-20 in PBS (PBS-Tween). Serum was diluted 1:200 in 0.5% (w/v) BSA-PBS and incubated in appropriate wells (100 μl/well for 2 h at 37° C), followed by washing 2 x with PBS-Tween and 1 x with PBS. Wells were again blocked with 0.5% (w/v) BSA-PBS (200 μl/well for 15 mins at 37° C) and washed 3 x with PBS-Tween before addition of the secondary antibody [HRPO-linked goat anti-mouse IgG (Sigma)] at a dilution of 1/20 000 in 0.5% (w/v) BSA-PBS (120 μl/well for 1 h at 37° C). The plates were washed again 2 x with PBS-Tween and 1x with PBS followed by addition of ABTS-H_2O_2 substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2] in 150 mM citrate phosphate buffer, pH 5.0 (150 μl/well for 10-15 min in the dark). The optical density of the plates was measured at A_{405} using an ELISA plate reader (FLUOStar OPTIMA, BMG Labtech).

3.3 RESULTS

3.3.1 Purification of inclusion bodies of recombinant α- and β-tubulin from pET28a.

Recombinant pET28a/α- and β-tubulin were expressed as insoluble inclusion bodies in *E. coli* host BL21 (DE3). The inclusion bodies of α- and β-tubulin contained in the insoluble fraction of the total lysate were purified by washes with 2 M urea and 20% sucrose and then solubilized with 6 M guanidine-HCl for further purification using nickel chelate chromatography (Sijwali *et al*., 2001). As shown in Fig. 3.3, minimal amounts of recombinant protein were lost during washing of the inclusion bodies.

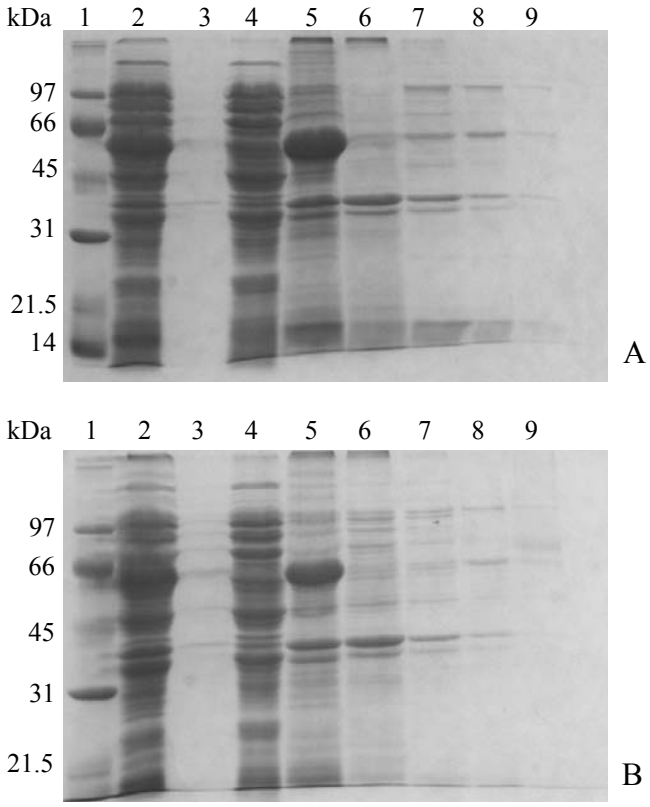

Figure 3.3: SDS-PAGE gel (10%) analysis of purification of recombinant pET28a/α- and β-tubulin expressed as inclusion bodies in E. coli BL21 (DE3). Panel A: α-tubulin. Lane 1, BioRad low molecular weight marker; lane 2, total lysate (15 µl); lane 3, cell pellet wash (15 µl); lane 4, soluble fraction of cell lysate (15 µl); lane 5, insoluble fraction of cell lysate (15 µl); lanes 6 and 7, washes of insoluble fraction with 2 M urea (15 µl); lanes 8 and 9, washes of insoluble fraction with 20% sucrose (15 µl). **Panel B: β-tubulin.** Lane 1, BioRad low molecular weight marker; lane 2, total lysate (15 µl); lane 3, cell pellet wash (15 µl); lane 4, soluble fraction of cell lysate (15 µl); lane 5, insoluble fraction of cell lysate (15 µl); lanes 6 and 7, washes of insoluble fraction with 2 M urea (15 µl); lanes 8 and 9, washes of insoluble fraction with 20% sucrose (15 µl). Proteins were stained with Coomassie R-250.

The results of the nickel affinity purification of α- and β-tubulin showed that fractions of both α- and β-tubulin were eluted with 0.5 M imidazole and contain relatively pure protein (Fig. 3.4) at a concentration of 1 – 1.5 mg/ml for both α- and β-tubulin as estimated by comparison with standard solutions of BSA and analysed on reducing SDS-PAGE (Fig. 3.5). The total yield per expression was approximately 3.6 mg for α-tubulin and 4.0 mg for β-tubulin.

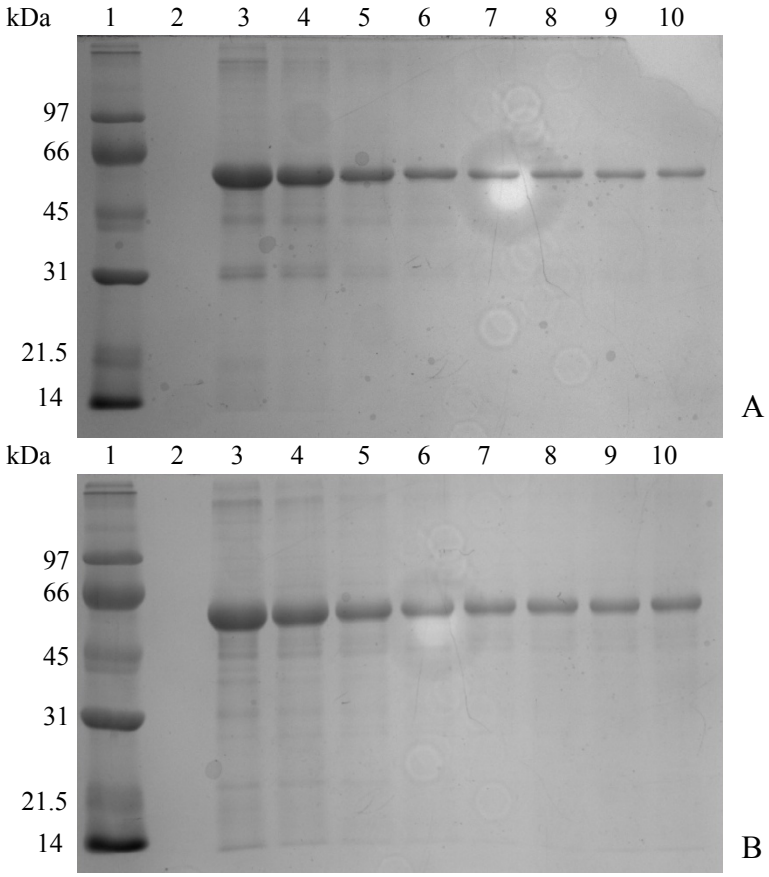

Figure 3.4: SDS-PAGE gel (10%) analysis of purification of recombinant pET28a/α- and β-tubulin by nickel-affinity chromatography using Sigma His-select resin. Panel A: α-tubulin elution fractions. Lane 1, BioRad low molecular weight markers; lane 2, column wash fraction (20 µl); lanes 3-10, elution fractions 1-8 (5 µl). **Panel B: β-tubulin elution fractions.** Lane 1, BioRad low molecular weight markers; lane 2, column wash fraction (20 µl); lanes 3-10 elution fractions 1-8 (5 µl). Proteins were stained with Coomassie R-250.

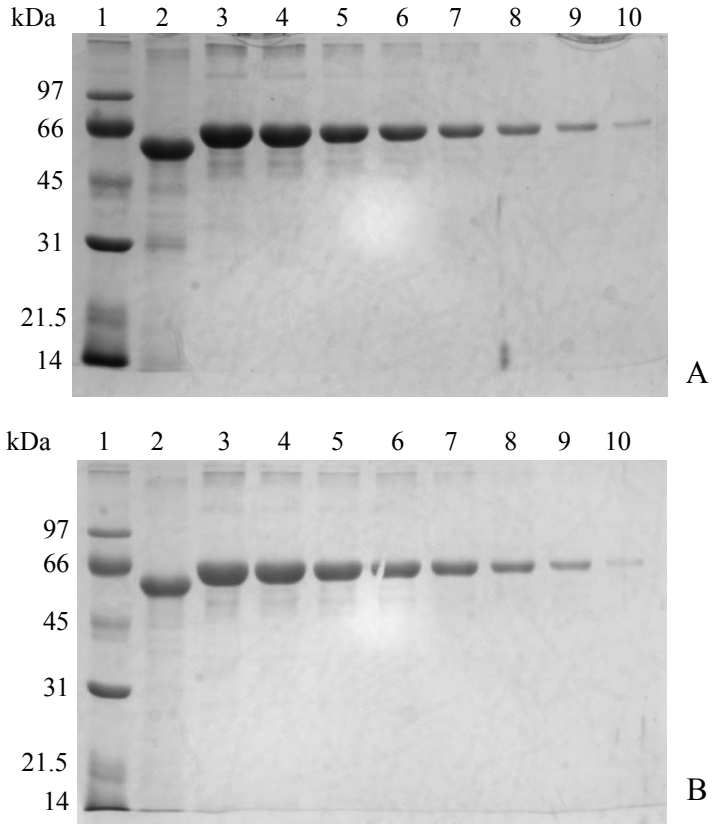

Figure 3.5: Estimation of concentration of α- and β-tubulin elution fraction 1 by comparison with standard solutions of BSA by reducing SDS-PAGE gel (10%) analysis. Panel A: α-tubulin and BSA standards. Lane 1, BioRad molecular weight markers; lane 2, α-tubulin elution fraction 1(5 µl); lanes 3-10, BSA at 2.0, 1.5, 1.0, 0.75, 0.5, 0.25, 0.125, 0.025 mg/ml (5 µl). **Panel B: β-tubulin and BSA standards.** Lane 1, BioRad molecular weight markers; lane 2, β-tubulin elution fraction 1(5 µl); lanes 3-10, BSA at 2.0, 1.5, 1.0, 0.75, 0.5, 0.25, 0.125, 0.025 mg/ml (5 µl). Proteins were stained with Coomassie R-250.

Purified α- and β-tubulin were run side-by-side to determine the size of each protein relative to the other (Fig. 3.6). Both appear to be the same size of 55 kDa determined by standard curve and compare favourably with the predicted sizes of 49.8 and 49.7 kDa for α- and β-tubulin respectively.

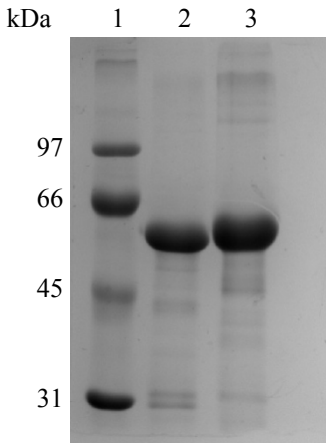

Figure 3.6: Comparison of recombinant pET28a/α- and β-tubulin by SDS-PAGE (10%) gel to determine their relative sizes. Lane 1, BioRad low molecular weight markers; lane 2, nickel affinity purified recombinant α-tubulin (5 µl); lane 3, nickel affinity chromatography purified recombinant β-tubulin (5 µl). Proteins were stained with Coomassie R-250.

3.3.2 Refolding of recombinant α- and β-tubulin

3.3.2.1 Refolding buffer method

Two methods were employed in attempting to refold recombinant α- and β-tubulin. The first method involved diluting the purified denatured protein 500x with a refolding buffer to approximately 20 µg/ml and then reconcentrating the protein after a 20 h incubation at 4° C (Sijwali *et al*., 2001). Due to the small size of the His-tag on α- and β-tubulin it was deemed unnecessary for its removal prior to refolding, as it is unlikely to interfere with refolding of the proteins. As can be seen in Fig. 3.7, the yields of refolded tubulin (lanes 5, 7 and 9) are lower than the starting concentration for both α-and β-tubulin. The refolded samples of α-tubulin produced aggregation in the form of high molecular weight structures seen at the top of the running gel (lanes 5, 7 and 9), which were also present in the non-refolded samples that were not reduced (lanes 6 and 8). Bands at 55 kDa were present in the reduced samples (lanes 4-5), as well as the non-reduced samples (lanes 6-9), however, bands were much smaller in the non-reduced samples. Boiling of samples did not reduce aggregation as compared to non-boiled samples (comparison of lanes 6 and 8 and of lanes 7 and 9).

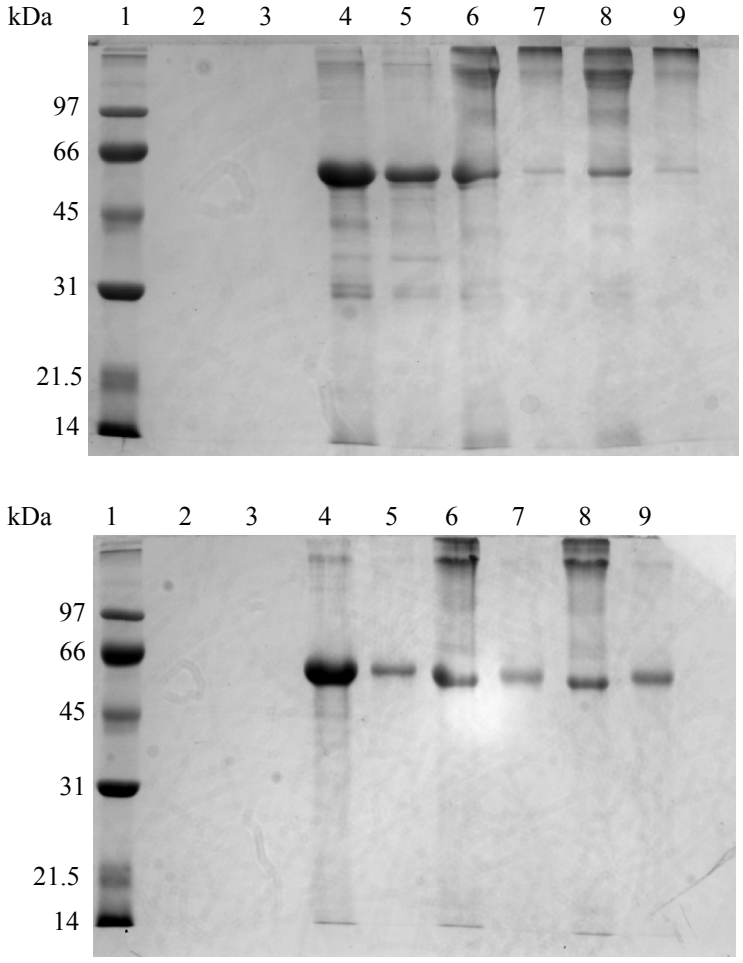

Figure 3.7: SDS-PAGE gel (10%) analysis of refolding of recombinant α- and β-tubulin by dilution and re-concentration in refolding buffer containing 1:0.5 mM reduced:oxidised glutathione. Panel A: analysis of α-tubulin refolding. Lane 1, BioRad low molecular weight markers; lanes 2-3, first and second filtrate (20 µl); lane 4, non-refolded, reduced and boiled (5 µl); lane 5, refolded, reduced and boiled (5 µl); lane 6, non-refolded, non-reduced and boiled (5 µl); lane 7, refolded, non-reduced and boiled (5 µl); lane 8, non-refolded, non-reduced and non-boiled (5 µl); lane 9, refolded, non-reduced and non-boiled (5 µl). **Panel B: analysis of β-tubulin refolding.** Lane 1, BioRad low molecular weight markers; lanes 2-3, first and second filtrate (20 µl); lane 4, non-refolded, reduced and boiled (5 µl); lane 5, refolded, reduced and boiled (5 µl); lane 6, non-refolded, non-reduced and boiled (5 µl); lane 7, refolded, non-reduced and boiled (5 µl); lane 8, non-refolded, non-reduced and non-boiled (5 µl); lane 9, refolded, non-reduced and non-boiled (5 µl). Proteins were stained with Coomassie R-250.

3.3.2.2 On-column refolding method

The second technique employed to refold recombinant α- and β-tubulin was adapted from Jang *et al*. (2008) and involved refolding the protein by applying a decreasing gradient of

urea to the protein whilst it remains bound to the nickel affinity column. The refolded protein was then eluted from the column with 500 mM imidazole. A low yield of eluted protein was observed for both α- and β-tubulin (Fig. 3.8) as compared to the yield obtained from direct nickel chelate purification (Fig. 3.4). The yield for α-tubulin was significantly lower than that obtained for β-tubulin. Concentration of α- and β-tubulin was estimated using the BCA™ assay to be 30 and 197 μg/ml respectively. High molecular weight structures were observed near the top of the gel for non-reduced samples (also observed for non-reduced samples in the refolding buffer method – Fig. 3.7, lanes 6-9). A band was also observed in non-reduced samples at a position slightly below that of reduced samples of tubulin. This mobility shift of non-reduced samples suggests that refolding of part of the total amount of protein in each sample was achieved.

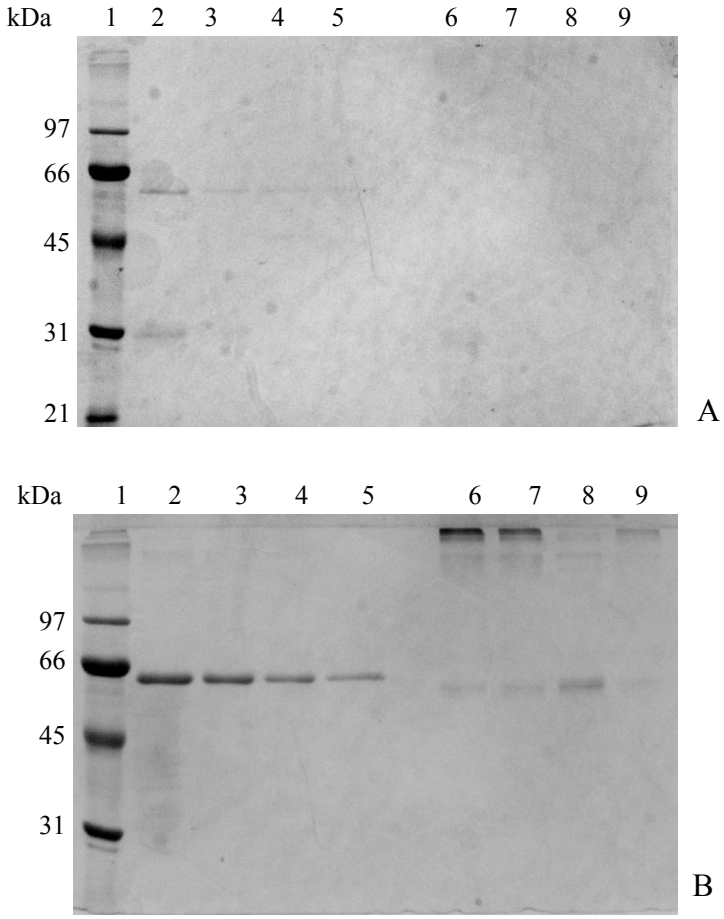

Figure 3.8: SDS-PAGE gel (10%) analysis of on-column refolding of recombinant α- and β-tubulin. Panel A: analysis of α-tubulin refolding. Lane 1, BioRad low molecular weight markers; lane 2-5, reduced elution fractions 1-4 (10 µl); lane 6-9, non-reduced elution fractions 1-4 (10 µl). **Panel B: analysis of β-tubulin refolding.** Lane 1, BioRad low molecular weight markers; lane 2-5, reduced elution fractions 1-4 (10 µl); lane 6-9, non-reduced elution fractions 1-4, (10 µl). Proteins were stained with Coomassie R-250.

3.3.3 Native microtubule renaturation

Native tubulin was purified from cultured *T. congolense* procyclics (strain IL 1180) according to the method of Lubega *et al*. (2002a) to be used as a control for immunisation along with recombinant α- and β-tubulin. A total number of 3.57 x 10^9 cells were obtained from 550 ml of culture and used for native tubulin isolation (Fig. 3.9). In the first step the soluble tubulin in the cell cytoplasm is removed by lysing the cells and removing the insoluble material (Fig. 3.9, Panel A, lane 2). The soluble tubulin monomers were then removed from the soluble cell lysate fraction by polymerisation with paclitaxel and

subsequent collection by centrifugation. The supernatant collected after centrifugation produced a band after western blot was performed indicating that a small amount of tubulin was present in this fraction, however, spill-over from lane 2 into lane 3 may have contributed to the detection of a band in this lane (Fig. 3.9, Panel B, lane 3). Microtubules were then depolymerised with 8 M urea and dialysed to renature the tubulin monomers, which were identified by western blot (Fig. 3.9, Panel B, lanes 8-10). The fractions obtained during isolation were probed with a mixture of anti-β-tubulin and anti-α-tubulin monoclonal antibodies. Although no bands were visible with Coomassie stain for any of the steps from the alkaline wash step (lanes 4-10), a band corresponding to 55 kDa was present in the western blot of the same gel (Fig. 3.9, Panel B, lanes 8-10). The final preparation shown in lane 10, representing renatured native tubulin, was concentrated to 270 µg/ml, as determined by the BCA™ assay (Section 3.2.7).

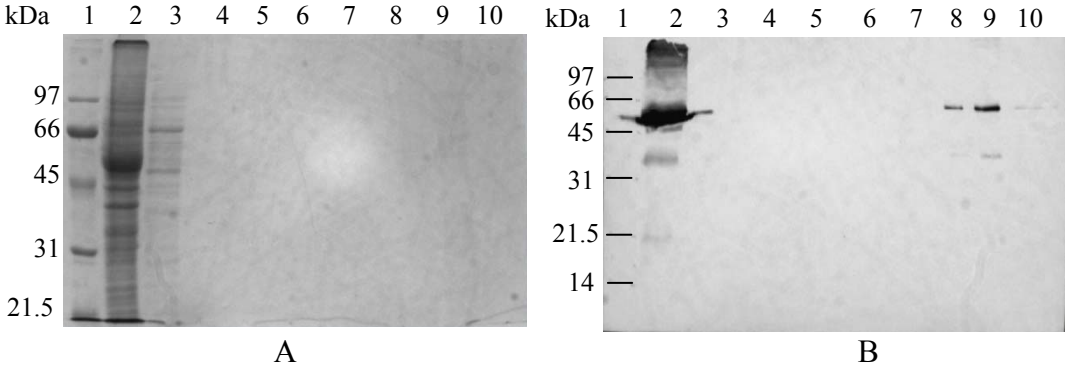

Figure 3.9: Analysis of native tubulin isolation from cultured *T. congolense* procyclics by SDS-PAGE (10%) gel and Western blot. Panel A: SDS-PAGE (10%) gel and panel B: Western blot of SDS-PAGE gel. Lane 1, BioRad low molecular weight markers; lane 2, cell lysate insoluble fraction (10 µl); lane 3, supernatant from 100 000 *g* centrifugation of soluble fraction; lane 4, alkaline wash fraction (200 µl); lanes 5-7, MES buffer filtrate fractions (200 µl); lane 8, renatured tubulin fraction containing aggregated tubulin (20 µl); lane 9, aggregated renatured tubulin fraction (20 µl); lane 10, renatured tubulin after removal of aggregated tubulin (20 µl). Proteins were stained with Coomassie R-250 in Panel A. Primary antibodies used in panel B were a mixture of anti-β-tubulin (KMX-1) and anti-α-tubulin (TAT-1) monoclonal antibodies at dilutions of (1:250) and (1:500) respectively. Secondary antibody was goat anti-mouse IgG HRPO-conjugate (Sigma) (1:3000 dilution). The reaction was developed using 4-chloro-1-naphthol/H_2O_2.

3.3.4 Immunisation of mice with recombinant α- and β-tubulin and native tubulin and challenge with *T. congolense* (strain IL1180).

Refolded α- and β-tubulin (Section 3.3.2) as well as native tubulin (Section 3.3.3) were investigated for their potential to provide immuno-protection to mice. Immunisation was carried out in 5 groups of 5 female BALB/c mice, receiving injections of either α-tubulin, β-tubulin, native tubulin, the unrelated protein, VP4AA, or PBS in the place of antigen, over 6 weeks and with a total of 3 immunisations for each of the respective antigens. An ELISA was performed in order to assess the level of antibody production in the various groups (Fig. 3.10).

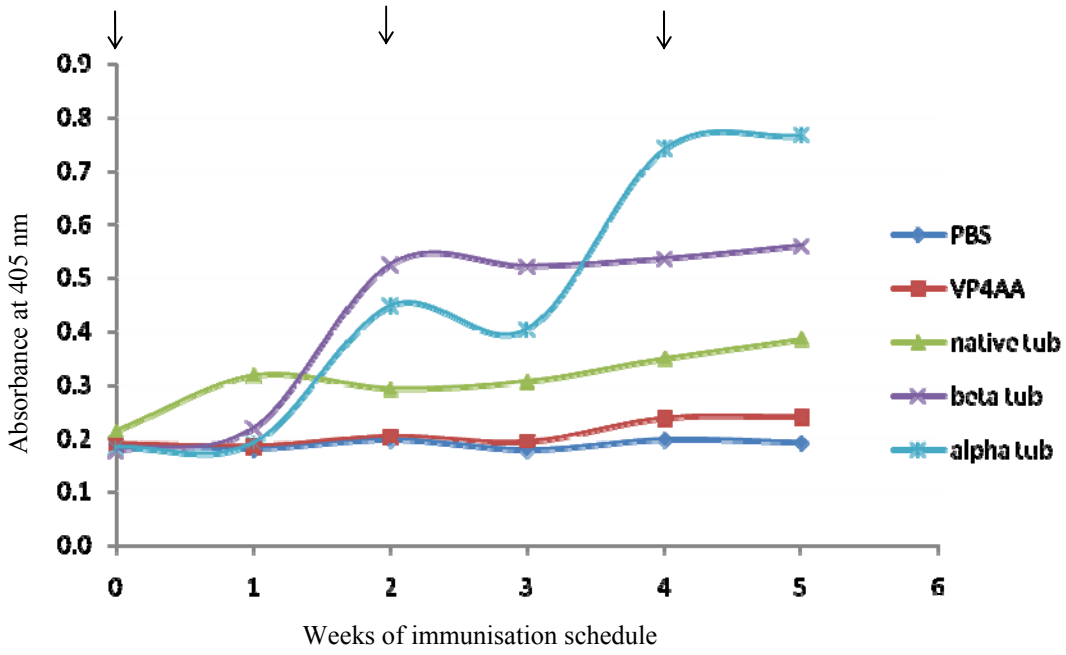

Figure 3.10: ELISA of immune response of mice immunised with α- and β-tubulin, native tubulin, VP4AA and PBS prior to challenge with T. congolense (strain IL 1180). Mice were immunised with α-tubulin (✳), β-tubulin (×), native tubulin (▲), VP4AA (■) and PBS (♦) at 20 µg per mouse per immunisation at weeks 0, 2 and 4 together with the adjuvants alum and saponin. ELISA plates were coated with α- and β-tubulin, native tubulin, VP4AA and no antigen (PBS) at 100 ng/well in the appropriate wells, and incubated with sera collected weekly and pooled for each group, at 1:200 in 0.5% (w/v) BSA-PBS (100 µ/well for 2 h at 37°C). The presence of antibodies was detected using HRPO-linked goat anti-mouse IgG at 1:20 000 in 0.5% (w/v) BSA-PBS (120 µl/well for 1 h at 37°C) and ABTS-H_2O_2 substrate solution (150 µl/well for 10-15 min at room temperature). Absorbance was measured at 405 nm, and data points were obtained by taking the average reading of duplicate wells. Arrows (↓) indicate the points where immunisations were given.

The results of the ELISA indicate that the highest titre of antibodies was produced for α-tubulin, followed by β-tubulin. Mice immunised with native tubulin produced a low but sustained response during the course of immunisation, while little immune response was observed for the VP4AA protein used as a control, as compared to the group which received PBS in place of an immunogen.

After completion of the immunisation schedule at week five, mice were infected with 10^3 parasites of *T. congolense* (strain IL 1180) from fresh blood obtained from an infected mouse and checked every two days for the presence of trypanosomes. Microscopic examination of blood smears revealed that parasites were present in all test and control animals by day 6 after challenge. The survival of mice in each group is graphed in Fig. 3.11. One mouse from the β-tubulin test group was found to be suffering from what appeared to be an ear infection, and was removed from the group prior to challenge, therefore the results of only four mice are shown for β-tubulin. As can be seen in Fig. 3.11, one mouse from the α-tubulin group died on day 14, a second mouse died on day 18, after which no further deaths occurred in this group. Three mice from the native tubulin group died on day 7, followed by a fourth mouse on day 17 and the fifth mouse by day 22. Two mice died on day 7 after challenge in the VP4AA group. One mouse from the PBS control group died on day 7 followed by another at day 11. Only the β-tubulin group had no deaths over the challenge period. Surviving mice of all groups showed sustained levels of parasitaemia up until the end of the monitoring period.

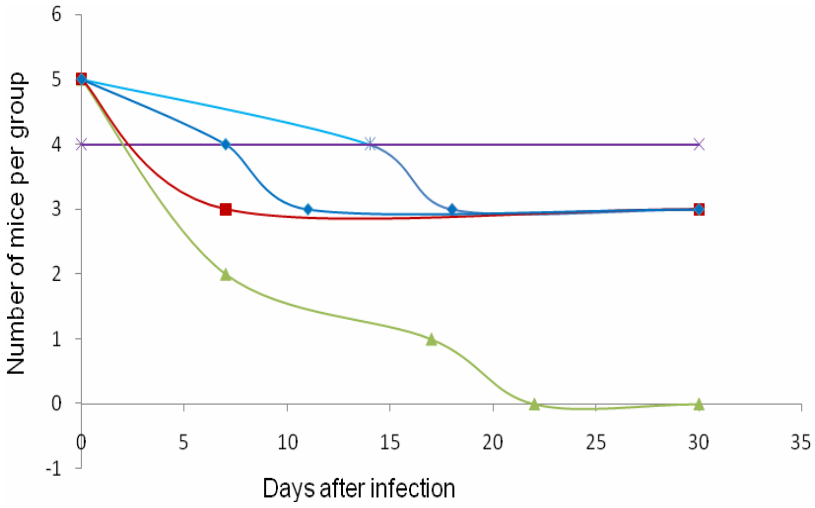

Figure 3.11: Graph of survival of mice after immunisation with recombinant α- and β-tubulin and native tubulin from T. congolense and challenge with T. congolense (strain IL1180). Mice immunised with α-tubulin (∗), β-tubulin (×), native tubulin (▲), VP4AA (■) and PBS (♦) were challenged with 10^3 parasites of *T. congolense* (strain IL1180) and survival of mice monitored over a 30 day period.

3.4 DISCUSSION

In the search for a vaccine against African trypanosomiasis, trypanosome tubulin proteins have been put forward as possible anti-parasite vaccine candidates. The observation that both native and recombinant tubulins are capable of providing efficient immuno-protection against an otherwise lethal infection in mice has supported further investigation of trypanosome tubulins as vaccine candidates (Lubega *et al*., 2002; Li *et al*., 2007).

In the present study, recombinant α- and β-tubulin were purified and solubilised according to the method of Sijwali *et al*. (2001). Recombinant proteins expressed in *E. coli* often form inclusion bodies *in vivo* that are nearly homogenously composed of the recombinant protein present as tightly packed, denatured proteins (Singh and Panda, 2005). In order for the recombinant protein in inclusion bodies to be used, it must be purified and refolded into its biologically active state, requiring complicated and lengthy procedures (Rudolph and Lilie, 1996). The one advantage of inclusion body formation is that purification of the recombinant protein is relatively simple; this is due to the observation that inclusion bodies consist almost solely of the recombinantly expressed protein and are resistant to

degradation by host cell proteases (Walsh, 2003). The purification of α- and β-tubulin by washing the insoluble fraction of the cell lysate with wash buffers containing 2 M urea and 20% sucrose in order to remove insoluble host cell proteins, was analysed by SDS-PAGE and found to be successful. A negligible amount of recombinant protein was lost during each wash, while insoluble host cell proteins were removed successfully with each wash step. Inclusion bodies were solubilised successfully using 6 M guanidine. This chaotrope completely denatures the proteins with a concomitant loss in secondary structure. Solubilised α- and β-tubulin were successfully purified by nickel chelate chromatography, to a final concentration of 1 – 1.5 mg/ml of relatively pure protein.

The first method applied in an attempt to refold recombinant α- and β-tubulin was adapted from Sijwali *et al*. (2001) and involved the dilution of the solubilised protein in a refolding buffer containing reduced and oxidised glutathione, glycerol and KCl, followed by re-concentration after a 20 h incubation period. The protein concentrated after refolding was found to have a molecular weight slightly above that of the denatured protein, which was unexpected as the refolded protein should be more compact than the denatured protein and therefore have a size shift giving the appearance of a slightly lower molecular weight compared to the denatured protein. The size shift observed may be due to the incorrect formation of the disulfide bonds in the refolded molecules, as non-native disulfide bonds have been found to occur during refolding, where there is a high pH and concentration of the protein (Singh and Panda, 2005). Aggregation in the form of high molecular weight structures was observed on non-reducing SDS-PAGE mainly for α-tubulin, but was also present in refolded β-tubulin samples. This aggregated protein can be explained as incorrectly refolded protein which formed inter-molecular disulfide bonds, and is not unexpected due to the observation that the yield of correctly refolded bioactive protein obtained from inclusion bodies is typically between 15-25% of the starting material (Singh and Panda, 2005). Difficulty in repeating the initial results obtained, the low yield of refolded tubulin and a size shift that did not comply with expectations, prompted the investigation of a different technique for protein refolding.

The on-column refolding technique was adapted from Jang *et al.* (2008), who had success with refolding of recombinant α- and β-tubulin from *Capsicum annuum* (pepper). This method relies on slow removal of the denaturant while the protein is bound to the nickel chelate resin by application of a decreasing gradient of urea. It was hoped that aggregation would be minimised as refolding would occur while the proteins were bound to the column, reducing the amount of protein-protein interaction possible as compared with refolding of proteins in solution. Refolding of β-tubulin was successful, as bands were observed in refolded samples on non-reducing SDS-PAGE that had an apparent molecular weight lower than that of the denatured protein, indicating that refolding had been successful resulting in a more compact structure. However, aggregation was still observed and may be due to incorrectly refolded molecules that aggregate upon elution from the column. A low yield of refolded protein was again observed, as compared to the yields of protein eluted during purification via nickel chelate chromatography under denaturing conditions. The lower yields were found to be due to the bound protein being washed off the column during the application of the refolding gradient, a problem not reported in the purification performed by Jang *et al.* (2008).

The yield of α-tubulin from on-column refolding was also lower than that obtained for β-tubulin, possibly due to the lower affinity of the His-tag on the α-tubulin for the nickel chelate resin as compared to that of β-tubulin. This is evident from a western blot which probed α- and β-tubulin with anti-His-tag monoclonal antibody, where α-tubulin produced a much weaker signal compared to β-tubulin, although the same amount of α- and β-tubulin was loaded onto the SDS-PAGE gel and electroblotted for the western blot. The binding of the His-tag on α-tubulin to the anti-His-tag antibody and to the nickel chelate resin was found to be weaker than for β-tubulin, resulting in a greater amount of protein being washed off the column during application of the refolding buffer gradient. The reason for the weaker binding of the α-tubulin to the His-Select affinity resin is unknown as both α- and β-tubulin were bound to the nickel affinity resin as solubilised, denatured protein. These proteins would have no or very little secondary structure due to solubilisation of the inclusion body protein with 6 M guanidine-HCl, a chaotropic agent (Singh and Panda,

2005). Thus, the His-tag could not be obscured by the conformation of the protein, as the secondary structure is absent and the hydrophobic regions of the recombinant protein are exposed.

Native tubulin was isolated from *T. congolense* procyclics grown in culture in order to provide a control for immunisation of mice with recombinant α- and β-tubulin and also to confirm previous results using native tubulin from *T. brucei* (Lubega *et al*., 2002a and b). The method of Lubega *et al*. (2002a), based on an earlier method by Stieger *et al*. (1984), was followed for the isolation of native tubulin. This method relies on the isolation of soluble tubulins in the cytoplasm, which may constitute a small fraction compared to the stable microtubules of the sub-pellicular cytoskeleton. The yield of renatured tubulin obtained from purification of native tubulin from *T. congolense* procyclics (strain IL 1180) was found to be small in comparison to the amount of tubulin present in the total lysate, as this fraction contains the cytoskeletal microtubules. Renatured native tubulin was not visible on SDS-PAGE stained with Coomassie R-250 whereas Lubega *et al*. (2002a) found renatured tubulin was visible with Coomassie staining. The differences in concentration of renatured tubulin recovered in the present study and by Lubega *et al*. (2002a) may be due to differences in the number of trypanosome cells used as starting material. Here, the starting material consisted of 3.57×10^9 *T. congolense* procyclics, whereas the number of blood stage *T. brucei* cells used by for tubulin purification by Lubega *et al*. (2002a) was not given. Despite not being visible with Coomassie staining, a band was observed for the renatured tubulin sample in a western blot probed with both anti-α-tubulin (TAT-1) and anti-β-tubulin (KMX-1) monoclonal antibodies, indicating that renatured tubulin was present. Another method has also been used to isolate native trypanosome tubulin which involves chromatographic purification of tubulin from a cell-free supernatant (MacRae and Gull, 1990). In this method, the tubulin containing fraction is concentrated and assembly/disassembly of tubulin using Mg^{2+} ions and GTP studied after the tubulin is eluted from the column.

In order to assess the immuno-protection potential of α- and β-tubulin, BALB/c mice were immunised separately with recombinant α- and β-tubulin, with control groups receiving native tubulin, VP4AA (an unrelated viral protein expressed in an identical way), or PBS mixed with saponin/alum adjuvant as both alum and saponin adjuvants are commonly used for livestock vaccines (Wilson-Welder *et al*., 2008). Sera from each group of mice were pooled and antibody production followed by ELISA prior to challenge. Antibody production was highest for α-tubulin, followed by β-tubulin and native tubulin. ELISA analysis showed that VP4AA did not produce a significant antibody response, as the antibody levels detected were as low as those observed for the PBS control group. This result was unexpected, as previous studies have shown that VP4AA is immunogenic in mice, and may be the result of error.

As an anti-parasite vaccine, both native tubulin and recombinant α- and β-tubulin from *T. congolense* (strain IL 1180) were not found to be protective against the development of parasitaemia after homologous challenge, as all mice in the different groups developed parasitaemia by day 6 after challenge. This contradicts previous research which indicated that both native tubulin (Lubega *et al*., 2002a and b) and recombinant β-tubulin (Li *et al*., 2007) could provide complete and partial immuno-protection to mice against otherwise lethal trypanosome infection, as complete and partial protection was observed in immunised mice in both of the above studies. Complete protection here refers to the absence of parasitaemia throughout the monitoring period after challenge, and partial protein refers to increased survival time of mice immunised as compared to controls following challenge. Complete and partial protection was observed in mice immunised with renatured native tubulin from *T. brucei* and challenged with *T. brucei, T. congolense* and *T. rhodesiense,* while denatured proteins had no effect. Groups of 15 mice were used for each test, where 36% showed partial and 64% total protection following *T. brucei* challenge and 27% showed partial and 73% total protection after *T. congolense* and *T. rhodesiense* challenge (Lubega *et al.*, 2002a). Surprisingly, and in contrast to the work of Lubega *et al*. (2002a), 100% of mice immunised with native tubulin from *T. congolense* in the present study succumbed to infection, whereas only 40% succumbed in the α-tubulin and other

control groups. The apparent poorer performance of native tubulin as compared to the control groups is unexpected and may be due to a pre-existing unknown factor within the group, such as illness, leading to immune system compromise.

Immunisation of BALB/c mice with recombinant β-tubulin from *T. evansi* provided immuno-protection levels of 83.3% against infection with T. *evansi*, 70% against infection with *T. equiperdum*, and 76.7% against infection with *T. brucei* in which no parasitaemia was observed over a 90 day monitoring period, where the number of animals in each test group are not given (Li *et al.*, 2007). The latent period and persistence span were also measured and it was found that mice which were immunised with recombinant β-tubulin and developed parasitaemia, did so later than unimmunised controls and had a notably longer survival period as compared to the controls (Li *et al.*, 2007). In the present study, the β-tubulin group had 100% survival at the end of the monitoring period, despite the presence of sustained parasitaemia. Partial protection against trypanosome infection using recombinant β-tubulin has been observed in mice (Li *et al.*, 2007) and also in goldfish, when immunised with recombinant β-tubulin from *T. danilewskyi*, a trypanosome species infective to fish species, and homologously challenged (Katzenback *et al.*, 2008).

The results obtained in the present study do not seem as promising as in previous studies of the immunotherapeutic potential of parasite cytoskeletal proteins. However, many findings have indicated that cytoskeletal proteins, and tubulins in particular, may be effective in immunotherapy against trypanosomiasis. In addition to the complete protection achieved in the aforementioned studies by Lubega *et al*. (2002a) and Li *et al*. (2007), immunisation of mice with a microtubule associated protein (MAP p15) resulted in complete protection against infection with *T. brucei* (Rasooly and Balaban, 2004). Over 50% of mice immunised with recombinantly expressed actin, a cytoskeletal protein from *T. evansi,* showed complete protection from lethal challenge with *T. evansi*, *T. equiperdum* and *T. b. brucei* (Li *et al.*, 2009). Paraflagellar rod (PFR) proteins from *T. cruzi*, the trypanosome species causing American trypanosomiasis, have been found provide up to 100% protection to mice against lethal challege with *T. cruzi* (Luhrs *et al.*, 2003).

As well as the ability of cytoskeletal proteins to induce protective immunity *in vivo*, antibodies against tubulin were found to inhibit parasite growth in culture. For example, immune sera from rabbits immunised with native tubulin from *T. brucei* were found to inhibit the growth of trypanosomes *in vitro* (Lubega *et al.*, 2002b). Similarly, affinity purified IgG from a rabbit immunised with recombinantly expressed α-tubulin from *T. danilewskyi* was found to inhibit the growth of this species in culture (Plouffe and Belosevic, 2006). In another study, recombinant β-tubulin from *Pseudocohnilembus persalinus*, a parasite causing the disease scuticociliatosis affecting cultivated marine fish, was used to immunise rats. The growth of ciliates was found to be inhibited when incubated with sera from the immunised rats, prompting the investigators to suggest β-tubulin as a vaccine candidate for scuticociliatosis (Kim *et al.*, 2006).

It has been found that BALB/c mice typically succumb to infection with *T. congolense* within ten days after challenge (Antoine-Moussiaux *et al.*, 2008). Some mouse strains are known to be resistant to trypanosome infection, in a similar way to certain breeds of cattle that are resistant to trypanosomiasis and it is also known that different isolates of *T. congolense* have varying degrees of virulence (Pinder *et al.*, 1986). Pinder *et al*. (1986) found that BALB/c mice are more susceptible to *T. congolense* infection than other strains such as C57B1/6, and died from day 8 onwards when inoculated with 10^4 cells of *T. congolense* strain Dinderesso/80/CRTA/3, a less virulent strain (Pinder *et al.*, 1986). Interestingly, in the present study 60% of the BALB/c mice immunised with α-tubulin test group and the control groups, immunised with VP4AA or PBS, survived past 30 days of the monitoring period when challenged with 10^3 cells of *T. congolense* strain IL 1180.

Despite the limited success of the present study as compared to previous investigations, much evidence exists of the immuno-protection potential of parasite cytoskeletal proteins. For this reason, further investigations of α- and β-tubulin from *T. congolense* may be warranted.

CHAPTER FOUR

GENERAL DISCUSSION

African trypanosomiasis affects in excess of 10 million km^2 of sub-Saharan Africa, resulting in agricultural and livestock losses in 37 countries, estimated as costing the continent roughly US$ 4.5 billion per annum (Kristjanson *et al.*, 1999; Antoine-Moussiaux *et al.*, 2009). Trypanosomes are transmitted by tsetse flies, and results in the disease known as sleeping sickness in humans, while in cattle and other ruminant livestock it is known as nagana. Trypanosomiasis is fatal in both humans and animals unless treatment is given.

Current efforts to control trypanosomiasis include chemotherapeutic methods, vector control and raising of trypanotolerant cattle. Treatment of the disease is limited to a handful of drugs which are either costly, or ineffective due to the emergence of drug resistance by the parasite (Geerts *et al.*, 2001). Control of the tsetse fly vector is mainly in the form of insecticide spraying, traps designed to catch tsetse flies and the release of sterile males. The above methods have had some success in various regions, but are limited in their feasibility due to factors including environmental concerns over insecticide spraying, as well as vector resistance, and logistical difficulty in covering the vast areas of the continent inhabited by tsetse (Mäser *et al.*, 2003; Delespaux *et al.*, 2008). Trypanotolerant cattle are able to tolerate infection with trypanosomes without developing disease symptoms. The disadvantage of trypanotolerant compared to susceptible breeds is their smaller size and lower milk production, which limits their usefulness (Murray *et al.*, 2004).

The above methods are unsatisfactory as a means of controlling African trypanosomiasis, therefore a vaccine against the disease is seen as the best solution. Conventionally, a vaccine is composed of the killed or attenuated pathogen, or pathogenic factors of the disease causing organism. The host then mounts an antibody response to the vaccine components, which allows the host to control an infection by that pathogen (Delves *et al.*, 2006). The surface of African trypanosomes is covered in a dense coat of protein known as variable surface glycoprotein (VSG), which possesses the inherent phenomenon of

antigenic variation (Turner, 1985; Pays and Nolan, 1998). Although VSG is highly immunogenic, it has been found to be impractical as a vaccine target, due to its antigenic variation, evidenced by the presence of over 1000 genes and pseudogenes in the trypanosome genome (Taylor and Rudenko, 2006). Due to the above considerations, alternative vaccine candidates have been sought. Two main avenues of vaccine research are currently being followed, namely the conventional (anti-parasite) vaccine approach and the anti-disease vaccine approach (Playfair *et al.*, 1991). The successes of previous studies provide hope that a vaccine against African trypanosomiasis may yet be found, despite the enormous difficulty posed by antigenic variation of VSG (Mkunza *et al.*, 1995; Lubega *et al.*, 2002a; Rasooly and Balaban, 2004; Li *et al.*, 2007).

The anti-disease vaccine approach aims to ameliorate the symptoms of the disease in the host by targeting pathogenic factors of the parasite, without necessarily ridding the host of the infection itself (Playfair *et al.*, 1991). This approach has met with some success, as the trypanosome cysteine peptidase, congopain, and serine peptidase, oligopeptidase B, have been identified as disease causing factors in animal African trypanosomiasis (Authie, 1994; Morty *et al.*, 2001; Lalmanach *et al.*, 2002; de Matos Guedes *et al.*, 2007; Bizaaré, 2008). Animals immunised with congopain have shown greater tolerance to trypanosome infection compared to controls (Authié *et al.*, 2001).

Although progress has been made in the area of an anti-disease vaccine (Authie, 1994; Magez *et al.*, 2002), some success has been obtained with the conventional vaccine approach as well. Antigens derived from the flagellar pocket region of the parasite have found to provide immuno-protection in mice against trypanosome challenge (Mkunza *et al.*, 1995; Radwanska *et al.*, 2000).

Tubulins and their associated proteins, known as microtubule associated proteins (MAPs), have been found to provide immuno-protection to otherwise lethal challenge by trypanosomes (Rasooly and Balaban, 2004). A MAP known as p52 was found to protect mice against infection with *T. brucei* (Balaban *et al.*, 1995), and both native and

recombinant MAP p15 were found to be highly protective to mice against infection with *T. b. brucei*, with no parasitaemia developing in all seven mice immunised with recombinant MAP p15 and seven out of eight mice immunised with native MAP p15 (Rasooly and Balaban, 2004). Although tubulin and MAPs are not exposed on the surface of the parasite, antibodies against these proteins may still be able to bind to their targets via entry into the cell possibly through the flagellar pocket region of the parasite: the site of endocytosis and exocytosis (Morgan *et al.*, 2002).

Previous studies investigating trypanosome tubulin as a vaccine candidate, have derived the tubulin from *T. brucei*, for native tubulin and *T. evansi*, for recombinant tubulin (Lubega *et al.*, 2002a; Li *et al.*, 2007). These species, however, are not as commercially important as *T. congolense* and *T. vivax*, the main species which cause disease in ruminant livestock (Lalmanach *et al.*, 2002). It was therefore considered to be of greater importance to investigate tubulin from *T. congolense* for its immuno-protective potential due to its greater economic relevance. Hence, in the present study, it was decided that recombinant expression of both α- and β-tubulin from *T. congolense* would be undertaken, due to the previous success achieved with recombinant β-tubulin from *T. evansi* (Li *et al.*, 2007). A related study found that the growth of *T. danilewskyi,* a trypanosome infective to certain fish species, was inhibited in the presence of affinity purified IgG from a rabbit immunised with recombinant α-tubulin from *T. danilewskyi* (Plouffe and Belosevic, 2006). Although both α- and β-tubulin have almost exactly the same molecular weight and identical tertiary structures, their amino acid sequences share only approximately 40% similarity (Nogales *et al.*, 1998), further strengthening the legitimacy for investigating both of these proteins.

PCR was used to amplify the α- and β-tubulin ORFs from *T. congolense* (strain IL 1180) genomic DNA, as the complete α- and β-tubulin ORFs were available on the database (GeneDB) and were not interrupted by introns in the genome. The almost total absence of introns in the genome is a feature of African trypanosomes which is unique amongst eukaryotic species (Perelman and Boothroyd, 1990; Cross, 2001; Donelson, 2003). The PCR resulted in products of approximately 1300 bp for both α- and β-tubulin, which

corresponds to their expected sizes of 1329 and 1356 bp respectively. Ligation of the α- and β-tubulin PCR amplicons into the T-vector, pTZ57R/T was successfully demonstrated by colony PCR, which uses cells transferred directly from a single colony of recombinant *E. coli* JM 109 as template DNA. If the ligation and transformation are successful, a PCR product will be produced at the expected size depending on the use of vector or specific primers.

The nucleotide sequence of the insert must be known before primers used to amplify the gene of interest are designed, so that restriction sites which are present in the insert sequence are not added to the primers. The presence of the correct restriction sites on the inserts was determined by digestion of the constructs by the respective restriction enzymes. Once the correct restriction sites had been verified, the constructs were sequenced and found to be free of frame-shift causing mutations.

Expression of recombinant α- and β-tubulin was attempted using both yeast and bacterial expression systems. The yeast expression system, pPICZαA28, was used for expression in the host *Pichia pastoris*. Ligation of the β-tubulin insert with the pPICZαA28 vector was not successful, due possibly to the absence of one of the necessary restriction sites on the vector, which would prevent the insertion of the β-tubulin insert during the ligation reaction and allow the vector to recircularize. The fault is more likely to lie with the pPICZαA28 vector in this case as the β-tubulin insert was successfully ligated with the pET28a vector using the same restriction sites.

Ligation of the α-tubulin insert into the pPICZαA28 vector was successful, and subsequent transfection of *P. pastoris* strain X33 was demonstrated by colony PCR. Despite verification of the presence of the insert by colony PCR, expression was unsuccessful. Expression may have failed due to unsuitable growth conditions or an incompatibility of *T. congolense* α-tubulin expression with the growth of *P. pastoris*. Linder *et al*. (1997), found that *P. pastoris* required contransformation with both α2- and β2-tubulin of *Reticulomyxa filosa* (freshwater amoeba) in order for detectable levels of α2-tubulin to be expressed, as

no expression of α2-tubulin was detected in *P. pastoris* cultures transformed with a single α2-tubulin construct (Linder *et al.*, 1997). Similarly, cotransformation of *P. pastoris* with *T. congolense* α- and β-tubulin constructs may be necessary for expression of these proteins in this system.

Successful expression of both α- and β-tubulin was achieved using the pET28a system in *E. coli* host strain BL21 (DE3) as fusion proteins with a 6xHis-tag with a size of approximately 55 kDa on reducing SDS-PAGE. Both α- and β-tubulin were expressed as insoluble inclusion bodies, and purified by nickel chelate chromatography at quantities of approximately 1 – 1.5 mg/ml. Western blot probing with anti-His-tag monoclonal antibody confirmed the presence of the His-tag on both α- and β-tubulin, however α-tubulin showed a weaker signal for the anti-His-tag antibody than β-tubulin. The reason for the weaker α-tubulin signal is not known, as both α- and β-tubulin have the same tertiary structure in the native protein, despite sharing only approximately 40% amino acid sequence identity (Nogales *et al.*, 1998). The proteins used for the blot were, however, denatured and would not possess their native structure. This would possibly allow the α-tubulin to assume a non-native structure, whereby the His-tag is obscured in such a way as to affect the binding of the anti-His-tag antibody to the His-tag on the protein. Western blot also confirmed that both α- and β-tubulin were recognised by monoclonal antibodies specific for each of these proteins.

In order to assess the immunoprotection potential of α- and β-tubulin in mice, it was necessary to refold the proteins, due to the expression of the above proteins as insoluble inclusion bodies, which must be completely denatured to allow purification. The need for refolded protein has been demonstrated by Lubega *et al*. (2002a) where refolded native *T. brucei* tubulin was able to provide protection to mice against challenge with *T. brucei*, *T. rhodesiense* and *T. congolense*, to a greater extent than denatured native tubulin. The study conducted by Li *et al*. (2007) also made use of refolded β-tubulin from *T. evansi* which was expressed similarly to α- and β-tubulin in the present study. In addition, recombinantly expressed α- and β-tubulin from *T. danilewskyi* for use in immune studies was refolded due

to its expression as inclusion bodies (Plouffe and Belosevic, 2006; Katzenback *et al.*, 2008). Proteins recombinantly expressed in *E. coli* typically aggregate in the form of insoluble inclusion bodies, where the recombinant protein is in a denatured, non-bioactive state (Rudolph and Lilie, 1996; Lilie *et al.*, 1998; Singh and Panda, 2005). Various refolding methods may be employed to refold the denatured recombinant protein into its native, bioactive state, and must be adapted and optimised for each different protein expressed (Singh and Panda, 2005). The underlying rationale for using refolded protein as opposed to denatured protein when assessing a protein as a vaccine candidate is the fact that in addition to linear epitopes, conformational epitopes are present in the three-dimensional, correctly folded structure of the bioactive proteins, which are not present in the denatured form of the same protein. The epitopes present on the refolded recombinant protein would be very similar to the epitopes of the native protein present in the parasite during an infection. For this reason, refolding of the recombinantly expressed α- and β-tubulin was attempted. However, epitopes present on the denatured protein may still be effective in generating antibodies capable of recognising the native protein. As an example, β-tubulin from *Pseudocohnilembus persalinus* was recombinantly expressed in *E. coli* as a fusion protein with glutathione *S*-transferase (GST), and used for immunisation of rats. The immune sera from the rats were found to recognise reduced recombinant GST-tagged β-tubulin from *P. persalinus* on a western blot, and was also found to have a higher parasiticidal effect on, and lower the proliferation of, cultured *P. persalinus* as compared to non-immune control sera (Kim *et al.*, 2006). Therefore, denatured α- and β-tubulin from *T. congolense* may also be used for immunisation, as it may still be capable of generating antibodies capable of recognising native protein present in the parasite.

Refolding of α- and β-tubulin was attempted through two methods. The first method involved dilution of the purified protein in a refolding buffer, containing reduced and oxidised glutathione (1:0.5 mM), glycerol as a cosolvent and KCl that functions as an aggregation suppressor, followed by reconcentration after a 20 h incubation at 4° C (Sijwali *et al.*, 2001). The second method involved refolding of the denatured proteins using a decreasing gradient of the denaturant, urea, while bound to a nickel chelate chromatography

column (Jang *et al.*, 2008). The first method produced limited success, as a large amount of protein formed aggregates as high molecular weight structures on non-reducing SDS-PAGE. Aggregation during *in vitro* refolding of proteins has been found to result from intermolecular interactions between exposed hydrophobic patches of partially folded protein molecules during the refolding process (Clark, 1998; Lilie *et al.*, 1998; Verma *et al.*, 1998). A small amount of protein was visible in non-reduced refolded samples at a molecular weight slightly higher than that of the reduced denatured samples. Differences in migration have been observed between reduced and non-reduced states of the same protein on SDS-PAGE, resulting in a gel-shift. This is thought to be due to differential binding of SDS to the protein in the different states, as an unreduced protein containing disulfide bonds is more compact, and does not bind as much SDS as the same protein in a reduced and denatured state, allowing the SDS more area on the protein to bind (Pitt-Rivers and Impiombato, 1968; Dunker and Kenyon, 1976; Rath *et al.*, 2009). As an example, an antibody fragment expressed in *E. coli* was found to have the appearance of a slightly higher molecular weight in the reduced state, as compared to the oxidised form of the same protein (Tavladoraki *et al.*, 1999). It would be expected therefore that α- and β-tubulin in their refolded states would have a more compact conformation due to the presence of multiple cysteine residues in the sequence of both proteins. Reproducible results were not obtainable with this method, and due to the high level of aggregation present, a second method was employed with the hope of reducing the amount aggregation during refolding.

The second method employed to refold α- and β-tubulin was adapted from Jang *et al.* (2008) and involved refolding of the denatured protein by applying a decreasing gradient of the denaturant, urea, to the protein while it was bound to a nickel chelate column by the His-tag present on the recombinant protein. It has been noted that aggregation is reduced when proteins are bound individually to the matrix of the chromatography column prior to refolding (Clark, 1998). The results of this method were considered more successful, although aggregation was observed as with the previous method. The protein bands observed on SDS-PAGE of non-reduced refolded samples were slightly smaller than those of the non-reduced denatured protein samples. This observation differs from the results of

the previous refolding method, which showed non-reduced refolded samples having the appearance of a higher molecular weight than non-reduced denatured samples. This result, however, agrees with the expectation that the non-reduced refolded protein would be more compact and therefore migrate faster on SDS-PAGE due to a decrease in the amount of SDS able to bind to the protein than the protein when in the reduced and denatured state, leading to a gel shift (Rath *et al.*, 2009). It was therefore concluded that the refolding achieved with the second method was more successful than the first method. Yields were lower for α-tubulin as compared to β-tubulin, due possibly to the weaker binding affinity of the α-tubulin His-tag to the nickel chelate resin. Lower yields were also obtained for eluted samples of refolded α- and β-tubulin compared to that obtained with nickel chelate chromatography under denaturing conditions, and may be attributed to a portion of the bound protein being washed off during application of the refolding gradient to the column. The amount of protein washed off the column could possibly be reduced by application of a smaller volume of refolding buffer gradient to the column at a slower flow rate to refold the bound denatured protein, or by starting the decreasing gradient of urea at a lower concentration. Further optimisation of the refolding protocol for both α- and β-tubulin is required to produce sufficient quantities of refolded protein for further study.

Native tubulin was isolated from cultured *T. congolense* procyclics (strain IL 1180) as a control for immunisation, due to its potential demonstrated by previous research that found native renatured tubulin of *T. brucei* to provide complete protection to mice against challenge with *T. brucei, T. congolense* and *T. rhodesiense* (Lubega *et al*., 2002a and b). The isolation was successful as evidenced by recognition of the renatured tubulin by anti-α and β-tubulin monoclonal antibodies. Low yields of native tubulin were obtained in comparison to the amount of tubulin detected by western blot in the cell lysate fraction in the form of sub-pellicular microtubules. This isolation technique relies on polymerisation of the soluble tubulin in the cell cytoplasm, as the pellicular microtubules have been noted to be largely resistant to depolymerisation by conventional methods (Stieger *et al.*, 1984). Polymerisation of the tubulin monomers with paclitaxel, a microtubule stabilising agent, was followed by depolymerisation and subsequent renaturation of the tubulin monomers by

dialysis. A low concentration of renatured tubulin was purified, which although was not visible with Coomassie staining, was detected in a western blot probed with anti-α-tubulin (TAT-1) and anti-β-tubulin (KMX-1) monoclonal antibodies, confirming the success of the isolation. The low yield of renatured native tubulin from *T. congolense* with this method as compared to the results of Lubega *et al*. (2002) may possibly be improved by increased sonication of the cell lysate in order to depolymerise the pellicular microtubules, as prolonged sonication has been noted as a means for disrupting trypanosome pellicular microtubules (Stieger *et al.*, 1984).

Immunisation of five groups of five BALB/c mice with the refolded recombinant α- and β-tubulin was carried out along with three controls comprising native tubulin, an unrelated protein VP4AA, and PBS (adjuvant only), along with the adjuvant, Adju-phos (alum) and saponin. An ELISA evaluation of antibody production over the immunisation schedule revealed that antibodies were produced for all immunogens except VP4AA, which showed antibody titres at the same level as for the PBS control group. As VP4AA is known to be immunogenic, the lack of antibodies detected in the ELISA may be as a result of too little coating of the wells with VP4AA or an insufficient amount of protein used for immunisation, although the same amounts were used as for the test proteins.

Subsequent challenge of all groups of mice with *T. congolense* (strain IL 1180) revealed that neither α-or β-tubulin or native tubulins were protective against infection, as mice in all groups developed parasitaemia. Three out five mice immunised with α-tubulin, VP4AA and PBS were surviving at the end of the monitoring period, while all mice immunised with native tubulin died within 22 days after infection. The poor performance of mice immunised with native tubulin may be as a result of a pre-existing condition within the group, resulting in immune system compromise, as this group performance was poorer than the control groups. For this reason, immunisation of mice with native tubulin from *T. congolense* should be repeated. No deaths were recorded at the end of the monitoring period in the group immunised with β-tubulin, despite all mice having sustained parasitaemia. The results suggest that β-tubulin may be able to provide partial immuno-

protection against trypanosome infection, as partial as well as complete protection was observed in mice immunised with β-tubulin from *T. evansi* and challenged with *T. evansi, T. equiperdum* and *T. brucei* (Li *et al.*, 2007). Partial protection was also observed in goldfish immunised with β-tubulin from T. *danilewskyi* when challenged with *T. danilewskyi* (Katzenback *et al.*, 2008).

The present study presents the first successful expression and purification of α- and β-tubulin from *T. congolense* in *E. coli*. Some success was achieved with refolding of both recombinant α- and β-tubulin into their native state, however, optimisation of refolding is required. Immunisation of BALB/c mice with α- and β-tubulin showed that β-tubulin is partially protective against infection with *T. congolense*. The present study confirms previous results that identify β-tubulin as having immunotherapeutic potential against parasite infection (Kim *et al.*, 2006; Li *et al.*, 2007; Katzenback *et al.*, 2008).

APPENDIX A

T. brucei α-tubulin (Tb927.1.2400)

ATGCGTGAGGCTATCTGCATCCACATTGGTCAGGCTGGTTGCCAGGTTGGTAACGCCTGCTGGGAATTGTTC
TGCCTGGAACACGGCATTCAACCCGATGGTGCGATGCCCTCTGACAAGACGATTGGCGTTGAGGATGATGC
GTTCAACACCTTCTTCTCTGAGACTGGTGCTGGCAAGCACGTTCCCCGCGCGGTGTTCTTGGACCTGGAGCC
AACAGTGGTGGATGAAGTGCGCACTGGCACGTACCGCCAGCTGTTCCACCCCGAGCAGCTGATCTCCGGCA
AGGAGGATGCGGCCAACAACTACGCTCGTGGCCACTACACCATTGGTAAGGAGATCGTCGACCTCTGCCTG
GACCGCATCCGCAAGCTCGCTGACAACTGCACTGGTCTTCAGGGCTTCCTCGTGTATCACGCCGTCGGCGGT
GGCACTGGTTCTGGCCTGGGTGCGCTGCTCTTGGAGCGCCTCTCCGTGGACTATGGCAAGAAGTCCAAGCTC
GGCTACACGGTGTATCCATCACCGCAGGTGTCGACGGCTGTCGTGGAGCCCTACAACTCTGTGCTCTCGACA
CACTCACTTCTGGAGCACACCGATGTTGCTGCGATGCTTGACAATGAAGCGATTTATGATTTGACTCGCCGC
AACCTCGATATTGAGCGCCCCACGTACACCAACCTGAACCGCCTCATCGGTCAGGTGGTTTCCTCGCTGACA
GCGTCCCTCCGCTTCGACGGTGCATTGAACGTGGATCTGACAGAGTTCCAGACAAACCTTGTGCCGTACCCA
CGTATCCACTTCGTGCTGACAAGCTATGCACCAGTCATCTCCGCAGAGAAGGCCTACCACGAGCAACTCTCT
GTCTCTGAGATCTCGAACGCTGTGTTTGAGCCCGCCTCCATGATGACAAAGTGCGACCCCCGCCACGGCAA
GTACATGGCGTGCTGCCTCATGTACCGTGGTGACGTTGTGCCAAAGGATGTGAATGCTGCCGTCGCGACCAT
CAAGACGAAGCGCACGATTCAGTTCGTGGACTGGTCTCCCACAGGCTTCAAGTGCGGTATCAACTACCAGC
CACCCACGGTGGTGCCAGGTGGTGACCTTGCCAAGGTGCAGCGCGCGGTATGCATGATCGCCAACTCCACG
GCCATCGCAGAGGTGTTCGCCCGTATTGACCACAAATTCGATCTCATGTACAGCAAGCGCGCCTTCGTGCAC
TGGTACGTCGGTGAGGGTATGGAAGAGGGTGAGTTCTCCGAGGCCCGTGAAGACCTTGCAGCACTTGAGAA
GGACTACGAAGAGGTTGGTGCCGAGTCCGCGGATATGGACGGTGAGGAGGATGTGGAGGAGTACTAG

T. congolense contig_001094

The highlighted sequence within in following sequence was the region located by a BLAST search using the *T. brucei* α-tubulin sequence as a query sequence.

CGTCCGTGTGCTCGAGCAGGGAGTGGGTGGAGAGCACCGAGTTGTATGGCTCCACCACGGCCGTCGACACC
TGCGGCGAGGGGTACACGGTGTAACCCAGCTTCGACTTCTTGCCGTAGTCCACGGAAAGGCGCTCCAGCAG
CAGTGCACCAAGGCCGGACCCAGTGCCACCACCAACGGCGTGGTACACCAGGAAGCCCTGGAGACCAGTG
CAGTTGTCGGCCAGCTTGCGGATGCGGTCAAGGCACAGGTCCACGATCTCCTTGCCAATGGTGTAGTGGCC
ACGGGCGTAGTTGTTGGCGGCGTCCTCCTTGCCGGAGATCAGCTGCTCGGGGTGGAACAGCTGGCGGTACG
TGCCGGTGCGGACCTCATCCACGACCGTGGGTTCCAGGTCGAGGAACACAGCACGAGGTACGTGCTTGCCG
GCGCCAGTCTCAGAGAAGAACGTGTTGAACGCATCGTCCTCAACGCCGATCGTTTTGTCCGAGGGCATCGC
GCCGTCGGGCTGGATTCCGTGCTCAAGGCAGAACAGCTCCCAGCACGCATTACCAACCTGGCAACCGGCCT
GACCAATGTGGATGCAGATAGCCTCGCGCATGGTGAATGAATAGGAGTGCTTTGTTGGGTGTTTGAAGGAT
CGCTTGGAAGTTTTACTGGGCACCGCGCGCAGGCAGGCATACAGACACGGTGGAAAAGAGAGAGAAAGAG
AGAAAAGGAAGGGTGATAAGCAAGAGCATGGGGGTACAGCAGCATTACCAAGTGGAACAGGGAAAGGAA
AGGGAAACAAAAGAAAAGAGGGAAAAGTCAGTGACTCATGTTCCCACGTGCACACACCAGAAAGGCATAC
GCATGGACAAACATCAACCAAAACAAAACAAAAAAGGTAAAAACAAGAGGAAAGAGAGAAAGGAAAAAG
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CAAAGGGGCAGCCACACACCCGCTAGAGTGGGAGTGATTCGTCGGCAACTGCCGTCTAGTACTGCTCCTCT
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CGTGACAGCCATCTTGAGGCCCTTGGGTGGGATGTCGCAAACAGAGGATTTGATGTTGTTGGGGATCCACTC
GATGAAGTAGGACGAGTTCTTGTTCTGCACGTTGAGCATCTGCTCGTCCACCTCCTTCGTCGACATGCGGCC
GCGGAAGAGCGCAGACGCTGTCAGGTAGCGGCCGTGGCGAGGATCGGCAGCTTGCATCATGTTTTTCGCAT
CGAACATCTGCTGTGTCAGCTCAGGCACGGAGAGGCCGCGGTACTGCTGAGAGCCACGGCTTGTCAACGGG
GCAAAGCCCATCATGAAGAAGTGCAGACGTGGGAACGGCACAAGGTTCACGGCAAGTTTGCGCAGGTCGG
AGTTGAGCTGACCGGGGAAGCGCAGGCAGCACGTCACGCCAGATACGACAGCGGACACGAGGTGGTTCAG

ATCGCCGAAGGTGGGTGTCGTCAGCTTGAGGGTACGGAAACAGATGTCATACAGAGCCCGTTGTCAATGCA
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AGAGACCTTGGGGGATGGGATGATGGAAAAGGTCATCATGATGCGATCGGGGTACTGTTCACGCAGCTTTG
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CGTCCGCCGGTCGCCTCATCGAAATATACATTAATGCGCTCCAGTTGCAGGTCAGAGTCACCCTGATAGGTG
CCAGTGGGATCCACACCGTGCTCATCGCTGATCACCTCCCAGAACTTGGAGCCAATCTGGTTGCCGCACTGG
CCAGCCTGAATACAAACGATCTCACGCATGATGGAATTGGATTAGTCTTCCTTGAAACGATTATAATCTTTC
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AGGCGGAGTGGGACAGGAAGAGGGAACACTTCATTAATGAGAGGAAACAAAACAAGATAAATAAAGAAA
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TCCACACAGACACACTTCTTTTCCTGTTCTCTAGTACTCCTCAACGTCCTCCTCGCCGTCCATGTCCGCAGAC
TCCGCACCGACCTCCTCGTAATCCTTCTCGAGGGCAGCGAGATCTTCACGGGCCTCGGAGAACTCACCCTCC
TCCATACCCTCACCAACATACCAGTGCACGAAGGCGCGCTTGCTGTACATGAGATCGAACTTGTGGTCAATG
CGCGCGAACACCTCCGCGATGGCAGTGGAGTTGGCAATCATGCACACCGCACGCTGCACCTTGGCGAGGTC
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ACGGCGTTCGAAATCTCCGACACGGAGAGCTGCTCGTGGTAAGCCTTCTCCGCAGAGATCACCGGCGCGTA
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CACGGGCGTAGTTGTTGGCGGCGTCCTCCTTGCCGGAGATCAGCTGCTCGGGGTGGAACAGCTGGCGGTAC
GTGCCGGTGCGGACCTCATCCACGACCGTGGGTTCCAGGTCGAGGAACACAGCACGAGGTACGTGCTTGCC
GGCGCCAGTCTCAGAGAAGAACGTGTTGAACGCATCGTCCTCAACGCCGATCGTTTTGTCCGAGGGCATCG
CGCCGTCGGGCTGGATTCCGTGCTCAAGGCAGAACAGCTCCCAGCACGCATTACCAACCTGGCAACCGGCC
TGACCAATGTGGATGCAGATAGCCTCGCGCATGGTGAATGAATAGGAGTGCTTTGTTGGGTGTTTGAAGGA
TCGCTTGGAAGTTTTACTGGGCACCGCGCGCAGGCAGGCATACAGACACGGTGGAAAAGAGAGAGAAAGA
GAGAAAAGGAAGGGTGATAAGCAAGAGCATGGGGGTACAGCAGCATTACCAAGTGGAACAGGGAAAGGA
AAGGAATTGTTTTTACCCCTCTGTTTATTCCGTTGTTGTGCCATTTTTTCTTTGGGTCAGATGTACAATGCTTT
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ACGGCTGTGAGTCCACTCGATGTGATTTCTTCGCCTGTTGTTCCGTTAACTATTTTGAGTCTTGGTAATGGTG
TCCATGGTCTTACCATTAGTCCTGAGTCACGTTGAACCCATGTGGCACCGTGGCGGTGTGAGCAGTGGTAGA
AACCGTGCTGCCTGGTTACGTCAATCATTTCTTTTCCACCCAAGGATATGGCGACCACTAGGAAATCGTGCC
TATGTTTTTGGTGGAACCGCTGAAGCAGATTCATAAATGCAAGTGAGCGCTCTGACTCTGTGAAAAAAAGT
AGTCCCACAAATCTACCTCGAAGGATATCCCTGTCGAAGGACTGCCCCGAAGGGCCTGTTATTTGAGCAAT
GTTTTTTAGATAGTCGTTGAGGGTCTCTTCGTGGGCAAACGGGGTGATGCGGCTGTCCAAGTTCTTTAGCTC
TTCCCTAAGTGCCGCTCGCTGTGTCGCTCCATCGCCGCCATATATTCGACTGTCCGGATCCTTTGGTCTAAAA
GTTGCTTCTACTGCTATCCTCATTAGCTTTGTTAGGGTCATTTGTTGTCGATCAATCATGGAAAAGTCGTTAA
CATTGAAGTGGCGTCTTAGCATTGGGCTCACTGGTGAACTATCGTGAGTGCCGCTGGTGCTACCGGCTGAGT
GAGCCTCCGTGCTTTGGTTACTGCGGAGGTTCGGAAAGGTGGCGACTTGCTGTAACCGTGGCTCTGTCCACC
CTCCCGGGCCACGACGGGCGGTGCGGAGGCTGAAGGCATTCCTTCGCATCATCGGTCACTTCACGATTTTAG
CGCCTCCGAGGGGGAAAAGCTTGTGAGAGAAGGCATGAAAAGGGAAAGAAAATTGCTGGTCAGT

APPENDIX B

The reverse complement of the above highlighted region was found, and identified as the *T. congolense* α-tubulin ORF via a multiple sequence alignment using ClustalX 2.0.10 software.

```
T.brucei        ATGCGTGAGGCTATCTGCATCCACATTGGTCAGGCTGGTTGCCAGGTTGGTAACGCCTGC 60
T.congo         ATGCGCGAGGCTATCTGCATCCACATTGGTCAGGCCGGTTGCCAGGTTGGTAATGCGTGC 60
                ***** ****************************** ***************** ** ***

T.brucei        TGGGAATTGTTCTGCCTGGAACACGGCATTCAACCCGATGGTGCGATGCCCTCTGACAAG 120
T.congo         TGGGAGCTGTTCTGCCTTGAGCACGGAATCCAGCCCGACGGCGCGATGCCCTCGGACAAA 120
                *****  ********** ** ***** ** ** ***** ** *********** *****

T.brucei        ACGATTGGCGTTGAGGATGATGCGTTCAACACCTTCTTCTCTGAGACTGGTGCTGGCAAG 180
T.congo         ACGATCGGCGTTGAGGACGATGCGTTCAACACGTTCTTCTCTGAGACTGGCGCCGGCAAG 180
                ***** ********** ************** ***************** ** ******

T.brucei        CACGTTCCCCGCGCGGTGTTCTTGGACCTGGAGCCAACAGTGGTGGATGAAGTGCGCACT 240
T.congo         CACGTACCTCGTGCTGTGTTCCTCGACCTGGAACCCACGGTCGTGGATGAGGTCCGCACC 240
                ***** ** ** ** ****** * ******** ** ** ** ******** ** *****

T.brucei        GGCACGTACCGCCAGCTGTTCCACCCCGAGCAGCTGATCTCCGGCAAGGAGGATGCGGCC 300
T.congo         GGCACGTACCGCCAGCTGTTCCACCCCGAGCAGCTGATCTCCGGCAAGGAGGACGCCGCC 300
                ************************************************** ** ***

T.brucei        AACAACTACGCTCGTGGCCACTACACCATTGGTAAGGAGATCGTCGACCTCTGCCTGGAC 360
T.congo         AACAACTACGCCCGTGGCCACTACACCATTGGCAAGGAGATCGTGGACCTGTGCCTTGAC 360
                ********** ******************** ********** ***** ***** ***

T.brucei        CGCATCCGCAAGCTCGCTGACAACTGCACTGGTCTTCAGGGCTTCCTCGTGTATCACGCC 420
T.congo         CGCATCCGCAAGCTGGCCGACAACTGCACTGGTCTCCAGGGCTTCCTGGTGTACCACGCC 420
                ************** ** ***************** ********** ***** ******

T.brucei        GTCGGCGGTGGCACTGGTTCTGGCCTGGGTGCGCTGCTCTTGGAGCGCCTCTCCGTGGAC 480
T.congo         GTTGGTGGTGGCACTGGGTCCGGCCTTGGTGCACTGCTGCTGGAGCGCCTTTCCGTGGAC 480
                ** ** ********** ** ***** ***** *****  ********** *********

T.brucei        TATGGCAAGAAGTCCAAGCTCGGCTACACGGTGTATCCATCACCGCAGGTGTCGACGGCT 540
T.congo         TACGGCAAGAAGTCGAAGCTGGGTTACACCGTGTACCCCTCGCCGCAGGTGTCGACGGCC 540
                ** ********** ***** ** ***** ***** ** ** *****************

T.brucei        GTCGTGGAGCCCTACAACTCTGTGCTCTCGACACACTCACTTCTGGAGCACACCGATGTT 600
T.congo         GTGGTGGAGCCATACAACTCGGTGCTCTCCACCCACTCCCTGCTCGAGCACACGGACGTT 600
                ** ******** ******** ******** ** ***** ** ** ******** ** ***

T.brucei        GCTGCGATGCTTGACAATGAAGCGATTTATGATTTGACTCGCCGCAACCTCGATATTGAG 660
T.congo         GCGGCCATGCTTGACAATGAGGCTATTTATGACTTGACGCGACGCAACCTGGACATTGAG 660
                ** ** ************** ** ******** ***** ** ******** ** ******

T.brucei        CGCCCCACGTACACCAACCTGAACCGCCTCATCGGTCAGGTGGTTTCCTCGCTGACAGCG 720
T.congo         CGCCCCACGTACACCAACCTGAACCGTCTCATCGGCCAGGTTGTCTCCTCGCTGACGGCG 720
                ************************** ******** ***** ** ********** ***

T.brucei        TCCCTCCGCTTCGACGGTGCATTGAACGTGGATCTGACAGAGTTCCAGACAAACCTTGTG 780
T.congo         TCCCTGCGCTTCGATGGTGCACTCAATGTGGACCTGACGGAGTTCCAGACCAACCTTGTG 780
                ***** ******** ****** * ** ***** ***** ********** *********

T.brucei        CCGTACCCACGTATCCACTTCGTGCTGACAAGCTATGCACCAGTCATCTCCGCAGAGAAG 840
T.congo         CCGTACCCACGTATTCACTTCGTGCTCACCAGCTACGCGCCGGTGATCTCTGCGGAGAAG 840
                ************** ********** ** ***** ** ** ** ***** ** ******

T.brucei        GCCTACCACGAGCAACTCTCTGTCTCTGAGATCTCGAACGCTGTGTTTGAGCCCGCCTCC 900
T.congo         GCTTACCACGAGCAGCTCTCCGTGTCGGAGATTTCGAACGCCGTGTTCGAGCCCGCGTCG 900
                ** ********** ***** ** ** ***** ******** ***** ******** **


T.brucei        ATGATGACAAAGTGCGACCCCCGCCACGGCAAGTACATGGCGTGCTGCCTCATGTACCGT 960
T.congo         ATGATGACCAAGTGCGACCCCCGCCACGGCAAGTACATGGCGTGCTGCCTCATGTACCGT 960
                ******** ************************************************
```

```
T.brucei        GGTGACGTTGTGCCAAAGGATGTGAATGCTGCCGTCGCGACCATCAAGACGAAGCGCACG 1020
T.congo         GGTGACGTTGTGCCCAAGGATGTGAACGCTGCCGTCGCGACCATCAAGACGAAGCGCACG 1020
                ************** *********** ********************************

T.brucei        ATTCAGTTCGTGGACTGGTCTCCCACAGGCTTCAAGTGCGGTATCAACTACCAGCCACCC 1080
T.congo         ATCCAGTTCGTCGACTGGTCTCCCACTGGCTTCAAGTGCGGCATCAACTACCAGCCCCCG 1080
                ** ******** ************** ************** ************** **

T.brucei        ACGGTGGTGCCAGGTGGTGACCTTGCCAAGGTGCAGCGCGCGGTATGCATGATCGCCAAC 1140
T.congo         ACGGTGGTGCCTGGTGGTGACCTCGCCAAGGTGCAGCGTGCGGTGTGCATGATTGCCAAC 1140
                *********** *********** ************** ***** ******** ******

T.brucei        TCCACGGCCATCGCAGAGGTGTTCGCCCGTATTGACCACAAATTCGATCTCATGTACAGC 1200
T.congo         TCCACTGCCATCGCGGAGGTGTTCGCGCGCATTGACCACAAGTTCGATCTCATGTACAGC 1200
                ***** ******** *********** ** *********** ******************

T.brucei        AAGCGCGCCTTCGTGCACTGGTACGTCGGTGAGGGTATGGAAGAGGGTGAGTTCTCCGAG 1260
T.congo         AAGCGCGCCTTCGTGCACTGGTATGTTGGTGAGGGTATGGAGGAGGGTGAGTTCTCCGAG 1260
                ********************** ** ************** ******************

T.brucei        GCCCGTGAAGACCTTGCAGCACTTGAGAAGGACTACGAAGAGGTTGGTGCCGAGTCCGCG 1320
T.congo         GCCCGTGAAGATCTCGCTGCCCTCGAGAAGGATTACGAGGAGGTCGGTGCGGAGTCTGCG 1320
                *********** ** ** ** ** ******** ***** ***** ***** ***** ***

T.brucei        GATATGGACGGTGAGGAGGATGTGGAGGAGTACTAG 1356
T.congo         GACATGGACGGCGAGGAGGACGTTGAGGAGTACTAG 1356
                ** ******** ******** ** ************
```

APPENDIX C

An alignment of the *T. congolense* α- and β-tubulin ORF sequences using ClustalX 2.0.10 software is given below.

```
T.c-alpha       ATGCGCGAGGCTATCTGCATCCACATTGGTCAGGCCGGTTGCCAGGTTGGTAATGCGTGC 60
T.c-beta        ATGCGTGAGATCGTTTGTATTCAGGCTGGCCAGTGCGGCAACCAGATTGGCTCCAAGTTC 60
                ***** ***    * ** ** **   *** ***  ***   **** ****      ** *

T.c-alpha       TGGGAGCTGTTCTGCCTTGAGCACGGAATCCAGCCCGACGGCGCGATGCCCTCGGACAAA 120
T.c-beta        TGGGAGGTGATCAGCGATGAGCACGGTGTGGATCCCACTGGCAC--CTATCAGGGTGACT 118
                ****** ** ** **  *********  *  * ***   *** *      *  **  *

T.c-alpha       ACGATCGGCGTTGAGGACGATGCGTTCAACACGTTCTTCTCTGAGACTGGCGCCGGCAAG 180
T.c-beta        CTGACCTGCAACTGGAGC---GCATT-AATGTATATTTCGATGAGGCGACCGGCGGACGC 174
                  ** * **     *  *   ** ** **    *  ***  **** *   ** ***

T.c-alpha       CACGTACCTCGTGCTGTGTTCCTCGACCTGGAACCCACGGTCGTGGATGAGGTCCGCACC 240
T.c-beta        TACGTGCCCCGCTCCGTGTTGATCGACCTCGAGCCCGGTACCATGGATTCTGTGCGCGCC 234
                 **** ** **  * *****  ******* ** ***     * *****   ** *** **

T.c-alpha       GGCACGTACCGCCAGCTGTTCCACCCCGAGCAGCTGATCTCCGGCAAGGAGGACGCCGCC 300
T.c-beta        GGCCCCTACGGCCAGATCTTCCGCCCGGATAACTTCATCTTTGGCCAGTCCGGCGCCGGC 294
                *** * *** ***** * **** *** **  *  * ****  *** **   * ***** *

T.c-alpha       AACAACTACGCCCGTGGCCACTACACCATTGGCAAGGAGATCGTGGACCTGTGCCTTGAC 360
T.c-beta        AACAACTGGGCGAAGGGCCACTACACTGAGGGCGCGGAGCTCATCGAC-TCCGTGCTGGA 353
                *******  **    ***********    ***  **** ** * *** *  *   **

T.c-alpha       CGCAT-CCGCAAGCTGGCCGACAACTGCACTGGTCTCCAGGGCTTCCTGGTGTACCACGC 419
T.c-beta        CGTGTGCTGCAAGGAGGCAGAGAGCTGCGACTGCCTGCAGGGTTTCCAGATCTGCCACTC 413
                **  * * *****  *** ** * ****    * ** ***** **** * * * **** *

T.c-alpha       CGTTGGTGGTGGCACTGGGTCCGGCCTTGGTGCACTGCTGCTGGAGCGCCTTTCCGTGGA 479
T.c-beta        CCTCGGTGGTGGCACGGGCTCTGGCATGGGAACGCTGCTCATCTCAAAGCTGCGTGAACA 473
                * * *********** ** ** *** * **  * *****  *       **    *   *
```

```
T.c-alpha       CTACGGCAAGAAGTCGAAGCTGGGTTACACCGTGTACCCCTCGCCGCAGGTGTCGACGGC 539
T.c-beta        GTACCCCGATCGCATCATGATGACCTTTTCCATCATCCCATCCCCCAAGGTCTCTGATAC 533
                 ***  * *       * * **   *   ** *   *** ** **  **** **     *

T.c-alpha       CGTGGTGGAGCCATACAACTCGGTGCTCTCCACCCACTCCCTGCTCGAGCACACGGACGT 599
T.c-beta        CGTCGTTGAGCCCTACAACACGACGCTGTCCGTGCACCAGCTGGTGGAGAACTCCGATGA 593
                *** ** ***** ****** **  *** ***   ***   *** * *** ** * ** *

T.c-alpha       TGCGGCCATGCTTGACAATGAGGCTATTTATGACTTGACGCGACGCAACCTGGACATTGA 659
T.c-beta        GTCGATGTGCATTGACAACGAGGCTCTGTATGACATCTGTTTCCGCACCCTCAAGCTGAC 653
                  **       ******* ****** * ****** *       **** ***  *  *

T.c-alpha       GCGCCCCACGTACACCAACCTGAACCGTCTCATCGGCCAGGTTGTCTCCTCGCTGACGGC 719
T.c-beta        GACACCCACCTTCGGCGATCTGAACCACCTCGTGTCCGCTGTCGTATCTGGCGTGACGTG 713
                *   ***** * *  * * *******  *** *   *   ** ** **     *****

T.c-alpha       GTCCCTGCGCTTCGATGGTGCACTCAATGTGGACCTGACGGAGTTCCAGACCAACCTTGT 779
T.c-beta        CTGCCTGCGCTTCCCCGGTCAGCTCAACTCCGACCTGCGCAAACTTGCCGTGAACCTTGT 773
                 * **********   ***   *****    ******    *  *       ********

T.c-alpha       GCCGTACCCACGTATTCACTTCGTGCTCACCAGCTACGCGCCGGTGAT---CTCTGCGGA 836
T.c-beta        GCCGTTCCCACGTCTGCACTTCTTCATGATGGGCTTTGCCCCGTTGACAAGCCGTGGCTC 833
                ***** ******* * ****** *  * *   ***  ** *** ***    *  **

T.c-alpha       GAAGGCTTACCACGAGCAGCTCTCCGTGTCGGAGATTTCGAACGCCGTGTTCGAGCCCGC 896
T.c-beta        TCAGCAGTACCGCGGCC---TCTCCGTGCCTGAGCTGACACAGCAGATGTTCGATGCGAA 890
                  **   **** **  *   ******** * *** *  *  *     *******  *

T.c-alpha       GTCGATGATGACCAAGTGC-GACCCCCGCCACGGCAAGTACATGGCGTGCTGCCTCATGT 955
T.c-beta        AAACATGATG-CAAGCTGCCGATCCTCGCCACGGCCGCTACCTGACAGCGTCTGCGCTCT 949
                    ****** * *  *** ** ** *********   *** ** *    *      * *

T.c-alpha       ACCGTGGTGACGTTGTGCCCAAGGATGTGAACGCTGCCGTCGCGACCATCAAGACGAAGC 1015
T.c-beta        TCCGCGGCCGCATGTCGACGAAGGAGGTGGACGAGCAGATGCTCAACGTGCAGAACAAGA 1009
                 *** **   * *   * * ***** *** ***      *    * * *  ***  ***

T.c-alpha       GCACGATCCAGTTCGTCGACTGGTCTCCCACTGGCTTCAAGTGCGGCATCAACTACCAGC 1075
T.c-beta        ACTCGTCCTACTTCATCGAGTGGATCCCCAACAACATCAAATCC----TCTGTTTGCGAC 1065
                 * **  * * *** **** ***   ****    * **** * *    **   *  *  *

T.c-alpha       CCCCGACGGTGGTGCCTGGTGGTGACCTCGCCAAGGTGCAGCGTGCGGTGTGCATGATTG 1135
T.c-beta        ATCCCACCCAAGGGCCTCAAGATGGC----------TGTCACGTTC----------ATCG 1105
                  ** **    * ****   * ** *          **   *** *          ** *

T.c-alpha       CCAACTCCACTGCCATCGCGGAGGTGTTCGCGCGCATTGACCACAAGTTCGATCTCATGT 1195
T.c-beta        GCAACAACACCTGCATCCAGGAGATGTTCCGCCGTGTGGGCGAGCAGTTCACGCTCATGT 1165
                 ****  ***   ****  **** *****   **  * * * *  *****   *******

T.c-alpha       ACAGCAAGCGCGCCTTCGTGCACTGGTATGTTGGTGAGGGTATGGAGGAGGGTGAGTTCT 1255
T.c-beta        TCCGTCGCAAGGCGTTCTTACACTGGTATACTGGTGAAGGTATGGACGAGATGGAGTTCA 1225
                 * *       ** *** * *********  ****** ******** ***   ******

T.c-alpha       CCGAGGCCCGTGAAGATCTCGCTGCCCTCGAGAAGGATTACGAGGAGGTCGGTGCGGAGT 1315
T.c-beta        CTGAGGCCGAATCCAACATGAACGATCTCGTGTCCGAGTACCAGCAGTACCAGGACGCCA 1285
                * ******       *  *    *  **** *   ** *** ** **  *   *  *

T.c-alpha       CTGCGGACATGGACGGCGAG---GAGGACGTTGAGGAGTACTAG 1356
T.c-beta        CGATTGAGGAGGAAGGTGAGTTTGATGAAGAGGAGCAGTACTAG 1329
                *    **   *** ** ***   ** ** *  *** ********
```

An alignment of the translated ORF's of T. congolense α- and β-tubulin sequences using ClustalX 2.0.10 software is given below.

```
beta            MREIVCIQAGQCGNQIGSKFWEVISDEHGVDPTGTYQGDSDLQLER--INVYFDEATGGR 58
alpha           MREAICIHIGQAGCQVGNACWELFCLEHGIQPDGAMPSDKTIGVEDDAFNTFFSETGAGK 60
                *** :**: **.* *:*.  **::. ***::* *:  .*. : :*   :*.:*.*: .*:

beta            YVPRSVLIDLEPGTMDSVRAGPYGQIFRPDNFIFGQSGAGNNWAKGHYTEGAELIDSVLD 118
alpha           HVPRAVFLDLEPTVVDEVRTGTYRQLFHPEQLISGKEDAANNYARGHYTIGKEIVDLCLD 120
                :***:*::**** .:*.**:*.* *:*:*:::* *:..*.**:*:**** * *::*  **

beta            VCCKEAESCDCLQGFQICHSLGGGTGSGMGTLLISKLREQYPDRIMMTFSIIPSPKVSDT 178
```

```
alpha           RIRKLADNCTGLQGFLVYHAVGGGTGSGLGALLLERLSVDYGKKSKLGYTVYPSPQVSTA 180
                   * *:.*  **** : *::*******:*:**:.:*  :* .:  : ::: ***:** :

beta            VVEPYNTTLSVHQLVENSDESMCIDNEALYDICFRTLKLTTPTFGDLNHLVSAVVSGVTC 238
alpha           VVEPYNSVLSTHSLLEHTDVAAMLDNEAIYDLTRRNLDIERPTYTNLNRLIGQVVSSLTA 240
                ******:.**.*.*:*::* :  :****:**:  *.*.:  **: :**:*:. ***.:*.

beta            CLRFPGQLNSDLRKLAVNLVPFPRLHFFMMGFAPLTSRGSQQYRGLSVPELTQQMFDAKN 298
alpha           SLRFDGALNVDLTEFQTNLVPYPRIHFVLTSYAPVISAEKAYHEQLSVSEISNAVFEPAS 300
                .*** * ** ** :: .****:**:**.: .:**: *  .  :. ***.*::: :*:. .

beta            MMQAADPRHGRYLTASALFRGRMSTKEVDEQMLNVQNKNSSYFIEWIPNNIKSSVCDIPP 358
alpha           MMTKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWSPTGFKCGINYQPP 360
                **  .*****:*::.. ::** : .*:*:  : .::.*.:  *::* *..:*..:   **

beta            --------KGLKMAVTFIGNNTCIQEMFRRVGEQFTLMFRRKAFLHWYTGEGMDEMEFTE 410
alpha           TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEGMEEGEFSE 420
                          :: ** :*.*.*.* *:* *:..:* **: ::**:***.****:* **:*

beta            AESNMNDLVSEYQQYQDATIEEEGEFDEEEQY 442
alpha           AREDLAALEKDYEEVGAESADMDGEEDVEEY- 451
                *..::  * .:*::    : : :** * **
```

A BLASTp search of the translated ORF of *T. congolense* α-tubulin was performed and the results yielded a match of 97% sequence identity with *T. brucei* α-tubulin.

GENE ID: 4357179 Tb927.1.2340 | alpha tubulin [Trypanosoma brucei TREU927]
(10 or fewer PubMed links)

Score = 887 bits (2291), Expect = 0.0
Identities = 451/451 (100%), Positives = 451/451 (100%), Gaps = 0/451 (0%)
Frame = +1

```
Query  1     MREAICIHIGQAGCQVGNACWELFCLEHGIQPDGAMPSDKTIGVEDDAFNTFFSETGAGK  180
             MREAICIHIGQAGCQVGNACWELFCLEHGIQPDGAMPSDKTIGVEDDAFNTFFSETGAGK
Sbjct  1     MREAICIHIGQAGCQVGNACWELFCLEHGIQPDGAMPSDKTIGVEDDAFNTFFSETGAGK  60

Query  181   HVPRAVFLDLEPTVVDEVRTGTYRQLFHPEQLISGKEDAANNYARGHYTIGKEIVDLCLD  360
             HVPRAVFLDLEPTVVDEVRTGTYRQLFHPEQLISGKEDAANNYARGHYTIGKEIVDLCLD
Sbjct  61    HVPRAVFLDLEPTVVDEVRTGTYRQLFHPEQLISGKEDAANNYARGHYTIGKEIVDLCLD  120

Query  361   RIRKLADNCTGLQGFLVYHAVgggtgsglgalllERLSVDYGKKSKLGYTVYPSPQVSTA  540
             RIRKLADNCTGLQGFLVYHAVGGGTGSGLGALLLERLSVDYGKKSKLGYTVYPSPQVSTA
Sbjct  121   RIRKLADNCTGLQGFLVYHAVGGGTGSGLGALLLERLSVDYGKKSKLGYTVYPSPQVSTA  180

Query  541   VVEPYNSVLSTHSLLEHTDVAAMLDNEAIYDLTRRNLDIERPTYTNLNRLIGQVVSSLTA  720
             VVEPYNSVLSTHSLLEHTDVAAMLDNEAIYDLTRRNLDIERPTYTNLNRLIGQVVSSLTA
Sbjct  181   VVEPYNSVLSTHSLLEHTDVAAMLDNEAIYDLTRRNLDIERPTYTNLNRLIGQVVSSLTA  240

Query  721   SLRFDGALNVDLTEFQTNLVPYPRIHFVLTSYAPVISAEKAYHEQLSVSEISNAVFEPAS  900
             SLRFDGALNVDLTEFQTNLVPYPRIHFVLTSYAPVISAEKAYHEQLSVSEISNAVFEPAS
Sbjct  241   SLRFDGALNVDLTEFQTNLVPYPRIHFVLTSYAPVISAEKAYHEQLSVSEISNAVFEPAS  300

Query  901   MMTKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWSPTGFKCGINYQPP  1080
             MMTKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWSPTGFKCGINYQPP
Sbjct  301   MMTKCDPRHGKYMACCLMYRGDVVPKDVNAAVATIKTKRTIQFVDWSPTGFKCGINYQPP  360

Query  1081  TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEGMEEGEFSE  1260
             TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEGMEEGEFSE
Sbjct  361   TVVPGGDLAKVQRAVCMIANSTAIAEVFARIDHKFDLMYSKRAFVHWYVGEGMEEGEFSE  420
```

```
Query  1261  AREDLAALEKDYEEVGAESADMDGEEDVEEY  1353
             AREDLAALEKDYEEVGAESADMDGEEDVEEY
Sbjct  421   AREDLAALEKDYEEVGAESADMDGEEDVEEY  451
```

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