INVESTIGATION OF THE MOLECULAR ADJUVANT POTENTIAL OF *TRYPANOSOMA CONGOLENSE* BiP/HSP70 USING CONGOPAIN AS MODEL ANTIGEN

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PREFACE

The experimental work described in this dissertation was carried out at the School of Biochemistry, Genetics and Microbiology, University of KwaZulu-Natal, Pietermaritzburg, from January 2009 to December 2010, under the supervision of Prof. Theresa Coetzer.

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

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Sabelo G. Hadebe (candidate)

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Prof. Theresa H. T. Coetzer (supervisor)

DEDICATION

I would like to dedicate this work to my mother and my late uncle (Bhekizenzo Hadebe).

DECLARATION - PLAGIARISM

I, Sabelo Goodman Hadebe declare that

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

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

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

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ABSTRACT

African animal trypanosomiasis is a major threat to African agriculture causing a loss estimated to 4.5 billion US$ per annum. *Trypanosoma congolense* is the major causative agent in African animal trypanosomiasis and is transmitted by tsetse flies of the *Glossina spp*. Congopain, a major cathepsin L-like cysteine peptidase in *T. congolense* is associated with trypanotolerance in N'Dama cattle and is a target for an anti-disease vaccine. It is suggested that trypanotolerant cattle control the disease by antibody mediated neutralisation of congopain, and that immunisation of cattle against congopain can mimic trypanotolerance resulting in minimised disease pathology. Susceptible cattle immunised with recombinant catalytic domain of congopain, C2, produced high levels of anti-congopain IgG specific antibodies against congopain, maintained weight and exhibited less severe anaemia. However, there was no effect on the establishment of *T. congolense* infection and acute anaemia development in trypanosusceptible cattle. It has been suggested that failure of congopain to give full protection of the host may be due to poor presentation to the immune system by conventional adjuvants used in previous studies.

The aim of the present study was to improve the presentation of the catalytic domain of congopain (C2) to the immune system, by linking it to the proposed molecular adjuvant, BiP, an ER localised HSP70. A further aim was to localise the domain(s) of BiP where the adjuvant properties reside. BiP consists of an ATPase domain (ATPD), a peptide binding domain (PBD) and a C-terminal domain (C-term). Consequently, BiP69, BiP69 lacking the C-terminal domain (BiP60), BiP coding fragments (ATPD, PBD and C-term) and the C2 coding sequence were amplified by PCR from either genomic *T. congolense* DNA or plasmid DNA. The PCR products were each sub-cloned into a pTZ57RT vector, and C2 cloned into a pET-28a expression vector. The BiP coding fragments were inserted into the recombinant pET-28a-C2 vector, resulting in pET-28a-BiP69-C2, pET-28a-BiP60-C2, pET-28a-ATPD-C2, pET-28a-PBD-C2 and pET-28a-C-term-C2 coding chimeras. The fusion proteins were expressed in an *E. coli* system as insoluble inclusion bodies at the expected sizes of 96 kDa (BiP69-C2), 88 kDa (BiP60-C2), 47 kDa (PBD-C2), 34 kDa (C-term-C2) and 27 kDa (C2). However, the ATPD-C2 fusion protein was expressed at a larger and smaller size in different attempts. Protein expression was confirmed by western blots using anti-BiP antibodies and anti-congopain N-terminal peptide antibodies.

Recombinantly expressed peptide binding domain (PBD)-C2, C-terminus-C2, BiP69-C2, BiP60-C2 chimeras and a BiP69 fusion protein were purified and refolded by a Ni-NTA based one-step on-column refolding method. Bacterial proteins co-purifying with BiP69-C2 and BiP60-C2 chimeras were removed by incubation with 5 mM ATP in the dissociation buffer, but poor yields resulted in using these chimeras as non-pure proteins. Immunisation of Balb/c mice with the BiP69-C2 fusion protein chimera induced a higher antibody response to C2 compared to immunisation with the BiP69/C2 mixture or with C2 in Adjuphos/Quil A. BiP69-C2 and PBD-C2 chimeras and BiP69/C2 mixture induced a robust antibody response to BiP69, but no correlation could be made with the contribution to control of parasitemia and disease induced pathology. Mice immunised with BiP69-C2 and PBD-C2 chimeras showed a better booster effect of *T. congolense* infection with higher anti-C2 antibody stimulation compared to control groups. Immunisation did not change the establishment of *T. congolense* infection and anaemia development in most immunised groups. However, mice immunised with the BiP69/C2 mixture and with the PBD-C2 chimera produced anti-C2 antibodies possible contributing to clearing parasites 10 days and 16 days earlier respectively, than mice immunised with BiP69-C2, C-term-C2 and BiP60-C2 chimeras and PBS, C2 and C2 in Adjuphos/Quil A control groups and showed no clinical symptoms of the disease. There was no significant difference in percentage mice survival between BiP-C2 chimera immunised mice and control groups immunised with C2 alone or with a mixture of Adjuphos/Quil A or immunised with PBS.

In the present study, it was shown that BiP69 has adjuvant effects when linked to C2 and that its peptide binding domain acts as an adjuvant. It is possible that the removal of the C-terminal domain reduced the adjuvant potency of the peptide binding domain suggesting a prominent role in the adjuvant effect of the BiP molecule. Finding the exact role of the C-terminal domain in the adjuvant effect of BiP would be of utmost interest, and would involve comparing anti-C2 antibody response produced by immunisation with C2 linked to the peptide binding domain with or without the C-terminal domain. Future work includes repeating this study in trypanosusceptible cattle to confirm these findings.

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LIST OF ABBREVIATIONS

2×YT	2 × yeast tryptone medium
3-D	three-dimensional
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
AMC	7-amino-4-methylcoumarin
BCA	bicinchoninic acid
BiP	immunoglobulin heavy-chain binding protein
Bis-Tris	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol
BMGY	buffered media glycerol yeast extract
BMM	buffered minimal media
BSA	bovine serum albumin
C2	catalytic domain of congopain
CP	cysteine protease
dNTP	deoxynucleotide triphosphate
DTT	dithiothreitol
E-64	*trans*-epoxysuccinyl-L-leucyl-amido(4-guanidino)butane
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme-linked immunosorbent assay
g	relative centrifugal force
HRPO	horse radish peroxidise
HSP70	heat shock protein 70
IgG	immunoglobulin G
IgM	immunoglobulin M
IgY	immunoglobulin Y
IPTG	isopropyl-beta-D-thiogalactopyranoside
kDa	kilodalton
MEC	molecular exclusion chromatography
M_r	relative molecular mass
OD_{600}	optical density at 600 nm
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis

PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
RT	room temperature
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TPP	three phase partitioning
VAT	variable antigen type
VSG	variable surface glycoprotein
YNB	yeast nitrogen base
YPD	yeast peptone dextrose media
Z	benzyloxycarbonyl

LITERATURE REVIEW

1.1 INTRODUCTION

African trypanosomiasis is a devastating disease in sub-Saharan Africa caused by parasites of the genus *Trypanosoma* (Sakurai *et al.*, 2008). It is transmitted via tsetse flies of the *Glossina* species from one mammalian host to another (Roditi and Lehane, 2008; Schofield and Kabayo, 2008). Current control strategies of the disease include chemotherapy and vector control, which have proven thus far to be unsatisfactory (Delespaux and de Koning, 2007; Schofield and Kabayo, 2008; Molyneux *et al.*, 2010). There is an urgent need for the development of a safe, effective solution. Antigenic variation is a result of the expression of different variable surface glycoproteins (VSGs) (Morrison *et al.*, 2009). These VSGs cover the surface of the parasite, enabling them to escape the immune system, and therefore it is difficult to develop a VSG based vaccine against trypanosomiasis (Kabayo, 2002; Barry and McCulloch, 2009). The concept of an "anti-disease vaccine" was developed, which targets the disease rather than the parasite (Playfair *et al.*, 1990). Congopain is a major cysteine peptidase and a pathogenic factor of *T. congolense*, and has consequently been identified as an anti-disease vaccine candidate (Authié, 1994; Authié *et al.*, 2001). The catalytic domain of congopain (C2) has poor immunogenicity and hence provides a good model for adjuvant assessment (Authié *et al.*, 2001; Boulangé *et al.*, 2001; Boulangé *et al.*, 2011). Adjuvants play a vital role in antigen presentation and in enhancing an immune response. Aside from conventional adjuvants, some biological molecules have been described more recently, that can target the antigen directly to the effector cells of the immune system (Kornbluth and Stone, 2006). Heat shock proteins are chaperones, that bind to nascent and misfolded proteins, and have recently been shown to have adjuvant properties when complexed to antigens (Li *et al.*, 2007a; Li *et al.*, 2009). Heat shock protein 70 is the best studied of the HSP family and activates monocytes, induces cytokine production and up-regulates co-stimulatory molecules (Wang *et al.*, 2002; Lehner *et al.*, 2004; Tobian *et al.*, 2005; Ge *et al.*, 2006; Li *et al.*, 2006). Immunoglobulin binding protein (BiP) is a member of the HSP70 family, residing in the endoplasmic reticulum (ER) (Munro and Pelham, 1986; Boulangé and Authié, 1994), and just like other HSP70 family members is expected to have adjuvant properties. It consists of an ATPase domain (ATPD), a peptide binding domain (PBD) and a C-terminal variable region

(Robert, 2003; Lehner *et al.*, 2004). It is unclear as to which domain of the BiP molecule has adjuvant properties (Wang *et al.*, 2002; Ge *et al.*, 2006; Li *et al.*, 2006). The objective of this study is to establish which of the *T. congolense* BiP domains bear adjuvant effects when complexed to C2, a model antigen. Constructs were prepared in which the three BiP domains, BiP60 devoid of the C-terminal region and full length BiP69 were individually linked to C2 and expressed as His-tagged fusion protein chimeras in a bacterial system. The purified fusion protein chimeras were used in immunisation experiments and the antibody response to C2 was evaluated before and after infection with trypanosomes.

1.2 TRYPANOSOMIASIS

African trypanosomes are insect-borne haemoflagellate protozoan parasites, that cause 'sleeping sickness' in humans, and 'nagana' in domesticated animals in sub-Saharan Africa (Molyneux *et al.*, 1996; Kabayo, 2002; Roditi and Lehane, 2008; Sakurai *et al.*, 2008). Sleeping sickness is endemic to approximately 36 countries in sub-Saharan Africa and it is estimated that about 60 million people are at risk of infection with approximately 300 to 500 000 people suffering from human African trypanosomiasis (Fèvre *et al.*, 2008; Welburn *et al.*, 2009). Nagana affects more than 50 million cattle each year, with more than 10 million km^2 of potential grazing land in 37 countries not utilisable for farming (Shaw, 2004).

African trypanosomes are transmitted to the mammalian host by blood feeding tsetse flies of the *Glossina spp* (Akoda et al., 2008; Masumu et al., 2010). *Trypanosoma brucei gambiense* and *T. b. rhodeisiense* are causative agents of human African trypanosomiasis while *T. congolense*, *T. vivax*, *T. b brucei* and *T. simiae* are causative agents of nagana (Taylor, 1998; Sakurai *et al.*, 2008). Other vectors identified in transmitting trypanosomes are *Tabanus*, *Haematopoda*, *Chrysops* and *Stomoxys spp*., which transmit *T. evansi* in Asia, Central and South America, the Middle East and Africa via their faeces, and *T. cruzi* and *Leishmania* in South America (Hargrove, 2004; Luckins and Dwinger, 2004; Miles *et al.*, 2004; Rogers and Robinson, 2004; Baral, 2010). Rarely, transmission can also be through breastfeeding and contaminated foods (Hargrove, 2004; Miles *et al.*, 2004). In cattle, trypanosomiasis is characterised by high fever, chronic anaemia, enlarged liver and spleen, loss of appetite that results in weight loss, reduced fertility and increased abortions (Murray *et al.*, 1982; Authié *et al.*, 2001; Naessens *et al.*, 2002). Immunosupression and susceptibility to secondary infections

is common, and these animals often die of congestive heart failure (Tabel *et al.*, 2000; Naessens, 2006; Baral, 2010).

African trypanosomiasis is therefore a major threat to both animal based agriculture and human health. It is estimated that more than 4.5 billion US$ per annum is lost to African agriculture as a result of trypanosomiasis (Kristjanson *et al.*, 1999; Antoine-Moussiaux *et al.*, 2009). Furthermore, this hinders socio-economic development in the continent as a whole (Bhalla, 2002; Shaw, 2004; Salifu *et al.*, 2010).

1.3 BIOLOGY OF AFRICAN TRYPANOSOMES

1.3.1 Life cycle

The life cycle of *T. congolense, T. brucei* and *T. vivax* (Fig 1.1) is spent within an insect vector (the tsetse fly), and the bloodstream of a host mammal (Vickerman, 1985). Parasites undergo many changes during transmission from the mammal host to the tsetse fly and these changes are crucial for adaptation to the different environmental conditions they encounter (Sharma *et al.*, 2009). Adaptations include morphological, biochemical and motility changes, e.g. parasites change from a slender to a stumpy form, and change their energy source from proline, the main source of energy in the insect midgut, to glucose in the host bloodstream (Hendriks *et al.*, 2000; Hill, 2003; Sharma *et al.*, 2009).

After ingestion by the tsetse fly, the quiescent blood stream forms differentiate into actively dividing procyclic forms inside the tsetse midgut. The procyclic, elongated forms have high mobility and migrate to the tsetse proboscis, hypopharynx and finally to the lumen of the salivary glands. In the salivary glands, cell division is completed by differentiation into short metacyclic, stumpy forms suitable for mammalian infection. The metacyclic stumpy forms, or epimastigotes, attach to the salivary glands by a flagellar membrane and cytoskeletal connections (Fig. 1.1) (Vickerman *et al.*, 1988; Hill, 2003; Matthews, 2005; Sakurai *et al.*, 2008). The adherent metacyclic forms differentiate into non-adherent, metacyclic infective forms by a process known as ‘metacyclogenesis’ (Sakurai *et al.*, 2008; Sharma *et al.*, 2009). Variant surface glycoproteins are expressed at this stage to prepare for bloodstream invasion of the mammal host (Matthews, 2005).

Upon biting of the mammalian host by the infected tsetse, the metacyclic infective forms are injected intradermally, where it causes skin lesions or 'chancre' (Tabel *et al.*, 2000). They then further differentiate and proliferate quickly into slender bloodstream forms. Eventually they enter the blood stream via lymphatic vessels and spread throughout the circulatory system (Tabel *et al.*, 2000; Matthews, 2005). The blood stream slender forms are slowly replaced by the non-proliferative stumpy forms, ensuring longer survival within the host and improving chance of transmission (Hendriks *et al.*, 2000; Matthews, 2005).

1.3.2 Morphology

The trypanosome cell body (Fig. 1.2) is elongated and consists of a well organised arrangement of microtubules (Vickerman, 1985; Woods *et al.*, 1989; Matthews, 2005). The microtubule skeleton is inter-connected and fixed to the membrane via cross-bridges (Vickerman *et al.*, 1988). The cell shape is held together by the microtubule cytoskeleton which remains intact throughout the trypanosomal cell cycle (Matthews, 2005). The single copy organelles like the flagellum, flagellar pocket, kinetoplast, mitochondrion and nucleus are positioned within the cytoskeleton and are concentrated at the posterior and central regions of the trypanosome cell. The flagellum, which runs to the anterior cell edge, is attached to the posterior mouth of the flagellar pocket. The flagellum is responsible for cell motility and consists of the microtubule 'axoneme', trypanin, and a paraflagellar rod, which all have been shown to contribute to motility (Woods *et al.*, 1989; Hutchings *et al.*, 2002; Hill, 2003; Vaughan and Gull, 2003). The flagellum is connected to the cell body in a region called the 'Flagellum Attachment Zone' (FAZ) by trypanin, a microtubule binding domain (Woods *et al.*, 1989; Hutchings *et al.*, 2002; Matthews, 2005). Trypanin has been shown to be important in giving direction during mobility (Hutchings *et al.*, 2002; Hill, 2003).

The mitochondrion is a single elongated structure running from the posterior to the anterior of the cell. The mitochondrion is active in the procyclic forms and generates energy through the mitochondrion based respiratory system, whereas it is less active and tubular in the bloodstream forms and lacks cristea (Matthews, 2005). The position of the kinetoplast depends on the stage in the life cycle of the trypanosome. In the bloodstream forms they are in the posterior end, whereas in the procyclic forms they are midway between the cell nucleus and

the posterior membrane, and in epimastogotes they are anterior to the central nucleus. The reasons for trypanosome shape change remain unclear, but it has been shown to be necessary for cell division in different life cycle stages (Matthews, 2005).

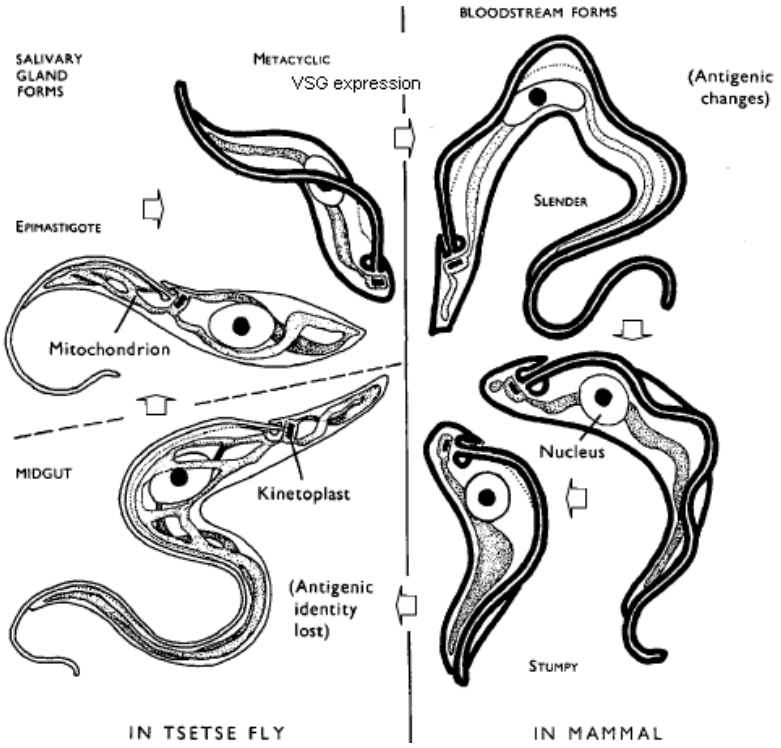

Figure 1.1: Life cycle of *Trypanosoma brucei* showing life cycle stages in the insect and mammalian host (Vickerman, 1969a).

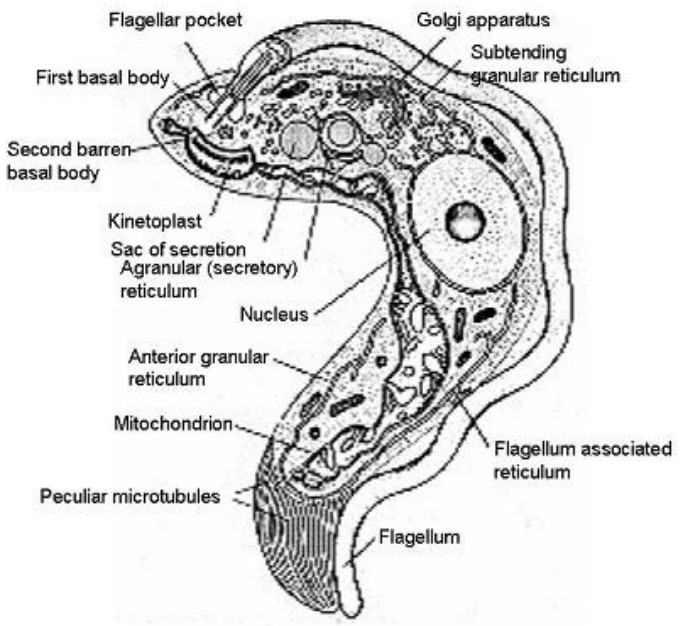

Figure 1.2: General structure of *Trypanosoma congolense* in bloodstream form (http://www.icp.ucl.ac.be/opperd/parasites/images/dia17 10.06.2009).

1.3.3 Host invasion

African trypanosomes are covered by variant surface glycoproteins which constitutes a highly antigenic surface coat. Trypanosomes have developed an efficient and complicated defence mechanism against the host immune response called 'antigenic variation' (Vickerman, 1969b; Donelson, 2003; Barry, 2004). 'Antigenic variation' is the phenomenon whereby a trypanosomal population spontaneously switches to expressing a new surface coat and continues to proliferate without being recognised by antibodies specific to the previous trypanosome population (Barry *et al.*, 1985; Barry, 1997). Each variant is distinct and is known as the 'Variant Antigen Type (VAT)'. It is the variation of these different VATs which provides protection against a specific host immune response (Vickerman, 1969b; Borst *et al.*, 1998; Barry *et al.*, 2003; Donelson, 2003).

1.3.4 Classification of trypanosomes

Trypanosome species have been classified on the basis of morphology, pathogenicity, host range, distribution and development cycle in the tsetse vector (Stevens and Brisse, 2004; Gibson, 2007). There have been many disputes over taxonomy of known trypanosome species, which has resulted in the demotion of some species to sub-species and the discarding of others (Haag *et al.*, 1998; Stevens and Brisse, 2004). Molecular taxonomy has brought new insights

in classification of tsetse transmitted trypanosome parasites (Stevens and Brisse, 2004; Njiru *et al.*, 2008b; Hide and Tait, 2009) Development of isoenzyme electrophoresis has enabled further and clearer characterisation of the trypanosome genus based on genetic variation of the 18S ribosomal RNA (Allsopp *et al.*, 1985; Hide *et al.*, 1990; Malele *et al.*, 2003). Levels of genetic divergence have been found in subgenera *Nannomonas* and *Duttonella*, while no clear boundaries are available in *Trypanozoon* subgenera. The taxonomy of trypanosomes is shown in Fig. 1.3. Trypanosomes belong to the phylum protozoa, family *Trypanosomatidae*, class *Zoomastigophora*, order *Kinetoplastida*, and genus *Trypanosoma* (Stevens and Brisse, 2004; Baral, 2010).

Trypanosomes are further sub-divided into two sections, Stercoraria and Salivaria based on the mode of transmission of metacyclic forms from vector to mammalian host. In Stercoraria, the metacyclic stage develops within the intestinal tract of the insect vector and is transferred through faeces. However, in the Salivaria, the metacyclic form is deposited in the proboscis of the vector and transmission occurs via saliva when the insect vector bites the mammalian host (Stevens and Brisse, 2004).

1.4 DIAGNOSIS OF AFRICAN TRYPANOSOMIASIS

Diagnosis of African trypanosomiasis has improved significantly in the past four decades. Currently, there are multiple diagnostic techniques for detecting African trypanosome infection (Eisler *et al.*, 2004; Chappuis *et al.*, 2005; Hide and Tait, 2009; Deborggraeve and Büscher, 2010). These techniques include clinical, parasitological, serological, and molecular methods (Eisler *et al.*, 2004). Early detection of trypanosomiasis allows for more effective control measures and is useful in studies of disease distribution, drug resistance, vector migration and eradication (Eisler *et al.*, 2004; Simarro *et al.*, 2008; Hide and Tait, 2009).

Clinical diagnosis is still regarded as the best available diagnostic method because of its simplicity and no requirement of specialist equipment, but is limited and requires verification by other diagnostic techniques. In parasitological detection methods, parasites can be directly detected in the host blood by microscopy, however this test is limited by low sensitivity at low parasitaemia levels (Uilenberg, 1998; Eisler *et al.*, 2004). In serological detection methods, antibodies specific to parasite surface proteins (Rebeski *et al.*, 2000) or trypanosomal specific

antigens (Rebeski *et al.*, 1999) are detected using tests such as indirect fluorescent antibody (IFA), enzyme linked immunosorbent assay (ELISA), and card agglutination test (CATT) (Eisler *et al.*, 1998; Hide and Tait, 2009; Radwanska, 2010). False positives and negatives, poor specificity, cross reactivity, lack of recombinant antigens and low sensitivity have been reported in tests for *T. congolense*, *T. vivax* and *T. brucei* (Masake and Vinand, 1991; Eisler *et al.*, 1998; Bossard *et al.*, 2010).

Molecular diagnosis methods by PCR have been useful where serological and parasitological diagnosis methods have failed, and have been shown to increase detection sensitivity by using DNA probes (Desquesnes and Dávila, 2002; Magona *et al.*, 2003; Njiru *et al.*, 2008a; Njiru *et al.*, 2008b; Deborggraeve and Büscher, 2010). This method identifies stable parasite genetic markers, which are present in all life cycle stages of trypanosomes, for example the internal transcribed spacer-1 (ITS-1) of highly conserved trypanosome ribosomal RNA (Masake *et al.*, 2002; Thumbi *et al.*, 2008). A more accurate trypanosomiasis diagnosis method is required if complete eradication or control of the disease is to be achieved. Although clinical and parasitological diagnosis methods are considered acceptable in field diagnosis, their low respective specificity and sensitivity, disqualify them as gold standard diagnostic methods. Single step ITS PCR is a promising alternative diagnostic method for animal trypanosomiasis, and in the near future may become the gold standard in epidemiology studies (Desquesnes and Dávila, 2002; Masake *et al.*, 2002; Thumbi *et al.*, 2008; Hide and Tait, 2009; Molyneux *et al.*, 2010).

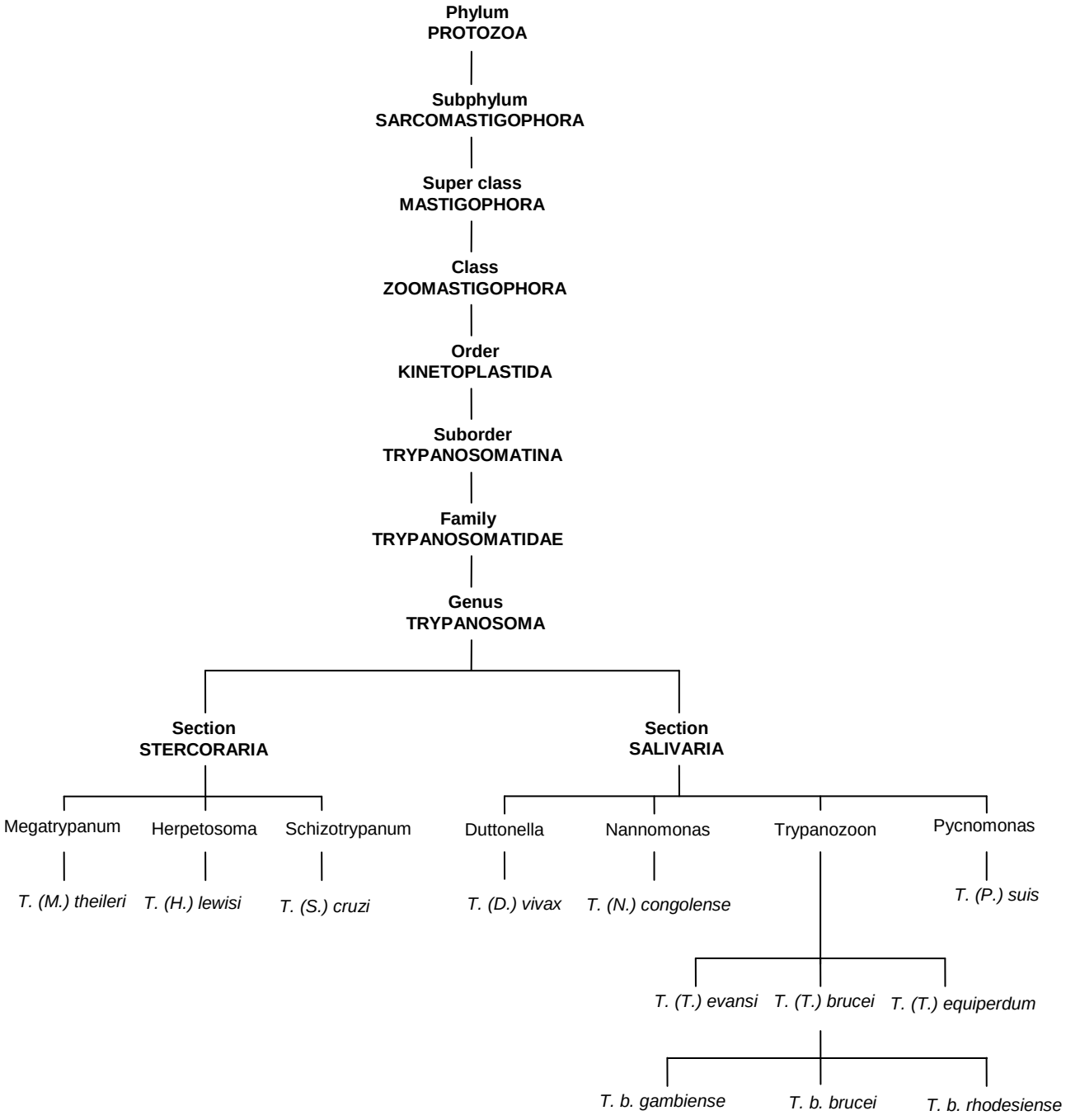

Figure 1.3: Classification of protozoan parasites belonging to the *Trypanosoma* genus. Adapted from Stevens and Brisse (2004).

1.5 CONTROL OF TRYPANOSOMIASIS

There have been various attempts to control trypanosome infection in Africa. Currently there are three control strategies: trypanocidal drugs (chemotherapy and chemoprophylaxis), breeding trypanotolerant cattle, and tsetse population control (McDermott and Coleman, 2001; Seed, 2001; Kgori *et al.*, 2006; Delespaux and de Koning, 2007; Molyneux *et al.*, 2010).

1.5.1 Vector control

Tsetse eradication attempts have included: clearing vegetation where tsetse flies rest, reducing host quantity (mostly wild mammals), development of odour baited tsetse fly traps, insecticides and sterilising male tsetse flies (Vreysen *et al.*, 2000; Aksoy, 2003; Welburn *et al.*, 2009). Initially insecticides proved to be successful due to the low population variability amongst tsetse and non-resistance. Unfortunately, non-selectivity of insecticide spraying, their toxicity and environmental contamination raised considerable safety concerns (Kgori *et al.*, 2006; Torr *et al.*, 2007). Fly traps and bait technology are manageable by small rural communities, but require commitment and integrated systems throughout the whole affected region (Eisler *et al.*, 2004; Vale and Torr, 2004; Van den Bossche and De Deken, 2004; Welburn *et al.*, 2009). The technique of sterilising male tsetse reduced the frequency of successful fertilisation of female flies and resulted in complete removal of tsetse flies from Zanzibar island (Vreysen *et al.*, 2000; Schofield and Maudlin, 2001; Aksoy, 2003). Limits to sustainability and monitoring allow re-infestation due to the rapid movement of tsetse communities from one place to the other. Tsetse eradication would require an integrated system throughout the sub-Saharan continent (Eisler *et al.*, 2004; Welburn *et al.*, 2009).

1.5.2 Trypanocidal drugs

Prophylactic and therapeutic trypanocidal drugs are the only effective methods used in the treatment and control of trypanosomiasis. In sub-Saharan Africa alone expenditure on trypanocidal drugs has been estimated at 13-35 million US$ per annum, and approximately 25-30 million drug doses are administered annually (Kristjanson *et al.*, 1999; Torr *et al.*, 2007). Currently there are three common trypanocidal drugs on the market: ‘Isometamidium’, ‘Homidium’ and ‘Diminazene’. The former two have prophylactic and therapeutic effects, whereas the latter only has therapeutic properties (Anene, 2001; Geerts *et al.*, 2001). These

drugs have been on the market for more than 50 years (Priotto *et al.*, 2006; Welburn *et al.*, 2009; Delespaux *et al.*, 2010; Molyneux *et al.*, 2010), and because of the slow progress in developing new drugs or alternative control strategies, this has led to monotherapy drug resistance and in some cases multi-drug resistance (Delespaux and de Koning, 2007; Delespaux *et al.*, 2008). Drug resistance has been reported in more than 17 African countries, and surveys conducted by the International Livestock Research Institute (ILRI) showed high incidence in East and West African countries (Delespaux *et al.*, 2008). Multi-drug resistance has been reported in Kenya and eastern Zambia (Mulugeta *et al.*, 1997; Delespaux and de Koning, 2007). The decentralisation of drugs to farmers and high cost of trypanocidal drugs has allowed suboptimal use by resource poor farmers, resulting in drug resistance (Anene, 2001; Geerts *et al.*, 2001; Welburn *et al.*, 2009). Mechanisms of trypanocidal drug resistance are not fully understood, but it is suspected that some animals are resistant to drugs simply because of a natural immunity (innate) or they are resistant due to prolonged exposure (acquired) (Holmes *et al.*, 2004). Cross-resistance and mutagenesis have not been excluded as mechanisms of resistance (Bray *et al.*, 2003; Delespaux *et al.*, 2010; Stewart *et al.*, 2010). Cross resistance may be due to the similarity between compound structures of drugs, so acquiring resistance to one may make the host resistant to multiple therapies (Delespaux *et al.*, 2010). Increasing drug dose and repeating treatments at short intervals are theoretical ways of dealing with drug and multi-drug resistance, however, this is limited by drug toxicity related side effects (Barrett *et al.*, 2004; Holmes *et al.*, 2004; Delespaux and de Koning, 2007).

Combination therapy has huge potential in bringing synergistic therapeutic effects, which may result in reduced drug dosage, increased efficacy, reduced resistance and minimised adverse effects (Anene, 2001; Delespaux and de Koning, 2007; Delespaux *et al.*, 2010).

With no sign of a successful anti-trypanosomiasis vaccine, and alarming levels of resistance to available drugs, there is an urgent need for the development of less host-toxic chemotherapeutic agents to fight the disease.

In this regard, proteins with prominent roles in the parasite life cycle e.g. major cysteine peptidases have been targeted for chemotherapeutic intervention (McGrath *et al.*, 1995; McKerrow *et al.*, 1995; Rosenthal *et al.*, 1996). The anti-parasitic effects of cysteine peptidase

inhibitors have been investigated in multiple protozoan parasites. Studies have shown that these inhibitors are capable of killing parasites or inhibiting specific active cysteine peptidases *in vivo* and/or *in vitro* e.g *T. cruzi* (Engel *et al.*, 1998), *T. congolense* (Mbawa *et al.*, 1992), *T. b brucei* (Troeberg *et al.*, 1999; Troeberg *et al.*, 2000), *P. falciparum* (Rosenthal *et al.*, 1996). Irreversible vinyl sulfones, peptidyl methylketones (CMK), fluoromethylketones (FMK) and chalcones have been shown to be lethal to bloodstream forms of *T. b brucei* parasites in culture at low concentrations (Troeberg *et al.*, 1999; Troeberg *et al.*, 2000).

Mice infected with *T. b brucei* and treated with micromolar amounts of vinyl sulfones, its derivatives or with small inhibitory molecules (Z-Phe-Ala-CNH_2), survived longer and cleared parasitaemia better than the untreated mice control group. Furthermore, chemotherapy altered trypanosomal cell division and morphology resulting in poor degradation of host proteins (Scory *et al.*, 2007; O'Brien *et al.*, 2008). Interestingly, some cysteine peptidase inhibitors have shown desirable pharmacokinetic and safety profiles in rodents, dogs and primates (Engel *et al.*, 1998; McKerrow, 1999; Keiser *et al.*, 2001; McKerrow *et al.*, 2008). These studies show cysteine peptidase inhibitors to have significant potential as chemotherapeutic agents in the treatment of diseases such as malaria, human and animal trypanosomiasis, leishmaniasis, schistosomiasis and Chagasis (Rosenthal *et al.*, 1996; McKerrow, 1999; Abdulla *et al.*, 2007; McKerrow *et al.*, 2008).

However, inhibition of host cysteine peptidases and the proteasome, and toxicity of fluoromethyl ketone derivatised peptidomimotopes have been a cause of concern in drug development. This has resulted in some irreversible inhibitors being withdrawn from drug development research/trials. It is still not understood, why some inhibitors are potentially lethal to livestock and humans, but have shown no toxic effects in rodents (McKerrow *et al.*, 2008).

Biochemical kinetics using specific inhibitors has paved a way of designing new synthetic drugs, which target and inhibit major cysteine peptidases of protozoan parasites. The crystal structures of cruzipain (McGrath *et al.*, 1995; Ljunggren *et al.*, 2007), rhodesain (Kerr *et al.*, 2009a; Kerr *et al.*, 2010), falcipain-2 (Kerr *et al.*, 2009b), and cathepsin B (Kerr *et al.*, 2010), have proved critical in understanding binding interactions of inhibitors to the active and

substrate binding sites of targeted peptidases. This has become the structural basis of designing new peptidomimotopes for use as chemotherapeutic agents.

1.5.3 Trypanotolerance

West African N'Dama cattle (*Bos taurus*) were introduced in Africa in 6000BC and through natural selection in the tsetse belt they have managed to attain a certain degree of tolerance against trypanosomal infection (Akol *et al.*, 1986; Naessens, 2006). This breed of cattle is able to survive without the aid of drug treatment in the humid and semi-humid areas of West and East Africa which have the highest incidence of trypanosomal infection (d'Ieteren *et al.*, 1998; Murray, 2004). Hence, trypanotolerance is described as the capacity to survive, and to remain productive after trypanosome infection (Murray *et al.*, 1982). Trypanotolerant N'Dama continues to gain weight, remain productive and develops a lesser severity of anaemia compared to the trypanosusceptible Zebu (*Bos indicus*), which often become weak, with high incidence of abortion and death (Authié *et al.*, 2001; Black *et al.*, 2001; Murray, 2004; Taylor and Authié, 2004; Naessens, 2006). Studies aimed at identifying the genetic markers responsible for trypanotolerance may bring new insights as to how resistance is induced (Naessens *et al.*, 2002; Naessens, 2006).

1.5.4 Vaccine against trypanosomiasis

Vaccination against trypanosomiasis is arguably the most desirable control method, with great potential impact. However, the antigenic variation of VSG coating the parasite has hampered attempts at producing an effective VSG based vaccine (Barry *et al.*, 1998; Donelson *et al.*, 1998; Donelson, 2003; Barry *et al.*, 2005). Vaccine development against disease remains an attractive approach to control with the increase in demand for safer and environmentally friendly control strategies (Authié, 1994; Taylor, 1998). An anti-disease vaccine strategy was developed as an alternative approach compared to methods mentioned in Section 1.5 for control of the disease (Playfair *et al.*, 1990; Authié, 1994). This strategy aims at inhibiting proteins implicated in pathogenesis, thereby halting disease progression. Invariant antigens are under research for their possible use in vaccine design. Invariable antigens expressed on the parasite surface and during the infection phase are often recognised by trypanotolerant cattle, and vaccination with these dominant proteins has been thought to be the optimal method for producing a sufficient immune response (Authié *et al.*, 1993b; Authié, 1994; Authié *et al.*,

2001). Partial protection has been obtained when cattle were vaccinated with flagellar pocket antigen fractions (Mkunza *et al.*, 1995) and purified tubulin (Lubega *et al.*, 2002; Li *et al.*, 2007b). Trypanosomal peptidases are also under investigation as anti-disease vaccine candidates, and there is promising evidence showing that immunisation with cysteine peptidases can confer significant protection against trypanosomiasis (Authié *et al.*, 2001), Chagasis (Schnapp *et al.*, 2002; Cazorla *et al.*, 2008a; Cazorla *et al.*, 2008b) and Leishmaniasis (Rafati *et al.*, 2006; Khoshgoo *et al.*, 2008).

1.6 KINETOPLASTID PAPAIN LIKE CYSTEINE PEPTIDASES

Peptidases are very important in protein catabolism, and are involved in many processes of living organisms. There are seven mechanistic classes of peptidases including aspartic, cysteine, glutamic, metallo, serine, threonine and mixed (Sajid and McKerrow, 2002; Caffrey and Steverding, 2009). Kinetoplastid cysteine peptidases are most important for survival, immunoevasion, enzyme activation, virulence, tissue and cellular invasion, hatching, development and moulting (Sajid and McKerrow, 2002; Mottram *et al.*, 2004; Caffrey and Steverding, 2009). Clan CA, family C1 cysteine peptidases are the most widely distributed among living organisms and are present in helminths (Robinson *et al.*, 2008; Smooker *et al.*, 2010), nematodes (Malagon *et al.*, 2010), bacteria (Shao *et al.*, 2002), protozoa (Sajid and McKerrow, 2002; Mottram *et al.*, 2004; Rosenthal, 2004; Ruszczyk *et al.*, 2008; Rodrigues *et al.*, 2010), plants (Solomon *et al.*, 1999) and mammals (Fernandes-Alnemri *et al.*, 1995). Kinetoplastid cysteine peptidases include cathepsin L-like and cathepsin B-like subfamilies, due to sequence identities with their mammalian homologues (Sajid and McKerrow, 2002). The main members of clan CA are lysosomal peptidases, optimally active in a weakly acidic and a reducing environment, and are sensitive to E-64 (Barrett *et al.*, 1982; Turk *et al.*, 2000; Lalmanach *et al.*, 2002; Serveau *et al.*, 2003). Lysosomal cysteine peptidases share similar amino acid sequences, structural folds and catalytic mechanisms. Their molecular weights range between 20 and 35 kDa (Turk *et al.*, 2000; Barrett, 2001; Sajid and McKerrow, 2002).

1.6.1 Congopain, a major cysteine peptidase of *Trypanosoma congolense*

Congopain is the major cysteine peptidase in *T. congolense* and belongs to the papain family (Authié *et al.*, 1992; Mbawa *et al.*, 1992; Lalmanach *et al.*, 2002). The 33 kDa peptidase is released in the bloodstream of the mammalian host. It has been implicated in host invasion, parasite development and differentiation, and plays a prominent role in mitigating the disease, producing a prominent antibody response in trypanotolerant cattle and also in susceptible cattle upon infection with *T. congolense* parasites (Authié *et al.*, 1993a; Authié, 1994; Authié *et al.*, 2001).

Two genes coding for *T. congolense* cathepsin L-like cysteine peptidase have been identified thus far. These genes are closely related and were named 'CP1' (Fish *et al*. 1995, EMBL accession number Z25813), and 'CP2' (Jaye *et al*. 1994, EMBL accession number L25130). Several polymorphic copies of the *T. congolense* cysteine peptidase gene are arranged in a tandem repeated array like those of *L. mexicana* (Souza *et al.*, 1992; Mottram *et al.*, 1997), *L. donovani* (Mundodi *et al.*, 2002; Hide and Bañuls, 2008) and *T. cruzi* (Lima *et al.*, 1994). There is emerging evidence for the existence of multiple variant genes, coding for cysteine peptidases in *T. congolense* (Kakundi, 2008; Mendoza-Palomares *et al.*, 2008; Pillay *et al.*, 2010).

More than 13 different genes have been identified coding for *T. congolense* cathepsin B-like peptidases and they have been grouped into clusters based on their catalytic triads (Mendoza-Palomares *et al.*, 2008). Some clusters have shown an unusual catalytic triad of Cys, Xaa and Asn. Two variants of congopain have been identified with substitutions in their catalytic triad where Cys is replaced by Ser and Asn is replaced by Tyr (Pillay *et al.*, 2010). These variants differ slightly in their substrate preferences, pH profiles and inhibitor kinetics compared to C2. It is likely that there are more cathepsin L-like peptidase gene copies embedded in this multiple tandem repeat array. There is 90% identity between the CP1 and CP2 gene, based on their amino acid sequences, although the N-terminal amino acid sequence and the catalytic domain residues differ (Boulangé *et al.*, 2001; Lalmanach *et al.*, 2002). The negative charge of CP2 relative to CP1 also accounts for their differences in kinetic parameters and pH activity profiles, suggesting distinct roles *in vivo* (Boulangé *et al.*, 2001).

Congopain has a similar sequence identity to cruzipain, a major cysteine peptidase in *T. cruzi*, sharing 54% ORF sequence identity (McKerrow *et al.*, 1995). The catalytic domain of congopain shares 68% sequence identity with that of cruzipain (Chagas *et al.*, 1997). A congopain 3-dimensional model has been constructed based on a cruzipain model and superimposition shows restricted specificity of congopain, with its catalytic domain buried inside a cleft between the R and L domains (McGrath *et al.*, 1995; Lecaille *et al.*, 2001). The structure of congopain is similar to that of cathepsin L-like enzymes and consists of two domains: the α-helical L-domain and the β-antiparallel sheet-containing R domain (Fig 1.4). The active site region is located in the V-shaped cleft between the R and L-domains and consists of the typical catalytic triad, Cys^{25}, His^{159} and Asn^{175} (papain numbering). These residues are highly conserved among cathepsin L-like enzymes. The catalytic triad, together with other substrate binding sites, determine substrate specificity (Turk *et al.*, 1998; Lecaille *et al.*, 2001; Lalmanach *et al.*, 2002; Akoda *et al.*, 2008).

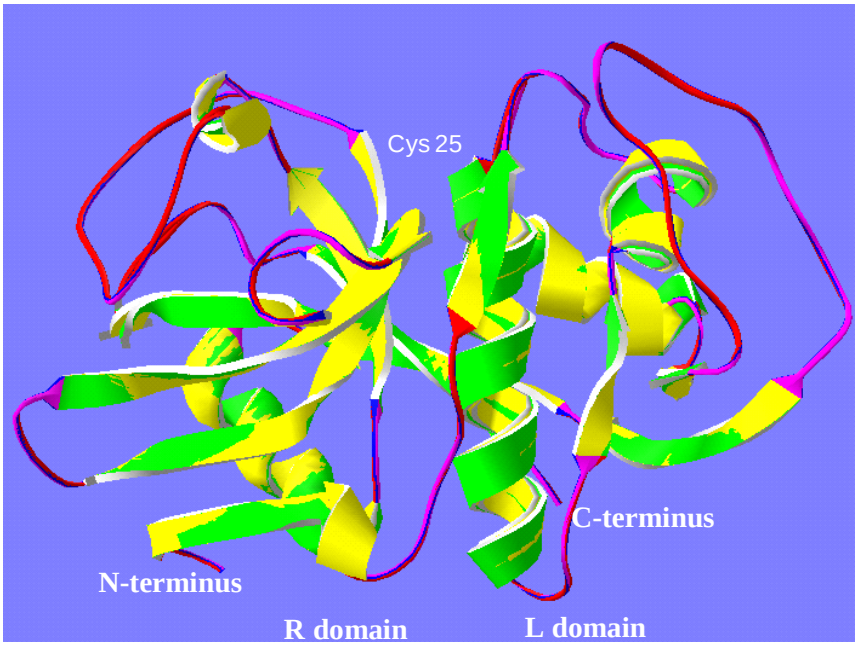

Figure 1.4: Predicted structure of the catalytic domain of congopain based on the sequence identity with cruzipain (Ndlovu, 2009). The 3-D model was coloured according to the secondary structure The green ribbon being congopain and yellow ribbon being cruzipain. The structure also shows α-helix L- domain and β-sheets R-domain. The v-shaped active site with the catalytic residues located between the R and L domains.

Congopain specificity is determined by the P_2/S_2 interaction (nomenclature according to Schechter and Berger (1967) and is shown to have preference towards bulky hydrophobic or aromatic residues in the P_2 site (Mbawa *et al.*, 1992; Turk *et al.*, 1998). The structure of

congopain is composed of a pre-domain, a pro-domain, central catalytic domain and a C-terminal extension (Authié, 1994). The catalytic domain (215 residues) is similar to mammalian cathepsin L, and differs from cruzipain in its enzymatic specificity, due to the presence of a Leu instead of Glu at position 205 in the S_2 subsite (McGrath *et al.*, 1995; Turk *et al.*, 1998; Caffrey and Steverding, 2009). The highly immunogenic C-terminal domain (105 residues) is linked to the catalytic domain by a poly-proline sequence, susceptible to proteolytic cleavage (Fig. 1.5). It has no effect on enzyme activity and its function is unknown (Chagas *et al.*, 1997; Boulangé *et al.*, 2001), although the *L. pifanoi* cathepsin L-like C-terminal extension has been linked to macrophage infection inside the host (Marin-Villa *et al.*, 2008). The pro-domain is involved in trafficking, secretion, and chaperoning the mature enzyme (Lalmanach *et al.*, 2002). The pro-domain contains the ERFNIN peptide embedded in the ER(F/W)N(I/V)N motif, specific to cathepsin L-like enzymes, and acts as a reversible competitive inhibitor of its mature enzyme (Lalmanach *et al.*, 1998). Synthetic peptides based on the pro-domain of cathepsin L-like enzymes, containing a common YHNGA sequence, inhibited purified congopain and cruzipan but did not inhibit lysosomal cathepsins L and B (Lalmanach *et al.*, 1998).

Congopain has been enzymatically characterised using conventional synthetic fluorogenic substrates (Authié *et al.*, 1992; Mbawa *et al.*, 1992; Chagas *et al.*, 1997). It shows proteolytic properties towards endopeptidyl substrates, preference to substrates with Arg or Lys in P_1 (Z-Arg-Arg-AMC), and hydrophobic amino acids in the P_2 site (Z-Phe-Arg-AMC) (Mbawa *et al.*, 1992; Chagas *et al.*, 1997; Gillmor *et al.*, 1997). Congopain is activated by reducing agents like DTT, L-cysteine and β-mercaptoethanol, irreversibly inhibited by E-64 and leupeptin, and reversibly inhibited by its natural inhibitors, cystatins and kininogens (Lalmanach *et al.*, 2002). Inhibition by natural inhibitors is similar to that observed in cruzipain, but congopain retains some of its activity in the host (Serveau *et al.*, 2003). Like mammalian cysteine peptidases, congopain is synthesised as an inactive zymogen that is activated into a mature form by autocatalytical cleavage and release of the pro-domain at acidic pH (pH<5.0) (Lalmanach *et al.*, 1998; Boulangé *et al.*, 2001; Lalmanach *et al.*, 2002; Serveau *et al.*, 2003). Congopain has been shown to be active at basic and neutral pH (8 and 6.5) (Boulangé *et al.*, 2001; Serveau *et al.*, 2003; Boulangé *et al.*, 2011).

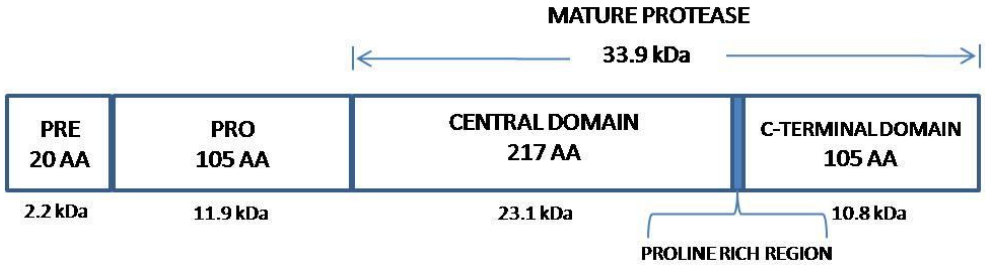

Figure 1.5: Schematic representation of *Trypanosoma congolense* cysteine peptidase (congopain). The structure is composed of the pre-domain, pro-domain, central catalytic domain, the poly-proline region and the C-terminal region according to the deduced amino acid sequence from Fish *et al*. (1995).

1.6.1.1 Congopain as a vaccine target

Trypanosomal peptidases are being investigated as possible anti-disease vaccine candidates (Authié, 1994). There is increasing evidence implicating congopain, a major cysteine peptidase of *T. congolense*, in the pathogenesis in trypanosomiasis (Authié, 1994; Authié *et al.*, 2001; Lalmanach *et al.*, 2002). Congopain has been shown to retain most of its activity at basic and neutral pH, suggesting its possible role in extracellular activities (Serveau *et al.*, 2003; Boulangé *et al.*, 2011). Trypanotolerant cattle produce a predominantly IgG response and proliferative response of lymph node cells to congopain, resulting in inhibition of enzyme activity, and thus neutralising its pathogenic effects (Authié, 1994; Lutje *et al.*, 1995a; Taylor, 1998). These findings imply that congopain is associated with trypanotolerance in cattle, and therefore, is an attractive target for an anti-disease vaccine strategy against bovine trypanosomiasis. It was proposed that an immune response targeting the catalytic domain of congopain (C2), produced through immunisation, could lead to the catalytic activity of congopain (C2) being completely inhibited by anti-congopain antibodies. A study using C2 as an antigen, expressed in the *Baculovirus* system (Boulangé *et al.*, 2001), showed that cattle were partially protected and mounted a highly specific IgG response to congopain. This suggests that antibody mediated inhibition of congopain activity could result in increased resistance to trypanosomiasis in susceptible cattle (Authié *et al.*, 2001). Studies have also shown that congopain has poor immunogenicity which results in only partial protection of cattle (Authié *et al.*, 2001; Boulangé *et al.*, 2001). Poor immunogenicity may be attributed to poor presentation of protective dimeric epitopes of congopain, poor delivery by the adjuvant and/or failure of the adjuvant to protect these protective dimeric epitopes within the host (Boulangé *et al.*, 2011).

1.7 ADJUVANTS

Vaccines would not be successful without the co-injection of adjuvants with the antigen to enhance humoral and/or cell mediated immune responses (Stills, 2005). All poorly immunogenic subunit and inactivated vaccines require immunostimulators or adjuvants to induce the desired type (cellular or humoral) and increased immune response (O'Hagan, 2001; O'Hagan *et al.*, 2001; Jansen *et al.*, 2006; Linghua *et al.*, 2006). The concept and use of adjuvants was discovered more than 90 years ago, and found to enhance the immune response of the host to the antigen (Schijns, 2001; Stills, 2005). Adjuvants are described as agents that stimulate the immune system response without acting as antigens (Singh and O'Hagan, 2003; Wilson-Welder *et al.*, 2008). Adjuvants increase the vaccine potency and reduce the amount of vaccine material required for disease protection; thereby decreasing toxicity, improving solubility and protecting conformation of the antigenic epitope (O'Hagan, 2001; Chamberlain and Gronvall, 2007). Adjuvants have five major mechanisms of action, which include, a depot effect, targeting of the antigen to immune stimulating cells, protection of the conformation of the antigen, presentation of the antigen to antigen presenting cells (APCs) and modulation of the type of immune response (Stills, 2005).

The depot effect protects the antigen from degradation, dilution and elimination by the host. It describes a process whereby the adjuvant localises and slowly releases an intact antigen, resulting in longer exposure of small amounts of antigen to the immune system. This results in host production of high levels of antibodies against the particular antigen (Stills, 2005). Adjuvants have specific features easily recognisable by the immune system, enhancing recognition and distribution of the antigen within the lymphatic system and spleen, resulting in stimulation of specific B- and T-cells (Janeway, 2001). Protection of the conformation of the antigenic epitope by the adjuvant is essential for proper immune induction (Chamberlain and Gronvall, 2007). Adjuvants have specific receptors on the APC surface and enhance presentation of the antigen to major histocompatibilty complex (MHC) molecules. Good adjuvants, used in therapeutic vaccines, induce protective immunity with minimal side effects, and protection is via humoral and/ or cell-mediated specific immune response (Stills, 2005). There are two types of adjuvants: conventional and molecular adjuvants.

1.7.1 Conventional adjuvants

Conventional adjuvants have been used effectively in traditional vaccines for years to enhance humoral and cell-mediated immunity adequate to confer protection. One of the first conventional adjuvants used successfully was an oil emulsigen with *Salmonella typhimurium* back in 1916. Later Freund's adjuvants were developed that included heat killed *Mycobacterium* (Jansen *et al.*, 2006). Conventional adjuvants are mostly chemically synthesised and include water-in-oil emulsions and oil-in-water emulsions. These two types differ slightly in oil content, antigen interaction and presentation, and viscosity (Jansen *et al.*, 2006). Some of the widely used water-in-oil emulsions include Freund's complete adjuvant and incomplete, Specol® (Prionics AG, Switzerland), RIBI® (Ribi ImmunoChem Research, Inc., Hamilton, MT, USA), Titermax® (CytRx Corp., Norcross, USA) which all stimulate the immune system in different ways. Currently, there are many water-in-oil adjuvants being tested in clinical trials as potential candidates in veterinary vaccines. The commonly known and probably most widely used conventional adjuvants are oil-in-water emulsigens. They include aluminium salts (Alum®) (Accurate Chemical & Scientific Co., USA), Syntex® (Chiron Corporation, USA) and Gerbu® (Biotechnik GmbH, Germany). Alum is the only conventional adjuvant that is permitted for use in both human and veterinary vaccines (O'Hagan *et al.*, 2001; Stills, 2005; Jansen *et al.*, 2006; Kornbluth and Stone, 2006). Though it is considered the safest and most desirable adjuvant so far, some studies have shown that it has poor adjuvanticity when co-administered with certain antigens and fails to induce cell-mediated, Th1 type and IgE mediated immune responses (Gupta, 1998).

Conventional adjuvants have been used effectively in traditional vaccines to enhance humoral and cell-mediated immunity adequate to confer protection, but the undesirable adverse side effects preclude their use in commercial vaccines (Gupta, 1998; Stills, 2005; Kornbluth and Stone, 2006; Linghua *et al.*, 2006). Most adjuvants lack good antigen presentation and distribution abilities and so there is poor recognition by the immune system. Furthermore, they induce side effects at the injection site including inflammation, lesions, granulomas, pain and late hypersensitivity, and thus are unsuitable for use in human and veterinary vaccines (Kornbluth and Stone, 2006). Therefore, there is an urgent need for much safer adjuvants inducing robust immune responses resulting in protection of the host.

1.7.2 Molecular adjuvants

There has been a great interest in the use of molecular adjuvants in vaccination. Molecular adjuvants include proteins, lipids, nucleic acids, carbohydrates and chemical compounds. They have specific receptors on the surface of APCs including dendritic cells (DCs) and macrophages (Stills, 2005). Molecular adjuvants maintain the antigen conformation, ensuring presentation of immunogenic epitopes to the immune system, resulting in improved magnitude and quality of the immune response (Kornbluth and Stone, 2006). They induce fewer side effects whilst prolonging antigen presentation to immune cells (Wilson-Welder *et al.*, 2008). This has brought about significant interest in identifying possible molecular adjuvants. One superfamily currently being investigated to this end is the heat shock proteins (Lindquist and Craig, 1988; Segal *et al.*, 2006).

1.8 HEAT SHOCK PROTEINS

Heat shock proteins (HSPs) are present in eukaryotes and prokaryotes and show a high degree of sequence phylogenetic conservation homology (Lindquist, 1986; Boulangé and Authié, 1994). Heat shock proteins are chaperones, binding to misfolded, non-native and nascent proteins, mediating their proper folding, trafficking, antigen processing and presentation (Lindquist and Craig, 1988; Hartl, 1996; Rico *et al.*, 1999; Nishikawa *et al.*, 2008). They are mainly released during periods of elevated stress (e.g. temperature change, UV light and chemical exposure) in order to protect cells from lethal damage (Hartl and Hayer-Hartl, 2002; Calderwood *et al.*, 2007). Furthermore, heat shock proteins have been shown to play a major role in stimulating the immune response directed towards tumour cell antigens, acting as adjuvants (Srivastava, 1994; Srivastava *et al.*, 1994; Udono *et al.*, 1994; Enomoto *et al.*, 2006). The heat shock protein family together with the grp family fall under the stress protein superfamily and are classified according to molecular weight, location and function (Lindquist and Craig, 1988). The heat shock protein family includes HSP27 (16-24 kDa), HSP60, HSP70 (68-73 kDa), HSP90 (85-90 kDa) and HSP110 (Pockley *et al.*, 2008), whereas the grp family includes gp96, GRP78, gp 170 and calreticulin (Table 1). These two families differ slightly in their location due to a retention signal on the C-terminus.

1.8.1 Heat shock protein 70

Heat shock protein 70 is the major and most studied of the HSP molecular chaperone family. The heat shock protein 70 sub-family includes 'Heat Shock Cognate' (HSC70), released under normal conditions, HSP70 induced under stressful conditions, and the endoplasmic reticulum (ER)-localised 'BiP' mainly responsible for housekeeping chaperoning functions (Munro and Pelham, 1986; Bangs *et al.*, 1993; Pidoux and Armstrong, 1993; Boulangé and Authié, 1994). Heat Shock Protein 70 has helped reveal characteristics of receptor mediated uptake by cells (e.g dendritic cells, macrophages) (Pockley *et al.*, 2008). HSP70 has both peptide dependent and independent immuno-stimulatory activities as it can be linked with antigens or injected along with antigens individually. It affects the adaptive immune system by direct presentation of antigenic peptides to APCs, or by stimulating antigen uptake via cross-presentation (Srivastava *et al.*, 1994; Nishikawa *et al.*, 2008). HSP70 activates monocytes, induces cytokines and up-regulates co-stimulatory molecules (Asea *et al.*, 2000; Moroi *et al.*, 2000; Wang *et al.*, 2001; Asea *et al.*, 2002; Mambula *et al.*, 2007).

Table 1: The location and function of molecular stress proteins (adapted from Calderwood *et al.*, 2007 and Pockley *et al.*, 2008).

Family	Location	Major Function (intracellular and extracellular)
HSP27*	Cytoplasm/ nucleus	Chaperone, anti-death and anti-inflammatory
HSP60*	Mitochondria	Chaperone, anti- and pro-inflammatory
HSP70*#	Cytoplasm/nucleus	Chaperone, pro-inflammatory, immunoregulatory
HSP90	Cytosol	Stabilise non-native, pro-immune
HSP110	Cytosol	Co-chaperone, pro-immune
Gp96/grp94	ER lumen	Substrate folding and stabilisation, pro-immune
GRP78/BiP	ER lumen	Chaperone, anti-inflammatory
Calreticulin	ER lumen	Chaperone, pro-inflammatory

* can be released under normal conditions

can be released under stress conditions

1.8.1.1 HSP70 structure and function

The structure of HSP70 consists of three functional domains (Fig. 1.6A): an N-terminal ATPase (44 kDa) domain, followed by a peptide binding domain (18 kDa) and a C-terminal (10 kDa) domain (Bukau and Horwich, 1998; Li *et al.*, 2006; Daugaard *et al.*, 2007). The structure of eukaryotic HSP70 is based on DnaK (Fig. 1.6B), an *E. coli* homolog of HSP70, the different domains of which have been crystallised independently (Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002). The ATPase domain has two lobes and a deep cleft binding ATP (Robert, 2003; Lehner *et al.*, 2004). Most substrate binding takes place in the ATP bound state, allowing opening of the peptide binding pocket. The substrate is stably bound to hydrolysed ATP (i.e. ADP plus free phosphate), which allows closing of the substrate binding pocket (Bukau *et al.*, 2000; Hartl and Hayer-Hartl, 2002; Bukau *et al.*, 2006).

The peptide/substrate binding domain is made up of a lower and upper, four stranded, anti-parallel β-sheet and a single α-helical sub-domain. The loops on the substrate binding site are positioned on the upper β-sheet, interacting with the substrate. The α-helical sub-domain has αA and βB helices which form hydrophobic side chains and interact with the β-sandwich (Robert, 2003; Lehner *et al.*, 2004).

The C-terminal domain of HSP70 is Gly/Pro rich, highly immunogenic and contains a localisation signal EEVD motif for cystosolic and nucleic HSPs. The motif enables binding to co-chaperones and other HSPs. The ER localised HSP70 members, like BiP/grp78, have an ER retention/retrieval signal or 'KDEL motif', which is highly conserved among ER resident proteins (Daugaard *et al.*, 2007). The variation/low degree of identity of the C-terminal region of the HSP70 sequence in different species has proved to be advantageous in vaccine development and has reduced the potential risk of autoimmune responses (Li *et al.*, 2006). The mechanism of how these three domains stimulate monocytes and dendritic cells is not clear. The C-terminal substrate binding domain has been shown to stimulate production of high concentrations of IgG2a and IgG3 subclasses of antibodies, and to attract immunological repertoires which enhance chemotactic cytokines (CC), chemokines, IL-2, TNF-α, nitric oxide and dendritic cell maturation (Wang *et al.*, 2002; Lehner *et al.*, 2004; Ge *et al.*, 2006; Li *et al.*, 2006; Zitzler *et al.*, 2008).

The ATPase domain has a stimulatory factor in antigen specific cytotoxic T cell induction, via the $CD4^+$ T cell independent pathway (Rico *et al.*, 1999; Nishikawa *et al.*, 2008; Pockley *et al.*, 2008; Zitzler *et al.*, 2008).

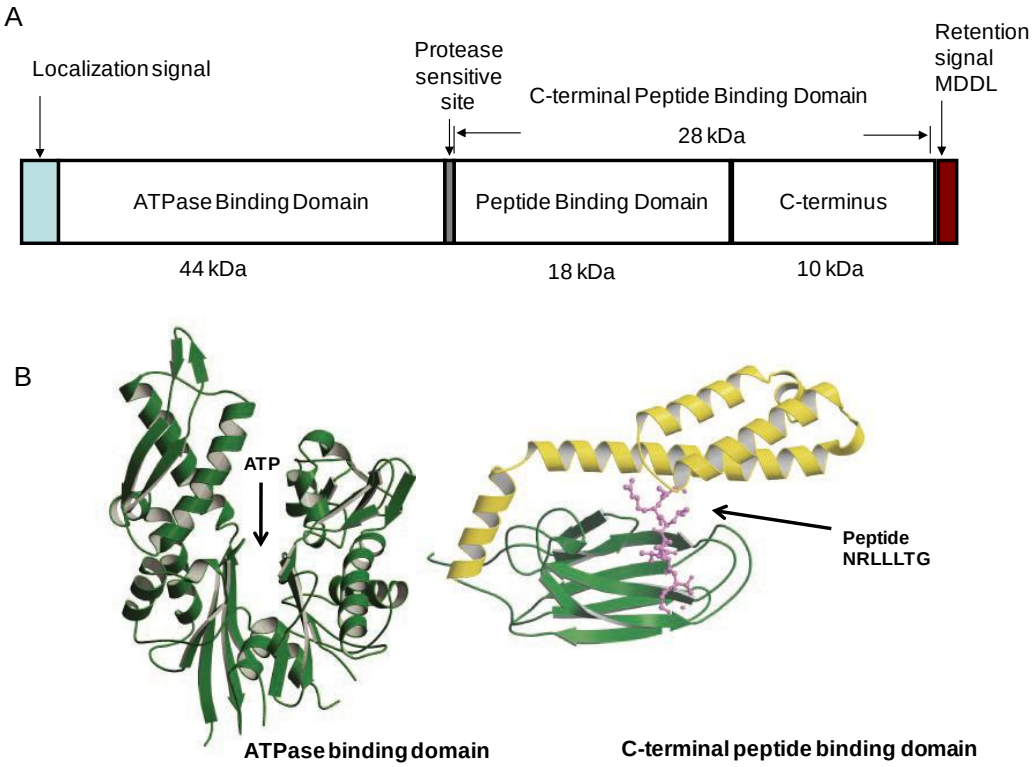

Figure 1.6: The structure of heat shock protein 70. A. The protein is composed of an N-terminal ATP binding domain, a peptide binding domain, a C-terminal variable region and the MDDL amino acid sequence adapted from Daugaard *et al*. (2007). **B**. Structures of the *E. coli* DnaK (HSP70) showing ATPase domain (green), and the peptide binding domain (yellow with green) and the α-helical latch of the peptide binding domain showing bound peptide (pink ball and stick model). ATP indicates the position of the ATP binding site (Hartl and Hayer-Hartl, 2002).

1.8.1.2 HSP70 binding receptors on Antigen Presenting Cells (APCs)

Heat shock protein 70 binds to antigenic peptides and the resulting HSP70 antigen-peptide complex is efficiently taken up by APCs possessing HSP70 receptors on their surfaces. These receptors include CD91 and 'lectin like-low density lipoprotein receptors 1 (LOX-1)' (Fig. 1.7) (Binder *et al.*, 2004; Massa *et al.*, 2005; Takemoto *et al.*, 2005; Calderwood *et al.*, 2007; Nishikawa *et al.*, 2008; Pockley *et al.*, 2008). CD91 is a member of the lipoprotein receptor family. It is expressed on fibroblasts, smooth muscle, macrophages and hepatocytes and is involved in HSP–antigenic peptide binding (Basu *et al.*, 2001). It has been suggested that CD91 is a common receptor for all immunogenic HSPs including HSP 60, 70, Gp96 and

calreticulin (Basu *et al.*, 2001). LOX-1 belongs to a scavenger receptor family, and recognises polyanions and HSP70 (Nishikawa *et al.*, 2008; Pockley *et al.*, 2008). It is expressed on endothelial cells, smooth muscle cells, fibroblasts, macrophages, and dendritic cells (Calderwood *et al.*, 2007). LOX-1 forms large complexes on the cell surface, and activation by lipopolysaccharides (LPS) can lead to secondary activation of Toll Like Receptor-2 (TLR-2) which signals multiple events of APC–T cell interaction (Asea *et al.*, 2002; Delneste *et al.*, 2002).

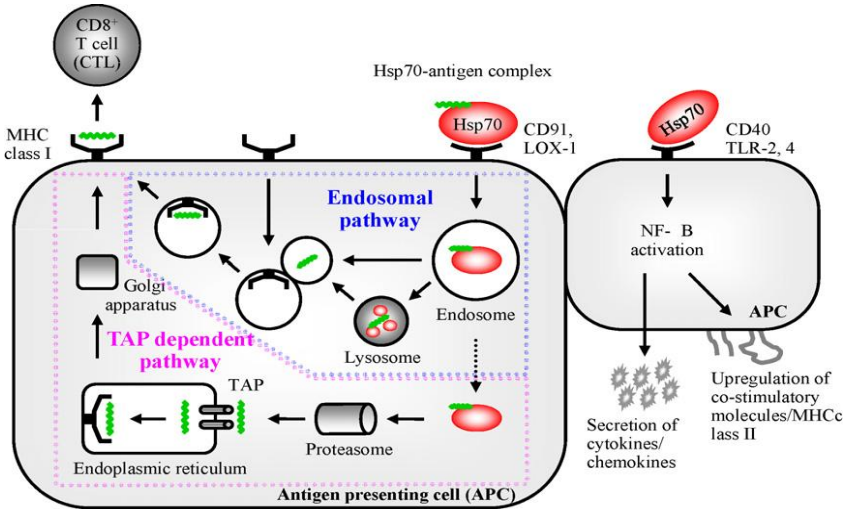

Figure 1.7: Heat shock protein 70-antigen complex uptake mechanism. HSP70-antigen complex is recognised by two receptors 'CD91' and 'LOX-1' and endocytosed for processing and presentation to MHC molecules. The internalised HSP70-antigen complex is processed by two pathways: the endosomal pathway and the TAP dependent pathway. HSP70 is recognised by co-stimulatory receptors such as CD40, TLR-2 and TLR-4 which signal activation of NF-κB, which induce cytokine and chemokine secretion (Nishikawa *et al.*, 2008).

1.8.1.3 Mechanisms of HSP70-antigen presentation

HSP70 binds to antigenic peptides through its C-terminal polypeptide-binding domain (Bukau and Horwich, 1998). HSP70-peptide complexes bind to APCs by binding to surface receptors such as CD91/LPR, LOX-1 and co-stimulatory receptors TLR (Moroi *et al.*, 2000; Basu *et al.*, 2001; Delneste *et al.*, 2002; Wang *et al.*, 2002; Nishikawa *et al.*, 2008). The HSP70-peptide complexes are internalised and processed within the APC endosomes (Fig. 1.7). The processed peptides are mainly loaded into MHC class II molecules for presentation to $CD4^+$ (helper) T-cells which stimulate and regulate the humoral immune response, i.e. antibody production (Udono *et al.*, 2001; Tobian *et al.*, 2005). It has been suggested that HSP70-peptide complexes could be miss-sorted from the endosomes into the cytoplasm for "cross-

presentation" by MHC class I molecules to $CD8^+$ (effector) T-cells (Robert, 2003; Nishikawa *et al.*, 2008). In the cytoplasm the HSP70-antigen complex is processed by the proteosome and the resulting peptide is transported by the transport associated protein (TAP) to the MHC class I molecule in the endoplasmic reticulum (Robert, 2003; Wang *et al.*, 2006b). The MHC-peptide complex molecule is trafficked to the cell surface of the APC for presentation. Cross-presented antigens are recognised by specific and $CD8^+$ (effector) T-cells which, when activated, elicit specific cell lysis (Blachere *et al.*, 1997; Massa *et al.*, 2005; Tobian *et al.*, 2005; Nishikawa *et al.*, 2008; Pockley *et al.*, 2008).

1.8.1.4 Adjuvant potential of HSP70

Heat shock proteins have been used successfully as adjuvants to improve the immune response in rodent trials against various antigens e.g. oncogenic peptides in a cancer vaccine (Srivastava, 1994; Udono *et al.*, 1994; Tamura *et al.*, 1997; Segal *et al.*, 2006; Zhang *et al.*, 2006), *Plasmodium falciparum* antigen in a malarial vaccine (Qazi *et al.*, 2005), viral proteins in Japanese Encephalitis virus, Foot and Mouth disease virus, Hantavirus, Hepatitis B virus, and HIV vaccines (Bogers *et al.*, 2004; Ge *et al.*, 2006; Li *et al.*, 2006; Wang *et al.*, 2006a; Li *et al.*, 2007a; Su *et al.*, 2007; Li *et al.*, 2009) and Chagas disease vaccine (Rico *et al.*, 1999; Planelles *et al.*, 2001; Morell *et al.*, 2006). In these studies heat shock proteins were co-injected with antigens, or as recombinant fusion proteins linked with the antigen. A more marked adjuvant effect was observed when HSP was covalently linked to the respective antigens.

A study was conducted to identify the domain of *Mycobacterium tuberculosis* HSP70 acting as adjuvant, and its role in enhancing the immune response when mixed or genetically linked to the major antigenic segment of Japanese Encephalitis Virus (JEV) "E protein" (Ge *et al.*, 2006). They also attempted to determine whether the truncated form of HSP70, i.e. peptide binding domain or (PBD), can act alone as efficiently as an adjuvant, as the whole HSP70 molecule. Results showed that linking E protein with PBD or HSP70 enhanced immunogenicity and elicited better humoral and cellular responses against E protein. Increased antigenicity was demonstrated with increased antibody titres, neutralising antibodies, increased lymphocyte proliferation, high levels of mIL-2 and mIFN-γ with E-PBD and E-HSP70 fusion proteins, when compared to E + HSP70, HSP70 alone and E protein alone.

Although, these fusion proteins were less effective compared to E protein administered with Freund's adjuvant, the significant adjuvant potential of *M. tuberculosis* HSP70 was clearly demonstrated.

It has also been thought that immunisation of mice with *T. cruzi* genes coding for KMP11 protein linked to HSP70, confers some degree of protection against late-stage Chagas disease in the absence of adjuvant (Planelles *et al.*, 2001). To confirm these findings Morell *et al*. (2006) conducted a study where *T. cruzi* paraflagellar rod proteins 2 and 3 (PFR-2 and PFR-3), a major component in the paraxial structure of the flagellum used for mobility and attachment to the host, were fused to the *T. cruzi* HSP70 gene. The resulting DNA plasmids were used in genetic immunisation of BALB/c and transgenic C57BL/6 mice.

Mice immunised with PFR-HSP70 fused genes had a significantly higher percentage of splenic cells expressing Th type 1 cytokines, IL-2 and IFN-γ, when compared to mice immunised with PFRs, HSP70 or vector alone. The presence of cytotoxic T lymphocytes (CTL) was measured as a marker indicating cellular lysis of target spleen cells (Jurkart-A2/k^d). Spleen cells in mice immunised with PFRs-HSP70 genes had the highest over-expression of PFRs and thus, highest CTL levels and cellular lysis. BALB/c mice immunised with PRFs and PFRs-HSP70 genes were infected with attenuated trypomastigote forms 10 weeks after their last immunisation. Mice immunised with PFRs and PFRs-HSP70 were able to control infection 21 to 28 days post-infection, whereas 25% of control mice immunised with vector alone or saline solution died at day 26.

1.8.2 Immunoglobulin Binding Protein (BiP).

Immunoglobulin binding protein (BiP) formerly known as grp78 (glucose regulatory protein 78) is a member of the HSP70 family of protein chaperones, and it resides in the cell ER. BiP is involved in translocation and trafficking of nascent secreted and membrane bound polypeptides from the cytoplasm into the ER, and also helps with their folding. BiP is an abundant protein that is expressed at increased levels in response to glucose deprivation, high levels of stress and is normally heat induced (Jensen *et al.*, 2002; Labriola *et al.*, 2011). BiP binds to newly synthesised polypeptides through cycles of binding ATP and releasing low protein affinity ADP (Vogel *et al.*, 1990; McCarty *et al.*, 1995; Hartl, 1996; Hartl and Hayer-

Hartl, 2002). *In vitro* BiP prefers heptapeptides with aliphatic residues, and normally binds to slow folding and multi-subunit proteins (Hellman *et al.*, 1999). BiP has been identified to be present in eukaryotes including rats (Munro and Pelham, 1986), humans (Panayi and Corrigall, 2006), nematodes (Heschl and Baillie, 1990), yeast (Vogel *et al.*, 1990; Pidoux and Armstrong, 1993), plants (Anderson *et al.*, 1994) and protozoan parasites including *P. falciparum* (Kumar *et al.*, 1991; Kumar and Zheng, 1992), *L. donovani* (Jensen *et al.*, 2002), *Babesia caballi* (Ikadai *et al.*, 2005), *T. brucei* (Bangs *et al.*, 1993), *T. cruzi* (Tibbetts *et al.*, 1994; Labriola *et al.*, 2011), and *T. congolense* (Boulangé and Authié, 1994). BiP of *T. congolense*, just like other HSP70 family proteins, has chaperone properties, binding to nascent polypeptides and being involved in their proper folding.

Trypanosoma congolense BiP is one the most abundant proteins expressed in all life cycle stages of the parasite (Authié *et al.*, 1993b; Boulangé and Authié, 1994; Grébaut *et al.*, 2009). A 69 kDa BiP was identified by blotting *T. congolense* lysates with sera from infected and uninfected cattle (Authié *et al.*, 1993b). Trypanotolerant and susceptible cattle showed differing antibody isotypes against 69 kDa BiP. This brought interest in identifying BiP as a possible antigen for use in both immunodiagnostics and an anti-disease vaccine strategy (Boulangé and Authié, 1994). The cDNA of BiP was immunoscreened and sequenced. It was found to be homologous to mammalian immunoglobulin binding protein, part of the heat shock protein 70 family, and named "R69BiP" (Boulangé and Authié, 1994). Its open reading frame of 2.35 kb, coding for 653 amino acid residues was identified and cloned in a pMAL expression vector system (Boulangé and Authié, 1994). The 69 kDa BiP was evaluated for its immunodiagnostic potential by cloning different fragments of its ORF and subsequently expressing them (Boulangé *et al.*, 2002). The different fragments of BiP were tested by dot blot technique, using anti-BiP antibodies raised in rabbits, and sera from infected and uninfected cattle. It was found that most antibodies recognised the species specific, variable C-terminal extension and had poor detection of primary infections of trypanosomiasis (Boulangé *et al.*, 2002). This diminished potential of BiP as an immunodiagnostic target was exacerbated by the highly conserved sequence identity of its ATPase and peptide binding domains to mammalian BiP (Boulangé and Authié, 1994). Development of an inhibition ELISA based on full length BiP, using a specific monoclonal antibody has shown improved sensitivity in secondary infection(s) of cattle in tsetse infested areas. This serological test is able to

differentiate between species, uses low cost reagents and is likely to be used in large scale screening of cattle (Bossard *et al.*, 2010).

BiP is also thought to have protective capabilities when mice are immunised with either recombinant BiP or a DNA coding for BiP (Jensen *et al.*, 2002). The protective efficiency of recombinant BiP co-administered with Freund's adjuvant and *L. donovani* DNA coding for BiP/Grp78 was tested separately in mice (Jensen *et al.*, 2002). Following immunisation, mice were infected with *L. donovani* promastigotes. Protective ability was measured by the number of skin lesions formed in each group and the time taken for healing. Mice immunised with recombinant BiP had significantly fewer lesions compared to controls, immunised with either GST or PBS. Only one BiP immunised mouse developed lesions, which healed after six weeks, whereas in control experiments six mice out of seven developed lesions requiring 7-10 weeks to heal. The parasitaemia burden in lymph nodes was cleared six weeks post-infection in vaccinated mice, except for one mouse which had a parasitaemia burden comparable to that of control mice at week ten. DNA vaccination showed similar results. Recombinant BiP and DNA coding for BiP were almost 100% protective against development of skin lesions suggesting that memory B- and T-cells developed, which elicited a rapid, strong immune response upon infection.

The present study was aimed at finding whether *T. congolense* BiP has adjuvant effects, and if so, which domain(s) would possess adjuvant properties. Congopain catalytic domain showing poor presentation to the immune system by conventional adjuvants, was to be used as a model antigen to assess the adjuvant properties of BiP.

1.9 OBJECTIVES OF THE STUDY

Previous studies in our laboratory, aimed at improving the immunogenicity of congopain, focussed on complexing bovine or rabbit α_2-macroglobulin (α_2M) with C2 (Huson *et al.*, 2009) or immunisation of C2 with different conventional adjuvants (Kateregga *et al*, In preparation[1]). Antibodies against C2 produced in rabbits immunised with a C2:α_2M complex consistently inhibited approximately 65% of C2 and congopain activity. However, antibodies against C2, produced in rabbits immunised with C2 alone, showed no inhibition of C2 or congopain. Although immunisation of cattle and mice with C2 co-administered with a conventional adjuvant (Quil A), gave a robust immune response inhibiting C2 and congopain activity, these antibodies failed to give full protection of the mammalian host upon infection with live parasites (Authié *et al*., 2001; Kateregga *et al*., In preparation). These findings show that there is an urgent need for a better delivery system, with improved targeting and presentation of C2 to immune cells, resulting in enhancement of the immune response and full protection of the host against trypanosomiasis.

The present study aimed to improve the delivery and presentation of C2 to the immune system by genetically linking C2 to BiP, a proposed molecular adjuvant. The first part of this study involved cloning of the congopain catalytic domain, C2, in a pET-28 expression vector, and subsequent sub-cloning each of the BiP fragments: ATPase domain, peptide binding domain (PBD), C-terminus as well as BiP69 and BiP60 into the same vector. The resulting His-tagged BiP-C2 fusion protein chimeras were expressed in an *E. coli* bacterial system (Chapter 2). The second part of this study focussed on purification of His-tagged BiP-C2 fusion protein chimeras and BiP69 followed by immunisation of BALB/c mice with the purified proteins, to assess the adjuvant potential of the BiP fragments when linked to C2 (Chapter 3). The anti-C2 antibody production, anaemia development, parasitemia levels, and percentage mice survival were compared between mice immunised with the BiP-C2 chimeras and mice immunised with C2 admixed with BiP69 or Adjuphos/Quil A. These results are finally discussed and possible future work proposed (Chapter 4).

[1] Kateregga, J., Lubega, G.W., Lindblad, E.B., Authié, E., Coetzer, T.H.T., Boulangé, A.F.V. Effect of GERBU®, Adjuphos®, purified saponin Quil A®, Freund's and α_2M adjuvants on the humoral immune response to congopain in mice and cattle.

2 CLONING AND EXPRESSION OF BIP-C2 FUSION PROTEIN CHIMERAS.

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ABSTRACT

Trypanosoma congolense is the major causative agent of African animal trypanosomiasis. The disease costs African agriculture an estimated 4.5 billion US$ per annum. Current control strategies are not effective and there is no vaccine against the disease. Congopain, a major cathepsin L-like cysteine protease of *T. congolense* is a target for an anti-disease vaccine, and showed partial protection in cattle. To improve the immunogenicity of C2, BiP, a 69 kDa HSP70 chaperone member, was considered as a molecular adjuvant. BiP comprises an ATPase domain (ATPD), a peptide binding domain (PBD) and a C-terminal domain (C-term). The aim of this study was to produce BiP-C2 fusion protein chimeras to identify the specific domain(s) of BiP bearing adjuvant effects. To this end BiP69, BiP69 lacking the C-terminal domain (BiP60), BiP coding fragments (ATPD, PBD and C-term) and congopain catalytic domain coding sequence (C2) were amplified by PCR from either genomic *T. congolense* DNA or plasmid DNA. The PCR products were each sub-cloned into a pTZ57RT vector, and C2 cloned into a pET-28a expression vector. The BiP coding fragments were inserted into the recombinant pET-28a-C2 vector, resulting in pET-28a-BiP69-C2, pET-28a-BiP60-C2, pET-28a-ATPD-C2, pET-28a-PBD-C2 and pET-28a-C-term-C2 coding chimeras. The fusion proteins were expressed in an *E. coli* system as insoluble inclusion bodies at the expected sizes of 96 kDa (BiP69-C2), 88 kDa (BiP60-C2), 47 kDa (PBD-C2), 34 kDa (C-term-C2) and 27 kDa (C2). However, the ATPD-C2 fusion protein was expressed at a larger and smaller size in different attempts. Protein expression was confirmed by western blots using anti-BiP antibodies and anti-congopain N-terminal peptide antibodies.

2.1 INTRODUCTION

Trypanosomiasis is a devastating disease affecting both man and animals mainly in Sub-Saharan Africa, South America and to a lesser extent Asia (Rogers and Robinson, 2004; Stevens and Brisse, 2004; Sakurai *et al.*, 2008). Sleeping sickness is caused by *Trypanosoma*

brucei gambiense and *T. b rhodeisiense*, while nagana is caused by *T. congolense*, *T. vivax* and to a lesser extent *T. b. brucei* (Hide, 1999; Cox, 2004; Stevens and Brisse, 2004). Nagana remains a major constraint to livestock-based agriculture and has significant impact on millions of people in developing African countries (Fèvre *et al.*, 2008; Hide and Tait, 2009) Current control strategies including trypanocidal drugs, tsetse control and trypanotolerant cattle are inefficient, due to drug resistance, environmental concerns and reduced economic production (Kabayo, 2002; Kgori *et al.*, 2006; Delespaux and de Koning, 2007; Torr *et al.*, 2007). One control strategy that remains most attractive with significant potential impact, if it could be developed, is vaccination (Welburn *et al.*, 2009; Baral, 2010). However, variable surface glycoproteins coating parasites hamper vaccine development (Barry and McCulloch, 2009; Morrison *et al.*, 2009; Baral, 2010). Alternative anti-disease strategies are being pursued where pathogenic factors are targeted for chemotherapy and/or vaccine development, to halt disease progression rather than killing the parasite (Playfair *et al.*, 1990; Playfair, 1991; Authié, 1994; Antoine-Moussiaux *et al.*, 2009; Baral, 2010).

In this context, kinetoplastid cysteine proteases are being considered as possible anti-disease vaccine candidates (Authié *et al.*, 2001; Rafati *et al.*, 2006; Cazorla *et al.*, 2008b). There is increasing evidence implicating congopain, a major cysteine proteinase of *T. congolense*, in the pathogenesis of trypanosomiasis (Authié, 1994; Authié *et al.*, 2001; Lalmanach *et al.*, 2002). Trypanotolerant N'Dama cattle produce prominent IgG responses against congopain (Authié, 1994; Taylor, 1998). This suggests that trypanotolerant cattle control the disease by antibody mediated neutralisation of congopain, and that immunisation of cattle with congopain can mimic trypanotolerance resulting in minimised disease pathology (Authié, 1994). Susceptible cattle immunised with recombinant catalytic domain of congopain (C2) produced high levels of anti-congopain IgG, maintained weight and exhibited less severe anaemia (Authié *et al.*, 2001). However, there was no effect on the establishment of *T. congolense* infection and acute anaemia development in trypanosusceptible cattle (Authié *et al.*, 2001). This study, also suggested that congopain is poorly presented by conventional adjuvants resulting in partial protection of cattle against trypanosome infection (Authié *et al.*, 2001; Boulangé *et al.*, 2001; Boulangé *et al.*, 2011).

Attempts to improve the effectiveness of congopain inhibition by antibodies have been made where bovine and rabbit α_2-macroglobulin were complexed with C2 in separate experiments (Huson *et al.*, 2009). Antibodies from rabbits immunised with a C2-α_2-macroglobulin complex inhibited approximately 65% of congopain activity whereas antibodies against C2 raised in rabbits immunised with C2 alone showed no significant inhibition of C2 or congopain activity (Huson *et al.*, 2009). Rabbit and bovine α_2-macroglobulin gave the same results. Immunisation of cattle with C2 co-administered in different conventional adjuvants mounted high antibody responses, but failed to mitigate disease progression (Kateregga *et al*. In preparation). These findings show that there is a need for an agent to improve delivery of C2 to the immune system resulting in enhanced immune response and full protection of the host against trypanosomiasis.

Adjuvants increase the vaccine potency and reduce the amount of vaccine material required for disease protection; thereby decreasing toxicity, improving solubility, and protecting immunogenic epitopes. Good adjuvants, used in therapeutic vaccines, induce protective immunity with minimal side effects, and protection is via humoral and/ or cell-mediated specific immune responses (O'Hagan *et al.*, 2001; Stills, 2005; Chamberlain and Gronvall, 2007). There is a demand for much safer, cheaper, efficient adjuvants, giving robust immune responses (O'Hagan, 2001; O'Hagan *et al.*, 2001; Singh and O'Hagan, 2003). Molecular adjuvants have recently gained popularity in eradication of diseases compared to conventional adjuvants due to their efficiency, safety and reasonably ease to produce (Kornbluth and Stone, 2006; Wilson-Welder *et al.*, 2008).

Heat Shock Proteins (HSPs) are highly conserved chaperones involved in protein trafficking and proper folding of misfolded and nascent peptides (Lindquist and Craig, 1988; Hartl, 1996; Hartl and Hayer-Hartl, 2002). They have also been shown to play a major role in stimulating the immune system towards complexed antigens or when co-injected with antigens without adjuvants (Qazi *et al.*, 2005; Li *et al.*, 2006; Li *et al.*, 2007a; Karyampudi and Ghosh, 2008). The best studied class of HSPs is HSP70 and it is classified according to its localisation i.e. in the endoplasmic reticulum (ER), cytosol or membrane bound, and its secretion during stressful or non-stressful conditions (Calderwood *et al.*, 2007; Pockley *et al.*, 2008; Määttänen *et al.*, 2010). Generally, the HSP70 structure is made up of a conserved N-terminal ATPase domain

(44 kDa), a peptide binding domain (18 kDa) and a variable C-terminal domain (10 kDa) which is species specific (Hartl and Hayer-Hartl, 2002; Robert, 2003; Lehner *et al.*, 2004; Daugaard *et al.*, 2007).

Immunoglobulin binding protein (BiP) is a member of the HSP70 family of chaperones, and resides in the ER (Vogel *et al.*, 1990; Panayi and Corrigall, 2006; Määttänen *et al.*, 2010). *Trypanosoma congolense* BiP is one the most dominant proteins expressed in all life cycle stages of the parasite (Boulangé and Authié, 1994; Grébaut *et al.*, 2009). A 69 kDa BiP was identified by blotting *T. congolense* lysates with sera from infected and uninfected cattle (Authié *et al.*, 1993b). Trypanotolerant and trypanosusceptible cattle showed differing antibody isotypes against the 69 kDa BiP, which brought interest in evaluating BiP as a possible antigen to be used in both immunodiagnostics and anti-disease vaccine strategies (Boulangé and Authié, 1994; Boulangé *et al.*, 2002; Bossard *et al.*, 2010).

The present study is aimed at producing recombinant BiP domain-C2 chimeras for evaluating the adjuvant potential of *T. congolense* BiP to enhance the immune response against congopain for improved disease control by the host. The catalytic domain of congopain has been shown to express in high amounts in the eukaryotic *P. pastoris* system from where it is easy to purify (Boulangé *et al.*, 2011). Efforts to express C2 in a bacterial system have proved difficult as the protease expresses as inclusion bodies, making it difficult to renature (Boulangé *et al.*, 2001). BiP has been expressed in a bacterial system as a soluble fusion protein with maltose binding protein, MBP, (Boulangé *et al.*, 2002). Expression of constructs of *T. congolense* BiP linked to C2 have been attempted in both bacterial and yeast systems (Ndlovu, 2009). BiP-C2 chimera-MBP fusion proteins were expressed as inclusion bodies in a bacterial system and proved difficult to purify and renature, while expression of the same chimeras in a yeast system was very poor (Ndlovu, 2009). The inclusion of the pro-domain of congopain in most constructs led to maturation of congopain into its catalytic active form during purification and this resulted in complete proteolysis of chimeras. In the present study the pro-domain of congopain was consequently omitted from all constructs. Since the C-terminal region of congopain is highly immunogenic, is unlikely to produce antibodies that can contribute to the inhibition of congopain activity and can misdirect the immune response away from the catalytic domain, it was also omitted from constructs.

Different domains of BiP i.e. the ATPase domain, the peptide binding domain, the C-terminal domain, full length BiP69 and BiP60 (BiP69 devoid of its C-terminal domain) were separately linked to the N-terminus of C2. Here the cloning of these constructs in a pET-28a expression vector and their expression as His-tagged fusion chimeras in a bacterial system is reported.

2.2 MATERIALS AND METHODS

Materials

Buffer salts and other commonly used chemicals were obtained from Merck (Johannesburg, South Africa) and Sigma-Aldrich (Munich, Germany) and were of the highest purity available. Coomassie blue R-250, 4-chloro-1-naphthol, L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane (E-64), ampicillin and kanamycin were purchased from Sigma (Munich, Germany). DNA markers: O'Gene® 1 kb mix, FastRuler® High range and Middle range molecular mass markers, EcoRI, NotI, NdeI, shrimp alkaline phosphatase (SAP), T4 DNA ligase, DNA dilution buffer, GeneJet® Plasmid Miniprep Kit, TranformAid® Bacterial Transformation kit, X-gal, IPTG and the pTZ57RT vector were obtained from Fermentas (Vilnius, Lithuania). The E.Z.N.A® gel extraction kit was purchased from peQLab (Enlangen, Germany) and the DNA clean and concentrator kit® from ZymoReseach (Orange, CA, USA). Competent *Escherichia coli* JM109, JM109 DE3 and BL21 DE3 were obtained from New England Biolabs (Ipswitch, MA, USA). The pET-28a expression vector was purchased from Novagen (Madison,WI, USA). *Taq* polymerase, 10 x PCR reaction buffer, 10 mM dNTP mix, and $MgCl_2$ were obtained from Solis Biodyne (Tartu, Estonia). Horse radish peroxidase-(HRPO) labelled horse anti-mouse IgG and HPRO-labelled rabbit anti-chicken IgY were obtained from Sigma. Leupeptin and 4-(2-aminoethyl)benzenesulfonylfluoride (AEBSF) were purchased from Roche (Germany). Biosciences Low molecular weight marker was obtained from GE Healthcare (South Africa). The pPic9-proC2 and pMAL-BiP69 (R69) plasmids were provided by Dr Alain Boulangé (University of KwaZulu-Natal). Chicken anti-congopain N-terminal peptide antibodies (Mkhize, 2003) and chicken anti-BiP antibodies were in house preparations. Mouse anti-BiP N-terminal monoclonal antibodies (M1D4) and mouse anti-BiP C-terminal (M1B2) monoclonal antibodies were a gift from Dr Boulangé.

Methods

2.2.1 Isolation of plasmid DNA

A glycerol stock of pPic9-proC2 was three-way streaked on a 2x YT plate [tryptone (16 g/L), yeast extract (10 g/L), NaCl (5 g/L), bacterial agar (15 g/L)] containing ampicillin (50 µg/ml) and the plate was incubated overnight at 37^{o}C. A single colony was selected from the plate and cultured overnight while shaken in liquid 2x YT medium [tryptone (16 g/L), yeast extract (10 g/L), NaCl (5 g/L)] containing ampicillin (50 µg/ml) at 37^{o}C. The plasmid DNA was isolated using GeneJet® plasmid DNA isolation kit (Fermentas) in accordance with the manufacturers' instructions. The plasmid DNA was analysed by electrophoresis on a 1% (w/v) agarose gel in TAE buffer [40 mM Tris-Cl, 1 mM Na_2EDTA, 0.06 % (v/v) glacial acetic acid, pH 7.4].

2.2.1 Agarose gel for DNA analysis

Agarose gel electrophoresis was used to separate and analyse DNA samples before visualisation under UV light. Agarose (1 g) was dissolved in 100 ml of 1 x TAE buffer [40 mM Tris-Cl, 1 mM Na_2EDTA, 0.06 % (v/v) glacial acetic acid, pH 7.4] and dissolved by heating in a microwave oven. Once cooled, ethidium bromide (0.5µg/ml) was added, and the solution poured into a casting tray. When the gel was set, it was placed in a tank filled with TAE buffer. DNA samples were mixed with loading solution [30 % (v/v) glycerol, 0.25 % (v/v) xylene, 0.25 % (w/v) bromophenol blue) and loaded in the wells. Electrophoresis was conducted at 80V for 35 min and the gel viewed and photographed under UV light using a Versadoc® imaging system (BioRad) using the Quantity one software from BioRad.

The molecular size of DNA bands were determined using a calibration curve prepared by plotting the relative mobility of DNA size markers against the log of their molecular weight (Fig. 2.1).

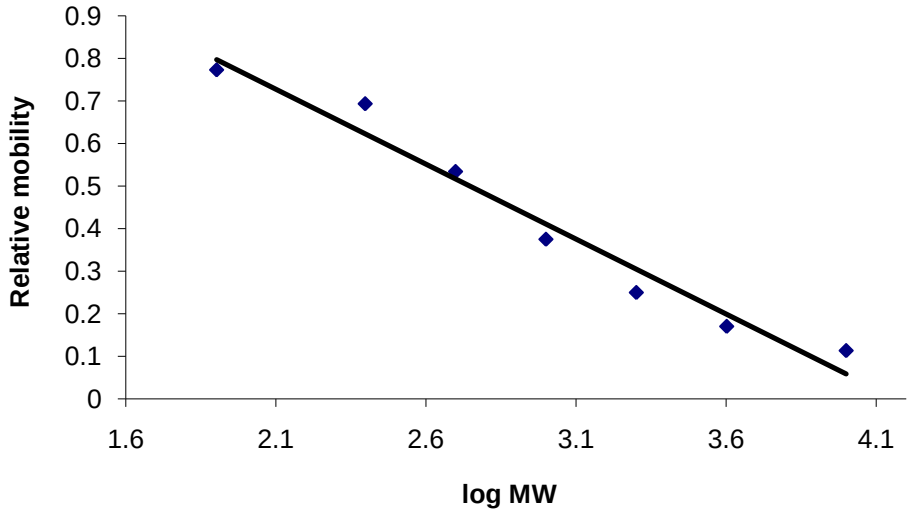

Figure 2.1: Standard curve of relative mobility of DNA size markers against the log of their molecular weight to determine size of DNA bands of unknown size. DNA Ladder O'Gene® Mix containing 10, 4, 2, 1, 0.5, 0.25 and 0.08 kb fragments (Fermentas) was used as a standard to plot the standard curve. The equation of the slope is y = -0.352x + 1.4666 with a correlation co-efficient of 0.965.

2.2.2 Amplification of C2 ORF using PCR

The plasmid DNA of pPic9-C2 (Fig. 2.2) was used as a template to amplify the gene coding for C2 using specific primers. Primers for C2 were designed in such a way that the sense primer would introduce an EcoRI restriction site (Roberts *et al.*, 2003) and the anti-sense primer would introduce a NotI restriction site with a stop codon (Fig 2.3; Table 2.1). The PCR reaction was carried out in a 20 µl reaction volume containing 25 mM $MgCl_2$, 1 x Taq polymerase buffer [80 mM Tris-Cl buffer, pH 9.4, 20 mM $(NH_4)_2SO_4$, 0.02% (v/v) Tween 20] (Solis Biodyne), 10 µM C2 Fw primer, 10 µM C2 Rv primer, 25 U Taq FirePol® DNA polymerase (Solis Biodyne), 20 mM dNTPs (Solis Biodyne). Amplification conditions for 25 cycles were as follows: initial denaturation (94^{o}C, 5 min), activation of Taq polymerase (94^{o}C, 30 s), primer annealing (56^{o}C, 30 s), extension (72^{o}C, 1 min) and final extension (72^{o}C, 7 min).

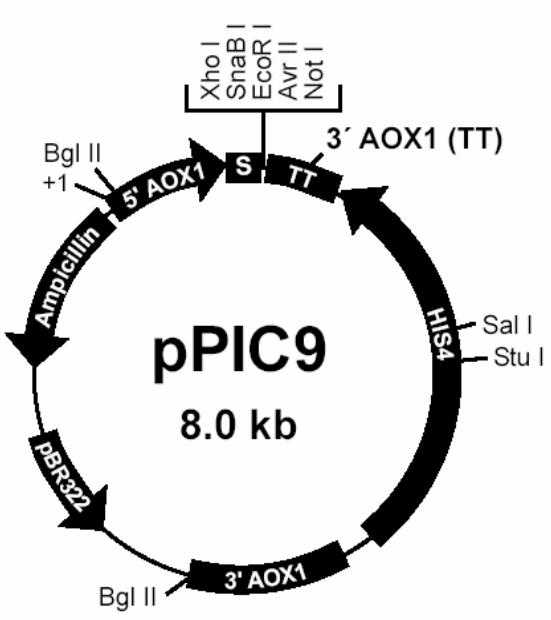

Figure 2.2: pPic9 expression vector map. ColE1 origin of replication (pBR322), 5' AOX1 fragment (5'AOX), multiple cloning sites, α-factor secretion signal (S), *c-myc* epitope (TT), ampicillin resistance gene, and the his4 gene for the selection by complementation in the his4 yeast strain (Invitrogen system manual).

Table 2.1: Sequences of primers used for amplification of C2 ORF by PCR.

Primer	Sequence	Restriction site
C2 Fw	CTGAATTCGCACCTGAGGCAGTTGACTGG	EcoRI
C2Rv	CCGCGGCCGCTTACGGAGTCGGGGGTGGAGGC	NotI

2.2.3 Cloning of C2 PCR product into pTZ57RT sub-cloning vector

A PCR product of 600 bp was amplified from a pPic9-proC2 template, as a single band and was cleaned using ZymoResearch®DNA clean and concentrator kit. The purified PCR product was ligated into the pTZ57RT sub-cloning vector (Fig 2.4) using DNA ligase, overnight at 4^{o}C. The ligated plasmid was transformed into *E. coli* JM109 cells using Bacterial TransformAid® kit (according to the manufacturers' instructions). The transformed cells were plated on 2xYT plates containing 50 µg/ml of ampicillin, 20 µg/ml of X-gal and 10 µg/ml of IPTG to allow blue and white screening of recombinants.

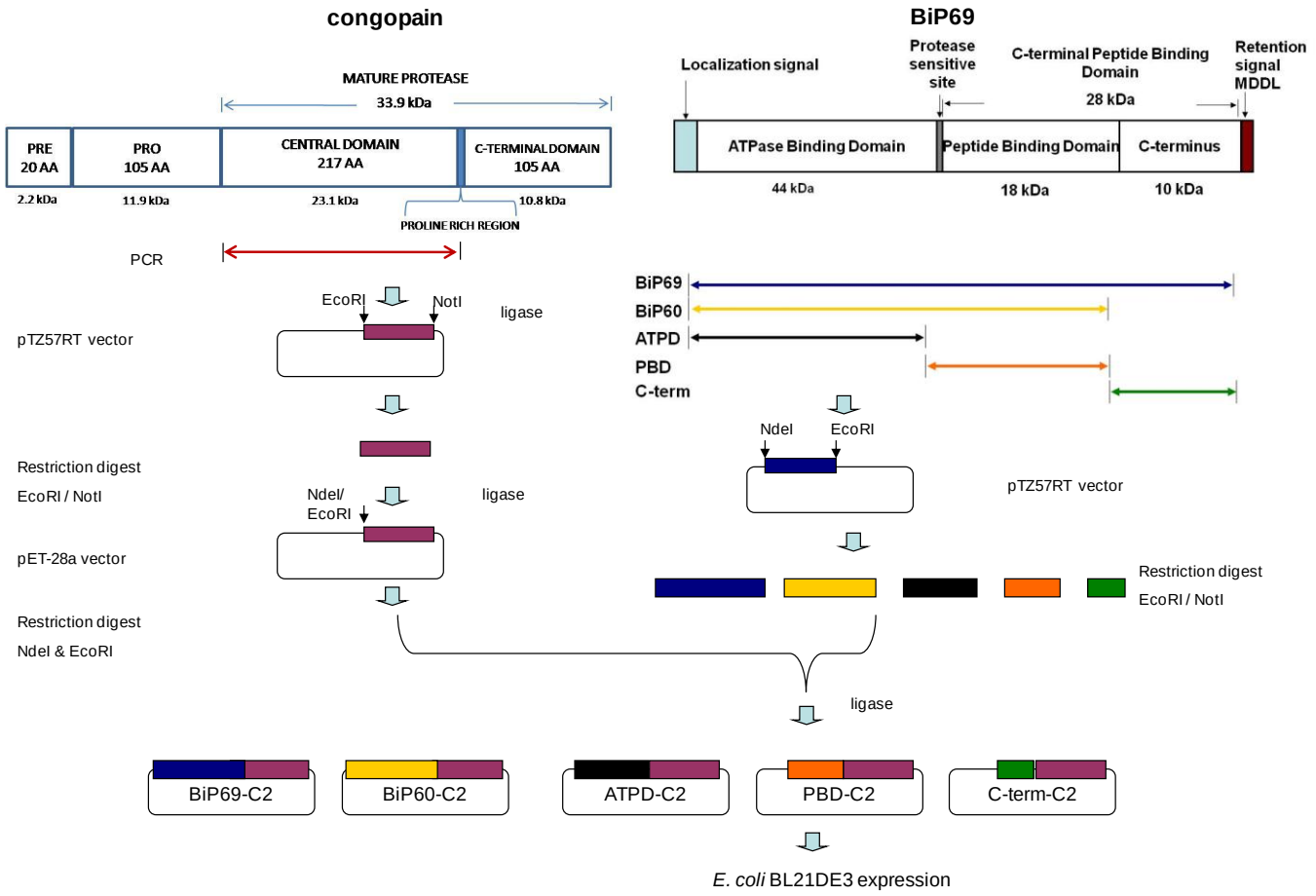

Figure 2.3: Scheme showing construction of His-tagged BiP-C2 fusion chimeras. Amplification of the catalytic domain of congopain (C2) from the pPic9-proC2 plasmid using catalytic domain primers. The amplicon (C2) was subsequently sub-cloned into a T-vector, recombination confirmed by restriction enzyme digestion and cloned into pET-28a. The BiP fragments were also amplified by PCR from *T. congolense* or pMAL-BiP69 plasmid, cloned into a T-vector, recombination confirmed by restriction digest and subsequently sub-cloned into recombinant pET-28a-C2 plasmid. The vertical arrows represent fragment being amplified, rectangular blocks represent cloning T-vector and pET-28a expression vector.

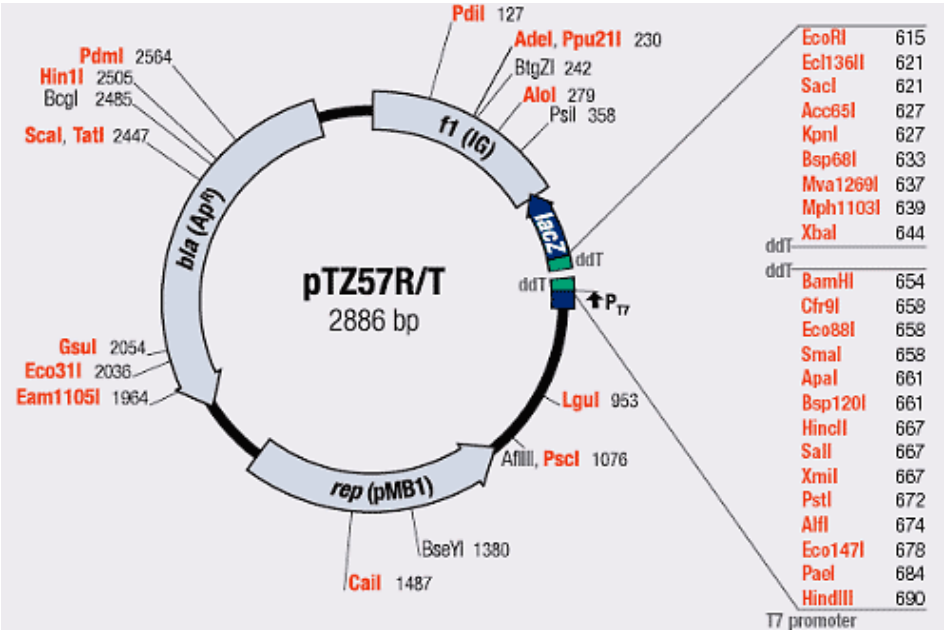

Figure 2.4: pTZ57RT sub-cloning vector map showing features of cloning efficiency. Multiple cloning sites, 5' and 3' dTT over hangs, lacZ gene (coding for blue and white colony screening), f1 (IG) region (for replication), ampicillin resistant gene [bla Ap^R] (selection of recombinants) and T7 promoter (Fermentas user's guide).

White colonies were selected for further screening to identify recombinants by colony PCR, using C2 specific primers (Table 2.1). The colony PCR was carried out in a 20 µl reaction volume using thermostable Taq polymerase. A template colony was picked and inoculated in the PCR mix, amplified using conditions as per Section 2.2.2. Recombinant colonies were grown at 37^{o}C overnight in 2xYT media containing ampicillin (50 µg/ml). Plasmid DNA of recombinant pTZ57RT-C2 was isolated using GeneJet® miniprep kit (Fermentas), and electrophoresed through a 1% (w/v) agarose gel. The pTZ57RT-C2 plasmid DNA was cut with EcoRI and NotI in Orange buffer to confirm recombinants (shown by the presence of the excised C2 insert of the expected size on an agarose gel). The dropped out C2 insert was excised from the agarose gel and purified using E.Z.N.A® pQEL gel extraction kit. The purified C2 product was ligated into the pET-28a vector. The pET-28a vector (Fig. 2.5) had previously been cut with EcoRI and NotI, dephosphorylated with 1U of shrimp alkaline phosphatase (SAP) (activated at 37^{o}C, 1 h and deactivated at 65^{o}C, 15 min) to circumvent re-circulisation or re-ligation, and purified with Zymoresearch® DNA clean and concentrator kit.

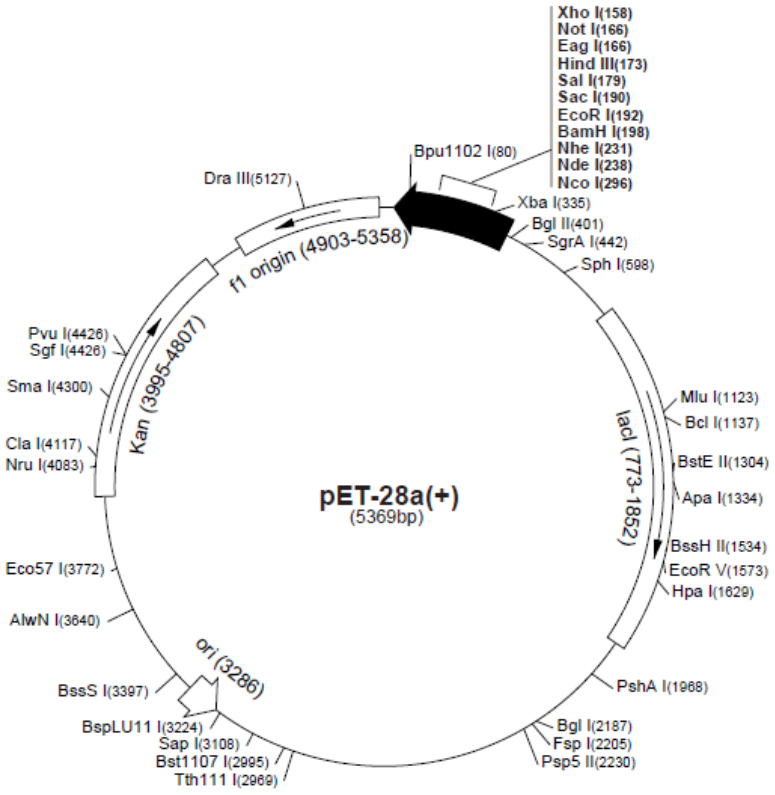

Figure 2.5: pET-28a expression vector map. The vector has a kanamycin resistance coding sequence, f1 origin of replication, T7 inducible promoter, T7 transcription start codon, multiple cloning site, *lac1* coding sequence, T7 terminator and N-terminal and C-terminal His-tag coding sequences, enabling easy purification by divalent ion affinity resins.

The ligated pET-28a-C2 plasmid DNA (50 ng) was transformed in E. cloni® cells (Lucigen) by heat shock (in accordance with manufacturers' instructions) (see Section 2.2.4).

2.2.4 Transformation of ligated pET-28a-C2 plasmid DNA into E. cloni® EXPRESS chemically competent cells using heat shock

Sterile culture tubes and the ligation mix (pET-28a-C2) were chilled on ice for 15 min. The E. cloni® EXPRESS cells (stored at -80^{o}C) were thawed completely on wet ice (10-20 min) and 15 µl added into a pre-chilled microfuge tube containing 50 ng of ligation mix. The microfuge tube was finger tapped briefly to mix the contents. The cells were incubated on ice for 30 min, heat shocked in a 42^{o}C water bath for 45 s and incubated on ice for 2 min before 985 µl of Expression Recovery Medium (Lucigen) was added at RT. The microfuge tubes with cells were shaken in a 37^{o}C incubator at 250 rpm for 1 hour before the cells were centrifuged (12 000 x g, 1 min, RT) and 800 µl of the supernatant discarded. The cell pellet was re-suspended

in 200 µl of the remaining recovery medium and cells plated on 2xYT plates containing kanamycin (33 µg/ml) and incubated overnight at 37^{o}C. Recombinant colonies were selected and screened by colony PCR using both C2 primers (Table 2.1) and pET vector universal primers (Table 2.2). Two recombinant colonies were selected and grown overnight in 2xYT media containing kanamycin (33 µg/ml) before plasmid DNA was isolated as described in Section 2.2.1.

Table 2.2: Sequences of primers used for screening of pET-28a recombinant colonies by PCR. The primers were designed based on the coding sequence of the 5' T7 promoter (pET Forward) and 3' T7 terminator (pET Reverse) flanking multiple cloning sites.

Primer	Tm (^{o}C)	Sequence (5'-3')
pET Fw	54	TAATACGACTCACTATAGGG
pET Rv	57	GCTAGTTATTGCTCAGCGG

The pET-28a-C2 plasmids were restricted with EcoRI and NotI in Orange buffer (Fermentas) to confirm recombinants. Successful restriction digest was analysed on 1 % (w/v) agarose gel and recombinants shown by the presence of the excised C2 insert of the expected size when viewed under UV light. Positive clones were further digested with NdeI and EcoRI in Orange buffer, dephosphorylated with SAP and purified using ZymoResearch® DNA clean and concentrator kit, for insertion/cloning of BiP fragments.

2.2.5 Cloning and expression of His-tagged BiP-C2 fusion protein chimeras

2.2.5.1 Isolation of genomic DNA from T. congolense IL1800 lysates and pMAL-R69 BiP plasmid DNA

Procyclic *T. congolense* parasites were cultured in 10 ml minimum essential medium (MEM) [25 mM HEPES Acid, 0.1 mM hypoxanthine, 2 mM glutamine, 10 mM proline, 3 mM cis-aconitate; 3 mM citrate] and containing 10% (w/v) foetal calf serum and were grown to 1 x 10^{7} parasites/ml. The medium containing parasites was centrifuged (1 600 x g, 10 min, RT) and the supernatant removed by decanting. The pellet was resuspended in 1 ml PBS and transferred to 4 x 1.5 ml microfuge tubes, and further diluted to 4 ml with PBS. Microfuge tubes were centrifuged (1 600 x g, 10 min, RT) and the supernatant removed. The pellet was resuspended by adding 150 µl TELT buffer (50 mM Tris-Cl, 62.5 mM EDTA, 2.5 M LiCl, 4%

(v/v) Triton X-100, pH 7.4), followed by incubation for 5 min on ice. A phenol:chloroform [150 µl, 1:1 (v/v)] mixture was added and incubated at RT for 5 min before the mixture was centrifuged (6 800 x g, 5 min) to separate nucleic acids from supernatant. The pellet was washed with 300 µl of 100% (v/v) ethanol and incubated for 5 min at RT followed by centrifugation (6 800 x g, 10 min) and the supernatant decanted carefully. The pellet was washed again with 100% (v/v) ethanol (1 ml) followed by centrifugation (6 800 x g, 5 min), the supernatant decanted again and the pellet incubated for 10 min at 37^{o}C and left to dry. The pellet was resuspended in 100 µl of TE buffer (100 mM Tris-Cl buffer, pH 7.4, 10 mM EDTA) with RNAse (20 µg/ml) followed by incubation for 45 min at 37^{o}C. The genomic DNA was stored at -20^{o}C until further use.

The pMAL-R69 BiP plasmid DNA was isolated from an overnight culture using the GeneJet® miniprep isolation kit (in accordance with the manufacturers' instructions). The pMAL-R69 BiP plasmid DNA or *T. congolense* genomic DNA (1 µl) was used as a template for amplification of different BiP fragments by PCR (Fig. 2.3). Primers for BiP fragments (BiP69, BiP60, ATPD, PBD and C-term) were designed in such a way that the forward primer would introduce an NdeI restriction site (CATATG) and the reverse primer introduce an EcoRI restriction site (GAATTC) (Table 2.3).

Table 2.3: Sequences of primers used for amplification of BiP fragments by PCR using *T. congolense* genomic DNA and pMAL-R69 BiP plasmid DNA as templates. The genomic DNA was isolated from T. congolense procyclic lysates, and plasmid DNA from pMAL- R69 BiP. The restriction enzyme sites are underlined.

Primer Direction	Tm (^{o}C)	Sequence (5' -3')
BiP NdeI Fw	70	CCCATATGGCGCCCGAGAGCGGCGGGAAG
BiP69 EcoR1 Rv	61	CTGAATTCAAGGTCATCCATGGGCTGCGG
BiP60 EcoRI Rv	46	GAGGAATTCCCGCGCCTCCACACGCTCCCC
ATPD EcoRI Rv	73	AAAGAATTCCACGCCCGCCAACCTCACTCT
PBD NdeI Fw	72	AAACATATGGTGCTGACCGGTGAGAGTGAG
C-term NdeI Fw	73	AAACATATGGGCGGCAAGCTCAGTGCTGAC

2.2.5.2 Sub-cloning of BiP fragments into pTZ57RT vector

The PCR products of BiP fragments: BiP69 (2 kb), BiP60 (1.8 kb), ATPD (1.2 kb), PBD (0.55 kb) and C-term (0.23 kb), were purified and concentrated using ZymoResearch® DNA clean and concentrator kit. The purified BiP fragments were ligated into sub-cloning vector pTZ57RT (Fig. 2.4) using T4 DNA ligase® (Fermentas) at 4^{o}C, overnight.

The ligated plasmids (pTZ57RT-BiP69, pTZ57RT-BiP60, pTZ57RT-ATPD, pTZ57RT-PBD, and pTZ57RT-C-term) were transformed separately into *E. coli* JM109 cells using Bacterial TransformAid® kit (in accordance with the manufacturers' protocol). The cells were plated on 2xYT plates in the presence of ampicillin (50 µg/ml), X-gal (20 µg/ml) and IPTG (10 µg/ml), for blue and white colony screening.

White colonies from each transformed ligated plasmid, were selected and screened by colony PCR for recombinants using insert specific primers (Table 2.3). The colony PCR reaction was carried out as shown in Tables 2.4 and 2.5. Recombinant colonies were grown overnight in the appropriate media with antibiotic present. The plasmid DNA was isolated using GeneJet® plasmid DNA isolation kit and analysed by 1% (w/v) agarose gel electrophoresis. Recombinant plasmids were digested with NdeI in Orange buffer (Fermentas) overnight at RT. The plasmids restricted were analysed by 1% (w/v) agarose gel and plasmids that showed linearisation with NdeI on the gel were further restricted with EcoRI restriction enzyme. The restricted plasmids with both restriction enzymes were analysed by 1% (w/v) agarose gel electrophoresis and recombinants shown by the presence of the excised BiP fragment insert of the expected size, thereby confirming recombinants in the pTZ57RT vector.

Table 2.4: PCR reaction components used for amplification of products from plasmid DNA templates.

PCR component	Volume (µl)	Final concentration
$MgCl_2$ (25 mM)	2	2.5 mM
PCR Buffer (10 x)	2	1 x
Fw primer (10 µM)	0.5	0.25 µM
Rev primer (10 µM)	0.5	0.25 µM
Taq	0.25	1U
dNTPs (20 µM)	0.5	0.5 µM
Sterile distilled H_2O	14.5	
Total volume	20	

Table 2.5: PCR reaction conditions for amplification of BiP fragments. A master mix containing PCR components (Table 2.4) was amplified in a 20 µl volume for 25 cycles. The conditions for initial denaturation (95°C, 5 min) and final extension (72°C, 7 min) were constant.

PCR conditions	Primer				
	BiPFw/ BiP69Rv	BiPFw/ BiP60Rv	BiPFw/ ATPDRv	PBDFw/ BiP60Rv	C-termFw/ BiP69Rv
Denaturation	95°C, 30 s	95°C, 30 s	95°C, 30 s	95°C, 30 s	95°C, 30 s
Annealing	56°C, 1 min	58°C, 1 min	65°C, 1 min	65°C, 30 s	55°C, 1 min
Elongation	72°C, 2 min	72°C, 2 min	72°C, 2 min	72°C, 1 min	72°C, 1 min

2.2.5.3 Cloning of BiP fragment into recombinant expression vector pET-28a-C2

The inserts dropped out by double digestion of the recombinant pTZ57RT vector plasmids were excised from the gel and purified using the E.Z.N.A® pQEL gel extraction kit and analysed on a 1% (w/v) agarose gel. The BiP fragments were ligated into pET-28a-C2 using a 3:1 insert to vector ratio. The vector was prepared as described in Section 2.2.4.

The ligated plasmids, pET-28a-BiP69-C2, pET-28a-BiP60-C2, pET-28a-ATPD-C2, pET-28a-PBD-C2 and pET-28a-C-term-C2, were transformed into E. cloni® cells as per Section 2.2.4. The recombinant colonies were screened by colony PCR using pET universal primers (Table 2.2) for C-term-C2 and PBD-C2, and BiP fragment insert specific primers for BiP69-C2, BiP60-C2 and ATPD-C2 (Table 2.3). Recombinant colonies were grown over-night in culture medium and plasmid DNA isolated. Recombinants were confirmed by digestion with NdeI and EcoRI in Orange buffer (to drop out BiP fragment insert) and digested with NdeI and NotI in Orange buffer (to drop out both BiP and C2 fragments). Recombinant plasmids were

transformed into *E. coli* BL21DE3 cells or *E. coli* JM109DE3 cells by electroporation (Section 2.2.5.4).

2.2.5.4 Transformation of pET-28a-BiP-C2 plasmids into E. coli BL21DE3 or JM109DE3 cells by electroporation

The recombinant plasmid DNA (2 µl) of pET-28a-BiP69-C2, pET-28a-BiP60-C2, pET-28a-ATPD-C2, pET-28a-PBD-C2, pET-28a-C-term-C2 and pET-28a-BiP69 were separately mixed with *E. coli* BL21DE3/JM109DE3 cells (50 µl), pre-chilled on ice. Each mixture was pipetted into an ice cold 1 mm Electroporator cuvette® and electroporated using the GenePulser® (BioRad) (settings: 2.5 kV, 25 µF, 200Ω). The cells were immediately resuspended in 2xYT medium (1 ml) without antibiotics and grown in culture tubes, in a shaking incubator at 250 rpm, for 1 hour at 37°C. The cultures were centrifuged (12 000 x g, 1 min) and 800 µl supernatant discarded. Pelleted cells were re-suspended in the remaining 200 µl supernatant and plated on 2xYT plates containing kanamycin (33 µg/ml) and incubated overnight at 37°C. Transformants in *E. coli* BL21DE3 or JM109DE3 strains were screened for recombinants by colony PCR (Section 2.2.3) Recombinant colonies were expressed in Terrific Broth with appropriate antibiotic (Section 2.2.5.5).

2.2.5.5 Expression of recombinant clones as His-tagged fusion proteins in Terrific Broth®

Recombinant clones in *E. coli* BL21DE3 or JM109DE3 strains were expressed in Terrific Broth® by auto-induction (Tartof and Hobbs, 1987; Studier, 2005) at 37°C, overnight. Following expression, the culture was centrifuged (6 000 x g, 10 min, RT) and the pellet lysed in lysis buffer [100 mM NaCl, 50 mM Tris-Cl, 0.5% (v/v) Triton X-100, lysozyme 20 µg/ml, pH 8.2] in a volume 1/20 of the initial culture volume. The lysate was left at RT for 15 min and a protease inhibitor cocktail was added prior to freezing. The lysate was thawed and sonicated (3 x 1 min) to break down nucleic acids and centrifuged (5 000 x g, 15 min, RT). The supernatant and pellet were separated and kept for SDS-PAGE analysis (Section 2.2.6). The bacterial pellet was resuspended in PBS, in a volume 1/40 of the initial culture volume.

2.2.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of proteins

Protein sample analysis was carried out in a 10% SDS-PAGE gel using a discontinuous Tris-Glycine system described by Laemmli (1970). SDS binds to protein molecules in a ratio of 1.4

g per g of protein and gives the protein a negative charge. When protein-SDS complexes are subjected to electrophoresis, they all have the same charge and therefore separate based only on differences in molecular weight (Dennison, 1999). Proteins were separated through a large pore stacking gel, pH 6.8 and a small pore running gel at pH 8.8, allowing clear separation of proteins of different sizes. A mini-PROTEAN 3® gel system (BioRad) was used to separate proteins. Samples from expression media containing BiP-C2 chimeras, BiP69 and C2 protein samples (pellets and supernatants) were mixed with reducing treatment buffer [125 mM Tris-Cl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol] in a 1:1 ratio and boiled for 5 min before loading. Low molecular weight marker (Amersham) were used to construct a calibration curve (Fig. 2.6). Electrophoresis was conducted in Tank buffer [250 mM Tris-Cl buffer, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3] at 18mA per gel. The proteins were stained with Coomassie brilliant blue R-250 [0.125 g (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid].

The molecular size of protein bands were determined using a calibration curve prepared by plotting the relative mobility of protein molecular weight standards against the log of their molecular weight (Fig. 2.6) (Dennison, 1999).

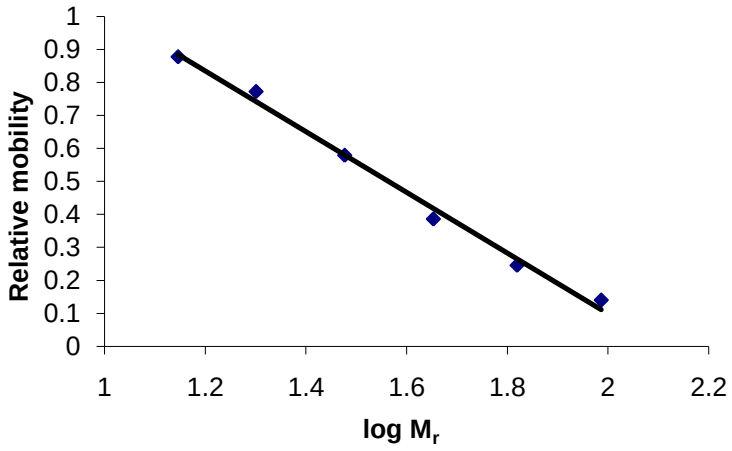

Figure 2.6: Standard curve of relative mobility of standard proteins against their log M_r, utilised to determine molecular weights of proteins of unknown size. Amersham Biosciences low molecular weight marker contains: phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (30 kDa), soybean trypsin inhibitor (20 kDa) and lysozyme (14 kDa). The equation of the slope is y = -0.9203x + 1.9394 with a correlation co-efficient of 0.9925.

2.2.7 Western blot using anti-BiP antibodies and anti-congopain N-terminal peptide antibodies

The proteins separated by SDS-PAGE (Section 2.2.6) were transferred to a nitrocellulose membrane for 1 h using a semi-dry blotter (Sigma Chemical Co., St. Louis, USA). Efficiency of blotting was confirmed by staining the gel with Ponceau S [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. The unoccupied sites on the nitrocellulose membrane were blocked for 1 h with 5% (w/v) low fat milk powder in TBS (20 mM Tris-Cl buffer, 200 mM NaCl, pH 7.4), followed by washing (3 x 5 min) with TBS. This was followed by incubation for 2 h with primary antibody [(affinity purified chicken anti-congopain N-terminal peptide antibody (1 µg/ml)] (for C2) or [(mouse anti-BiP M1D4 monoclonal antibody (1: 2000) or chicken anti-BiP antibody (1 µg/ml)] (for BiP), diluted in 0.5% (w/v) BSA in TBS. Following washing in TBS (3 x 5 min) nitrocellulose was incubated for 1 h at RT with HRPO-linked secondary antibody [(rabbit anti-chicken IgY 1:12 000 (Sigma) or rabbit anti-mouse IgG 1:10 000 (Sigma)], diluted in 0.5% (w/v) BSA in TBS. Following washing in TBS (3 x 5 min) the nitrocellulose membrane was immersed in 4-chloro-1-naphthol/H_2O_2 substrate solution [0.006% (w/v) 4-chloro-1-naphthol, 0.1% (v/v) methanol, and 0.0015% (v/v) H_2O_2]. This was incubated in the dark and the reaction was terminated by rinsing the membrane in distilled water, once bands were visible.

2.3 RESULTS

2.3.1 Amplification of BiP fragments and congopain catalytic domain (C2) by PCR

The pMAL-BiPR69 plasmid DNA (Boulangé and Authié, 1994) and *T. congolense* genomic DNA were isolated and analysed on an agarose gel (Fig. 2.7). Plasmid DNA of pMAL-BiPR69 migrated above 10 kb and genomic DNA migrated above 5 kb. Isolated plasmid DNA or genomic DNA was used as the template in a PCR reaction to amplify BiP fragments.

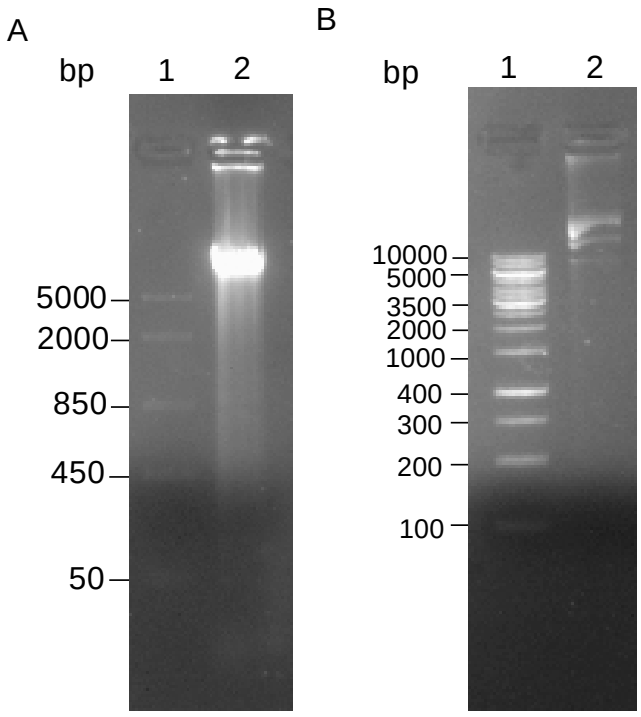

Figure 2.7: Isolation of genomic DNA from procyclic *T. congolense* and plasmid DNA from pMAL-BiPR69 culture. Genomic *T. congolense* DNA isolated using the phenol:methanol precipitation method (**A**), and R69BiP plasmid DNA isolated using GeneJet® miniprep kit (**B**) were electrophoresed on a 1% (w/v) agarose gel in 1 x TAE buffer. A: lane 1, DNA Ladder FastRuler® Middle range (Fermentas); lane 2, genomic *T. congolense* DNA. B: Lane 1, DNA Ladder O'Gene® 1kb mix (Fermentas); lane 2, pMAL-BiPR69 plasmid DNA.

Different BiP coding fragments were amplified using specific primers (Table 2.3) and analysed on a 1% (w/v) agarose gel (Fig. 2.8). BiP69 coding fragment was amplified as a 2 kb amplicon using BiP NdeI Forward and BiP69 EcoRI Reverse primers (Fig. 2.8, lane 2). BiP60 coding fragment amplified as a 1.8 kb product using BiP NdeI Forward and BiP60 EcoRI Reverse primers (Fig. 2.8, lane 3). ATPase Domain coding fragments was amplified as a 1.2 kb PCR product using BiP NdeI Forward and ATPD EcoRI Reverse primers (Fig. 2.8, lane 4). The peptide binding domain coding fragment was amplified as a 0.55 kb product using PBD NdeI Forward and BiP60 EcoRI Reverse primers (Fig. 2.8, lane 5), and lastly the C-terminus

coding fragment was amplified as a 0.23 kb PCR product using C-term NdeI Forward and BiP69 EcoRI Reverse primers (Fig. 2.8, lane 6).

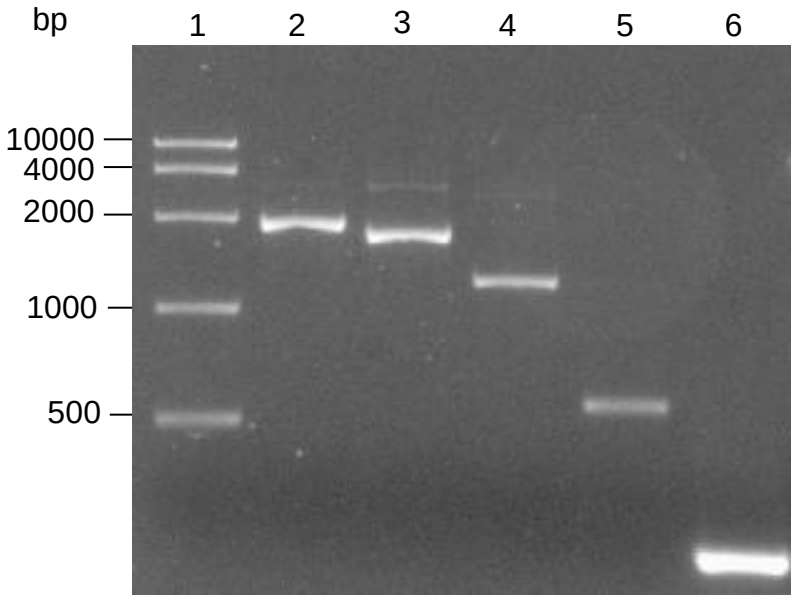

Figure 2.8: Amplification of BiP fragments by PCR using specific primers. BiP69 plasmid was used as a template to amplify BiP69, BiP60, ATPD, PBD and C-term fragments and the PCR products were cleaned with DNA clean and concentrator kit. PCR products were electrophoresed on a 1% (w/v) agarose gel. Lane 1, DNA Ladder FastRuler®, High Range (Fermentas); lane 2, BiP69; lane 3, BiP60; lane 4, ATPD; lane 5, PBD; lane 6, C-term.

The pPic9-proC2 construct had been cloned and expressed previously in a *P. pastoris* system (Boulangé *et al.*, 2011). Plasmid DNA of pPic9-proC2 was isolated and used as a template to amplify the catalytic domain of congopain, C2. A single band at 600 bp was observed by electrophoresis on a 1% (w/v) agarose gel (Fig. 2.9, lane 2), corresponding to the expected size of the catalytic domain coding fragment of congopain (C2).

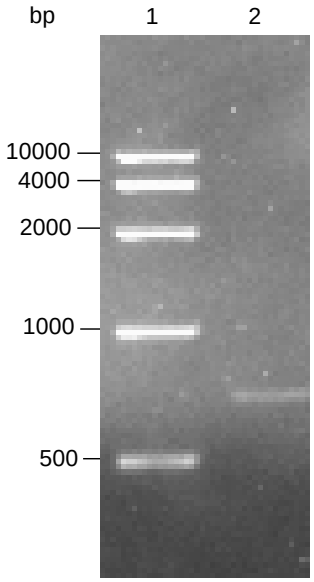

Figure 2.9: Amplification of C2 by PCR using specific primers. pPic9-ProC2 plasmid was used as a template to amplify C2, and the PCR product was cleaned with DNA clean and concentrator kit and analysed by electrophoresis on a 1% (w/v) agarose gel in 1 x TAE buffer. Lane 1, DNA Ladder FastRuler®, High Range (Fermentas); lane 2, C2.

2.3.2 Cloning of BiP fragments and the catalytic domain of congopain (C2) into a pTZ57RT vector

The amplified PCR products of BiP coding fragments, (namely BiP69, BiP60, ATPD, PBD, and C-terminus) together with that of C2 were purified and ligated into a pTZ57R/T vector and transformed into *E. coli* JM109 cells. Following blue-white colony selection, recombinant clones were screened by colony PCR (results not shown). Two recombinant clones (indicated as 1 and 2 in figure legend of Fig. 2.10) from each ligation mix were grown in appropriate media, plasmid DNA isolated and double restricted: plasmid DNA of pTZ57RT-BiP69, pTZ57RT-BiP60, pTZ57RT-ATPD, pTZ57RT-PBD, and pTZ57RT-C-term with NdeI and EcoRI, and C2 plasmid DNA with EcoRI and NotI. Double digestion of BiP coding fragments inserted in the pTZ57RT vector, showed the pTZ57RT vector at 3 kb and BiP coding fragments at 2 kb for BiP69 (Fig. 2.10, Panel A, lanes 3 and 5), 1.8 kb for BiP60 (Fig. 2.10, Panel A, lanes 7 and 9), 1.2 kb for ATPD (Fig. 2.10, Panel B, lanes 3 and 5), 0.55 kb for PBD (Fig. 2.10, Panel B, lanes 7 and 9), 0.23 kb for C-term (Fig. 2.10, Panel B, lanes 11 and 13). Some plasmids were not completely digested after incubation overnight at 25^{o}C and this was shown by triple bands representing no digestion, partial digestion and complete digestion (Fig.2.10, Panel A, lanes 2 & 5 and Panel B, lane 5).

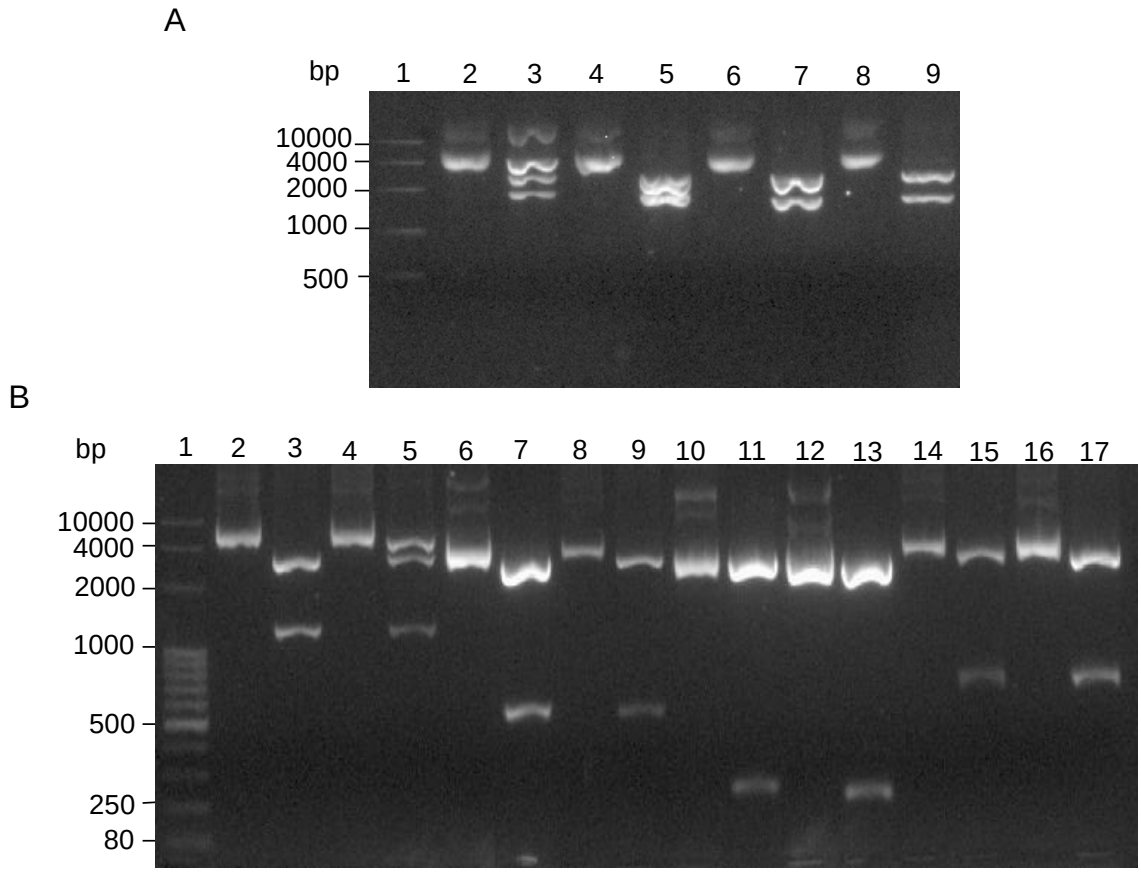

Figure 2.10: Restriction digests of BiP-coding domains in T-vector. All BiP fragments in pTZ57R/T were double digested with NdeI and EcoRI (or EcoRI and NotI for C2) overnight at RT. The plasmid DNA was electrophoresed on a 1% (w/v) agarose gel. A: Lane 1, DNA ladder FastRuler® (High range, Fermentas); lane 2, BiP69 1 uncut; lane 3, BiP69 1 cut; lane 4: BiP69 2 uncut; lane 5: BiP69 2 cut; lane 6, BiP60 1 uncut; lane 7, BiP60 1 cut; lane 8: BiP60 2 uncut; lane 9: BiP60 2 cut. B: Lane 1, DNA Ladder (O'Gene® Mix, Fermentas); lane 2, ATPD 1 uncut; lane 3: ATPD 1 cut; lane 4: ATPD 2 uncut; lane 5: ATPD 2 cut; lane 6: PBD 1 uncut; lane 7: PBD 1 cut; lane 8, PBD 2 uncut; lane 9: PBD 2 cut; lane 10: C-term 1 uncut; lane 11: C-term 1cut; lane 12: C-term 2 uncut; lane 13: C-term 2 cut; lane 14, C2 uncut; lane 15: C2 cut; lane 16, C2 uncut and lane 17, C2 cut.

Double digestion of pTZ57RT-C2 plasmids with EcoRI and NotI showed the C2 insert at the expected size of 0.6 kb (Fig. 2.10, Panel B, lanes 15 and 17) and pTZ57RT vector at the expected size of 3 kb. This confirmed successful sub-cloning of all BiP coding fragments and C2 coding fragment into the pTZ57RT vector. The recombinant plasmids were sent for sequencing (SegoliLab Unit, ILRI, Kenya), to verify that no unwanted mutations had been introduced by the Taq polymerase enzyme that is not a high fidelity DNA polymerase enzyme and is likely to introduce mutations [estimated at a rate of 1 nucleotide per cycle of approximately 1-2/12000 (Solis Biodyne)].

2.3.3 Cloning of C2 coding sequence into a His-tagged pET-28a expression vector

The pET-28a expression vector (Fig. 2.11, Panel A, lane 2) and recombinant catalytic domain (C2) coding sequence in a pTZ57RT vector (Fig. 2.11, Panel A, lane 4) were digested with EcoRI and NotI. The digested plasmids of both pET-28a (Fig. 2.11, Panel A, lane 3) and C2 (Fig. 2.11, Panel A, lane 5) were analysed by 1% (w/v) agarose gel electrophoresis, and DNA bands corresponding to the expected sizes cut out and purified. The C2 coding sequence had an expected size of 0.6 kb (Fig. 2.11, Panel B, lane 2) and the pET-28a expression vector an expected size of 5.39 kb (Fig. 2.11, Panel B, lane 3).

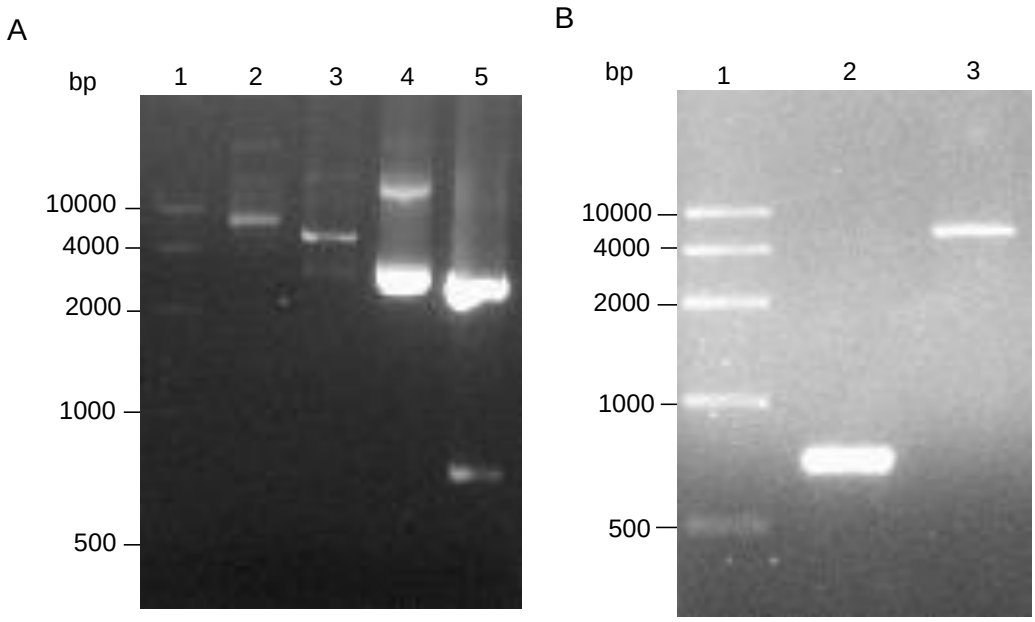

Figure 2.11: Restriction digests of C2 coding sequence in a pTZ57RT vector and pET-28a expression vector and their purification. The plasmids were double digested with EcoRI and NotI, electrophoresed on a 1% (w/v) agarose gel (A), and then gel purified for ligation (B). A: lane 1, DNA Ladder FastRuler®, High range (Fermentas); lane 2, pET-28a vector uncut; lane 3, pET-28a cut; lane 4, C2 coding sequence in pTZ57RT vector uncut; lane 5, C2 coding sequence in pTZ57RT vector cut. B: lane 1, DNA Ladder Fast Ruler®, High range (Fermentas); lane 2, C2 coding sequence purified; lane 3, pET-28a purified.

The purified insert (C2) and vector (pET-28a) were ligated and transformed into chemically competent E. cloni® cells. The recombinant colonies were screened by colony PCR using pET universal forward and reverse primers. The resulting PCR products, migrated higher than the expected size of 0.6 kb (Fig. 2.12, lanes 2, 3 and 5), due to the extra 200 bases amplified by pET primers on the 5' and 3' ends of the pET-28a vector. Two recombinant colonies were selected, grown in appropriate media, and plasmid DNA isolated.

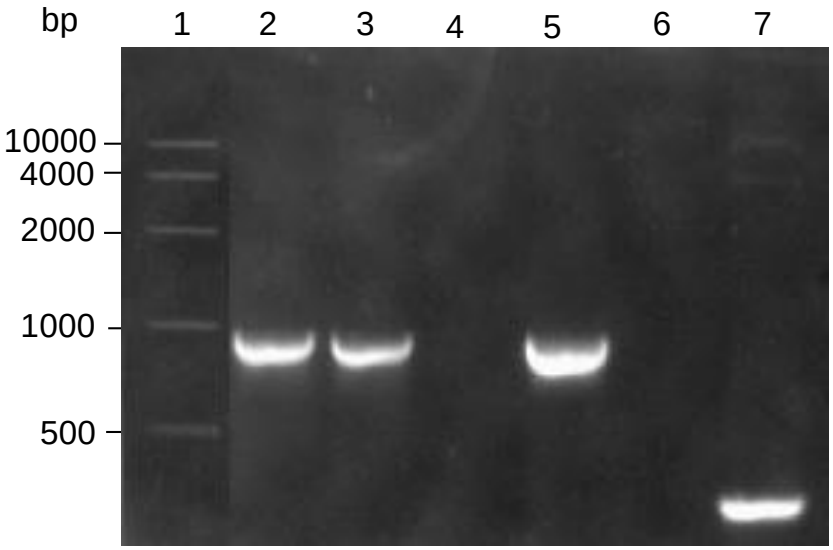

Figure 2.12: Screening of the pET-28-C2 recombinant colonies by colony PCR using pET universal primers. The reaction mix was analysed on a 1% (w/v) agarose gel in 1 x TAE buffer. Lane 1, DNA Ladder, FastRuler® High range (Fermentas); lanes 2-6, colonies 1-5; lane 7, positive control pET-28a plasmid DNA.

Neither single restriction digest of pET-28a-C2 plasmid DNA EcoRI and NotI (Fig. 2.13, lanes 4 and 5 respectively) nor double digest with NdeI and EcoRI (Fig. 2.13, lane, 6), released the C2 insert, although EcoRI completely linearised the plasmid. However, restriction digest with EcoRI and NotI released the C2 insert at the expected size of 600 base pairs (Fig 2.13, lane 7).

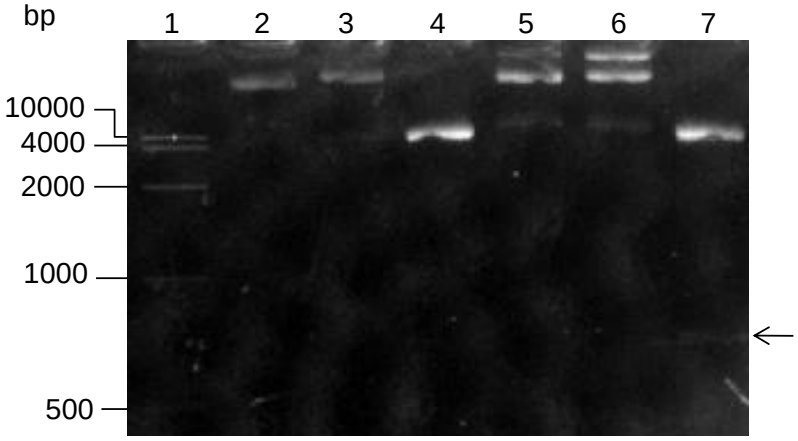

Figure 2.13: Single and double restriction digest of recombinant pET-28-C2 with various restriction enzymes. The recombinant pET-28a-C2 plasmid was either single digested with EcoRI or NotI, or double digested with a combination of NdeI and EcoRI, or EcoRI and NotI. The digested plasmid was analysed by 1% agarose gel electrophoresis. Lane 1, DNA Ladder FastRuler® High range (Fermentas); lane 2, pET-28a plasmid; lane 3, pET-28-C2 plasmid; lane 4, pET-28-C2 cut with EcoRI; lane 5, pET-28-C2 cut with NotI; lane 6, pET-28-C2 cut with NdeI and EcoRI; and lane 7, pET-28-C2 cut with EcoRI and NotI. The arrow points to the 600 bp C2 insert.

The NdeI and EcoRI double digested recombinant pET-28a-C2 plasmid was dephosphorylated and purified. BiP fragments, previously cut with NdeI and EcoRI and purified, were ligated into pET-28a-C2 and the ligation mixture was transformed into chemical competent E. cloni® cells.

Recombinant clones were screened by colony PCR (results not shown). Resulting positive clones were grown in appropriate media and plasmid DNA was isolated. The plasmid DNA of each construct (BiP69-C2, BiP60-C2, ATPD-C2, PBD-C2 or C-term-C2) was confirmed by restriction digest with NdeI and EcoRI to release BiP inserts (Fig. 2.14, lanes 3, 6, 9, 12 and 15), and also with NdeI and NotI to release BiP-C2 fragment (Fig 2.14, lanes 4, 7, 10, 13 and 16). The sizes of inserts were confirmed by comparison with results of previous restriction digests (Fig. 2.10). Uncut plasmids migrate slower in an agarose gel and so different conformations of plasmids can be seen as a faint band above the major band (Fig. 2.14, lanes 8, 11 and 14). Some of the plasmids were not completely digested by restriction enzymes when left overnight at 25^{o}C. This is shown by double bands between 5 kb and 8 kb, and they differ in size depending on the construct (Fig 2.14, lanes 6, 7, 9, 10 and 13).

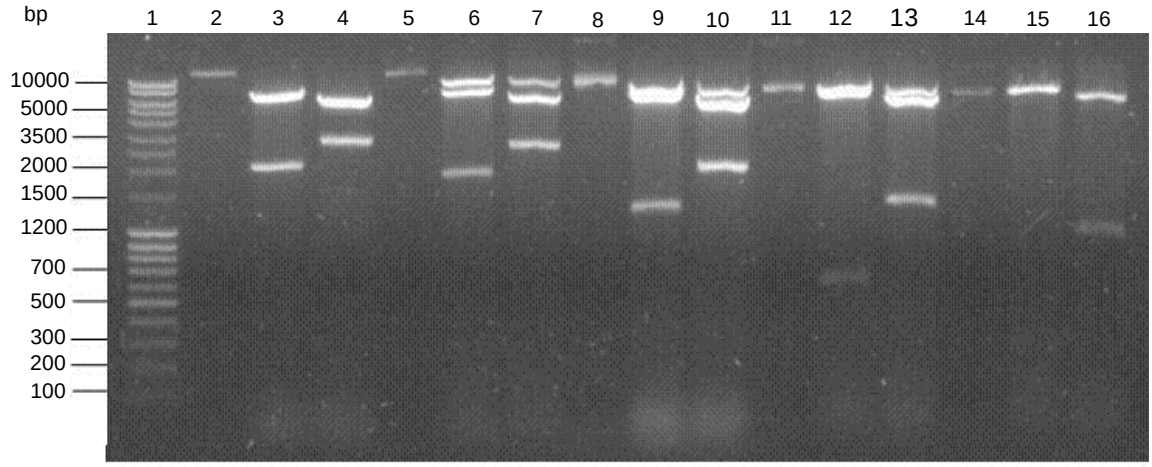

Figure 2.14: Restriction digest of recombinant plasmids of BiP-C2 coding fragments in a pET-28a vector. Recombinant plasmid DNA of BiP69-C2, BiP60-C2, ATPD-C2, PBD-C2 and C-term-C2 were double digested with NdeI and EcoRI, or NdeI and NotI overnight at RT. The plasmids were analysed by 1% agarose gel electrophoresis. Lane 1, DNA ladder O'Gene® Mix Mass Ruler (Fermentas); lane 2, BiP69-C2 uncut; lane 3, BiP69-C2 cut NdeI & EcoRI; lane 4, BiP69-C2 cut NdeI & NotI; lane 5, BiP60-C2 uncut; lane 6, BiP60-C2 cut NdeI & EcoRI; lane 7, BiP60-C2 cut NdeI & NotI; lane 8, ATPD-C2 uncut; lane 9: ATPD-C2 cut NdeI & EcoRI; lane 10, ATPD-C2 cut NdeI & NotI; lane 11, PBD-C2 uncut, lane 12, PBD-C2 cut NdeI & EcoRI; lane 13, PBD-C2 cut NdeI & NotI; lane 14, C-term-C2 uncut; lane 15, C-term-C2 cut NdeI & EcoRI; lane 16, C-term-C2 cut NdeI & NotI.

The recombinant plasmids of pET-28a-BiP69-C2, pET-28a-BiP60-C2, pET-28a-ATPD-C2, pET-28a-PBD-C2 and pET-28a-C-term-C2 were transformed into *E. coli* BL21DE3 or JM109DE3 cells by electroporation and expression conducted in Terriffic Broth. The cells were lysed and the pellet separated from the supernatant to determine the solubility of the expressed protein (Fig 2.15).

His-tagged BiP-C2 fusion chimeras were expressed as insoluble proteins at the expected sizes (Fig. 2.15): BiP69-C2, 96 kDa (lane 2), BiP60-C2, 88 kDa (lane 4), PBD-C2, 47 kDa (lane 8) and C-term-C2, 34 kDa (lane 11). The His-tagged ATPD-C2 fusion protein chimera was expressed at a higher molecular weight than expected: i.e 88 kDa (lane 6). BiP69 was expressed as an insoluble 73 kDa fusion protein (lane 13) and C2 was expressed as an insoluble 27 kDa fusion protein (lane 14).

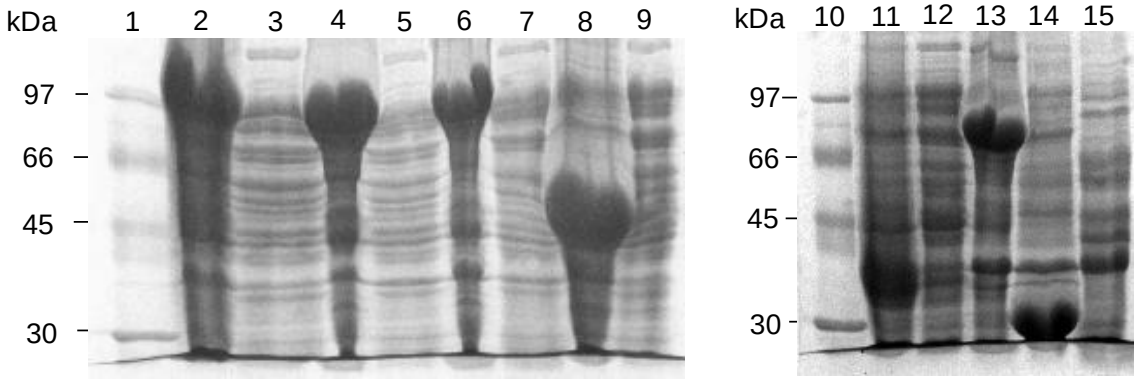

Figure 2.15: Expression of His-tagged BiP-C2 fusion protein chimeras in Terrific Broth overnight at 37°C. A single colony of transformed *E. coli* BL21DE3 strain was inoculated in Terrific Broth, and allowed to express overnight. Lane 1 and 10, Biosciences low molecular weight markers; lane 2, BiP69-C2 pellet; lane 3, BiP69-C2 supernatant; lane 4, BiP60-C2 pellet; lane 5, BiP60-C2 supernatant; lane 6, ATPD-C2 pellet; lane 7, ATPD-C2 supernatant; lane 8, PBD-C2 pellet; lane 9, PBD-C2 supernatant; lane 11, C-term-C2 pellet; lane 12, C-term-C2 supernatant; lane 13, BiP69 pellet; lane 14, C2 pellet; lane 15, *E. coli* BL21DE3 cells. Lysate samples (PBS resuspended pellet and supernatant) were mixed with equal volumes of reducing treatment buffer before boiling for 5 min. Protein expression was analysed using reducing 10% SDS-PAGE and staining with Coomassie blue R-250.

Expression of soluble proteins has been achieved when culturing temperatures were lowered, allowing slow expression of target protein and proper folding by the *E. coli* chaperone machinery (Baneyx, 1999; Joseph and Andreotti, 2008). Overexpression of His-tagged BiP-C2 fusion protein chimeras when cultured at 37°C resulted in protein aggregation, forming inclusion bodies. Inclusion bodies are very difficult to work with as they require solubilisation with high concentrations of detergents and often require a renaturation step, which would

usually result in the loss of some of the target protein. To avoid this scenario, His-tagged BiP-C2 fusion protein chimeras' expression was attempted at lower temperatures. Expression of His-tagged BiP-C2 chimeras at 16^{o}C for 24 h had no effect on protein solubility (Fig. 2.16). Expression at 22^{o}C did not improve solubility for most protein chimeras either (Fig. 2.17), except for BiP69 which had partially soluble protein (Fig. 2.17 Panel B, lane 2).

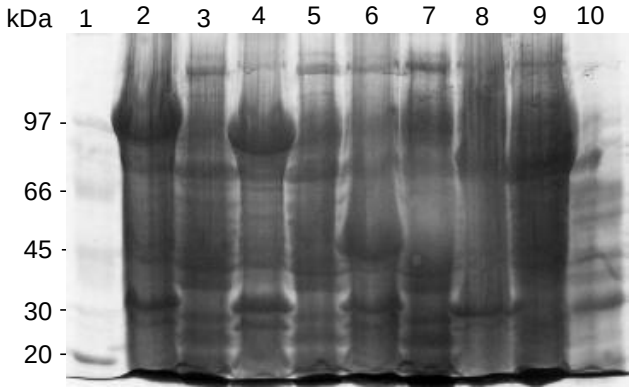

Figure 2.16: Expression of His-tagged BiP-C2 fusion protein chimeras in Terrific Broth at 16^{o}C for 24 h. A single colony from BiP69-C2, BiP60-C2, PBD-C2 or BiP69 recombinant *E. coli* BL21DE3 clones was picked from a plate and inoculated in Terrific Broth. The colony was cultured at 37^{o}C overnight and the culture diluted (1:50) in new Terrific Broth, before being further cultured for 24 h at 16^{o}C. Protein expression and solubility was analysed on a 10% SDS-PAGE gel, followed by staining in Coomassie blue R-250. Lane 1, Biosciences low molecular weight marker (Amersham); lane 2, BiP69-C2 pellet; lane 3, BiP69-C2 supernatant; lane 4, BiP60-C2 pellet; lane 5, BiP60-C2 supernatant; lane 6, PBD-C2 pellet; lane 7: PBD-C2 supernatant; lane 8, BiP69 pellet; lane 9, BiP69 supernatant; lane 10, non-recombinant *E. coli* BL21DE3cells.

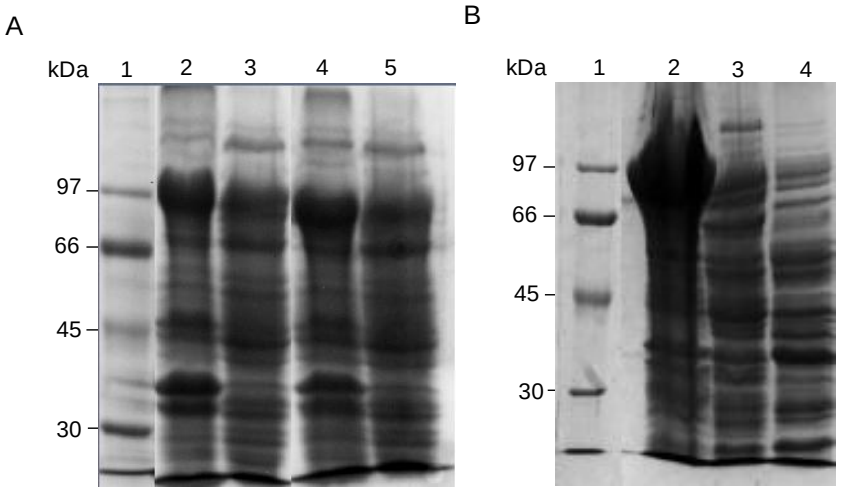

Figure 2.17: Expression of His-tagged BiP-C2 fusion protein chimeras in Terrific Broth at 22^{o}C for 18 h. A single colony selected from His-tagged BiP69-C2 and BiP60-C2 recombinant *E. coli* BL21DE3 clones (**A**) and BiP69 recombinant *E. coli* BL21DE3 clones (**B**) were inoculated in Terrific Broth media and grown for 18 h at 22^{o}C. Protein expression and solubility was analysed on a 10% SDS-PAGE gel followed by staining in Coomassie blue R-250. A: lane 1, Biosciences molecular weight marker (Amersham); lane 2, BiP69-C2 pellet; lane 3, BiP69-C2 supernatant; lane 4, BiP60-C2 pellet; lane 5, BiP60-C2 supernatant. B: Lane 1, Biosciences low molecular weight marker; lane 2, BiP69 pellet; lane 3, BiP69 supernatant; lane 4, *E. coli* BL21DE3 cells.

Failure to express soluble His-tagged BiP-C2 fusion protein chimeras at lower temperatures, meant that proteins required purification under denaturing conditions, and refolding of denatured proteins to their native form for immunisation experiments. The expression of His-tagged BiP-C2 fusion protein chimeras was confirmed by western blot. Proteins separated on a SDS-PAGE gel were transferred onto a nitrocellulose membrane and probed with chicken anti-BiP IgY antibodies and chicken anti-congopain N-terminal peptide antibodies, to confirm that both BiP and C2 were present in each of the chimeras (Fig 2.18).

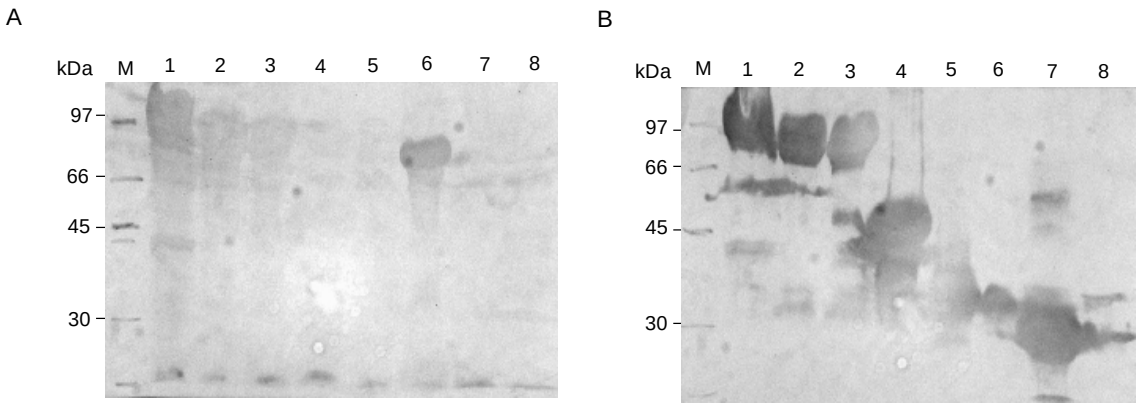

Figure 2.18: Western blot analysis of expressed BiP-C2 chimeras using chicken anti-congopain N-terminal peptide and chicken anti-BiP antibodies. Expressed His-tagged BiP-C2 chimeras were probed with anti-BiP (**A**) and anti-congopain N-terminal peptide (**B**) antibodies (1 µg/ml) and HRPO linked rabbit anti-chicken antibodies (1:10 000) used as a secondary antibody. A & B: M, Biosciences molecular weight marker; lane 1, BiP69-C2 pellet; lane 2, BiP60-C2 pellet; lane 3, ATPD-C2 pellet; lane 4, PBD-C2 pellet; lane 5, C-term-C2 pellet; lane 6: BiP69 pellet; lane 7, C2 pellet; lane 8, *E. coli* BL21DE3 cells.

Anti-BiP antibodies (Fig. 2.18, Panel A) were able to detect BiP69-C2 (lane 1), BiP60-C2 (lane 2), ATPD-C2 (lane 3) and BiP69 (lane 6) but could not detect PBD-C2 and C-term-C2 (lanes 4 and 5). This can be explained by the fact that the anti-BiP antibodies recognise N-terminal epitopes of BiP, and thus cannot detect the variable C-terminal and conserved peptide binding domains. Anti-congopain N-terminal peptide antibodies detected expression of all His-tagged BiP-C2 fusion protein chimeras (Fig. 2.18, Panel B). Non-specific interaction is observed for both anti-BiP and anti-congopain N-terminal peptide antibodies, as bands at smaller sizes were detected. Some of these bands are attributable to the degradation of fusion protein chimeras by bacterial proteases.

Efforts to re-clone ATPD-C2 to obtain a chimera of the expected size using new ATPD clones were unsuccessful, as some clones either did not express or expressed at 50 kDa, a much smaller size than expected (Fig 2.19, Panel A, lanes 2-5). Probing of His-tagged ATPD-C2 fusion protein chimera by western blot confirmed that only the ATPD part of the ATPD-C2 chimera was being expressed at 50 kDa (Fig. 2.19, Panel B, lanes 7 and 8). Probing with anti-congopain N-terminal peptide antibodies did not detect C2 as a fused chimera at 70 kDa as expected but only C2 at 27 kDa (Fig 2.19, Panel C, lane 13). Sequencing of the pET-28a-ATPD-C2 plasmid DNA did not show any mutations or insertion of a stop codon causing incomplete expression of the chimera.

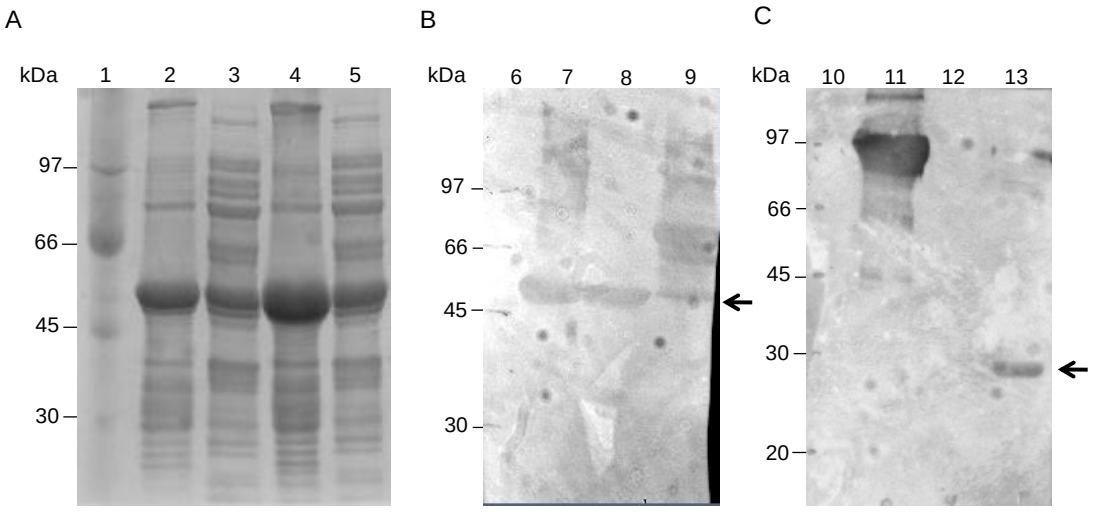

Figure 2.19: Expression of ATPD-C2 in Terrific Broth and analysis with western blotting using anti-BiP and anti-congopain N-terminal peptide antibodies. Recombinant clones of ATPD-C2 were expressed in Terrific Broth and analysed using 10% SDS-PAGE and staining with Coomassie R-250 dye (Panel A). Expressed ATPD-C2 chimeras were probed with mouse anti-BiP M1D2 monoclonal antibody at 1:2 000 dilution (Panel B) and chicken anti-congopain N-terminal peptide antibodies at 1 µg/ml (Panel C). Goat anti-mouse IgG HRPO conjugated (1:10 000) and rabbit anti-chicken IgY-HRPO (1:10 000) were used as secondary antibodies. A: lane 1, Biosciences molecular weight marker (Amersham); lane 2, ATPD-C2 pellet in PBS clone 1; lane 3, ATPD-C2 supernatant clone 1; lane 4, ATPD-C2 pellet in PBS clone 2; lane 5, ATPD-C2 supernatant clone 2. B: Western blot analysis of ATPD-C2 chimera using anti-BiP M1D2 antibodies; lane 6, Biosciences molecular weight marker (Amersham); lane 7, ATPD-C2 pellet in PBS; lane 8, ATPD-C2 supernatant; lane 9, BiP69 pellet control. C: lane 10, Biosciences molecular weight marker (Amersham); lane 11, BiP69-C2 control; lane 12, ATPD-C2 supernatant; lane 13, ATPD-C2 pellet in PBS. The arrows show the respective proteins of interest.

Cloning of all BiP fragments, namely BiP69, BiP60, ATPD, PBD and C-terminus as well as congopain catalytic domain (C2) into the pTZ57RT vector was successful. All constructs were sent for sequencing, and confirmed to be the expected sizes with no mutations. C2 was successfully sub-cloned into a pET-28a expression vector, followed by cloning of BiP fragments into the same recombinant vector. His-tagged BiP-C2 fusion protein chimeras, His-

tagged C2 and BiP69 fusion proteins were successfully expressed in auto-inducing, glucose rich media (Terrific Broth) as insoluble inclusion bodies. Over- expression was observed at *E. coli* optimum growing temperature (37^{o}C) and attempts to express these His-tagged fusion proteins at lower temperatures as soluble proteins were unsuccessful. The expressed proteins were confirmed to have both BiP and C2 segments, by western blot using chicken anti-BiP and anti-congopain N-terminal peptide antibodies.

2.4 DISCUSSION

African trypanosomiasis still remains a major problem in Sub-Saharan Africa (Simarro *et al.*, 2008; Hide and Tait, 2009; Welburn *et al.*, 2009; Molyneux *et al.*, 2010). The emergence of drug- and multi-drug resistance threatens livestock production and has already caused a decline in productivity (Bray *et al.*, 2003; Shaw, 2004; Delespaux and de Koning, 2007; Delespaux *et al.*, 2008). With no sign of new drugs on the market and delays in finding alternative and safe control methods such as vaccination, trypanosomiasis eradication remains difficult (Bhalla, 2002; Vale and Torr, 2004; Welburn *et al.*, 2009). The concept of an anti-disease vaccine strategy, targeting the disease rather than the parasite, was developed aiming to inhibit invariant antigens implicated in pathogenesis (Playfair *et al.*, 1990; Authié, 1994; Antoine-Moussiaux *et al.*, 2009). Congopain, implicated in the development of anaemia in the host (Authié, 1994) and recognised by sera from trypanotolerant cattle, is one of the invariant antigens under investigation for the development of an anti-disease vaccine (Authié *et al.*, 2001). The catalytic domain of congopain expressed in *Baculovirus,* has been used in immunisation studies in cattle (using RWL proprietary adjuvant from SmithKline-Beecham) (Authié *et al.*, 2001). Although this adjuvant induced higher and more sustained antibody titres in cattle against congopain and reduced host symptoms were observed (e.g. cattle maintained weight with milder anaemia) upon infection with *T. congolense* IL 3000 parasites, there was no difference in the development of infection when compared to non-immunised cattle (Authié *et al.*, 2001).

The objective of the present study was to improve the immune response against C2/congopain by using a chaperone BiP as a molecular adjuvant, with the hope of raising robust humoral or cell mediated immune response capable of completely inhibiting C2 activity and reducing disease pathology. C2 has been complexed or conjugated with other adjuvants, namely RWL

proprietary adjuvant (Authié *et al.*, 2001), α_2-macroglobulin (Huson *et al.*, 2009), Adjuphos and Quil A (Bizaaré, 2008; Kateregga *et al*. In preparation), ISA206 oil-in-water (Alain Boulangé pers. comm.), all with a hope of improving the immunogenicity of C2 and ideally attaining full protection of the host upon trypanosome infection. Antibodies from mice immunised with C2, co-administered with alum, or combination of Adjuphos and Quil A, and rabbits immunised with a C2-α_2-macroglobulin complex, inhibited between 65-100% of C2/congopain activity (Bizaaré, 2008; Huson *et al.*, 2009). However, only partial protection was provided when the animals were challenged with *T. congolense* (Bizaaré, 2008; Kateregga *et al*. In preparation).

In the present study, the results of construction of congopain catalytic domain (C2) fused to various domains of BiP, and their subsequent expression in a bacterial system is reported. In the design of these constructs, a truncated form of congopain, i.e. the catalytic domain (C2) was chosen, because it was found that chimeras containing the pro-domain of congopain have a propensity to cleave the chimeras while expressing in *P. pastoris* (Ndlovu, 2009). Although, it has been shown that the pro-domain is vital in the proper folding of the protease and maintenance of the inactive zymogen state (Lalmanach *et al.*, 1998; Korde *et al.*, 2008; Lanfranco *et al.*, 2008), its proteolytic activity during protein expression and purification (Ndlovu, 2009), resulted in its removal from all constructs. *T. congolense* BiP, like other HSP70 members, has three functionally distinct domains: an N-terminal ATPase domain, the C-terminal peptide binding domain and a C-terminal domain. How these different domains stimulate the immune response is not fully understood (Robert, 2003; Lehner *et al.*, 2004). However, Wang and co-workers (2002) and Li *et al*. (2006) have shown that truncated *Mycobacterium* HSP70 C-terminal peptide binding domain can stimulate C-C chemokine and cytokine production, tumor necrosis factor, IL-12, NO and dendritic cell maturation.

To identify which domain of BiP has adjuvant potential, these domains of *T. congolense* BiP were fused to C2 in frame on the N-terminus, and expressed as recombinant fusion protein chimeras in a His-tagged pET-28a vector. It has been shown that antigens genetically fused to HSP70 are better delivered and presented to APCs, resulting in increased immune response when compared to antigens complexed to HSP70 or co-administered along with HSP70 (Ge *et al.*, 2006; Li *et al.*, 2006; Morell *et al.*, 2006; Li *et al.*, 2007a). His-tagged BiP-C2 fusion

protein chimeras, namely full length BiP69-C2, BiP60-C2 (BiP69 devoid of the C-terminus), N-terminal ATPD-C2, C-terminal PBD-C2 and C-term-C2, were expressed as insoluble proteins retained in inclusion bodies. The C-terminus of *T. congolense* BiP has been shown to be highly immunogenic, with specific epitopes not found in other organisms, including trypanosonomal species (Bangs *et al.*, 1993; Boulangé *et al.*, 2002). The role played by the immunogenic 10 kDa C-terminal extension was also investigated, firstly by linking it to C2 alone and secondly by excluding it from the BiP60-C2 and PBD-C2 constructs. Full length BiP69 and its truncated forms have been expressed as recombinant MBP fusion proteins in a pMAL bacterial system (Boulangé *et al.*, 2002). Although truncated forms of BiP expressed predominantly as soluble proteins, full length BiP was expressed mostly as insoluble protein. This is consistent with studies reporting expression of other kinetoplastids BiP homologs in *T. b. brucei* and *L. donovani*, as insoluble proteins in *E. coli* (Bangs *et al.*, 1993; Jensen *et al.*, 2002).

Attempts to express and purify MBP fusion protein chimeras of full length BiP69 and its truncated form BiP60, genetically linked to C2, were unsuccessful (Ndlovu, 2009). This was attributed to the expression of chimeras as insoluble inclusion bodies, and difficulty in purification using amylose affinity chromatography, as higher and lower molecular mass bacterial proteins co-purified (Boulangé *et al.*, 2002; Ndlovu, 2009). This expression as insoluble protein was thought to be the result of poor solubility of MBP fusion protein chimeras in a pMAL vector. Therefore, a vector with a small carrier protein was proposed, which would not interfere with immunisation studies, and would allow easy purification. An expression vector coding for polyhistidines is the preferred choice of carrier protein in protein purification (Arnau *et al.*, 2006a; Arnau *et al.*, 2006b). A small His-tag on fusion proteins often does not interfere with enzymatic characterisation of proteases (Morty *et al.*, 2005). This is confirmed by studies on recombinant hamster BiP expressed in *E. coli* with six His-residues on the N-terminus, which showed no interference with ATPase activity (Wei and Hendershot, 1995).

All His-tagged BiP-C2 chimeras were expressed as insoluble inclusion bodies in *E. coli*. Protein aggregation can be due to a number of factors including specific DNA sequences likely to code for amino acid residues that form intermolecular bonds/interactions during

structural protein synthesis, over-loading of the chaperone or refolding capacity of the expression host (Sijwali *et al.*, 2001) and a change in primary protein structure, resulting from mutations (Baneyx, 1999; Ho *et al.*, 2003; Jhamb *et al.*, 2008).

This meant that, a difficult purification procedure would be necessary, requiring solubilisation and refolding of each protein to its native state. His-tagged C2 and BiP69 fusion proteins were expressed at their respective expected sizes and with the exception of the His-tagged ATPD-C2 fusion protein chimera, all other His-tagged BiP-C2 fusion protein chimeras were expressed at their expected sizes. Detection by western blotting using anti-BiP and anti-congopain N-terminal peptide antibodies confirmed that all chimeras contained the required BiP domain and C2. The anti-BiP antibodies could not detect PBD-C2 and C-term-C2 chimeras, as they appear to recognise only epitopes on the N-terminus of the BiP69 molecule. The non-specific binding observed may be due to cross-reactivity of the chicken anti-BiP antibodies with bacterial proteins. The recombinant BiP protein used for antibody production was cut out from an SDS-PAGE gel and it is possible that some bacterial proteins were also cut out and injected together with BiP, resulting in antibodies that cross-react with bacterial proteins. The anti-congopain N-terminal peptide antibodies detected all His-tagged BiP-C2 fusion protein chimeras expressed at their expected sizes.

The His-tagged ATPD-C2 fusion protein chimera was expressed at an unexpected size of 88 kDa, and the construct was thus sent for sequencing. Sequencing results revealed that a random DNA sequence had been incorporated into the construct, so it was re-amplified and re-cloned. Following re-cloning, the His-tagged ATPD-C2 fusion protein chimera was expressed as a partially soluble fusion protein chimera at a size of 50 kDa, much lower than the 66 kDa expected. Expression of His-tagged ATPD-C2 as a smaller protein could not be explained, but after probing with antibodies in western blotting, it was shown that mouse anti-BiP antibodies detected the ATPase domain of the BiP molecule, in the 50 kDa protein. Probing with anti-congopain N-terminal peptide antibodies, detected a 27 kDa protein band in the soluble fraction that was possibly C2. The behaviour of ATPD-C2 fusion protein could not be explained, so it was omitted from the vaccination experiment. Furthermore, the ATPase domain has been shown in previous studies not to play a role in adjuvanticity of HSPs (Wang *et al.*, 2002; Ge *et al.*, 2006; Li *et al.*, 2006).

It has been shown that protein aggregation in *E. coli* can be reduced by culturing at lower temperature since it slows down the rate of protein expression, and thereby improving folding and increasing yield of soluble protein (Baneyx, 1999; Joseph and Andreotti, 2008). Attempts to express these fusion protein chimeras at lower temperatures (22^{o}C and 16^{o}C) were unsuccessful except for BiP69 which expressed some soluble protein at 22^{o}C. Formation of inclusion bodies could be attributed to the bacterial chaperone system (DnaK-DnaJ, GroEL/ES) being inactive at lower temperatures and thus unable to facilitate refolding of expressed proteins (Joseph and Andreotti, 2008). Competent cells like the ArticExpress® system (Stratagene) have a chaperone system that can maintain activity at much lower temperatures (Joseph and Andreotti, 2008), and this system could be evaluated in future for the expression of these His-tagged BiP-C2 fusion protein chimeras.

BiP69 is an ER localised soluble protein shown by its localisation tetrapeptide MDDL, and is not secreted in the cytoplasm during normal non-stress conditions (Bangs *et al.*, 1993; Boulangé and Authié, 1994; Bangs *et al.*, 1996; Jensen *et al.*, 2002; Ikadai *et al.*, 2005). However, with increased stress levels e.g. a significant drop in temperature, it can be partially secreted into the cytoplasm to assist with folding and trafficking of misfolded proteins (Vogel *et al.*, 1990; Kumar *et al.*, 1991; Pidoux and Armstrong, 1993; Boulangé *et al.*, 2002; Jensen *et al.*, 2002). This was observed when full length BiP was expressed at 22^{o}C, but it could not assist in refolding the whole chimera.

In the present study the congopain catalytic domain was successfully cloned into a pTZ57RT vector and subsequently sub-cloned into the pET-28a expression vector. The various BiP fragments were successfully sub-cloned into the pET-28a-C2 recombinant vector. His-tagged full length BiP69-C2, BiP60-C2, PBD-C2, C-term-C2, full length BiP69 and C2 transformed in *E. coli* BL21DE3 were over-expressed in inclusion bodies in *E. coli*. Lowering culturing temperatures had no effect in attaining soluble proteins, but full length BiP was partially soluble at 22^{o}C. Antibodies against BiP recognised N-terminal epitopes of recombinant BiP while antibodies directed against N-terminal peptide of congopain recognised all expressed recombinant proteins. These His-tagged fusion proteins will be purified and refolded for vaccination studies.

3 PURIFICATION OF *TRYPANOSOMA CONGOLENSE* BIP-C2 FUSION PROTEIN CHIMERAS AND TESTING THEIR VACCINE POTENTIAL IN MICE.

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ABSTRACT

Kinetoplastid cysteine peptidases have been a target for chemotherapy and vaccine development against parasitic diseases. Congopain, the cysteine peptidase of *Trypanosoma congolense*, is associated with trypanotolerance in N'Dama cattle and is a target for an anti-disease vaccine strategy. In order to improve the presentation of the catalytic domain of congopain (C2) to the immune system, it was linked to the proposed molecular adjuvant, BiP. A further aim was to localise the domain(s) of BiP where the adjuvant properties reside. Consequently recombinantly expressed peptide binding domain (PBD)-C2, C-terminus-C2, BiP69-C2 and BiP60-C2 chimeras as well as a BiP69 fusion protein were purified and refolded by a Ni-NTA based one-step on-column refolding method. Bacterial proteins co-purifying with BiP69-C2 and BiP60-C2 chimeras were removed by incubation with 5 mM ATP in the dissociation buffer. Immunisation of Balb/c mice with the BiP69-C2 fusion protein chimera induced a higher antibody response to C2 compared to control groups. BiP69-C2 and PBD-C2 chimeras and a mixture of BiP69 and C2 induced a robust antibody response to BiP69. Mice immunised with BiP69-C2 and PBD-C2 chimeras showed a better booster effect upon infection with *T. congolense* compared to control groups. Mice immunised with a mixture of BiP69 and C2 and with the PBD-C2 chimera cleared parasites and maintained very low levels of parasites 10-15 days earlier than mice immunised with other BiP-C2 chimeras and control groups and showed no clinical symptoms of the disease. There was no significant difference in percentage survival between BiP-C2 chimera immunised mice and control groups immunised with C2 alone or with a mixture of Adjuphos/Quil A or immunised with PBS.

3.1 INTRODUCTION

Trypanosoma congolense is the most prevalent and detrimental trypanosome species causing African animal trypanosomiasis (Hide, 1999). The disease costs to African agriculture are estimated to be more than 4.5 billion US$ per annum (Kristjanson *et al.*, 1999). The clinical symptoms in livestock infected with the disease include anaemia, weight loss, high fever and increased foetal abortion rates (Murray *et al.*, 1982; Büscher and Lejon, 2004; Taylor and Authié, 2004).

Control strategies include vector control using insecticides, insect traps, trypanocidal therapeutic drugs, clearing of forests and the use of trypanotolerant cattle. These strategies are largely ineffective or cause huge environmental concerns (Kabayo, 2002; Vale and Torr, 2004; Van den Bossche and De Deken, 2004; Welburn *et al.*, 2009). Chemotherapy is the only available method of controlling established disease, but is limited by drug resistance and the absence of new drugs (Barrett *et al.*, 2004; Holmes *et al.*, 2004; Barrett *et al.*, 2007; Delespaux and de Koning, 2007; Mamoudou *et al.*, 2008) . There is currently no effective vaccine against African trypanosomiasis, due to difficulties in its development (Welburn *et al.*, 2009; Molyneux *et al.*, 2010). This is largely due to the highly variable surface glycoproteins covering the surface of the parasite, which enables it to evade the host immune system (Barry *et al.*, 1985; Barry and McCulloch, 2009; Morrison *et al.*, 2009).

An anti-disease vaccine strategy was a concept proposed for design of a malarial vaccine, which aimed to control the disease rather than the parasite (Playfair *et al.*, 1990). The strategy targets invariant antigens implicated in pathogenesis, and immunisation with these antigens is thought to protect animals by inducing a protective immune response (Playfair, 1991; Authié *et al.*, 1993a; Authié, 1994; Authié *et al.*, 2001). Partial protection has been achieved when immunising with a *T. b. rhodesiense* flagellar pocket antigen fraction (Mkunza *et al.*, 1995), *T. b. brucei* and *T. evansi* tubulin (Lubega *et al.*, 2002; Li *et al.*, 2007b), microtubule associated proteins (Rasooly and Balaban, 2004) and congopain of *T. congolense* (Authié *et al.*, 2001).

Congopain, a major cysteine peptidase of *T. congolense*, is a target for an anti-disease vaccine, as it has been implicated in natural trypanotolerance of West African N'Dama cattle. Immunisation of cattle with the catalytic domain of congopain (C2) partially protected naïve

cattle, by mounting an antibody response that inhibited congopain activity (Authié *et al.*, 2001). Failure of congopain to fully protect the host against *T. congolense* infection has been attributed to its poor immunogenicity and insufficient presentation and delivery of its protective epitopes by conventional adjuvants (Authié *et al.*, 2001; Lalmanach *et al.*, 2002; Boulangé *et al.*, 2011). Sera from trypanotolerant cattle consistently recognised the dimeric form of congopain, found at physiological pH (Serveau *et al.*, 2003; Boulangé *et al.*, 2011) and thought to contain the protective epitopes. In order to improve the immunogenicity of congopain, chimeras were produced with the molecular adjuvant, BiP, an ER localised HSP70 of *T. congolense*.

Heat shock proteins are members of the "stress protein" superfamily (Pockley *et al.*, 2008) and are chaperones involved in folding of newly synthesised and misfolded proteins, protein trafficking and degradation (Lindquist and Craig, 1988; Hartl, 1996; Hartl and Hayer-Hartl, 2002; Bukau *et al.*, 2006). Heat Shock Protein 70 (HSP70) is believed to play a vital role in regulation of the immune response, showing potential as an adjuvant in subunit vaccine development (MacAry *et al.*, 2004; Tobian *et al.*, 2005; Su *et al.*, 2007; Li *et al.*, 2009). This potential is exhibited by the HSP70 chaperoning of exogenous complexed peptides and internalisation by antigen presenting cells, through a receptor mediated endocytosis pathway (Lehner *et al.*, 2004; Javid *et al.*, 2007; Karyampudi and Ghosh, 2008; Nishikawa *et al.*, 2008). The internalised antigens are presented to MHC class I and II molecules eliciting T lymphocyte mediated immunity (Blachere *et al.*, 1997; Ge *et al.*, 2006; Li *et al.*, 2006; Li *et al.*, 2007a; Nishikawa *et al.*, 2008).

The structure of HSP70 comprises a 44 kDa N-terminal ATPase domain, an 18 kDa C-terminal peptide binding domain and a 10 kDa C-terminal extension (Bukau and Horwich, 1998; Hartl and Hayer-Hartl, 2002; Wang *et al.*, 2002; Lehner *et al.*, 2004; Javid *et al.*, 2007). The ATPase and peptide binding domains are highly conserved across all species with sequence identity ranging from 45-70%. The C-terminal domain is highly variable and differs between species (Boulangé and Authié, 1994; Ikadai *et al.*, 2005). It has been shown that mycobacterial HSP70 has adjuvant effects when linked to the major antigenic segment of Japanese encephalitis virus, antigenic epitopes of foot and mouth diseases virus, hantaviral nucleocapsid protein of Hantavirus, and the hepatitis B surface protein of Hepatitis B virus,

resulting in significantly enhanced humoral and cellular responses (Ge *et al.*, 2006; Li *et al.*, 2006; Li *et al.*, 2007a; Su *et al.*, 2007).

Wang *et al*. (2002), did a study where either mycobacterial HSP70 or the C-terminal peptide binding domain (HSP70 $_{359-610}$) was covalently linked to synthetic peptides, and the resulting HSP-peptide complexes were used for the immunisation of rhesus macaques primates and Balb/c mice. This induced maturation of dendritic cells and stimulation of Th1 type polarised cytokines shown by higher serum IgG2a and IgG3 antibodies, as well as significantly higher induction of IL-12, TNF-α and NO and reduced autoantibody responses. It was also shown that removal of the ATPase domain of mycobacterial HSP70 was necessary for effective stimulation of monocytes to produce IL-12, TNF-α, NO and C-C chemokine.

In this chapter humoral immune response of Balb/c mice when immunised with BiP-C2 fusion protein chimeras is reported. Different BiP-C2 fusion protein chimeras were previously constructed and recombinantly expressed (Chapter 2). Here the purification and refolding of His-tagged BiP69-C2, BiP60-C2, PBD-C2, and C-term-C2 fusion protein chimeras and BiP69 from inclusion bodies is described. Balb/c mice were immunised with each purified BiP-C2 chimera, C2 alone, or mixed with BiP69 or conventional adjuvants and the antibody response followed by ELISA. Mice were challenged by *T. congolense* infection and a possible booster effect of infection on antibody levels, parasitemia and disease progression monitored to evaluate the adjuvant potential of the different BiP domains.

3.2 MATERIALS AND METHODS

Materials

Buffer salts and other common chemicals were obtained from Saarchem (South Africa), Merck (Germany) and Sigma-Aldrich (Germany) and were of the highest purity available.

His-select™ Nickel affinity resin (Ni-NTA), His select™ Cobalt affinity resin (Co-NTA), Guanidine-HCl and Sephacryl S-300HR resin were obtained from Sigma (Munich, Germany). Chicken anti-congopain N-terminal peptide IgY antibodies (Mkhize, 2003) and chicken anti-BiP IgY antibodies were in-house antibodies. Mouse anti-BiP N-terminal monoclonal antibodies (M1D4) and mouse anti-BiP C-terminal (M1B2) monoclonal antibodies were a gift

from Dr A Boulangé. Horse anti-mouse IgG-HPRO conjugate and HPRO conjugated goat anti-mouse IgG antibodies were obtained from Jackson Immunochemicals (USA), and rabbit anti-chicken IgY-HPRO conjugate from Sigma (Steinhem, Germany). Nitrocellulose membrane was purchased from GE Healthcare (Frieburg, Germany) and Vacutainer™ tubes from BD Vacutainer Systems (UK). Nunc-Immuno™ 96-well plates were from Nunc Intermed (Denmark). EndoTrap® columns were obtained from Profos AG (Regensburg, Germany). 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] (ABTS) was obtained from Roche (Germany). Procedures using animals received ethical approval from the University of KwaZulu-Natal animal ethics committee (reference number 069/2010/Animal). Pathogen free Balb/c mice were obtained from National Health Laboratory Services (Johannesburg, South Africa). Adjuphos® and Quil A® adjuvants were a gift from Erik Lindlad, Brenntag Biosector, Frederikssund, (Denmark).

Methods

3.2.1 Washing of insoluble inclusion bodies of His-tagged fusion proteins

The pellet obtained from separating insoluble and soluble recombinantly expressed His-tagged fusion protein (Section 2.2.6.5) after lysis with lysis buffer was resuspended in PBS and centrifuged (10 000 x g, 15 min, RT). The resulting pellet was washed twice with 1% (v/v) Triton X-100, twice with water and twice with 1 M urea with centrifugation (10 000 x g, 5 min, RT) after each wash. The supernatants were kept for SDS-PAGE analysis (Section 3.2.8). The resulting insoluble inclusion bodies were purified and solubilised following a method by Sijwali *et al*. (2001). The cell pellet was resuspended and washed twice with wash buffer 1 [2 M urea, 20 mM Tris-Cl, 2.5% (v/v) Triton X-100, pH 8.0] and centrifuged (12 000 x g, 30 min, 4^{o}C) between each wash. The resulting pellet was washed twice with wash buffer 2 [20% (w/v) sucrose, 20 mM Tris-Cl, pH 8.0] and centrifuged (12 000 x g, 30 min, 4^{o}C) between each wash. Finally the pellet was solubilised with solubilisation buffer (6 M guanidine-Cl, 20 mM Tris-Cl, 500 mM NaCl, 10 mM imidizole, pH 8.0) overnight at RT. Solubilised sample was centrifuged (27 000 x g, 30 min, 4^{o}C) and the resulting supernatant loaded onto a 1 ml His-select™ Nickel affinity column (Sigma).

3.2.2 Purification of His-tagged fusion proteins

Insoluble His-tagged fusion proteins were first purified under denaturing conditions using a His-select™ Nickel affinity column. Small protein amounts and poor protein refolding limited this method and a one-step on-column refolding method was pursued which allows easy purification and refolding of bound proteins (Glynou *et al.,* 2003; Lemercier *et al.,* 2003). Partially soluble His-tagged BiP69 fusion protein was purified under native conditions using a His-select™ Nickel affinity column.

3.2.2.1 Purification of insoluble His-tagged fusion proteins under denaturing conditions

The insoluble fusion protein chimeras were purified under denaturation conditions on a His-select™ Nickel affinity column according to the manufacturer's protocol (Sigma) with minor modifications involving washing with 20 mM imidazole to wash off non-specifically bound proteins and elution with 250 mM imidazole in a lower pH (6.3) buffer, followed by a further decrease in pH of the elution buffer to 4.6 with the omission of imidazole to elute strongly bound His-tagged proteins. Prior to use the column was equilibrated with 3 column volumes of Buffer A (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 8.0). The solubilised sample was circulated over the column for an hour at RT or overnight at 4^{o}C, and the unbound fraction kept. The Ni-NTA column was washed with 3 column volumes of Buffer A and washes collected in 1.5 ml aliquots. The column was washed with 20 mM imidazole in Buffer A, followed by washing with 3 column volumes of Buffer B (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 6.3). The fusion protein was eluted with 250 mM imidazole in Buffer B followed by a final elution with Buffer C (100 mM NaH_2PO_4, 10 mM Tris-Cl, 8 M urea, pH 4.6).

3.2.2.2 Purification of His-tagged fusion proteins by an on-column refolding method

The His-select™ Nickel affinity column (1 ml) was washed with distilled water, recharged with 0.1 M NiCl, further washed with 5 ml distilled water before equilibrating with 5 ml solubilisation buffer (6 M guanidine-Cl, 500 mM NaCl, 20 mM Tris-Cl, 10 mM imidazole, 2 mM β-mercaptoethanol, pH 8.0). The solubilised protein (5 ml) was mixed with resin in a 15 ml conical tube and allowed to bind for 1 hour at RT or overnight at 4^{o}C with rotation. The resin-protein mixture was packed into 2 ml Pierce® column and the flow-through was

collected. The column was washed with 5 ml solubilisation buffer followed by a wash with 5 ml Wash buffer (6 M urea, 500 mM NaCl, 20 mM Tris-Cl, 20 mM imidazole, 2 mM β-mercaptoethanol, pH 8.0). The bound protein was refolded while absorbed on the column using a linear gradient of 6-0 M urea (30 ml, flow rate, 0.7 ml/min). The bound protein was further washed with 5 ml of Wash buffer without urea. The refolded protein was eluted using a linear gradient of 20 to 500 mM imidazole in elution buffer (500 mM NaCl, 50 mM Tris-Cl, 1 mM β-mercaptoethanol, pH 8.0) in a total volume of 20 ml. The washes and eluted fractions were kept for analysis by reducing SDS-PAGE (Section 3.2.8).

3.2.2.3 Purification of soluble His-tagged fusion proteins under native conditions

The manufacturer's protocol (Sigma) was used for purification of soluble protein using a Ni-NTA resin. His-tagged BiP69 soluble protein (5 ml) was mixed with 1 ml of His select™ Nickel affinity resin in a 15 ml conical tube, and binding effected overnight at 4^{o}C by end-over-end rotation or rotation for 1 hour at RT. The mixture was packed into a column (2 ml). The flow-through was collected and the column washed twice with 4 ml wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidizole, pH 8.0) and 1.5 ml samples collected. The column was finally eluted with four column volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0). All collected samples were analysed by SDS-PAGE (Section 3.2.8).

3.2.3 Purification of His-tagged fusion proteins by molecular exclusion chromatography (MEC)

A Sephacryl S-300 HR column (1.25 x 83 cm) was equilibrated with 50 mM Tris-Cl buffer, pH 7.5 and calibrated with a mixture of 2 mg/ml blue dextran (> 2 000 kDa) and 5 mg/ml each of bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (34.3 kDa), soy bean trypsin inhibitor (21 kDa), and lysozyme (14 kDa). The fractions were eluted with 50 mM Tris-Cl buffer and 2 ml fractions collected. The absorbance values at 280 nm (A_{280nm}) were taken for each sample to construct a standard curve (Fig. 3.1). The calibration curve was constructed by determining the K_{av} of each standard protein. The void volume (V_o) of the column was determined using the elution volume (V_e) of blue dextran. The availability constant (K_{av}) was calculated based on the following equation: $K_{av} = V_e - V_o/V_t - V_o$, where K_{av} = availability constant; V_e = elution volume; V_o = void volume and V_t = total column

volume (Dennison, 1999). The K_{av}-values were used to plot a standard curve of K_{av} vs log M_r. The protein sample (BiP69-C2 or BiP60-C2) was concentrated by dialysis against PEG 20 000 to a volume equivalent to 2.5% of the column volume and loaded onto the column. Fractions of 2 ml each were collected, their A_{280nm} monitored, and samples corresponding to the peaks of absorption at 280 nm analysed by reducing SDS-PAGE.

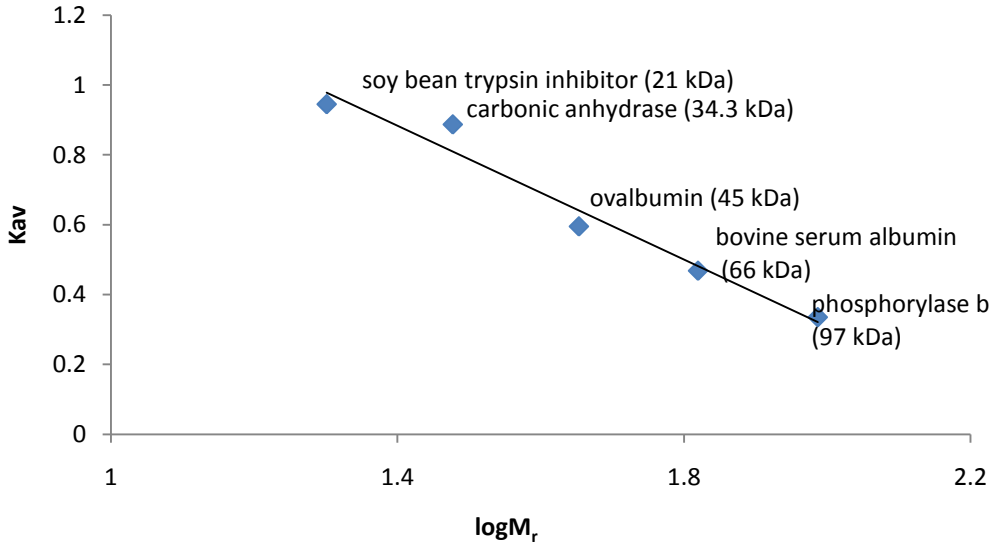


Figure 3.1: Fischer's plot of availability constant (K_{av}) of each standard protein against log M_r used to estimate M_r of proteins. The calibration mixture was applied to a Sephacryl S-300 HR column (1.25 × 83 cm) and eluted with 50 mM Tris-Cl buffer, pH 7.5 (25 $ml.h^{-1}$, 4ºC). The availability constant (K_{av}) was determined for each protein standard. The equation of the slope is y = -0.9567x + 2.2222 with correlation co-efficient of 0.9656.

3.2.4 Refolding of His-tagged fusion protein chimeras by stepwise dilution

The denatured samples of each chimera eluted from the Ni-NTA column were pooled, the resulting sample reduced by addition of 10 mM DTT. The reduced protein was dialysed in a 10 kDa cut-off dialysis tube against 1 L of renaturation buffer (50 mM L-arginine, 20 mM Tris-Cl buffer, pH 8.5, 20% (v/v) glycerol, 4 M urea, 0.05 mM reduced glutathione, 0.1 mM oxidized glutathione, 1 mM EDTA, 1 µM E-64, 1 µM leupeptin, 1 µM AEBSF) for 16 h at 4^{o}C. The urea concentration in the renaturation buffer was changed to 2 M and finally omitted in each successive dialysis step. The refolded protein was concentrated using ultrafiltration (Amicon concentrator, Millipore, Rockford, USA) with a 30 kDa cut-off membrane.

3.2.5 Removal of co-purifying bacterial proteins by washing with dissociation buffer

Removal of co-purifying bacterial proteins from His-tagged BiP69-C2 and BiP60-C2 fusion protein chimeras was achieved by a minor modification of the on-column refolding method using Ni-NTA affinity resin. Denatured His-tagged BiP-C2 fusion protein chimera was mixed with 2 ml of the Ni-NTA affinity resin and allowed to bind by mixing at 250 rpm either for 1 h at RT or overnight at 4^{o}C. The on-column refolding protocol (Amersham) was followed using a 50 mM phosphate buffer, pH 8.0 containing (150 mM NaCl, 20 mM imidazole and 2 mM β-mercaptoethanol). The column was incubated with dissociation buffer (20 mM HEPES, 10 mM $MgCl_2$, 150 mM NaCl, 50 mM KH_2PO_4, 5 mM ATP, pH 7.0) for 2 h at 4^{o}C and further washed with phosphate buffer before elution with 500 mM imidazole in elution buffer (50 mM Tris-Cl, pH 7.4, 0.5 M NaCl, 1 mM β-mercaptoethanol).

3.2.6 Expression of the catalytic domain of congopain (C2) in *Pichia pastoris*

A glycerol stock of *P. pastoris* GS115 containing pPic9-C2 was streaked on a YPD plate [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose and 15 g/L bacteriological agar] containing ampicillin (50 µg/ ml) and incubated for two days at 30^{o}C. A single colony was picked and grown in 100 ml of YPD medium [1% (w/v) yeast extract, 2% (w/v) peptone, 2% (w/v) dextrose] with 50 µg/ml of ampicillin to prevent contamination. The culture was allowed to grow for 2-3 days at 30^{o}C in an orbital shaking incubator. These cells were transferred to 1 L of buffered glycerol BMGY medium [1% (w/v) yeast extract, 2% (w/v) peptone, 100 mM potassium phosphate buffer, pH 6.5 and 1.34% (w/v) yeast nitrogen base (YNB)] in a baffled flask. The culture was allowed to grow for a further 2 days at 30^{o}C in a shaking incubator to increase biomass. The cells were harvested by centrifugation (5 000 x g, 10 min, 4^{o}C) and resuspended in 500 ml of buffered minimal medium (BMM) [100 mM potassium phosphate buffer, pH 6.5, 1.34% (w/v) YNB, 0.00004% (w/v) biotin and 0.5% (v/v) methanol]. The culture was grown in a baffled 2 L flask for a further 4-6 days in a 30^{o}C shaking incubator to allow expression of C2. Methanol [0.5% (v/v)] was added daily during the expression period to induce continuous expression of C2 in *P. pastoris.* Cells were pelleted by centrifugation (5000 x g, 10 min, RT) and the supernatant kept at -20^{o}C, as *P. pastoris* strains secretes protein of interest into the medium (Aloulou *et al.*, 2006).

3.2.7 Purification of C2

3.2.7.1 Three Phase Partitioning (TPP)

The recombinantly expressed C2 containing supernatant (500 ml) was thawed and filtered through Whatman No 1 filter paper. Three phase partitioning (Pike and Dennison, 1989) was used as an initial method for concentrating and purification of recombinant C2. The pH of the supernatant was lowered to pH 4.2 with phosphoric acid to effect autocatalytical maturation of C2 to the active form of the peptidase. Tertiary butanol [30% (v/v), 250 ml] was added to 500 ml of supernatant and C2 was precipitated out into the third phase by adding ammonium sulfate [40% (w/v), 300 g)]. The salt was completely dissolved by stirring and the mixture was centrifuged (6 000 x g, 10 min, 4^{o}C) using a pre-chilled swing out rotor. The precipitated protein containing layer was collected at the interface between the aqueous and tertiary butanol layers and redissolved in phosphate buffered saline (PBS) [100 mM Na_2HPO_4, 2 mM KH_2PHO_4, 2.7 mM KCl and 137 mM NaCl, pH 7.2]. The protein was dialysed against several changes of PBS for 48 h at 4^{o}C using 10 kDa cut-off snake Skin® dialysis tubing (Rockford, USA), to remove any residual traces of tertiary butanol and salts. Reducing SDS-PAGE analysis of a C2 sample purified by TPP revealed that a further purification step was required to remove higher molecular weight proteins.

3.2.7.2 Ion exchange chromatography

A column (0.75 x 10 cm) was packed with Sephadex C-25 resin (Sigma) and equilibrated with 50 mM sodium acetate buffer, pH 4.2 (25 ml.h^{-1}, 4^{o}C). The dialysed C2 protein sample (5 ml) was loaded and circulated through the column overnight at 4^{o}C. The column was further washed with sodium acetate buffer until the A_{280} reached baseline. Five column volumes of a 0 - 1 M NaCl gradient in sodium acetate buffer was used to elute bound protein and elution was monitored by measuring A_{280}. The samples representing peak absorbance values were analysed by reducing 12% SDS-PAGE. Fractions containing purified C2 were pooled and concentrated using ultrafiltration (10 kDa cut-off Centricon YM-10, Rockford, USA).

3.2.8 SDS-PAGE for analysis of proteins

Analysis of protein samples was carried out in a 10% SDS-PAGE gel using a discontinuous Tris-Glycine system described by Laemmli (1970). SDS tightly binds to protein molecules and

gives the protein a negative charge. When negatively charged protein-SDS complexes are subjected to electrophoresis, they experience the same current and are separated based solely on molecular weight (Dennison, 1999). Proteins were separated through a large pore stacking gel, pH 6.8, and a small pore running gel at pH 8.8, allowing clear separation of proteins of different sizes. A mini-PROTEAN 3® gel system (BioRad) was used to separate proteins. His-tagged BiP-C2 fusion protein chimeras, BiP69 or C2 protein samples were mixed with an equal volume of reducing treatment buffer [125 mM Tris-Cl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) β-mercaptoethanol] and boiled for 5 min before loading. Low molecular weight markers (Amersham) comprising phosphorylase b (97 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (34.3 kDa), soy bean trypsin inhibitor (21 kDa) and lysozyme (14 kDa) were used to construct a calibration curve and included on all gels as a M_r reference. Electrophoresis was conducted in Tank buffer [250 mM Tris-Cl buffer, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3] at 20 mA per gel. The proteins were stained with Coomassie brilliant blue R-250 [0.125 g (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v) glacial acetic acid]. The molecular size of protein bands were determined using a calibration curve prepared by plotting the relative mobility of protein molecular weight standards against the log of their molecular weight (Fig. 3.2) (Dennison, 1999).

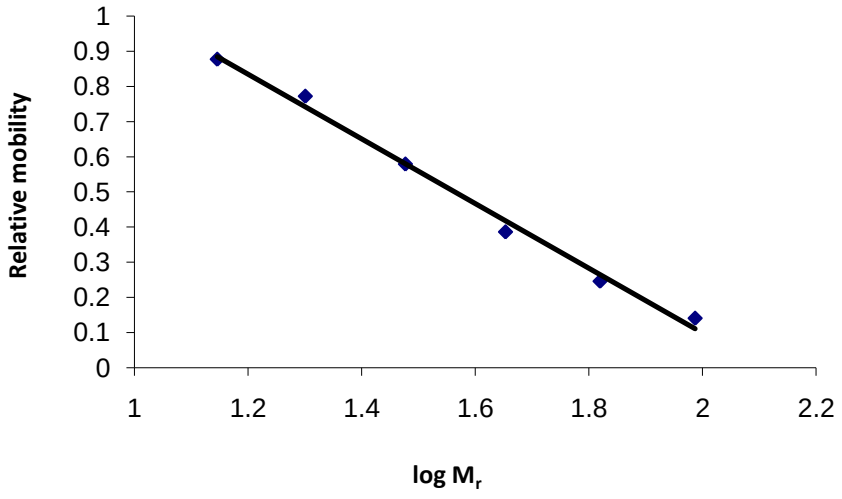


Figure 3.2: Calibration curve of relative mobility of standard proteins against their log M_r used to determine M_r of proteins of unknown size. Biosciences low molecular weight markers (Amersham) contains: phosphorylase b (97 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (34.3 kDa), soybean trypsin inhibitor (21 kDa) and lysozyme (14 kDa). The equation of the slope is y = -0.9203x + 1.9394 with a correlation co-efficient of 0.9925.

The detection limit for Coomassie blue is approximately 50-100 ng of protein. When more sensitive protein detection was required, a silver stain method with a detection limit of 1-10 ng described by Blum *et al.* (1987) was employed. Proteins with low concentration for detection were concentrated with a SDS/KCl precipitation method before loading.

Silver staining

Briefly, the gel was soaked in fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.05% (v/v) 37% formaldehyde] in a clean glass Petri dish overnight followed by washing in washing solution 1 [50% (v/v) ethanol] (3 x 20 min). The gel was then soaked in pretreatment solution [0.02% (w/v) $Na_2S_2O_3.5H_2O$] for 1 min, rinsed in dH_2O (3 x 20 s), and soaked in impregnation solution [0.2% (w/v) $AgNO_3$, 0.075% (v/v) 37% formaldehyde] (20 min). The gel was rinsed in dH_2O (3 x 20 s) and immersed in developing solution [6% (w/v) Na_2CO_3, 0.0004% (w/v) $Na_2S_2O_3.5H_2O$, 0.05% (v/v) 37% formaldehyde] until the protein bands became visible. The gel was washed in dH_2O (2 x 2 min) and development stopped by immersing in stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] (10 min) before being washed in washing solution 2 [50% (v/v) methanol] for 20 min.

3.2.9 Protein quantification assay

Protein concentration was determined using two methods, namely BCA™ Protein Assay kit (Pierce, Rockford, IL, USA) and direct visualisation of protein bands on an SDS-PAGE gel and comparing with BSA protein standards of known concentration also separated on SDS-PAGE under reducing conditions. For the BCA protein assay, reagent A was mixed with reagent B, in a 50:1 ratio to prepare the working reagent. Bovine serum albumin protein standards (25 – 2 000 µg/ml) were each mixed with working reagent (200 µl) in a Nunc® 96 microtiter plate and incubated at 37^{o}C for 30 min. The bicinchoninic acid in the working reagent interacts with proteins producing a purple coloured product which is measured at 595 nm. A standard curve was constructed plotting absorbance at 595 nm vs standard BSA concentrations (Fig. 3.3).

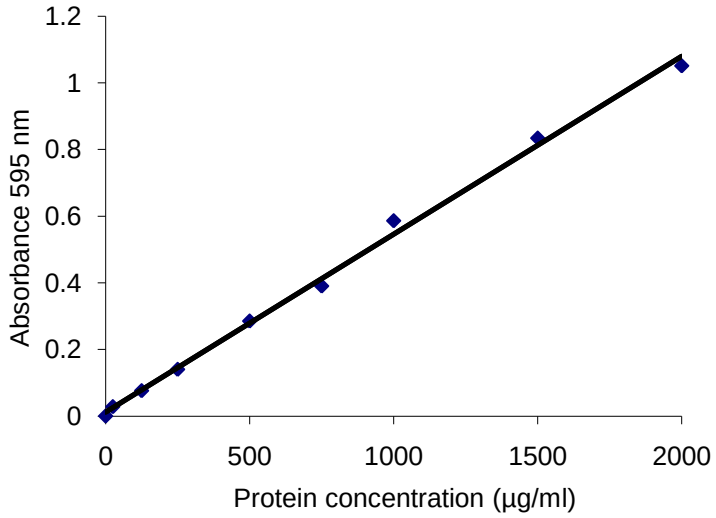


Figure 3.3: Calibration curve to quantify protein samples unknown concentration. The BCA protein assay was used to determine the absorbance of BSA standard protein samples (25 - 2 000 µg/ml) and a standard curve constructed. The equation of the slope is y = 0.0005x + 0.0119 with a correlation co-efficient of 0.9968.

Quantification of proteins by direct visualisation was achieved by separating standard amounts of BSA (25 - 2000 µg/ml) on a 10% SDS-PAGE gel under reducing conditions followed by staining with Coomassie blue R-250. The concentration of proteins of unknown concentration was estimated by comparing protein band intensity against known protein standards (Fig. 3.4).

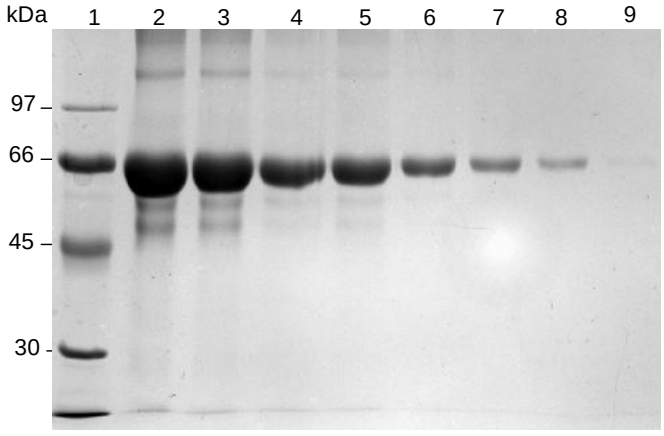


Figure 3.4: Determination of BSA protein concentration by direct visualisation on a SDS-PAGE gel. The BSA protein standards (2 000 - 25 µg/ml) were mixed with an equal volume of reducing treatment buffer and boiled for 5 min before separation on a reducing 10% SDS-PAGE gel. Lane 1, Biosciences Low molecular weight marker (Amersham); lane 2, 2000 µg/ml; lane 3, 1500 µg/ml; lane 4, 1000 µg/ml; lane 5, 750 µg/ml; lane 6, 500 µg/ml; lane 7, 250 µg/ml; lane 8, 125 µg/ml and lane 9, 25 µg/ml. After electrophoresis the gel was stained with Coomassie blue R-250.

3.2.10 Western blot using either anti-BiP, anti-congopain N-terminal peptide or anti-His-tag antibodies

Proteins separated by SDS-PAGE were transferred to a nitrocellulose membrane (Amersham Biosciences) as described by Towbin *et al*. (1979) for 1 h using a dry blotter (BioRad, Hercules, CA, USA) at 17 V, maximum current. The nitrocellulose membrane was transiently stained with Ponceau S [0.1% (w/v) Ponceau S in 1% (v/v) glacial acetic acid] to mark the positions of molecular mass standards and destained by adding a drop of 1 M NaOH to the distilled H_2O used for rinsing the membrane. The unoccupied sites on the nitrocellulose membrane were blocked for 1 h with low fat milk powder in Tris Buffered Saline [5% (w/v) low fat milk powder, 20 mM Tris-Cl, 200 mM NaCl, pH 7.4], followed by washes (3 x 5 min) with TBS. This was followed by incubation for 2 h with primary antibody (affinity purified chicken anti-congopain N-terminal peptide antibodies (1 µg/ml) for C2 and mouse anti-BiP M1D4 or M1B2 monoclonal antibodies (1: 2000) or chicken anti-BiP antibodies (1 µg/ml) for BiP or HPRO-conjugated mouse anti-His-tag monoclonal antibodies (1: 500), diluted in 0.5% (w/v) BSA in TBS. This was followed by washes of the nitrocellulose membrane with TBS (3 x 5 min) before it was incubated for 1 h with HRPO-conjugated secondary antibody diluted in 0.5% (w/v) BSA in TBS (rabbit anti-IgY-HPRO conjugate or horse/goat anti-mouse IgG-HPRO conjugate). The membrane was washed again in TBS (3 x 5 min) before being developed in substrate solution [0.06% (w/v) 4-chloro-1-naphthol, 0.1% (v/v) methanol, 0.0015% (v/v) hydrogen peroxide (H_2O_2) in TBS]. This was left to develop in the dark until protein bands were visible.

3.2.11 Endotoxin removal by EndoTrap®

In the present study, endotoxins were removed from recombinantly expressed bacterial proteins before immunisation of mice using a polymaxin B based endotoxin affinity resin, EndoTrap® (Profos AG, Germany), according to the manufacturer's specifications.

3.2.12 Immunisation of mice

Mice were kept under pathogen free conditions in the Animal House in the School of Biological and Conservation Sciences, Pietermaritzburg campus, University of KwaZulu-Natal. Six to eight weeks old female Balb/c mice (40) were put in 8 cages of 5 mice each. All mice were injected intraperitoneally (i.p.), except for C2, mixed with Adjuphos/Quil A which

was injected sub-cutenously. Insulin syringes (1 ml) with 27 guage needles (Terumo™) were used to inject mice on days 0, 7 and 14 at two sites in the abdomen or on the back under the skin. Mice were immunised either with PBS, C2 alone, C2 mixed with adjuvant mixture [Quil A (30 µg) and Adjuphos (1:1 ratio in volume)] in PBS, C2 mixed with BiP69 protein, PBD-C2, C-term-C2, BiP69-C2 or BiP60-C2. All mice were immunised with 0.87 µmole of protein per mouse in a final volume of 100 µl. PBS was injected in a final volume of 100 µl. The mice were boosted with the same dose of each immunogen and were bled through the tail once per week and blood collected into heparinised capillary tubes. Capillaries were centrifuged in a micro-centrifuge for 5 min. The capillaries were broken at the interface between blood cells and plasma, and the plasma collected in microfuge tubes.

3.2.13 Measuring antibody production by Enzyme Linked Immunosorbent Assay (ELISA)

To follow antibody production in mice, 96-well microtitre plates (Nunc Intermed, Denmark) were coated with 150 µl per well of either of either recombinant C2 (1 µg/ml) or recombinant BiP69 (1 µg/ml) diluted in PBS overnight at 4^{o}C. The unoccupied spaces of coated wells were blocked with 0.5% (w/v) BSA-PBS (200 µl per well) at 37^{o}C for 1 h followed by washing 3 times with 0.1% (v/v) PBS-Tween. Mouse plasma from each group was diluted 1:100 with 0.5% (w/v) BSA-PBS and incubated in the wells (100 µl per well) for 2 h at 37^{o}C. This was followed by washing 3 times with 0.1% (v/v) PBS-Tween before incubation with 120 µl per well of HPRO-conjugated goat anti-mouse IgG antibodies [diluted 1 in 10 000 in 0.5% (w/v) BSA-PBS] at 37^{o}C for 1 h. Following 3 times washing with 0.1% (v/v) PBS-Tween, the plate was incubated in the dark with 150 µl per well of ABTS/ H_2O_2 substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in 0.15 M citrate-phosphate buffer, pH 5.0] for 15 min at RT. Once the green colour had developed sufficiently, the absorbance was read at 405 nm using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany).

3.2.14 Infection of immunised mice with *T. congolense* parasites

To determine protection efficacy of immunised mice, they were challenged by infection with blood stream forms of a chronic *T. congolense* IL1180 strain. Firstly, blood of Balb/c mice infected with *T. congolense* IL1180 strain was collected at high parasitemia levels by cardiac puncture. Whole blood was centrifuged (2 000 x g, 5 min, RT), serum diluted with phosphate

saline glucose [57 mM Na_2HPO_4, 3 mM NaH_2PO_4, 42 mM NaCl, 50 mM glucose and 1 mM hypoxanthine] in 50% (v/v) glycerol and live parasites counted using a Neubauer haemocytometer or counts per field of view using light microscopy at 20 x magnification (Paris *et al.*, 1982). Immunised mice were infected with 10^3 parasites per ml, 8 weeks after the last booster injection. Blood was collected every week from the tail and centrifuged (2 000 x g, 5 min). Parasites were counted using a Neubauer haemocytometer once every 3 days for the first 30 days and thereafter once every 4 days to estimate parasitemia burden. When necessary plasma was diluted with PSG to aid parasite counts. Packed cell volume (PCV) percentage was monitored to assess anaemia and disease progression, by measuring the proportion of blood volume that is occupied by red blood cells using capillary microhaematocrit centrifugation. The survival time and disease clinical symptoms after challenge were also monitored. Anti-C2 antibody levels were monitored over an 8 week period after the last boost to determine when antibody levels decreased sufficiently to perform the challenge with parasites. This was done to ensure that a possible booster effect of infection could be observed.

3.2.15 Statistical analysis

A two-way ANOVA test was used to determine wether there was a significant difference in the number of parasites over time post-infection between the experimental and control groups. Log Rank Student's *t*-test was used to determine if there was a significant difference in the percentage of mice survival between experimental and control groups. A *p* value of 0.05 or less was considered significant. GraphPad Prism 5 software was used for analysis.

3.3 RESULTS

3.3.1 Washing of inclusion bodies from insoluble protein

The BiP-C2 chimeras were expressed as insoluble proteins in inclusion bodies (shown in Fig. 2.15). A washing step was required to remove bacterial proteins expressed along with the target His-tagged BiP-C2 fusion protein chimeras (Fig. 3.5).

Upon washing with detergents all His-tagged BiP-C2 fusion protein chimeras seemed to be partially solubilised especially BiP69-C2 (96 kDa), BiP60-C2 (88 kDa) and BiP69 (73 kDa) (Fig. 3.5, Panels A, B and E, lanes 5-9). Some of the BiP-C2 inclusion bodies in the final pellet were also lost as a result of the washes (Fig. 3.5, Panels A-E, lanes 5-16). Most of BiP69 was completely solubilised when washed with 1 M urea (Fig. 3.5, Panel E, lane 12) showing that it is partially soluble. Washes with buffers 1 (containing urea and Triton X-100) and 2 (containing sucrose) significantly reduced the amount of bacterial proteins especially in the case of the PBD-C2 (47 kDa) and C-term-C2 (34 kDa) chimeras (Fig. 3.5, Panels C and D, lanes 5-16). All BiP-C2 chimeras were completely solubilised by 8 M urea (Fig. 3.5, Panels A-E, lane 17).

3.3.2 Purification of His-tagged BiP-C2 fusion protein chimeras using Ni-NTA affinity chromatography under denaturing conditions

Purification of solubilised His-tagged BiP69-C2 and BiP60-C2 fusion protein chimeras, by Nickel affinity chromatography under denaturing conditions, resulted in co-purification of bacterial proteins (Fig. 3.6, Panels A and B, lanes 7-14). In contrast, co-purifying bacterial proteins were successfully removed from His-tagged PBD-C2 and His-tagged C-term-C2 fusion protein chimeras by washes in buffers of decreasing pH and increasing imidazole concentration (Fig. 3.6, Panel C, lanes 12-14 and Panel D, lanes 9 and 10). Most His-tagged BiP-C2 fusion protein chimeras seemed to bind poorly to the nickel resin. Most of the protein either did not bind (Fig. 3.6, Panels A-D, lane 2) or was removed during washing steps, with 20 mM imidazole (Fig. 3.6, Panel A, lanes 3-10, Panel B, lanes 3-9 and Panel C, lanes 3-10).

Small amounts of the His-tagged BiP69-C2 fusion protein chimera were obtained and it co-eluted with bacterial proteins. The His-tagged BiP60-C2 fusion protein chimera also co-eluted with bacterial proteins and both chimeras thus required further purification steps. The His-

tagged PBD-C2 and His-tagged C-term-C2 fusion protein chimeras were eluted as pure proteins (Panel C, lanes 10, 12-14 and Panel D, lanes 9 and 10) and only required refolding.

3.3.3 Refolding of denatured His-tagged BiP-C2 fusion protein chimeras by step-wise dialysis method against refolding buffers

Since His-tagged PBD-C2 and C-term-C2 were successfully purified under denaturing conditions these proteins were refolded by step-wise dilution against refolding buffers with different urea concentrations, chaotrophic detergents and salts which promote refolding. Refolding of His-tagged BiP69-C2 and BiP60-C2 containing co-purifying bacterial proteins was also attempted to assess whether co-purifying bacterial proteins were associated with their denatured form.

Upon refolding by dialysis, aggregated proteins were observed when samples were reduced and boiled before SDS-PAGE analysis as shown by protein bands above 97 kDa (Fig. 3.7, Panel A-C, lanes 3-5). However, this was not observed when protein samples were not reduced or boiled (Fig. 3.7, Panel A-C, lanes 6-9). Refolding of BiP-C2 fusion protein chimeras by dialysis in refolding buffer resulted in significant losses in protein and did not diminish co-purifying bacterial proteins shown by major bands at 90 kDa, 75 kDa, 50 kDa an some minor bands between 30 and 90 kDa (Fig. 3.7, Panels A-C, lanes 2-9). Refolding by step-wise dialysis required big vessels, large amounts of refolding buffer which requires expensive reagents i.e. L-arginine and glutathione (reduced and oxidized) and a further concentration step by ultrafitration, which escalated protein refolding and purification costs. Purified denatured His-tagged BiP69 and PBD-C2 chimera were not refolded by this method due to these reasons. Other purification and refolding methods were pursued which were less costly, more effective and less time consuming e.g. on-column refolding based on gel filtration (Section 3.3.4).

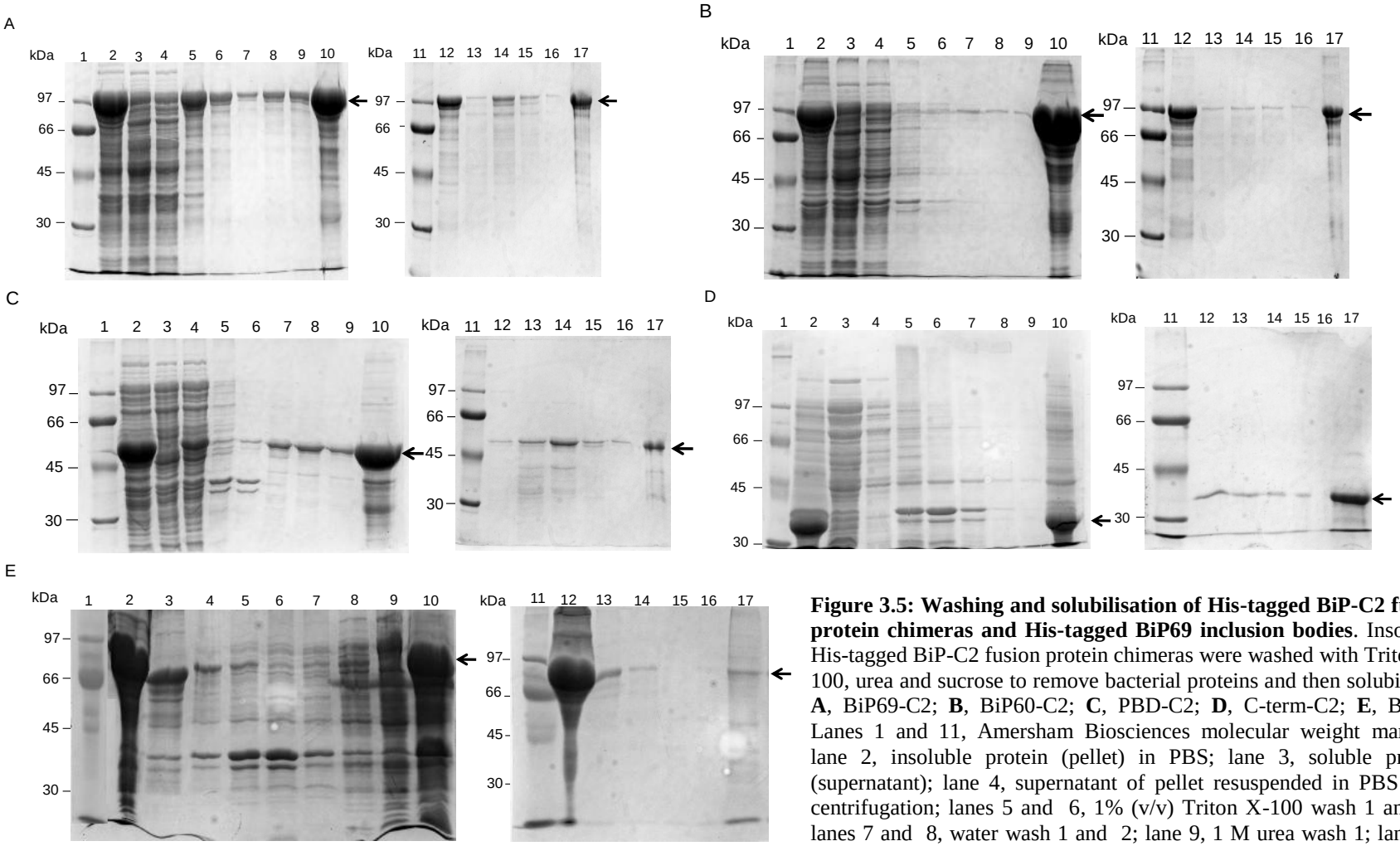


Figure 3.5: Washing and solubilisation of His-tagged BiP-C2 fusion protein chimeras and His-tagged BiP69 inclusion bodies. Insoluble His-tagged BiP-C2 fusion protein chimeras were washed with Triton X-100, urea and sucrose to remove bacterial proteins and then solubilised. **A**, BiP69-C2; **B**, BiP60-C2; **C**, PBD-C2; **D**, C-term-C2; **E**, BiP69. Lanes 1 and 11, Amersham Biosciences molecular weight markers; lane 2, insoluble protein (pellet) in PBS; lane 3, soluble protein (supernatant); lane 4, supernatant of pellet resuspended in PBS after centrifugation; lanes 5 and 6, 1% (v/v) Triton X-100 wash 1 and 2; lanes 7 and 8, water wash 1 and 2; lane 9, 1 M urea wash 1; lane 10, inclusion bodies re-suspended in 1 M urea; lane 12, 1 M urea supernatant; lanes 13 and 14, wash buffer 1; lanes 15 and 16, wash buffer 2; lane 17, inclusion bodies solubilised with 6 M guanidine-Cl. The protein samples were mixed with an equal volume of reducing treatment buffer and boiled before being analysed by reducing 10% SDS-PAGE and stained with Coomassie blue R-250. The arrows show the respective proteins of interest.

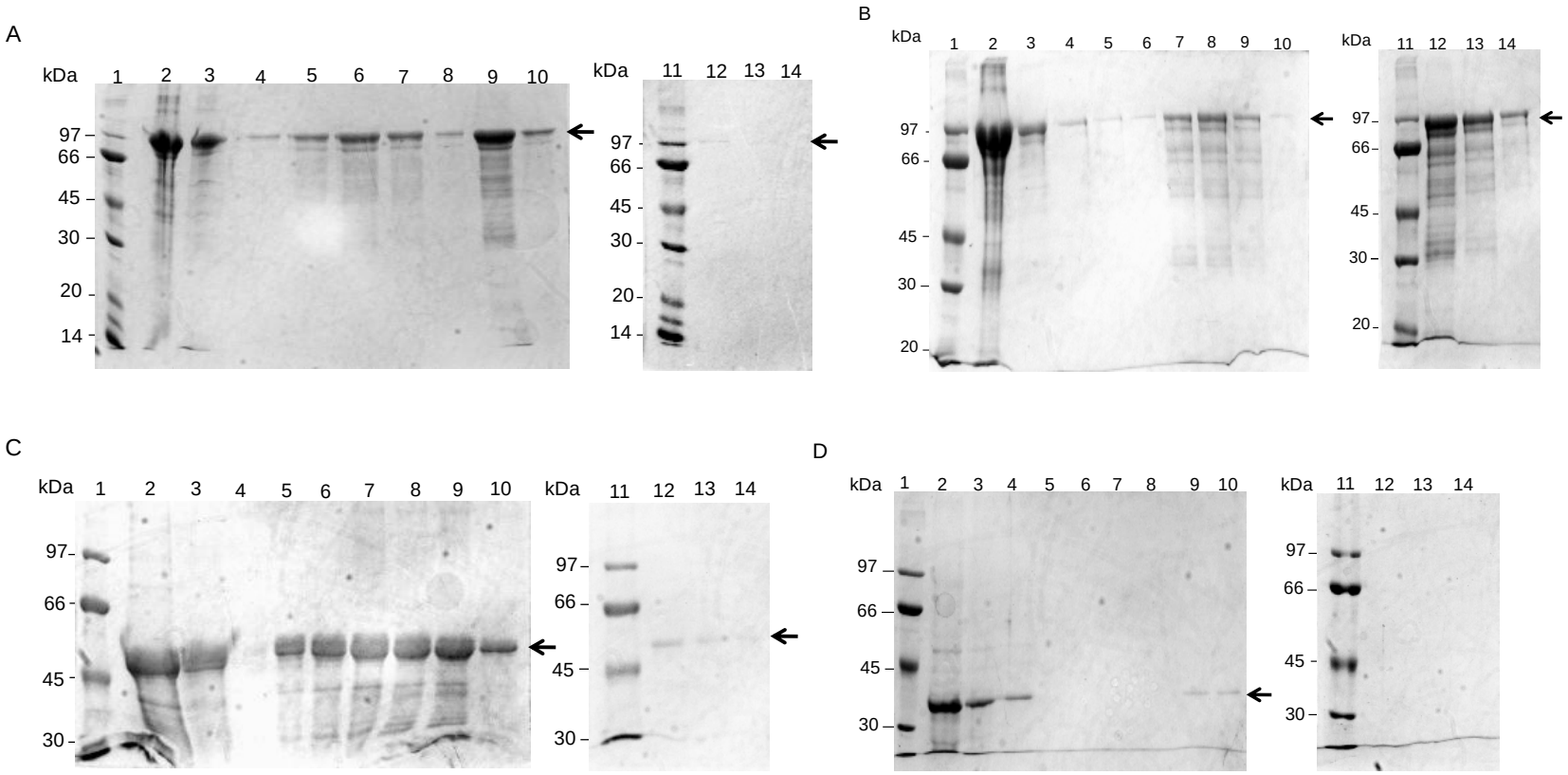


Figure 3.6: Purification of His-tagged BiP-C2 chimeras using Ni-NTA affinity column under denaturing conditions. Solubilised His–tagged protein: **A**, BiP69-C2; **B**, BiP60-C2; **C**, PBD-C2; **D**, C-term-C2 was adsorbed to the Ni-affinity resin. Following washes with buffer of progressively decreasing pH, containing 20 mM imidazole, protein was eluted with 250 mM imidazole. Lanes 1 and 11, Amersham Biosciences molecular weight marker; lane 2, unbound fraction; lanes 3 and 4, pH 8 buffer wash; lanes 5 and 6, 20 mM imidazole in pH 8 buffer wash; lanes 7 and 8, pH 6.3 buffer wash; lanes 9 and 10, elution with 250 mM imidazole in pH 6.3 buffer; lanes 12, 13 and 14, pH 4.6 buffer elutions. The protein samples were mixed with an equal volume of reducing treatment buffer boiled, and analysed by 10% SDS-PAGE and stained with Coomassie blue R-250. The arrows show the respective proteins of interest.

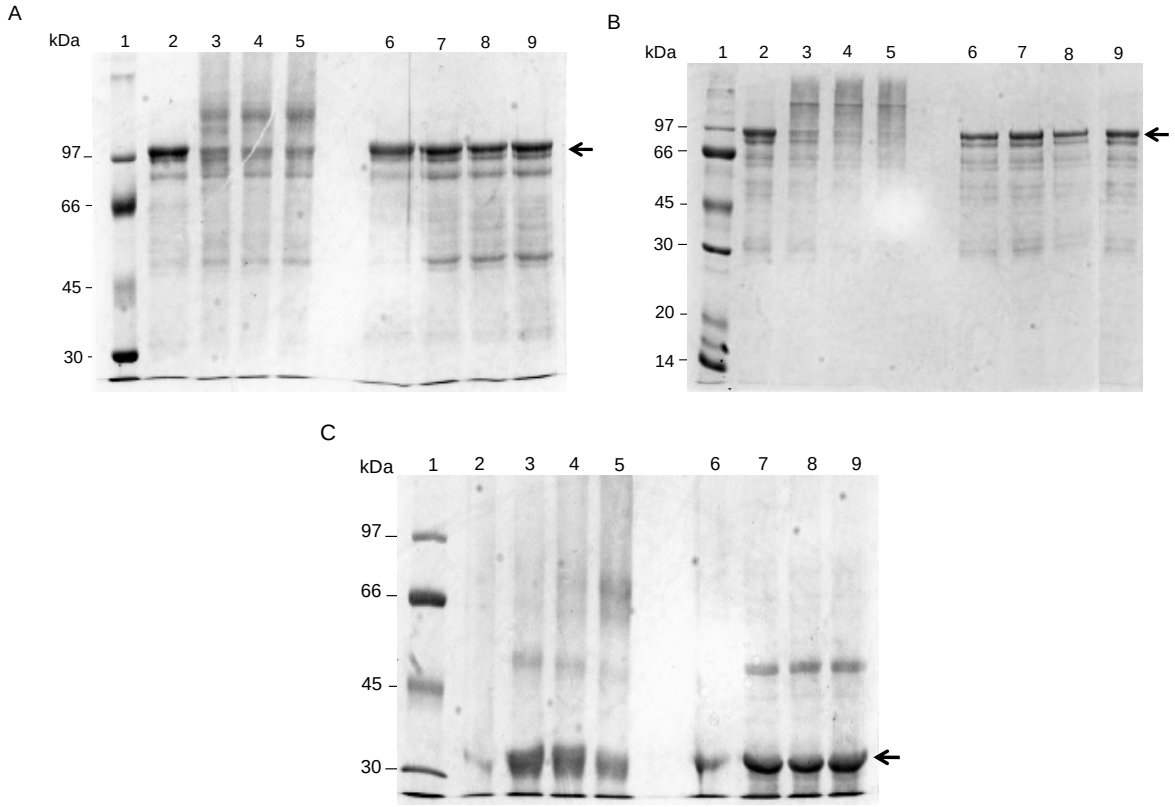


Figure 3.7: Refolding of BiP-C2 chimeras by step-wise dialysis method in refolding buffer with different concentrations of urea. Protein fractions eluted from Ni-chelate affinity columns (**A**, BiP69-C2; **B**, BiP60-C2; **C**, C-term-C2) were pooled, reduced with 10 mM DTT and subsequently dialysed against refolding buffer with decreasing urea concentrations. Lane 1, Amersham Biosciences molecular weight marker; lanes 2 and 6, sample in 4 M urea; lanes 3 and 7, sample in 2 M urea; lanes 4 and 8, sample in 1 M urea; lanes 5 and 9, sample in 0 M urea. Protein samples in lanes 2 - 5 were reduced and boiled and protein samples in lanes 6 - 9 were not boiled and not reduced and all samples were analysed by 10% SDS-PAGE under reducing conditions and stained with Coomassie blue R-250. The arrows show the respective proteins of interest.

3.3.4 On-column refolding and purification of His-tagged BiP-C2 fusion protein chimeras using urea and imidazole gradients

Each solubilised His-tagged BiP-C2 fusion protein chimera was adsorbed to a Ni-affinity resin. The bound protein was then washed and refolded using a decreasing urea gradient, and eluted using an increasing imidazole gradient (Fig. 3.8). On-column refolding and purification did not reduce the number of co-purifying bacterial proteins in samples containing His-tagged BiP69-C2 and BiP60-C2 fusion protein chimeras (Fig. 3.8, Panel A, lanes 10, 12-14; Panel B, lanes 13-16). On-column refolding and purification of His-tagged PBD-C2, His-tagged C-term-C2 and His-tagged BiP69 fusion proteins using Nickel affinity chromatography was successful, resulting in the complete removal of co-purifying bacterial proteins (Fig. 3.8, Panel C, lanes 3-5; Panel D, lanes 3-6 and Panel E, lane 12). Although both His-tagged PBD-C2 and

His-tagged C-term-C2 fusion protein chimeras were eluted before elution with the imidazole gradient, they were washed off as pure proteins with 20 mM imidazole (Fig. 3.8, Panels C and D, lanes 3-5). Although the His-tagged BiP69 fusion protein was eluted at low concentration with 20 mM imidazole, it was refolded and purified to homogeneity (Fig. 3.8, Panel E, lane 12).

3.3.5 Purification of His-tagged fusion protein chimeras by molecular exclusion chromatography

Purification of His-tagged BiP-C2 fusion protein chimeras with Ni^{2+}/Co^{2+}-affinity resins was unsuccessful as bacterial proteins were co-purifying with the target His-tagged fusion protein. To circumvent this, Sephacryl S-300 HR MEC, that separates proteins based on molecular weight, was attempted to remove the co-purifying lower molecular weight bacterial proteins. Proteins eluted as two peaks of 97 kDa and 66 kDa (Fig. 3.9, Panel A). Fractions corresponding to the absorbance peaks were analysed by reducing 10% SDS-PAGE (Fig. 3.9, Panel B). Separation of His-tagged BiP60-C2 fusion protein (97 kDa, arrow in Fig. 3.9) on molecular exclusion chromatography failed to remove bacterial co-purifying proteins at 66 kDa, 45 kDa and 30 kDa (Fig. 3.9, Panel B, lanes 2-15). Bacterial proteins co-eluting with the BiP60-C2 fusion protein required a different approach to remove bacterial proteins (Section 3.3.6).

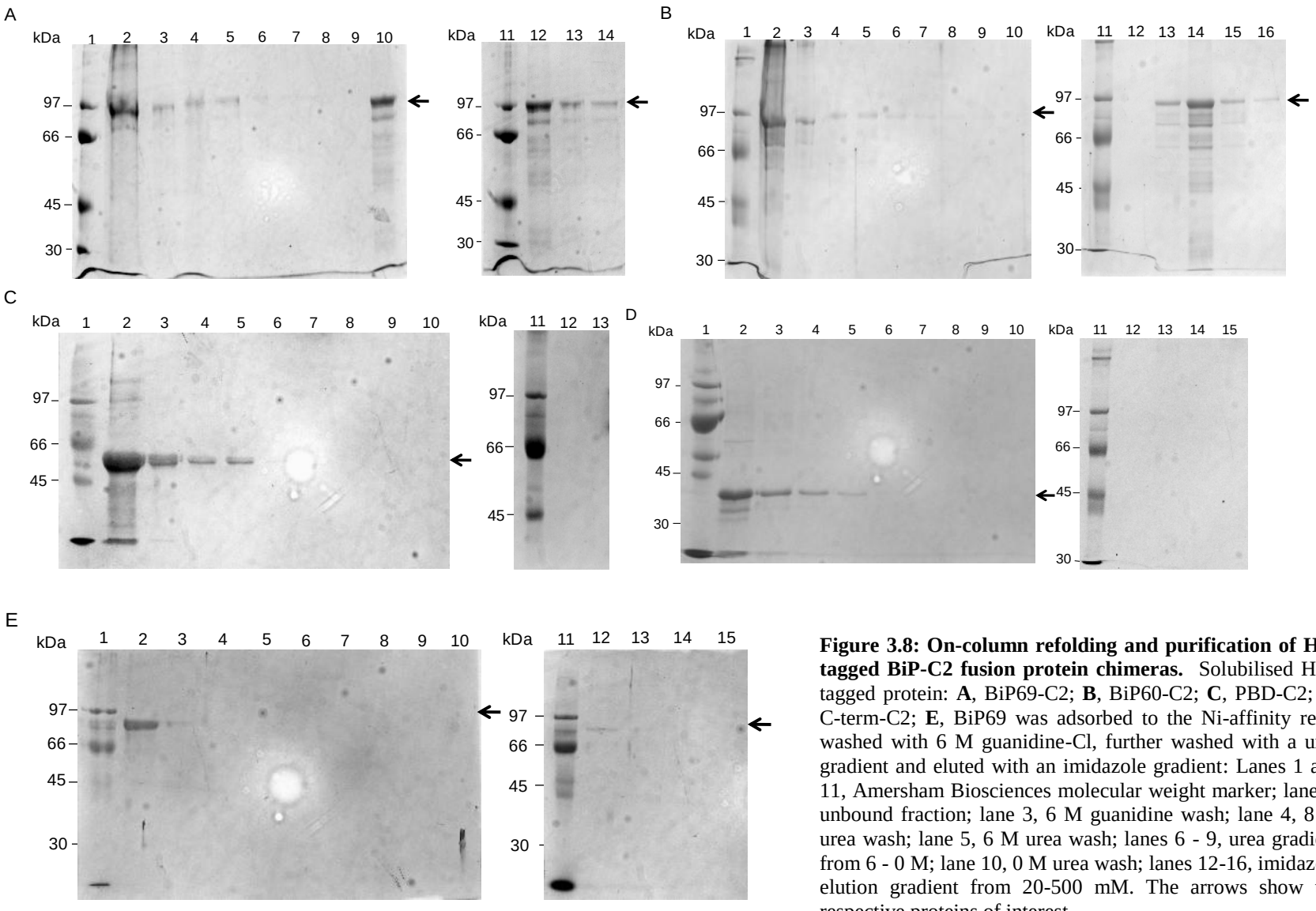


Figure 3.8: On-column refolding and purification of His-tagged BiP-C2 fusion protein chimeras. Solubilised His–tagged protein: **A**, BiP69-C2; **B**, BiP60-C2; **C**, PBD-C2; **D**, C-term-C2; **E**, BiP69 was adsorbed to the Ni-affinity resin washed with 6 M guanidine-Cl, further washed with a urea gradient and eluted with an imidazole gradient: Lanes 1 and 11, Amersham Biosciences molecular weight marker; lane 2, unbound fraction; lane 3, 6 M guanidine wash; lane 4, 8 M urea wash; lane 5, 6 M urea wash; lanes 6 - 9, urea gradient from 6 - 0 M; lane 10, 0 M urea wash; lanes 12-16, imidazole elution gradient from 20-500 mM. The arrows show the respective proteins of interest.

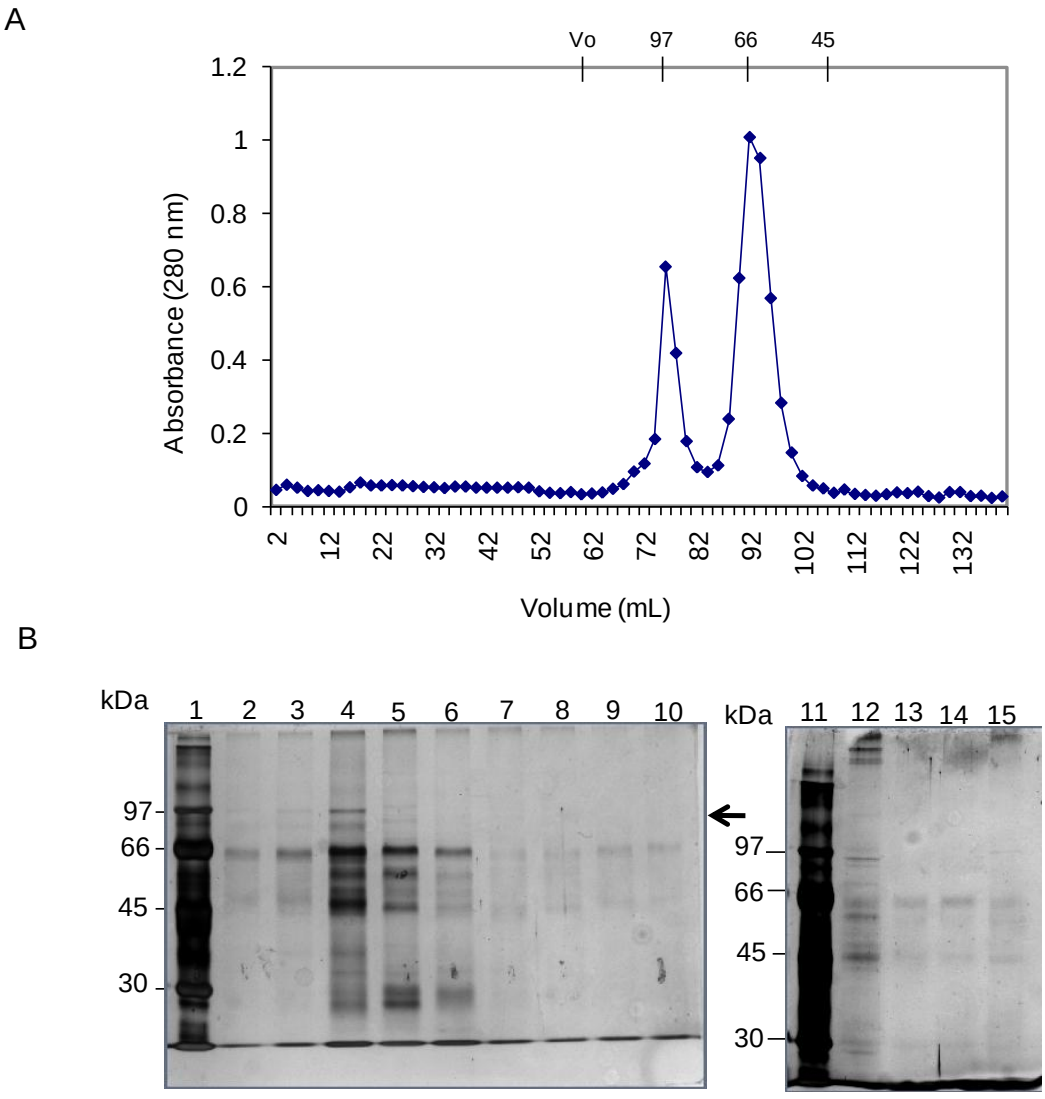


Figure 3.9: Purification of the His-tagged BiP60-C2 fusion protein chimera by molecular exclusion chromatography. A: Elution profile of BiP60-C2 on the Sephacryl S-300HR column (1.25 x 83 cm) equilibrated with 50 mM Tris-Cl buffer, pH 7.5. A total of 74 fractions (2 mL each) were collected at a flow rate (2.4 ml/min) and absorbance readings taken at 280 nm. **B**: Fractions 36-50 corresponding to 72 - 100 ml were further analysed by 10% reducing SDS-PAGE and silver stained. Samples were concentrated using SDS/KCl precipitation method, mixed with an equal volume of reducing treatment buffer and boiled before loading. Lanes 1 and 11, Biosciences low molecular weight marker (Amersham); lanes 2-16, peak fractions from Sephacryl S-300 HR (see Panel A). The arrows show the respective proteins of interest.

3.3.6 Removal of co-purifying bacterial proteins by washing with dissociation buffer

Removal of co-purifying bacterial proteins was achieved when denatured His-tagged BiP69-C2 (Fig. 3.10, Panel A) and BiP60-C2 (Fig. 3.10, Panel B) fusion protein chimeras were allowed to bind for a longer time period to the Ni-NTA resin, incubated in dissociation buffer [20 mM HEPES, 5 mM ATP, 10 mM $MgCl_2$, 150 mM KCl, pH 7.0] for 2 h at 4^{o}C (Fig. 3.10, Panels A and B, lane 10) and refolded by a urea gradient in phosphate buffer (Panels A and B, lanes 5-9). These co-purifying bacterial proteins were suspected to be bacterial chaperones and co-chaperones which tend to bind target proteins especially proteins with similar properties e.g. BiP (Joseph and Andreotti, 2008). Protein yields were too small, but were relatively pure compared to other methods (Panel A, lanes 12-14 and Panel B, lane 13). Attempts to improve protein yields by binding protein to Ni-NTA for longer and using different buffers was unsuccessful.

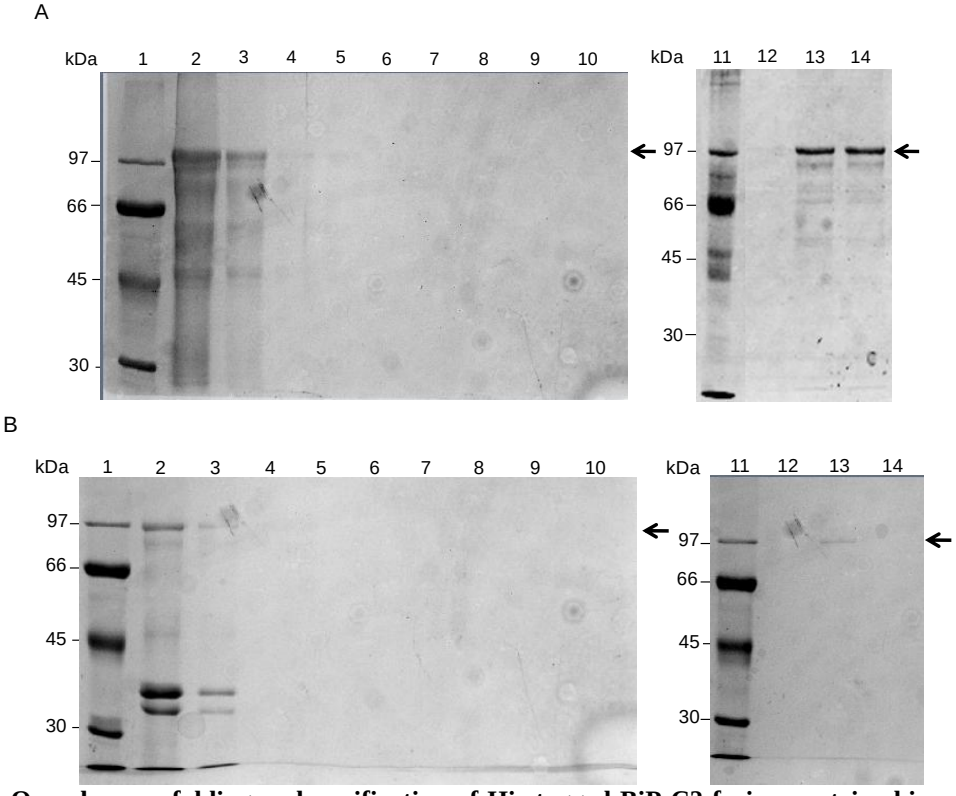


Figure 3.10: On-column refolding and purification of His-tagged BiP-C2 fusion protein chimeras using a Nickel affinity resin, with removal of co-purifying bacterial proteins by 5 mM ATP wash. The Nickel-NTA resin was mixed with 4 M urea solubilised BiP69-C2 (**A**) or BiP60-C2 (**B**) protein (4 mL), and left to bind. The bound protein in the column was refolded with refolding buffer using a urea gradient from 4 - 0 M, before elution with imidazole and analysis on a 10% SDS-PAGE gel followed by staining with Coomassie R-250. Lanes 1 and 11, Amersham Biosciences Low molecular weight marker; lane 2, unbound fraction; lane 3, 4 M urea wash; lanes 4-9, urea gradient from 4-0 M; lane 10, dissociation buffer wash; lane 12, phosphate buffer wash; lanes 13 and 14, elution with 500 mM imidazole in elution buffer. The arrows show the respective proteins of interest.

3.3.7 Purification of His-tagged BiP69 fusion protein under native conditions.

Most of the His-tagged BiP69 fusion protein was solubilised by washing the pellet with 1 M urea buffer (Fig. 3.5, Panel E, lane 12). The solubilised protein was refolded by dialysis against PBS and purified on a Ni-NTA affinity resin. Soluble His-tagged BiP69 fusion protein was purified successfully under native conditions with relatively pure and high protein yields (Fig. 3.11, lanes 6 and 7). Most of bacterial co-purifying proteins were removed by washes before dialysis and also during purification on the column. Purification of soluble His-tagged BiP69 directly from expressed culture mediums was ineffective as significant amounts of bacterial proteins were co-purifying with BiP69 (results not shown).

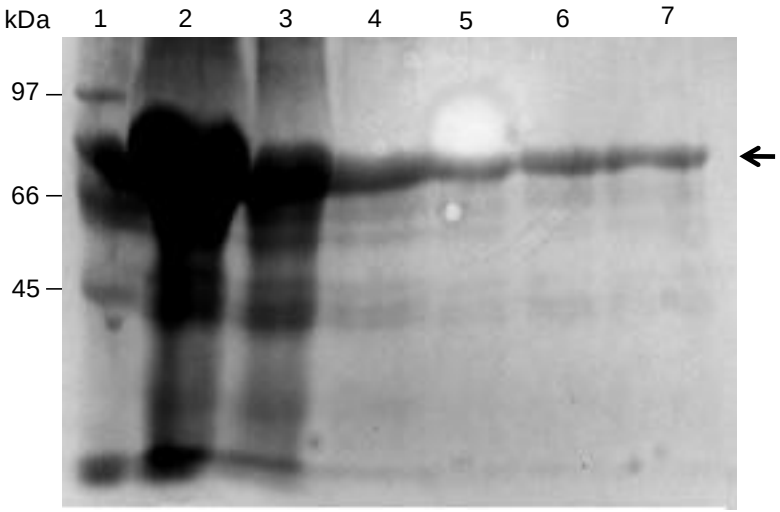


Figure 3.11: Purification of His-tagged BiP69 fusion protein under native conditions. Soluble His-tagged BiP69 fusion protein was allowed to bind with the Ni^{2+} affinity resin overnight at $4^{o}C$. The flow through was collected and the column washed twice with Tris-Cl buffer and eluted with 250 mM Imidazole. Lane 1, Bioscience Low molecular weight marker (Amersham); lane 2, BiP69 dialysed protein; lane 3, unbound material; lane 4, wash buffer 1; lane 5, wash buffer 2; lane 6, elution 1 with 250 mM imidazole in elution buffer; lane 7, elution 2 with 250 mM imidazole in elution buffer. The samples were mixed with an equal volume of reducing treatment buffer, boiled and analysed on a 10% SDS-PAGE gel before staining in Coomassie blue R-250. The arrows show the respective proteins of interest.

3.3.8 Purification of C2 by three phase partitioning and ion exchange chromatography

C2 expressed in large volumes of *P. pastoris* supernatant, was concentrated using three phase partitioning (TPP). This step was also suppose to serve as a purifying step to remove some expressed extracellular yeast proteins, but was unsuccessful as yeast proteins at 22 kDa and 38 kDa were observed after concentration (Fig. 3.12, lane 3). C2 was further purified by removing contaminating yeast proteins using Sephadex C-25 ion exchange chromatography (Fig. 3.13, Panel A). Purification of C2 was successful as contaminating higher molecular weight yeast proteins were successfully removed by low salt concentrations, followed by C2 elution at about 0.3 M NaCl (Fig. 3.12, Panel A). Analysis on reducing 12% SDS-PAGE gel showed C2 migrating at 27 kDa as expected (Panel B, lanes 3-10), but a further lower molecular weight band was observed at 20 kDa and is due to the autocatalytic activity of the peptidase that results in the production of the mature 23 kDa peptidase.

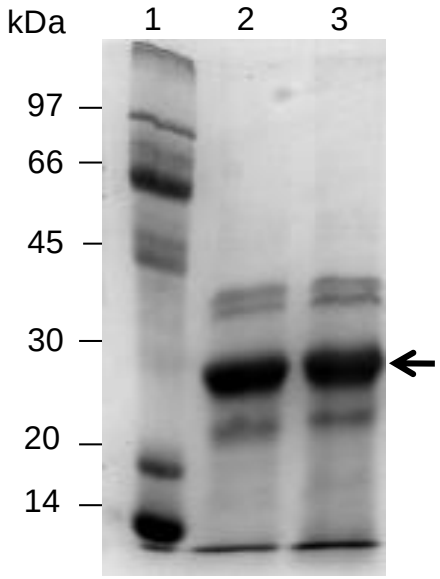
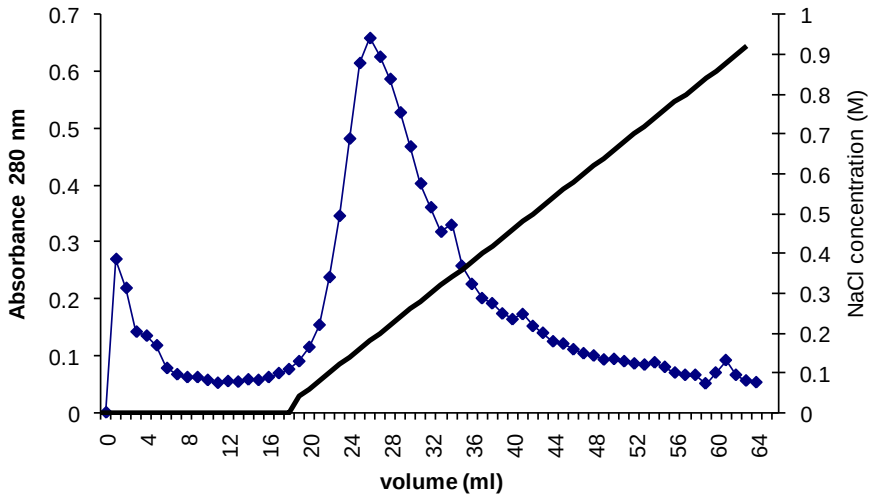


Figure 3.12: Analysis of expression of C2 in *Pichia pastoris* and subsequent precipitation by TPP from culture supernatants. C2 supernatants were precipitated by TPP using 40% (w/v) NH_2SO_4 and 30% (v/v) t-butanol. Lane 1, Biosciences low molecular weight marker; lane 2, C2 supernatant from culture (10 µl); lane 3, C2 precipitated sample after TPP, dialysed and re-suspended in PBS (10 µl). Samples were mixed with an equal volume of reducing treatment buffer, boiled before loading and analysed by 12% SDS-PAGE before staining in Coomassie blue R-250. The arrows show the respective proteins of interest.

A



B

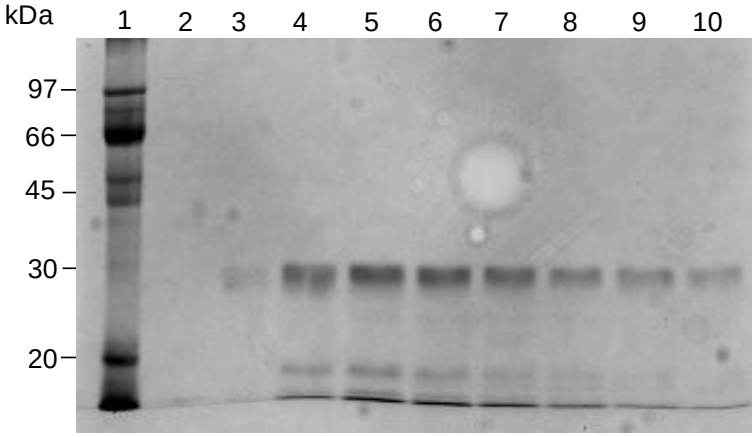


Figure 3.13: Purification of C2 by ion exchange chromatography on a Sephadex C-25 column. **A:** Elution of C2 on a Sephadex C-25 column (0.75 x 10 cm, 0.41 ml/min) with a 0-1 M NaCl gradient in 50 mM Na-acetate buffer pH 4.2. The protein elution profile was constructed by measuring absorbance at 280 nm for each fraction. The purity of proteins (20-42 ml) was assessed by 12% reducing SDS-PAGE and staining with Coomassie blue R-250. **B**: Lane 1, Biosciences low molecular weight marker (Amersham); lane 2, unbound fraction; lanes 3-10, fractions from elution peak at 22-42 ml.

3.3.9 Western blotting to detect purified proteins

The identity of His-tagged BiP-C2 fusion protein chimeras, His-tagged BiP69 and C2 purified from *P. pastoris* was confirmed by western blotting using chicken anti-congopain N-terminal peptide antibodies that detected all BiP-C2 chimeras (Fig. 3.14, Panel A), mouse anti-BiP monoclonal antibodies (M1D4 and M1B2) that detected all N-terminal and C-terminal epitopes in the BiP fusion proteins (Fig. 3.14, Panel B), and mouse anti-His-tag monoclonal

antibodies that detected all His-tagged fusion proteins (Fig. 3.14, Panel C). Purified His-tagged BiP69-C2, 96 kDa (Panel A, lane 1), His-tagged BiP60-C2, 88 kDa (Panel A, lane 2), His-tagged PBD-C2, 47 kDa (Panel A, lane 3), and His-tagged C-term-C2, 34 kDa (Panel A, lane 4) and C2 (Panel A, lane 5) were detected at the expected sizes using anti-congopain antibodies. The same fusion proteins were detected at the expected sizes using anti-BiP monoclonal antibodies (Panel B, lanes 1-4 and lane 5, BiP69) with non-specific binding or degraded fusion protein chimeras observed in BiP69-C2 and BiP60-C2 fusion protein chimeras at 45 kDa, 66 kDa, 80 kDa (Panel A and B, lanes 1 and 2), and in PBD-C2 fusion protein chimera at 35 and 30 kDa (Panel A, lane 4). All chimeras were confirmed to have their His-tag intact, after purification at 96 kDa (BiP69-C2, lane 1), 88 kDa (BiP60-C2, lane 2), 47 kDa (PBD-C2, lane 3) and 34 kDa (C-term-C2, lane 4).

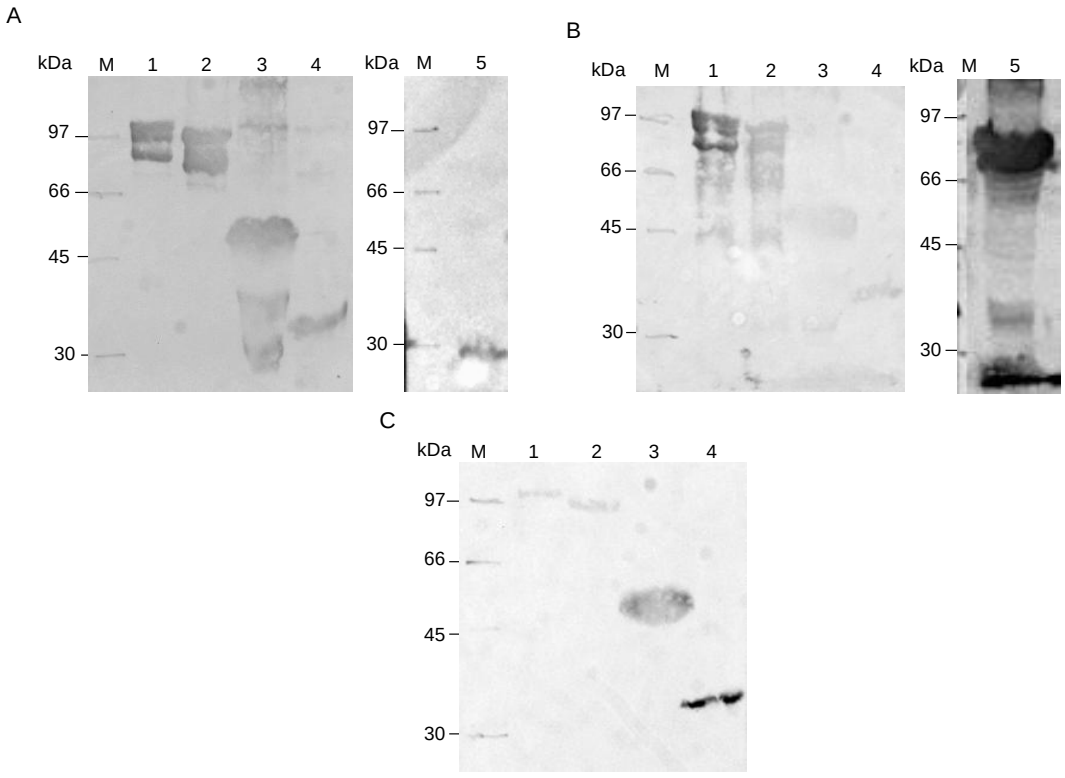


Figure 3.14: Western blot analysis of purified of BiP-C2, BiP69 and C2 fusion proteins using chicken anti-congopain peptide, mouse anti-BiP monoclonal antibodies (M1B2 and M1D4) and mouse anti-His-tag monoclonal antibodies. Purified His tagged BiP-C2 fusion protein chimeras and C2 protein were probed with anti-congopain peptide (**A**), anti-BiP (**B**) and anti-His-tag (**C**) antibodies and either HPRO linked rabbit anti-chicken antibodies (1:10 000) or HPRO-conjugated horse anti-mouse antibodies used as a secondary antibody. M, Biosciences molecular weight marker; lane 1, BiP69-C2; lane 2, BiP60-C2; lane 3, PBD-C2; lane 4, C-term-C2; lane 5, C2 or BiP69.

3.3.10 Determination of antibody response against C2 using an ELISA

Eight groups of Balb/c mice were immunised with C2 alone, a mixture of C2 with Adjuphos/ Quil A, BiP69-C2, BiP60-C2, PBD-C2, C-term-C2 or a mixture of BiP69 and C2. The C2 specific antibody response was followed by ELISA using pooled mouse plasma collected every week from each group of mice (Fig. 3.15). All groups of mice produced C2-specific antibodies after the first boost (week 1), except those immunised with PBS or C-term-C2. BiP69-C2 produced higher levels of C2-specific antibodies compared to the other responding groups, followed by groups immunised with C2 in Adjuphos/Quil A, BiP69/C2 mixture, PBD-C2 and C2 alone. Upon infection with *T. congolense* at week 12, all mice showed a short-lived booster effect on antibody levels 7-14 days post infection, matching the observation of circulating parasites in the blood 6-12 days after infection as observed by microscopy. Mice immunised with BiP69-C2 and PBD-C2 showed the highest booster effect upon infection, suggesting better activation of memory B cells. Antibody response was only followed until week 14 for mice immunised with BiP60-C2 as all mice died.

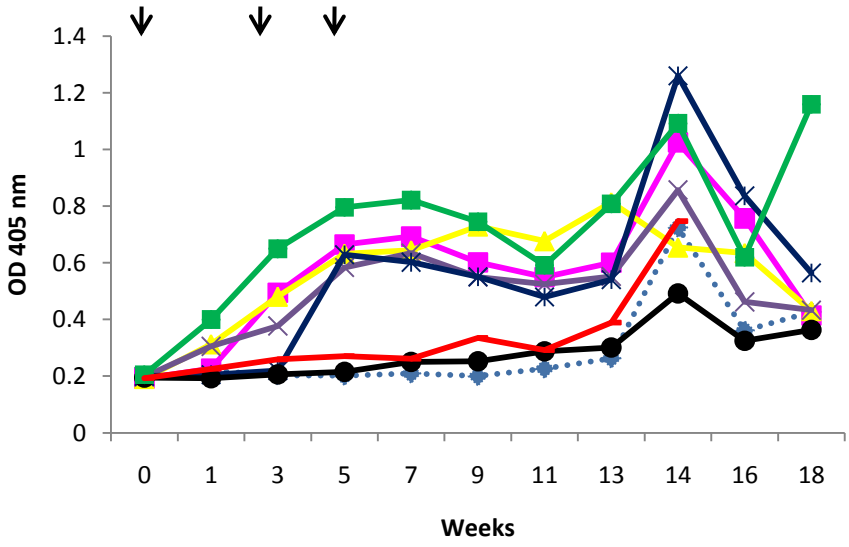


Figure 3.15: ELISA showing the anti-C2 antibody response of Balb/c mice when C2 was immunised with BiP-C2 chimeras and C2 with adjuvant. Antibody response was monitored for BiP69-C2 (), BiP60-C2 (), PBD-C2 (), C-term-C2 (), BiP69/C2 mixture (), C2 in Adjuphos/Quil A mix (), C2 alone () and PBS () immunised mice. Microtitre plates were coated with C2 and incubated with pooled mouse plasma samples collected weekly. Goat anti-mouse IgG conjugated to HRPO followed by ABTS/H_2O_2 substrate solution were used in the detection of mouse antibodies. Absorbance was read at 405 nm and represents the average of three replicates of each sample. Arrows represent time of immunisation and boosters.

Mice immunised with BiP69-C2 gave a higher antibody response compared to those immunised with C2 alone and C2 administered with a mixture of Adjuphos/Quil A. Mice started producing anti-C2 antibodies by week 2, just after the first booster injection in all groups. Antibody production seems to peak at week 8 in most cases. Mice injected with PBS or immunised with C-term-C2 produced no antibodies against C2, while mice immunised with the BiP69/C2 mixture, C2 in Adjuphos/Quil A mixture and PBD-C2 showed relatively similar antibody responses. Mice immunised with BiP60-C2 had a low specific antibody response against C2 compared to those immunised with C2 alone, C2 in Adjuphos/Quil A or the BiP69/C2 mixture.

Mice immunised with BiP69-C2, and PBD-C2 chimeras and as well as the BiP69/C2 mixture produced higher BiP69-specific antibody responses (Fig. 3.16). Mice immunised with BiP60-C2 produced a significantly lower anti-BiP69 antibody response. All mice immunised with C2 in the absence of BiP69 (C2 alone, C2 with Adjuphos/Quil A, C-term-C2 or PBS) produced no specific antibody response to BiP69 (Fig. 3.16). All groups showed a short-lived 'booster effect of infection' on antibody levels 7-14 days post infection, matching the observation of circulating parasites in the blood 6-12 days after infection as observed by microscopy. Mice immunised with BiP60-C2 died at week 14 corresponding to 7-14 days post infection.

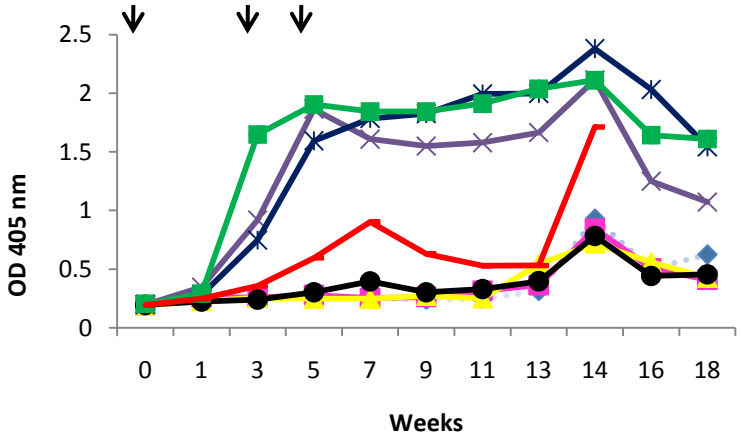


Figure 3.16: ELISA showing anti-BiP69 antibody response of Balb/c mice immunised with BiP-C2 chimeras. Antibody response was monitored for BiP69-C2 (), BiP60-C2 (), PBD-C2 (), C-term-C2 (), BiP69/C2 mixture (), C2 in Adjuphos/Quil A (), C2 alone () and PBS () immunised mice. Microtitre plates were coated with BiP69 and incubated with pooled mouse plasma samples collected weekly. Horse anti-mouse IgG conjugated to HRPO followed by ABTS/H_2O_2 substrate solution were used in the detection of mouse antibodies. Absorbance was read at 405nm and represents the average of three replicates. Arrows represent time of immunisation and boosters.

3.3.11 Infection and mice survival

Immunised mice were infected with *T. congolense* IL1180, which causes a mild chronic infection, 8 weeks after the last booster injection and parasitaemia monitored. In all groups of mice parasites were only detected at day 5 post infection (Fig. 3.17, Panels A-E). Parasitaemia peaked at day 10 post infection and thereafter all groups seemed to control infection as parasites were cleared from the blood stream with successive waves of parasitaemia observed throughout the infection period in all groups. Mice immunised with PBD-C2 cleared circulating parasites in the bloodstream 10 days earlier compared to PBS and C2 groups (Panel C). Mice immunised with the BiP69/C2 mixture controlled parasitaemia levels 16 days earlier compared to PBS and C2 groups (Panel E). Control (PBS), C2, C2 in Adjuphos/Quil A, BiP69-C2 (Panel A), BiP60-C2 (Panel B) and C-term-C2 (Panel D) immunised groups failed to keep low parasitaemia levels throughout the infection period. Mice immunised with BiP60-C2 showed a delayed infection, but did not survive the first peak of parasitaemia. The ability of mice to control parasitaemia after the first wave of parasitaemia correlated with their ability to control anaemia.

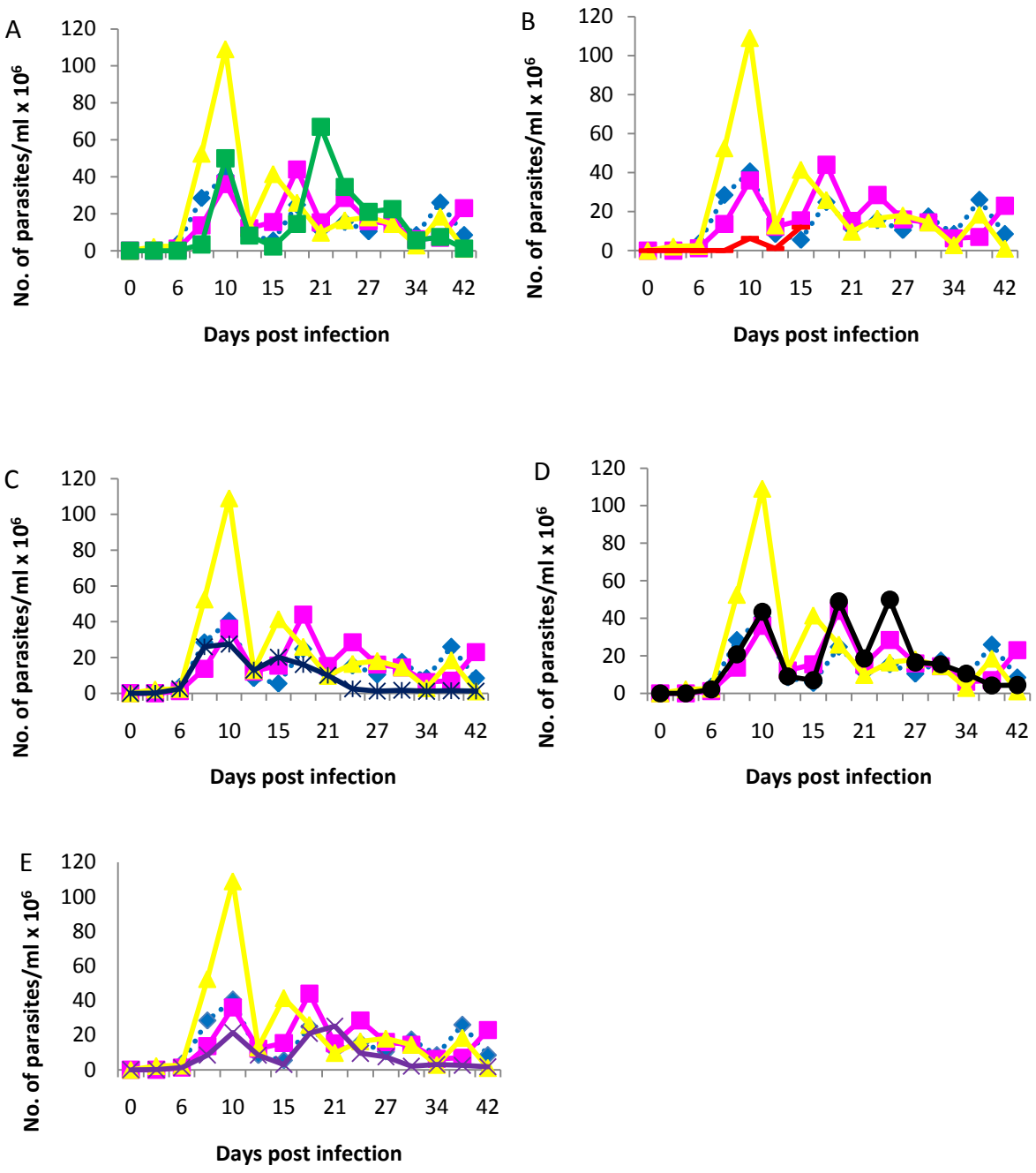


Figure 3.17: Parasitaemia levels of infected, immunised Balb/c mice. All groups were infected with *T. congolense* IL1180 at week 12. Parasitaemia of immunised mice **A**, BiP69-C2 (green); **B**, BiP60-C2 (red); **C**, PBD-C2 (dark blue); **D**, C-term-C2 (black); **E**, BiP69/C2 mixture (purple) compared to control groups PBS (), C2 () and C2 in Adjuphos/Quil A () was monitored by counting the number of parasites circulating in the bloodstream using a haemocytometer. Parasites were first counted once every two days, and then twice a week. Statistical evaluation on number of parasites days post infection between PBS and experimental groups revealed *p*-values > 0.05 (two-way ANOVA test) and were considered not significant.

Mice controlled infection by also controlling anaemia which was measured as the volume occupied by red blood cells. At the peak of parasitaemia on day 10 post infection all groups

had the lowest volume of red blood cells, and the levels recovered after parasite clearance from the blood stream (Fig. 3.18). Most groups consistently maintained lower anaemia levels, but did not a make full recovery. The BiP60-C2 group did not recover from anaemia with a PCV less than 30% at day 15 post infection. As a result all mice in this group died at day 16 and 17 post-infection.

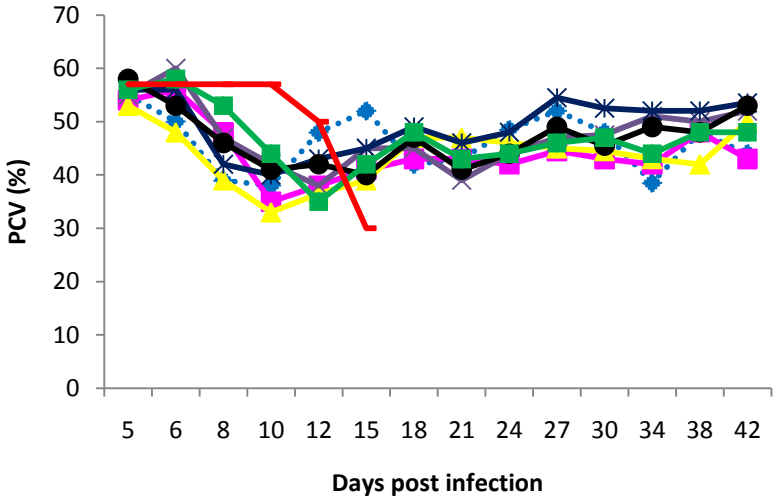


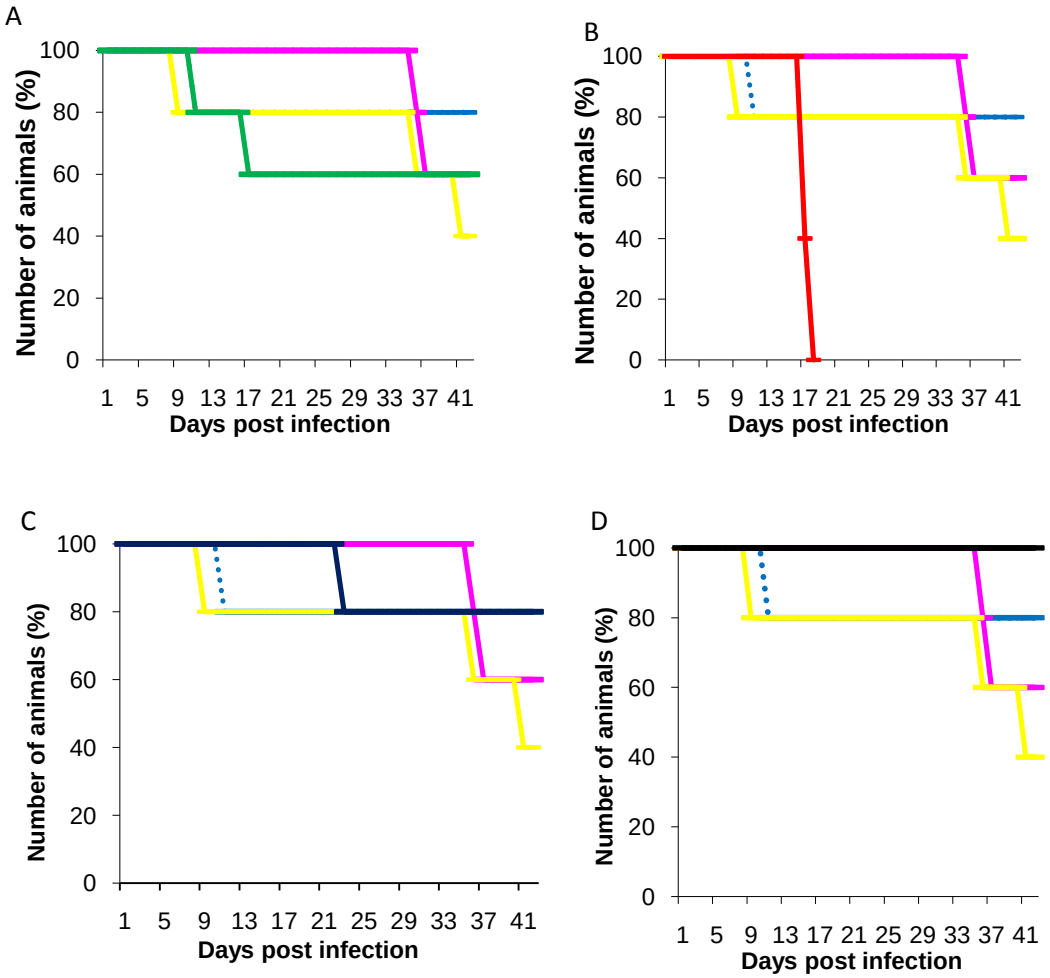
Figure 3.18: Disease progression of immunised mice after infection shown by percentage of packed cell volume (PCV). Immunised mice were infected with *T. congolense* IL1180 at week 12 and anaemia levels monitored by measuring PCV. Percentage PCV of BiP69-C2 (), BiP60-C2 (), PBD-C2 (), C-term-C2 (), BiP69 and C2 mixture (), C2 in Adjuphos/Quil A (), C2 alone () and PBS () immunised mice was monitored by centrifuging pooled blood from each group of mice followed by measuring volume occupied by red blood cells in capillary tubes. Measurements were done in triplicate.

There was no difference in mice survival when comparing the PBS control group and the BiP-C2 chimera immunised mice (Fig. 3.19 and Table 3.1). In the group immunised with C2 alone 60% of the mice died at days 36 and 37 post-infection, while 60% of those immunised with C2 co-administered with Adjuphos/Quil A died at days 9, 36 and 41 post infection. In the group immunised with BiP69-C2 40% of mice died at day 11 and 17 post infection (Fig. 3.19, Panel A). In the group immunised with PBD-C2 only 20% of the mice died at day 10. No mice died in the C-term-C2 and BiP69/C2 mixture immunised groups (Panel D and E). All surviving mice were humanely killed at day 42 post infection due to severe pathological effects of the disease.

Table 3.1: Number of immunised mice surviving in days post infection following *T. congolense* IL1180 infection.

Groups	Death (dpi)	Survival number	% Survival
PBS	11	4	80
C2	36, 37	3	60
C2+Adjuv	9, 36, 41	2	40
BiP69+C2	nd	5	100
PBD-C2	23	4	80
C-term-C2	nd	5	100
BiP69-C2	11, 17	3	60
BiP60-C2	17, 18	9	0

nd- no death
dpi- days post infection



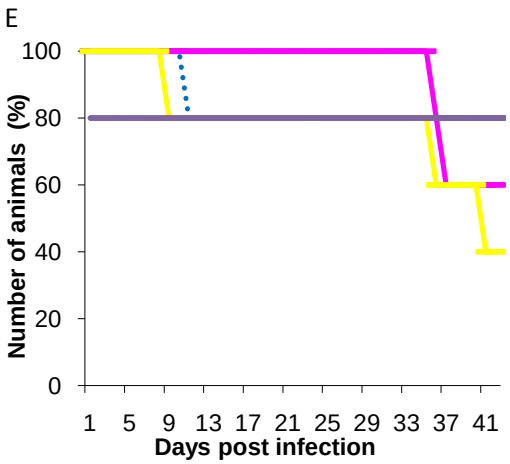


Figure 3.19: Kaplan-Meier curve showing survival of immunised mice after challenge with *T. congolense* IL1180. Comparison of survival of Balb/c mice immunised with **A**, BiP69-C2 (green); **B**, BiP60-C2 (red); **C**, PBD-C2 (dark blue); **D**, C-term-C2 (black); **E**, BiP69 and C2 mixture (purple) with control groups: PBS (), C2 () and C2 in Adjuphos/Quil A (). Number of mice per group were expressed as percentage each group had five mice, with each mouse representing 20%. Percentage survival was recorded after intraperitoneal infection with 1000 living parasites. Statistical evaluation on median survival times of PBS and experimental groups revealed *p*-values > 0.05 (Log rank, student's *t*-test) and were considered not significant.

3.4 DISCUSSION

Kinetoplastid cysteine peptidases have been a target for both vaccine development and chemotherapy (Caffrey and Steverding, 2009). Vaccination of rodents with DNA coding for cysteine peptidases or with recombinant cysteine peptidases co-administered with or without adjuvants has shown partial or full protection of the host upon infection (Authié *et al.*, 2001; Cazorla *et al.*, 2008b; Fedeli *et al.*, 2010). Vaccination with cysteine peptidases I, II and III of *L. infantum*, *L. dovonani* and *L. braziliense* respectively confer protection to susceptible mice against cutaneous and visceral leishmaniasis displaying high IgG2a and IgG1 antibody response, reduced parasite load and tissue damage (Rafati *et al.*, 2006; Khoshgoo *et al.*, 2008; Rafati *et al.*, 2008). Vaccine development using cruzipain, a major cysteine peptidase in *T. cruzi*, displayed protective immunity when a prime-boosting immunisation was used inducing strong specific IgG titres, reduced tissue damage and lower levels of parasitaemia when compared to controls (Cazorla *et al.*, 2008a; Cazorla *et al.*, 2008b).

Congopain, a major cysteine peptidase in *T. congolense*, induces antibody production in N'Dama cattle and is thought to be involved in trypanotolerance mitigating disease pathology and is thus a target for an anti-disease vaccine strategy. Full protection of rodents or cattle has

never been achieved when vaccinating with congopain or its catalytic domain (Authié *et al.*, 2001; Bizaaré, 2008). It is still not clear as to why congopain fails to give complete protection like other kinetoplastid cysteine peptidases and it is suspected that it is poorly presented to the immune system by the conventional adjuvants used in previous vaccination studies (Alain Boulangé pers. comm.). In order to improve the delivery of the catalytic domain of congopain to the immune system it was linked to BiP69, an ER localised HSP70 thought to have molecular adjuvant effects. A construct was prepared for the recombinant expression of fusion protein comprising the individual BiP domains linked to C2 in a His-tagged pET-28a vector. His-tagged fusion protein chimeras were over-expressed in a bacterial system as insoluble inclusion bodies. This part of the study was concerned with solubilising, refolding and purification of the BiP-C2 chimera fusion proteins and evaluating them in a mouse vaccination and challenge experiment.

Inclusion body formation might have been promoted by the high number of cysteine residues in the BiP-C2 fusion protein chimera sequences as was suggested by Jungbauer and Kaar (2007). Inclusion bodies are less likely to be degraded and are easier to separate from soluble bacterial proteins by centrifugation (Katoh and Katoh, 2000; Singh and Panda, 2005). Washing of His-tagged fusion protein chimeras with chaotrophic detergents removed some bacterial proteins, although some target protein loss also occurred, thereby decreasing final yields. Some membrane-bound contaminating bacterial proteins are known to be difficult to remove even with stronger detergents and increased concentrations of detergents (Clark, 2001).

His-select Nickel affinity resins are commonly used in purification of His-tagged proteins, although relatively poor binding and low yields has been reported (Lichty *et al.*, 2005). In the present study most protein was eluted by washes with low concentrations of imidazole and buffers of slightly deceasing pH. Changing the time allowed for adsorption to the resin, concentration of denatured protein loaded on the column and/or temperature at which these steps were conducted, did not improve binding capacity of His-tagged fusion proteins. Purification under denaturing conditions resulted in purification of PBD-C2 and C-term-C2 chimeras to homogeneity but with poor yields, while BiP69-C2 and BiP60-C2 chimeras co-eluted with bacterial proteins.

Refolding of denatured fusion proteins, purified under denaturing conditions, was first attempted by a stepwise dialysis method. Low yields of refolded inclusion bodies by batch dialysis were obtained for all four chimeras attempted. The method was too lengthy requiring at least four days, it required expensive reagents and a further purification and concentration steps to remove co-purifying bacterial proteins thereby escalating refolding costs as is often is the case for inclusion bodies (Li *et al.*, 2004; Jungbauer and Kaar, 2007). The co-purifying bacterial proteins in BiP69-C2 and BiP60-C2 chimeras could have promoted protein aggregation during the refolding step, thereby reducing folding yields as suggested by Singh and Panda (2005).

The one-step, on-column refolding method is based on immobilised nickel affinity chromatography and allows simultaneous purification and refolding of His-tagged fusion protein by a gradual decrease in denaturant concentration (Glynou *et al.*, 2003; Lemercier *et al.*, 2003). High yields of pure refolded C-term-C2 and PBD-C2 chimeras were obtained using this approach. However, bacterial proteins co-eluted with BiP69-C2 and BiP60-C2 chimeras. His-tagged BiP69 fusion protein was successfully purified, but with poor yield. A one-step on-column purification and refolding method was shown to give better recovery of folded protein compared to the dilution and dialysis methods. This method resulted in improved protein refolding, in a shorter time and with reduced cost of protein purification and refolding. Protein recovery was comparable to yields obtained in folding of a His-tagged *T. brucei* exopolyphosphatase (Lemercier *et al.*, 2003), Toc75 (Rogl *et al.*, 1998) and apoaequorin (Glynou *et al.*, 2003) using one-step immobilised metal affinity chromatography.

Attempts to remove co-purifying bacterial proteins by gel filtration and ion exchange chromatography were unsuccessful. It was then suspected that the co-eluting bacterial host proteins might be attached to target proteins by non-covalent interactions requiring a different purification approach. Host-encoded proteins, e.g. metal binding protein (WHP) (Wulfing *et al.*, 1994) and apoaequorin (Sly D) (Inouye *et al.*, 1989) are histidine rich and have a high affinity for divalent cations and tend to bind to Ni-NTA resins becoming difficult to remove (Glynou *et al.*, 2003). It is possible that some of the co-purifying bacterial proteins could be WHP and Sly D proteins, observed at 27 and 29 kDa respectively, however the presence of higher molecular weight co-purifying proteins does not support this idea. It was also suspected

that some of the co-purifying bacterial proteins were host co-chaperones like DnaK and DnaJ binding to target proteins as suggested by Rial and Ceccarelli (2002). Removal of co-purifying bacterial proteins has been achieved when fusion proteins were incubated with dissociation buffer with varying ATP concentrations (Rohman and Harrison-Lavoie, 2000; Rial and Ceccarelli, 2002; Joseph and Andreotti, 2008). Incubation of nickel bound His-tagged BiP60-C2 and His-tagged BiP69-C2 fusion protein chimeras with dissociation buffer containing 5 mM ATP, removed bacterial co-purifying proteins. However, poor adsorption of the His-tagged fusion protein to the immobilised nickel affinity resin was observed, resulting in reduced recovery. Changing various parameters in an attempt to improve adsorption of His-tagged fusion proteins to the nickel affinity resin was not successful and did not improve protein yields. His-tagged BiP69-C2 and His-tagged BiP60-C2 fusion protein chimeras were purified and refolded using an on-column refolding method, but were used as non-pure proteins for immunisation due to co-purifying bacterial proteins.

Cysteine peptidases have been expressed as active enzyme in both bacterial (Eakin *et al.*, 1992; McGrath *et al.*, 1995; Sanderson *et al.*, 2000) and yeast systems (Aoki *et al.*, 2003; Aloulou *et al.*, 2006; Pillay *et al.*, 2010; Boulangé *et al.*, 2011). The catalytic domain of congopain, C2, was expressed in *P. pastoris* and purified by three phase partitioning and ion exchange chromatography to homogeneity for the vaccination study. The His-tagged BiP69 fusion protein was purified successfully under native conditions using nickel affinity resin to homogeneity for the vaccination study.

The identity of the purified PBD-C2 and C-term-C2 chimeras, BiP69 and C2 fusion proteins and the non-purified BiP69-C2 and BiP60-C2 chimeras were confirmed by western blotting using anti-BiP and anti-congopain N-terminal peptide antibodies. Non-specific interactions observed in the western blots of non-purified BiP69-C2, BiP60-C2 chimeras and purified PBD-C2 chimera are suspected to be degradation products from the unstable chimeras. This may especially be the case for bands smaller than 30 kDa which are thought to be products of C2 hydrolysis. It is also possible that these chimeras were cleaved by the maturing C2 peptidase which is part of the chimera, resulting in degradation products as C2 has been shown to retain its full activity at physiological pH (Boulangé *et al.*, 2011).

After removal of endotoxins, purified PBD-C2 and C-term-C2 chimeras, BiP69 and C2 and the non-purified BiP69-C2 and BiP60-C2 chimeras were used to immunise Balb/c mice. Inbred, susceptible Balb/c mice are widely used and provide an economical alternative model for research, especially when studying direct effects of trypanosomal specific antibodies and their role in parasite clearance and anaemia control (Taylor, 1998; Taylor and Authié, 2004; Naessens, 2006; Antoine-Moussiaux *et al.*, 2008).

Since African trypanosomes are extracellular haemoparasites, the humoral immune response of the host is the most dominant upon infection. Host antibodies can be protective: mediating parasite clearance, neutralising toxic products released by parasites and clearing immune complexes formed by the complement system (Taylor and Authié, 2004). Trypanotolerant N'Dama cattle produce long-lived IgG responses to VSG, congopain and BiP69, implicating these antigens in trypanotolerance (Authié *et al.*, 1993b; Lutje *et al.*, 1995b; Taylor, 1998). This is confirmed by the secretion of IgM antibodies and proliferative T-cell response to VSG, congopain and BiP69, by activated spleen cells from susceptible cattle *in vitro* (Lutje *et al.*, 1996; Taylor *et al.*, 1996; Taylor, 1998).

In the present study, immunisation of *T. congolense* susceptible Balb/c mice with a BiP69-C2 chimera produced a higher level of specific anti-C2 antibodies compared to immunisation with C2 mixed with BiP69 or conventional adjuvants. These results are comparable to those reported for vaccination of rodents using recombinant Hepatitis B virus surface protein and major antigenic segment of Japanese encephalitis virus E-protein linked to mycobacterial HSP70 and its C-terminal peptide binding domain, where they induced higher antibody titres compared to HSP70 mixed with antigens. The C-terminal peptide binding domain has been thus suggested as possible domain with adjuvant effect (Wang *et al.*, 2002; Ge *et al.*, 2006; Li *et al.*, 2006). In the present study PBD-C2 induced moderate levels of anti-C2 antibodies, slightly lower levels than with BiP69-C2, but better in controlling parasitaemia burden and reducing disease induced pathology. This group also showed a better booster effect of *T. congolense* infection shown by much higher anti-C2 antibody stimulation. Booster effect of *T. congolense* infection have been shown in trypanotolerant N'Dama cattle three years post infection producing specific anti-congopain and BiP69 antibodies (Lutje *et al.*, 1995b; Lutje *et al.*, 1996). Immunisation did not change *T. congolense* infection and anaemia development in

most groups showing a similar pattern of infection and blood parameter to what was shown in a previous study (Kateregga *et al*. In preparation). It is possible that the removal of the C-terminal domain reduced adjuvant potency of the peptide binding domain, suggesting a prominent role in the adjuvant effect of the BiP molecule. This has not been shown in other studies, however the epitopes binding to antigen presenting cells have been localised on the C-terminal peptide binding domain (Zitzler *et al.*, 2008). It would be necessary to find the exact role of the C-terminal domain in adjuvant effect by comparing peptide binding domain with or without the C-terminal domain.

In leishmaniasis the prime-boost vaccination with cysteine peptidase type I, II and III of *L. infatum* co-administered with either CpG-deoxyoligonucleotide (ODN) or Montanide 720 induced a strong antibody response and gave significant protection shown by reduced footpad lesions and liver parasite load (Rafati *et al.*, 2006; Khoshgoo *et al.*, 2008). In Chagas' disease, vaccination with cruzipain mixed with either macrophage activating lipopeptide-2 (MALP-2) or CpG- ODN induced a strong cruzipain specific IgG antibody response that was adequate in controlling *T. cruzi* infection shown by reduced parasite load and tissue damage (Cazorla *et al.*, 2008a; Cazorla *et al.*, 2008b). Furthermore, there was a significant percentage mice survival in groups prime-boosted with recombinant cruzipain protein admixed with MALP-2 (Cazorla *et al.*, 2008b). In the present study no significant differences in percentage mice survival could be deduced as only 20% of the mice in the PBS control group died upon infection, suggesting that the *T. congolense* strain IL1180 used is not particularly pathogenic in mice.

Leishmania major HSP70 and its peptide binding domain induced a strong humoral immune response in Balb/c mice, however, this failed to reduce lymph node parasitaemia burden and dermal pathology (Rafati *et al.*, 2007). These observations suggested that HSP70 is not involved in control of cutaneous leishmaniaisis in rodents. In contrast earlier findings on *L. donovani* showed that ER-localised BiP co-administered with Freund's adjuvant played a role in controlling parasitaemia burden in lymph nodes and foot pad dermal pathology leading to control of cutaneous leishmaniasis (Jensen *et al.*, 2002). In the present study mice immunised with BiP69-C2, and PBD-C2 chimeras as well as a BiP69/C2 mixture induced a robust humoral immune response, but no correlation could be made with the contribution to control

of parasitaemia levels and reduced disease pathology as the BiP69-C2 chimera immunised group succumbed to severe clinical disease pathology on day 27 after *T. congolense* challenge.

Survival is an imprecise parameter to assess vaccine induced protective antibody mediated immunity in mice challenged with *T. brucei* or *T. congolense* (Antoine-Moussiaux *et al.*, 2008). Susceptible Balb/c mice do not necessarily die from uncontrolled anaemia or high parasitaemia, but they can die from systemic inflammatory response syndrome, renal failure, central nervous system invasion and opportunistic infections as a result of immuno-depression (Stijlemans *et al.*, 2007; Antoine-Moussiaux *et al.*, 2008; Radwanska *et al.*, 2008; Noyes *et al.*, 2009). In addition, death can also be dependent on mouse strain, trypanosome species and strain and diseases stage (Antoine-Moussiaux *et al.*, 2008; Noyes *et al.*, 2009).

Mechanisms underlying control of trypanosomal infections in rodents and cattle are not well understood with few authors suggesting immune cytokines such as INF-γ, TNF-α, NO and B-cells secreting IgG1, IgG2a and IgG3a as being crucial factors in phagocytosis, parasite clearance, long lasting immunity and control of infection (Lutje *et al.*, 1995b; Taylor, 1998; Magez *et al.*, 2006). In the present study it was shown that the anti-C2 antibodies produced by immunisation with PBD-C2 were likely to be involved in clearing of parasitaemia and disease induced pathology, showing similar observations to trypanotolerant cattle.

In conclusion, these results showed that vaccination of Balb/c mice with a BiP69-C2 chimera induces an increased IgG antibody response compared to vaccination with C2 alone, or with C2 mixed with Adjuphos/Quil A, BiP69 mixed with C2 and the PBD-C2 chimera. Mice immunised with BiP69-C2 and PBD-C2 chimeras showed a better booster effect upon infection with *T. congolense* compared to control groups. There was no significant difference in the percentage mice surviving between controls and BiP69-C2 chimera immunised mice. Mice immunised with a BiP69/C2 mixture and with a PBD-C2 chimera cleared parasites and maintained very low levels of parasites 10-15 days earlier and showed no clinical symptoms of the disease. The role of the C-terminus on the adjuvant effect of HSP70 still needs to be further investigated. Now that its has been established that BiP69 has adjuvant effects and that its PBD region acts as an adjuvant alone, it would be interesting to repeat this study in trypanosusceptible cattle to confirm these findings.

4 GENERAL DISCUSSION

African animal trypanosomiasis (nagana) is a devastating disease which remains a major constraint to livestock based agriculture and significantly impacts on millions of people in sub-Saharan Africa (Büscher and Lejon, 2004; Hide and Tait, 2009; Welburn *et al.*, 2009; Salifu *et al.*, 2010). The disease cost in this region is estimated to be more than $ 5 billion US per annum (Kristjanson *et al.*, 1999; Shaw, 2004). The disease is caused by parasites of the genus *Trypanosoma* and transmitted via tsetse flies of the *Glossina* species from one host to another (Akoda *et al.*, 2008; Roditi and Lehane, 2008; Schofield and Kabayo, 2008; Masumu *et al.*, 2010). In cattle, trypanosomiasis has characteristic symptoms of high fever, chronic anaemia, enlarged liver and spleen, loss of appetite and subsequent weight-loss, reduced fertility, foetal abortion(s), and immunosupression that results in susceptibility to opportunistic infections (Murray *et al.*, 1982; Taylor and Authié, 2004).

Current control strategies include trypanocidal drugs, tsetse control and trypanotolerant cattle, none of which is entirely satisfactory. This is due to increasing drug resistance, the deleterious environmental impact of the insecticides and the small size of trypanotolerant cattle make their use not economically viable (Kgori *et al.*, 2006; Delespaux and de Koning, 2007; Torr *et al.*, 2007; Schofield and Kabayo, 2008). Hence, there is an urgent need for the development of new, effective control strategies. Variable surface glycoproteins (VSGs) covering the parasite is subject to antigenic variation, hindering vaccine development (Barry and McCulloch, 2009; Morrison *et al.*, 2009). Vaccine development targeting parasite invariant antigens such as *T. b. rhodeisiense* flagellar pocket antigens (Mkunza *et al.*, 1995) and *T. b. brucei* and *T. evansi* tubulin (Lubega *et al.*, 2002; Li *et al.*, 2007b) have been shown to give partial protection, although attempts to reproduce these results in other trypanosomal species were unsuccessful. A concept called ‘anti-disease vaccine’ was developed which aims at targeting the disease pathology rather than the parasitic infection itself (Playfair *et al.*, 1990; Schofield, 2007). Invariant antigens released by parasites during infection or upon parasite death have been implicated in pathogenesis, and their inhibition is thought to have potential in inducing host protection (Playfair, 1991; Authié, 1994; Authié *et al.*, 2001; Antoine-Moussiaux *et al.*, 2009).

Cysteine peptidases of kinetoplastids are targeted for vaccine development and chemotherapy. Vaccination with DNA coding for cysteine peptidases or cysteine peptidases recombinant

proteins, with or without co-administered adjuvants, has been shown to provide significant protection of mammalian hosts from disease upon infection (Authié *et al.*, 2001; Cazorla *et al.*, 2008a; Fedeli *et al.*, 2010; Kedzierski, 2010). The prime-boost vaccination with cysteine peptidase type I, II and III of *L. infatum* admixed with either CpG- deoxyoligonucleotide (ODN) or Montanide 720 induced strong antibody response and gave significant protection of mice against cutaneous leishmaniasis, as shown by reduced footpad lesions and liver parasite burden (Rafati *et al.*, 2006; Khoshgoo *et al.*, 2008). Vaccination with cruzipain in either macrophage activating lipopeptide-2 (MALP-2) or CpG-ODN induced a strong cruzipain specific IgG antibody response capable of reducing parasite load and tissue damage in Chaga's disease (Cazorla *et al.*, 2008a; Cazorla *et al.*, 2008b). There was a significant percentage mice survival in groups prime-boosted with recombinant cruzipain protein admixed with MALP-2 (Cazorla *et al.*, 2008b).

Trypanotolerant N'Dama cattle produce prominent IgG responses against congopain (Authié, 1994; Taylor, 1998). This suggests that trypanotolerant cattle control the disease by antibody mediated neutralisation of congopain, and that immunisation of trypanosusceptible cattle with congopain may induce an immune response mimicking natural trypanotolerance, resulting in minimised disease pathology (Authié, 1994). Trypanosusceptible cattle immunised with the recombinant catalytic domain of congopain (C2) co-administered with the RWL proprietary adjuvant from SmithKline-Beecham mounted a high C2-specific IgG response that inhibited congopain activity. Immunisation had no effect on the establishment of *T. congolense* infection and acute anaemia development, but immunised animals showed faster recovery and high blood parameter during the chronic phase (Authié *et al.*, 2001). Immunisation of rabbits with C2 complexed to bovine or rabbit α_2-macroglobulin (Huson *et al.*, 2009), and mice with C2 co-administered with Adjuphos and Quil A (Bizaaré, 2008; Kateregga *et al*. In preparation), raised antibodies capable of inhibiting between 65 and 100% of C2 activity. Immunised mice were partially protected when challenged with *T. congolense* developing less severe chronic anaemia and reduced parasitemia burden compared to controls (Kateregga *et al*. In preparation). C2-immunised animals showed a better control of the disease and eventually of infection during chronic phase of the disease, but the effect remains limited. This has been associated with poor presentation of the native form of C2 to immune system by

conventional adjuvants. There is a need to improve C2 presentation to attain better immune responses mimicking that in trypanotolerant cattle.

The objective of this study was first to improve the presentation of the native form of the catalytic domain of congopain (C2), by using the chaperone BiP (an ER localised HSP70) as a molecular adjuvant. Secondly, it was aimed to identify the specific domain of BiP where the adjuvant effect resides through analysing anti-C2 immune response in mice. Thirdly, it was aimed to compare anti-C2 antibody response raised by conventional adjuvants (Adjuphos/Quil A mixture) with that raised by the molecular adjuvant BiP, and lastly to compare BiP69 fused to C2 with C2 co-administered with BiP69. BiP-C2 chimeras were first constructed by cloning C2 in a pET-28a expression vector before inserting BiP fragments namely BiP69, BiP60, PBD and C-term into the same recombinant plasmid. The various BiP-C2 fusion protein chimeras were expressed as insoluble proteins in inclusion bodies, purified and refolded using an on-column refolding method. Co-purifying bacterial proteins were removed by washing BiP69-C2 and BiP60-C2 chimeras with 5 mM ATP. However, low yields limited immunisation with pure protein. Purified recombinant proteins were used to immunise Balb/c mice in the hope of raising a robust antibody response to C2, capable of reducing its activity.

The heat shock protein 70 has been shown to display adjuvant effects, inducing both humoral and cell-mediated immunity to complexed antigens. The immune response induced by HSP70 is of type 1 cytokine polarised, although type 2 favoured cytokine responses are observed with some antigens. The type 1 cytokines are predominantly INF-γ, IL-2, TNF, as well as IgG2a and IgG2b, which all play key roles in pro-inflammatory and/or anti-inflammatory immune responses, that result in enhanced immunity to various disease pathogens (Planelles *et al.*, 2001; Wang *et al.*, 2002; Lehner *et al.*, 2004; Ge *et al.*, 2006; Wang *et al.*, 2006b; Karyampudi and Ghosh, 2008).

The immunogenic potential of HSP70 in a vaccine strategy has been explored in *L. major* and *L. donovani* (Jensen *et al.*, 2002; Rafati *et al.*, 2007). Jensen *et al.*, (2002) previously showed that BiP, an ER localised HSP70 of *L. donovani,* when used in prime-boosting experiments in murine models enhanced the healing of lesions when compared to controls, suggesting that HSP70 can itself act as antigen in a vaccine strategy by stimulating an appropriate immune

response. In contrast, *L. major* HSP70 and its truncated forms were used in a prime-boost vaccination experiment and failed to reduce necrotic foot-pad lesions and lymph node parasitaemia burden. However, truncated C-terminus peptide binding domain and full length HSP70-immunised mice did induce a strong humoral IgG1 and IgG2 immune response before and after infection (Rafati *et al.*, 2007). This work suggested that *L. major* HSP70 is not involved in inducing immune protection against cutaneous leishmaniasis.

Host immune responses are crucial in controlling parasite load, parasite responses disease pathology and cell/host/parasite death (Stijlemans *et al.*, 2007; Antoine-Moussiaux *et al.*, 2009; Baral, 2010). Host antibodies have been shown to play a major role in parasite control during trypanosome infection by interaction with surface proteins of parasites (Namangala *et al.*, 2000; Magez *et al.*, 2006; Naessens, 2006; Pays, 2006; Magez *et al.*, 2008; Radwanska *et al.*, 2008). The key role of IgG in *T. congolense* and *T. brucei* parasite clearance has been confirmed in knock-out mouse studies (Namangala *et al.*, 2000; Magez *et al.*, 2006; Stijlemans *et al.*, 2007; Magez *et al.*, 2008). IgM was found not to have a role in parasite clearance in both *T. congolense* and *T. brucei* (Magez *et al.*, 2006; Magez *et al.*, 2008), but was found to play a role in *T. evansi* parasite clearance (Baral *et al.*, 2007). In *T. congolense* clearance, the mechanism is thought to be IgG-mediated phagocytosis of opsonised parasites by macrophages and Kupffer cells (Shi *et al.*, 2004).

In the present study, most of immunised mice groups controlled the first wave of parasitaemia effectively, indicating better opsonisation of parasites by anti-C2 antibodies. Most groups including those immunised with PBS, C2 alone, C-term-C2 and BiP69-C2, developed severe pathology demonstrated by hair loss, and increased morbidity by day 25 post infection. The experiment had to be terminated due to increasing adverse pathology, clinical symptoms and immune suppression, common symptoms observed in cattle suffering from the disease at the later stages (Murray *et al.*, 1982). Mice immunised with PBD-C2 and the BiP69/C2 mixture showed no clinical symptoms of the disease upon *T. congolense* infection, suggesting that anti-C2 antibodies produced were involved in controlling disease induced clinical symptoms. Significant differences in the percentage of mice surviving could not be deduced between mice immunised with BiP-C2 chimeras and control mice, due to the chronicity of the disease

with *T. congolense* IL1180 strain infection, as suggested in previous studies (Antoine-Moussiaux *et al.*, 2008; Noyes *et al.*, 2009).

In the present study, immunisation with BiP69-C2, and PBD-C2 chimeras as well as the BiP69/C2 mixture produced a robust antibody response to BiP69, but no correlation could be made on its contribution to control of parasitaemia levels or reduction of disease pathology, as the BiP69-C2 group succumbed to severe clinical disease pathology. This suggests that it is possible that BiP and its fragments may have played some role in controlling parasitaemia, however its involvement was in the capacity of an adjuvant rather than acting as an antigen itself.

Radwanska *et al.*, (2008) showed that *T. brucei* infections cause a rapid loss of splenic marginal zone B cells, hindering plasma cell differentiation and their specific protective antibody secretion induced by vaccination. These findings are worrying for the development of an anti-disease vaccine, which relies on raising a robust humoral antibody response to inhibit circulating parasite invariant antigens and the production, and later activation, of viable memory B cells for controlling disease upon secondary infections. In this study a significant booster effect of infection with *T. congolense* was observed when immunising with PBD-C2 and BiP69-C2 suggesting activation of memory B cells secreting anti-C2 antibodies. This is promising as memory B cells secreting specific antibodies involved in disease control are crucial in attaining a successful anti-disease vaccination.

Mice experimentally infected with *T. congolense* IL1180, do not necessarily die from uncontrolled anaemia or high parasitaemia, they rather develop chronic infection lasting for months (Noyes *et al.*, 2009). During the chronic phase of the disease mice seem to eventually die from systemic inflammatory response syndrome, renal failure, central nervous system (CNS) invasion and opportunistic infections as a result of immune-depression (Stijlemans *et al.*, 2007; Antoine-Moussiaux *et al.*, 2008; Noyes *et al.*, 2009). Mortality generally depends on mouse strain, trypanosome species and strain and the disease stage (Antoine-Moussiaux *et al.*, 2008; Noyes *et al.*, 2009). Thus, survival is not an accurate parameter to evaluate the efficacy of a vaccine in inducing protective antibody mediated immunity in mice.

The role of mouse models in studying trypanotolerance and vaccine development against trypanosomiasis has been questioned (Naessens, 2006; Antoine-Moussiaux *et al.*, 2008; Noyes *et al.*, 2009). The susceptibility and tolerance of certain strains of mice to particular strains of trypanosomes complicates this model and its use in studying trypanosomiasis and mechanisms invoking trypanotolerance (Antoine-Moussiaux *et al.*, 2008; Noyes *et al.*, 2009; Baral, 2010). Nevertheless, mouse models are convenient to use, as they provide an economical alternative model for research, especially when studying the direct effects of specific antibodies and their role in parasite clearance and anaemia control. A major obstacle with the use of murine models is the lack of physiological correlation with N'Dama cattle, in their development of trypanotolerance, making it hard to draw conclusions based on mouse models for use in cattle. Genetic crossbred mouse strains are promising for finding suitable murine models mimicking trypanotolerant West African N'Dama cattle (Antoine-Moussiaux *et al.*, 2008; Namangala *et al.*, 2009).

Some authors speculate that cysteine peptidases may be involved in facilitating trypanosomes' entry into the CNS in *T. brucei* species. Down regulation of brucipain by RNAi showed a reduced permeability of the blood brain barrier to parasite penetration (Nikolskaia *et al.*, 2006; Abdulla *et al.*, 2008). The recent identification of multi-copy genes of cathepsin L-like and B-like cysteine peptidase variants which possess slightly differing catalytic triads and kinetic constants (Mendoza-Palomares *et al.*, 2008; Pillay *et al.*, 2010), suggests that these variants might also play essential but different roles in pathogenesis and parasite survival inside host, and that immunisation with one variant may not be sufficient in attaining complete inhibition of other circulating variants upon infection with *T. congolense*. Gene disruption technology of the cysteine peptidases has not been possible, due to difficulties with *in vitro* culturing of the parasite bloodstream forms where these peptidases are up-regulated (Mottram *et al.*, 1996). Recently, a completed *T. congolense in vitro* life cycle culture provides new prospects for gene knockout and -down regulation technology of genes in blood stream forms that should contribute to identifying possible functions of cysteine peptidases, and provide a safe *in vitro* testing environment for possible chemotherapeutic agents against *T. congolense* (Coustou *et al.*, 2010).

In addition to the targeting of cysteine peptidases in vaccine development, they have also been targets of chemotherapy. Drugs currently available on the market are either no longer effective due to drug resistant parasites or they are too toxic for use e.g. first line therapy in human infection with severe *T. b. rhodeisiense*, with the arsenic based compound Melarsoprol, carries a 5% mortality rate from severe encephalitis which is entirely treatment (not disease) related. Cysteine peptidase inhibitors are being screened as possible chemotherapeutic agents for the treatment of trypanosomiasis and some are showing good pharmacokinetic and safety profiles in rodents, canines and primates (McKerrow *et al.*, 2008; Kerr *et al.*, 2009a; Mott *et al.*, 2009). The anti-parasitic effects of cysteine peptidase inhibitors e.g. micromolar concentrations of irreversible vinyl sulfones, peptidyl methylketones fluoromethylketones its derivatives and small inhibitory molecules (Z-Phe-Ala-CNH_2) are capable of killing parasites or inhibiting specific active cysteine peptidase enzymes *in vivo* and/or *in vitro*. Mice treated with these inhibitors cleared parasitaemia, altered trypanosomal cell division and morphology, with less degradation of host proteins and longer host survival (Scory *et al.*, 2007; Abdulla *et al.*, 2008; O'Brien *et al.*, 2008). The advancement in obtaining crystal structures of *T. brucei* cathepsin B and of rhodesain, *P. falcipurum* falcipain-2 and falcipain-3 (Kerr *et al.*, 2009b; Kerr *et al.*, 2010), *L. major* oligopeptidase B (McLuskey *et al.*, 2010) and congopain of *T. congolense* in progress (Prof. T. Coetzer pers. comm.) are promising avenues in designing new peptidomimotopes which could potentially be used as chemotherapeutic agents.

In summary, in this study it was shown that BiP/HSP70 of *T. congolense* has an adjuvant effect and that its peptide binding domain acts as an adjuvant alone, though with reduced effectiveness as compared to whole BiP. This study also showed that BiP linked to C2 gives better antibody response compared to simply mixing C2 with BiP or with conventional adjuvants (Adjuphos/Quil A). In addition, immunisation with PBD-C2 chimera raised an anti-C2 antibody response able to clear circulating parasites. Immunisation did not affect establishment of *T. congolense* infection, but mice immunised with the PBD-C2 chimera and a mixture of BiP69 and C2 did not develop clinical symptoms of the disease upon infection with *T. congolense*, thereby mimicking trypanotolerant cattle. It is possible that the removal of the C-terminal domain reduced adjuvant potency of the peptide binding domain suggesting an important role in the adjuvant effect of the BiP molecule. Finding the exact role of C-terminal

domain in the adjuvant effect of BiP would involve comparing anti-C2 antibody response produced by immunisation with C2 linked to the peptide binding domain with or without the C-terminal domain. Ultimately, it would be of utmost interest to repeat this study in trypanosusceptible cattle to confirm these findings. This could then allow PBD or BiP69 to be used as general adjuvants to fuse with various pathogenic factors, in order to achieve full protection against the disease in susceptible cattle as observed in trypanotolerant N'Damas.

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APPENDIX A

Tabular results of statistical analysis using 2way ANOVA test comparing number of parasites days post infection between PBS group and experimental groups. The statistics were done using GraphPad Prism software.

Source of Variation	% of total variation	P value		
Parasites	13.55	0.0003		
Days	43.05	< 0.0001		
Source of Variation	P value summary	Significant?		
Parasites	***	Yes		
Days	****	Yes		
Source of Variation	Df	Sum-of-squares	Mean square	F
Parasites	7	4.185e+015	5.979e+014	4.370
Days	14	1.330e+016	9.500e+014	6.944
Residual	98	1.341e+016	1.368e+014	
Number of missing values	0			
Bonferroni multiple comparisons	Number of comparisons: 105			

PBS vs C2

Days	PBS	C2	Difference	95% CI of diff.	Difference	t	P value	Summary
0	0.0	0.0	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
5	500000	0.0	-500000	-6.030e+007 to 5.930e+007	-500000	0.03023	P > 0.05	ns
6	4.000e+006	1000000	-3.000e+006	-6.280e+007 to 5.680e+007	-3.000e+006	0.1814	P > 0.05	ns
8	3.000e+007	1.000e+007	-2.000e+007	-7.980e+007 to 3.980e+007	-2.000e+007	1.209	P > 0.05	ns
10	4.000e+007	4.000e+007	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
12	9.000e+006	1.000e+007	1000000	-5.880e+007 to 6.080e+007	1000000	0.06045	P > 0.05	ns
15	5.500e+006	1.550e+007	1.000e+007	-4.980e+007 to 6.980e+007	1.000e+007	0.6045	P > 0.05	ns
18	2.480e+007	4.400e+007	1.920e+007	-4.060e+007 to 7.900e+007	1.920e+007	1.161	P > 0.05	ns
21	1.130e+007	1.530e+007	4.000e+006	-5.580e+007 to 6.380e+007	4.000e+006	0.2418	P > 0.05	ns
24	1.550e+007	2.850e+007	1.300e+007	-4.680e+007 to 7.280e+007	1.300e+007	0.7859	P > 0.05	ns
27	1.050e+007	1.600e+007	5.500e+006	-5.430e+007 to 6.530e+007	5.500e+006	0.3325	P > 0.05	ns
30	1.750e+007	1.450e+007	-3.000e+006	-6.280e+007 to 5.680e+007	-3.000e+006	0.1814	P > 0.05	ns
34	8.500e+006	6.250e+006	-2.250e+006	-6.205e+007 to 5.755e+007	-2.250e+006	0.1360	P > 0.05	ns
38	2.600e+007	7.000e+006	-1.900e+007	-7.880e+007 to 4.080e+007	-1.900e+007	1.149	P > 0.05	ns
42	8.500e+006	2.300e+007	1.450e+007	-4.530e+007 to 7.430e+007	1.450e+007	0.8766	P > 0.05	ns

PBS vs C2+Adjuv								
Days	PBS	C2+Adjuv	Difference	95% CI of diff.	Difference	t	P value	Summary
0	0.0	0.0	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
5	500000	2.200e+006	1.700e+006	-5.810e+007 to 6.150e+007	1.700e+006	0.1028	P > 0.05	ns
6	4.000e+006	2.500e+006	-1.500e+006	-6.130e+007 to 5.830e+007	-1.500e+006	0.09068	P > 0.05	ns
8	3.000e+007	5.000e+007	2.000e+007	-3.980e+007 to 7.980e+007	2.000e+007	1.209	P > 0.05	ns
10	4.000e+007	1.000e+008	6.000e+007	200122 to 1.198e+008	6.000e+007	3.627	P < 0.05	*
12	9.000e+006	1.000e+007	1000000	-5.880e+007 to 6.080e+007	1000000	0.06045	P > 0.05	ns
15	5.500e+006	4.000e+007	3.450e+007	-2.530e+007 to 9.430e+007	3.450e+007	2.086	P > 0.05	ns
18	2.480e+007	3.000e+007	5.200e+006	-5.460e+007 to 6.500e+007	5.200e+006	0.3144	P > 0.05	ns
21	1.130e+007	1.000e+007	-1.300e+006	-6.110e+007 to 5.850e+007	-1.300e+006	0.07859	P > 0.05	ns
24	1.550e+007	2.000e+007	4.500e+006	-5.530e+007 to 6.430e+007	4.500e+006	0.2720	P > 0.05	ns
27	1.050e+007	2.000e+007	9.500e+006	-5.030e+007 to 6.930e+007	9.500e+006	0.5743	P > 0.05	ns
30	1.750e+007	1.000e+007	-7.500e+006	-6.730e+007 to 5.230e+007	-7.500e+006	0.4534	P > 0.05	ns
34	8.500e+006	3.000e+006	-5.500e+006	-6.530e+007 to 5.430e+007	-5.500e+006	0.3325	P > 0.05	ns
38	2.600e+007	2.000e+007	-6.000e+006	-6.580e+007 to 5.380e+007	-6.000e+006	0.3627	P > 0.05	ns
42	8.500e+006	1000000	-7.500e+006	-6.730e+007 to 5.230e+007	-7.500e+006	0.4534	P > 0.05	ns

PBS vs BiP69+C2								
Days	PBS	BiP69+C2	Difference	95% CI of diff.	Difference	t	P value	Summary
0	0.0	0.0	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
5	500000	50000	-450000	-6.025e+007 to 5.935e+007	-450000	0.02720	P > 0.05	ns
6	4.000e+006	1000000	-3.000e+006	-6.280e+007 to 5.680e+007	-3.000e+006	0.1814	P > 0.05	ns
8	3.000e+007	9.000e+006	-2.100e+007	-8.080e+007 to 3.880e+007	-2.100e+007	1.270	P > 0.05	ns
10	4.000e+007	2.000e+007	-2.000e+007	-7.980e+007 to 3.980e+007	-2.000e+007	1.209	P > 0.05	ns
12	9.000e+006	8.000e+006	-1000000	-6.080e+007 to 5.880e+007	-1000000	0.06045	P > 0.05	ns
15	5.500e+006	3.000e+006	-2.500e+006	-6.230e+007 to 5.730e+007	-2.500e+006	0.1511	P > 0.05	ns
18	2.480e+007	2.100e+007	-3.800e+006	-6.360e+007 to 5.600e+007	-3.800e+006	0.2297	P > 0.05	ns
21	1.130e+007	2.520e+007	1.390e+007	-4.590e+007 to 7.370e+007	1.390e+007	0.8403	P > 0.05	ns
24	1.550e+007	9.500e+006	-6.000e+006	-6.580e+007 to 5.380e+007	-6.000e+006	0.3627	P > 0.05	ns
27	1.050e+007	7.400e+006	-3.100e+006	-6.290e+007 to 5.670e+007	-3.100e+006	0.1874	P > 0.05	ns
30	1.750e+007	2.050e+006	-1.545e+007	-7.525e+007 to 4.435e+007	-1.545e+007	0.9340	P > 0.05	ns
34	8.500e+006	3.000e+006	-5.500e+006	-6.530e+007 to 5.430e+007	-5.500e+006	0.3325	P > 0.05	ns
38	2.600e+007	2.500e+006	-2.350e+007	-8.330e+007 to 3.630e+007	-2.350e+007	1.421	P > 0.05	ns
42	8.500e+006	1.750e+006	-6.750e+006	-6.655e+007 to 5.305e+007	-6.750e+006	0.4081	P > 0.05	ns

PBS vs PBD-C2								
Days	PBS	PBD-C2	Difference	95% CI of diff.	Difference	t	P value	Summary
0	0.0	0.0	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
5	500000	50000	-450000	-6.025e+007 to 5.935e+007	-450000	0.02720	P > 0.05	ns
6	4.000e+006	3.000e+006	-1000000	-6.080e+007 to 5.880e+007	-1000000	0.06045	P > 0.05	ns
8	3.000e+007	3.000e+007	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
10	4.000e+007	3.000e+007	-1.000e+007	-6.980e+007 to 4.980e+007	-1.000e+007	0.6045	P > 0.05	ns
12	9.000e+006	1.000e+007	1000000	-5.880e+007 to 6.080e+007	1000000	0.06045	P > 0.05	ns
15	5.500e+006	2.010e+007	1.460e+007	-4.520e+007 to 7.440e+007	1.460e+007	0.8826	P > 0.05	ns
18	2.480e+007	1.600e+007	-8.800e+006	-6.860e+007 to 5.100e+007	-8.800e+006	0.5320	P > 0.05	ns
21	1.130e+007	1.000e+007	-1.300e+006	-6.110e+007 to 5.850e+007	-1.300e+006	0.07859	P > 0.05	ns
24	1.550e+007	2.450e+006	-1.305e+007	-7.285e+007 to 4.675e+007	-1.305e+007	0.7889	P > 0.05	ns
27	1.050e+007	1.250e+006	-9.250e+006	-6.905e+007 to 5.055e+007	-9.250e+006	0.5592	P > 0.05	ns
30	1.750e+007	2.000e+006	-1.550e+007	-7.530e+007 to 4.430e+007	-1.550e+007	0.9370	P > 0.05	ns
34	8.500e+006	1.150e+006	-7.350e+006	-6.715e+007 to 5.245e+007	-7.350e+006	0.4443	P > 0.05	ns
38	2.600e+007	1.450e+006	-2.455e+007	-8.435e+007 to 3.525e+007	-2.455e+007	1.484	P > 0.05	ns
42	8.500e+006	1.250e+006	-7.250e+006	-6.705e+007 to 5.255e+007	-7.250e+006	0.4383	P > 0.05	ns

PBS vs C-term-C2								
Days	PBS	C-term-C2	Difference	95% CI of diff.	Difference	t	P value	Summary
0	0.0	0.0	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
5	500000	50000	-450000	-6.025e+007 to 5.935e+007	-450000	0.02720	P > 0.05	ns
6	4.000e+006	2.000e+006	-2.000e+006	-6.180e+007 to 5.780e+007	-2.000e+006	0.1209	P > 0.05	ns
8	3.000e+007	2.000e+007	-1.000e+007	-6.980e+007 to 4.980e+007	-1.000e+007	0.6045	P > 0.05	ns
10	4.000e+007	4.000e+007	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
12	9.000e+006	9.000e+006	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
15	5.500e+006	7.000e+006	1.500e+006	-5.830e+007 to 6.130e+007	1.500e+006	0.09068	P > 0.05	ns
18	2.480e+007	4.900e+007	2.420e+007	-3.560e+007 to 8.400e+007	2.420e+007	1.463	P > 0.05	ns
21	1.130e+007	1.860e+007	7.300e+006	-5.250e+007 to 6.710e+007	7.300e+006	0.4413	P > 0.05	ns
24	1.550e+007	5.000e+007	3.450e+007	-2.530e+007 to 9.430e+007	3.450e+007	2.086	P > 0.05	ns
27	1.050e+007	1.650e+007	6.000e+006	-5.380e+007 to 6.580e+007	6.000e+006	0.3627	P > 0.05	ns
30	1.750e+007	1.550e+007	-2.000e+006	-6.180e+007 to 5.780e+007	-2.000e+006	0.1209	P > 0.05	ns
34	8.500e+006	1.050e+007	2.000e+006	-5.780e+007 to 6.180e+007	2.000e+006	0.1209	P > 0.05	ns
38	2.600e+007	4.200e+006	-2.180e+007	-8.160e+007 to 3.800e+007	-2.180e+007	1.318	P > 0.05	ns
42	8.500e+006	4.450e+006	-4.050e+006	-6.385e+007 to 5.575e+007	-4.050e+006	0.2448	P > 0.05	ns

PBS vs BiP69-C2								
Days	PBS	BiP69-C2	Difference	95% CI of diff.	Difference	t	P value	Summary
0	0.0	0.0	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
5	500000	0.0	-500000	-6.030e+007 to 5.930e+007	-500000	0.03023	P > 0.05	ns
6	4.000e+006	1000	-3.999e+006	-6.380e+007 to 5.580e+007	-3.999e+006	0.2418	P > 0.05	ns
8	3.000e+007	3.000e+006	-2.700e+007	-8.680e+007 to 3.280e+007	-2.700e+007	1.632	P > 0.05	ns
10	4.000e+007	5.000e+007	1.000e+007	-4.980e+007 to 6.980e+007	1.000e+007	0.6045	P > 0.05	ns
12	9.000e+006	8.000e+006	-1000000	-6.080e+007 to 5.880e+007	-1000000	0.06045	P > 0.05	ns
15	5.500e+006	2.000e+006	-3.500e+006	-6.330e+007 to 5.630e+007	-3.500e+006	0.2116	P > 0.05	ns
18	2.480e+007	1.000e+007	-1.480e+007	-7.460e+007 to 4.500e+007	-1.480e+007	0.8947	P > 0.05	ns
21	1.130e+007	7.000e+007	5.870e+007	-1.100e+006 to 1.185e+008	5.870e+007	3.549	P > 0.05	ns
24	1.550e+007	3.000e+007	1.450e+007	-4.530e+007 to 7.430e+007	1.450e+007	0.8766	P > 0.05	ns
27	1.050e+007	2.000e+007	9.500e+006	-5.030e+007 to 6.930e+007	9.500e+006	0.5743	P > 0.05	ns
30	1.750e+007	2.000e+007	2.500e+006	-5.730e+007 to 6.230e+007	2.500e+006	0.1511	P > 0.05	ns
34	8.500e+006	6.000e+006	-2.500e+006	-6.230e+007 to 5.730e+007	-2.500e+006	0.1511	P > 0.05	ns
38	2.600e+007	7.000e+006	-1.900e+007	-7.880e+007 to 4.080e+007	-1.900e+007	1.149	P > 0.05	ns
42	8.500e+006	1000000	-7.500e+006	-6.730e+007 to 5.230e+007	-7.500e+006	0.4534	P > 0.05	ns

PBS vs BiP60-C2								
Days	PBS	BiP60-C2	Difference	95% CI of diff.	Difference	t	P value	Summary
0	0.0	0.0	0.0	-5.980e+007 to 5.980e+007	0.0	0.0	P > 0.05	ns
5	500000	0.0	-500000	-6.030e+007 to 5.930e+007	-500000	0.03023	P > 0.05	ns
6	4.000e+006	0.0	-4.000e+006	-6.380e+007 to 5.580e+007	-4.000e+006	0.2418	P > 0.05	ns
8	3.000e+007	1000	-3.000e+007	-8.980e+007 to 2.980e+007	-3.000e+007	1.814	P > 0.05	ns
10	4.000e+007	6.000e+006	-3.400e+007	-9.380e+007 to 2.580e+007	-3.400e+007	2.055	P > 0.05	ns
12	9.000e+006	1000000	-8.000e+006	-6.780e+007 to 5.180e+007	-8.000e+006	0.4836	P > 0.05	ns
15	5.500e+006	1.000e+007	4.500e+006	-5.530e+007 to 6.430e+007	4.500e+006	0.2720	P > 0.05	ns
18	2.480e+007	0.0	-2.480e+007	-8.460e+007 to 3.500e+007	-2.480e+007	1.499	P > 0.05	ns
21	1.130e+007	0.0	-1.130e+007	-7.110e+007 to 4.850e+007	-1.130e+007	0.6831	P > 0.05	ns
24	1.550e+007	0.0	-1.550e+007	-7.530e+007 to 4.430e+007	-1.550e+007	0.9370	P > 0.05	ns
27	1.050e+007	0.0	-1.050e+007	-7.030e+007 to 4.930e+007	-1.050e+007	0.6348	P > 0.05	ns
30	1.750e+007	0.0	-1.750e+007	-7.730e+007 to 4.230e+007	-1.750e+007	1.058	P > 0.05	ns
34	8.500e+006	0.0	-8.500e+006	-6.830e+007 to 5.130e+007	-8.500e+006	0.5139	P > 0.05	ns
38	2.600e+007	0.0	-2.600e+007	-8.580e+007 to 3.380e+007	-2.600e+007	1.572	P > 0.05	ns
42	8.500e+006	0.0	-8.500e+006	-6.830e+007 to 5.130e+007	-8.500e+006	0.5139	P > 0.05	ns

APPENDIX B

Tabular results of statistical analysis using Log rank test comparing median survival days post infection between PBS group and experimental groups. The statistics were done using GraphPad Prism software.

Days	PBS	C2+Adj
0	100	100
9		80
11	80	
36		60
42	80	60

Survival of PBS vs C2Adj:# of subjects at risk

Days	PBS	C2+Adj
0	5	5
9		5
11	5	
36		4
42	4	3

Survival of PBS vs C2Adj:Data summary

	PBS	C2+Adj
Number of rows	10	10
# of blank lines	5	5
# rows with impossible data	0	0
# censored subjects	4	3
# deaths/events	1	2
Median survival	Undefined	Undefined

Survival of PBS vs C2Adj:Curve comparison

	Data Set-A
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	0.4132
df	1
P value	0.5203
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	0.4098
df	1
P value	0.5221
P value summary	ns
Are the survival curves sig different?	No
Median survival	
PBS	Undefined
C2+Adj	Undefined
Hazard Ratio	
Ratio	0.4753
95% CI of ratio	0.04921 to 4.591

Survival of PBS vs C2:Survival proportions

Survival of PBS vs C2:Curve comparison

Days	PBS	C2
0	100	100
11	80	
36		80
37		60
42	80	60

Survival of PBS vs C2:# of subjects at risk

Days	PBS	C2
0	5	5
11	5	
36		5
37		4
42	4	3

Survival of PBS vs C2:Data summary

	PBS	C2
Number of rows	10	10
# of blank lines	5	5
# rows with impossible data	0	0
# censored subjects	4	3
# deaths/events	1	2
Median survival	Undefined	Undefined

	Data Set-A
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	0.2645
df	1
P value	0.6071
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	0.1475
df	1
P value	0.7009
P value summary	ns
Are the survival curves sig different?	No
Median survival	
PBS	Undefined
C2	Undefined
Hazard Ratio	
Ratio	0.5515
95% CI of ratio	0.05710 to 5.327

Survival of PBS vs BiP69+C2:Survival proportions

Days	PBS	C2
0	100	100
11	80	
42	80	100

Survival of PBS vs BiP69+C2:# of subjects at risk

Days	PBS	C2
0	5	5
11	5	
42	4	5

Survival of PBS vs BiP69+C2:Data summary

	PBS	C2
Number of rows	10	10
# of blank lines	5	5
# rows with impossible data	0	0
# censored subjects	4	5
# deaths/events	1	0
Median survival	Undefined	Undefined

Survival of PBS vs BiP69+C2:Curve comparison

	Data Set-A
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	1
df	1
P value	0.3173
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	1
df	1
P value	0.3173
P value summary	ns
Are the survival curves sig different?	No
Median survival	
PBS	Undefined
C2	Undefined
Hazard Ratio	
Ratio	7.389
95% CI of ratio	0.1466 to 372.4

Survival of PBS vs PBD-C2:Survival proportions

Days	PBS	C2
0	100	100
11	80	
23		80
42	80	80

Survival of PBS vs PBD+C2:# of subjects at risk

Days	PBS	C2
0	5	5
11	5	
23		5
42	4	4

Survival of PBS vs PBD+C2:Data summary

	PBS	C2
Number of rows	10	10
# of blank lines	5	5
# rows with impossible data	0	0
# censored subjects	4	4
# deaths/events	1	1
Median survival	Undefined	Undefined

Survival of PBS vs PBD+C2:Curve comparison

	Data Set-A
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	0.006211
df	1
P value	0.9372
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	0.02222
df	1
P value	0.8815
P value summary	ns
Are the survival curves sig different?	No
Median survival	
PBS	Undefined
C2	Undefined
Hazard Ratio	
Ratio	1.118
95% CI of ratio	0.06935 to 18.03

Survival of PBS vs C-term-C2:Survival proportions

Days	PBS	C2
0	100	100
11	80	
42	80	100

Survival of PBS vs C-term-C2:# of subjects at risk

Days	PBS	C2
0	5	5
11	5	
42	4	5

Survival of PBS vs Cterm-C2:Data summary

	PBS	C2
Number of rows	10	10
# of blank lines	5	5
# rows with impossible data	0	0
# censored subjects	4	5
# deaths/events	1	0
Median survival	Undefined	Undefined

Survival of PBS vs Cterm-C2:Curve comparison

	Data Set-A
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	1
df	1
P value	0.3173
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	1
df	1
P value	0.3173
P value summary	ns
Are the survival curves sig different?	No
Median survival	
PBS	Undefined
C2	Undefined
Hazard Ratio	
Ratio	7.389
95% CI of ratio	0.1466 to 372.4

Survival of PBS vs BiP69-C2:Survival proportions

Days	PBS	BiP69-C2
0	100	100
11	80	80
17		60
42	80	60

Survival of PBS vs BiP69-C2:# of subjects at risk

Days	PBS	BiP69-C2
0	5	5
11	5	5
17		4
42	4	3

Survival of PBS vs BiP69-C2:Data summary

	PBS	BiP69-C2
Number of rows	10	10
# of blank lines	5	5
# rows with impossible data	0	0
# censored subjects	4	3
# deaths/events	1	2
Median survival	Undefined	Undefined

Survival of PBS vs BiP69-C2:Curve comparison

	Data Set-A
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	0.36
df	1
P value	0.5485
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	0.2647
df	1
P value	0.6069
P value summary	ns
Are the survival curves sig different?	No
Median survival	
PBS	Undefined
BiP69-C2	Undefined
Hazard Ratio	
Ratio	0.4868
95% CI of ratio	0.04633 to 5.114

Survival of PBS vs BiP60-C2:Survival proportions

Days	PBS	BiP60-C2
0	100	100
11	80	80
17		60
18		60
42	80	

Survival of PBS vs BiP60-C2:# of subjects at risk

Days	PBS	BiP60-C2
0	5	5
11	5	5
17		4
18		2
42	4	

Survival of PBS vs BiP60C2:Data summary

	PBS	BiP60-C2
Number of rows	10	10
# of blank lines	5	5
# rows with impossible data	0	0
# censored subjects	4	3
# deaths/events	1	2
Median survival	Undefined	Undefined

Survival of PBS vs BiP60C2:Curve comparison

	Data Set-A
Comparison of Survival Curves	
Log-rank (Mantel-Cox) Test	
Chi square	0.36
df	1
P value	0.5485
P value summary	ns
Are the survival curves sig different?	No
Gehan-Breslow-Wilcoxon Test	
Chi square	0.2647
df	1
P value	0.6069
P value summary	ns
Are the survival curves sig different?	No
Median survival	
PBS	Undefined
BiP60-C2	Undefined
Hazard Ratio	
Ratio	0.4868
95% CI of ratio	0.04633 to 5.114