FUNCTIONAL EXPRESSION OF *TRYPANOSOMA CONGOLENSE* PYROGLUTAMYL PEPTIDASE TYPE I AND DEVELOPMENT OF REVERSE GENETICS TOOLS

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(DVM)

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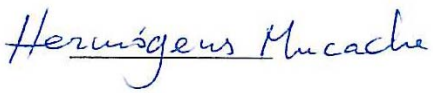
PREFACE

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 2008 to March 2010, under the supervision of Prof. Theresa Coetzer and co-supervision of Prof. Alain Boulangé.

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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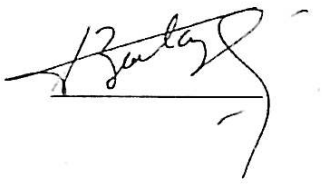

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DECLARATION-PLAGIARISM

I, Hermogenes Neves Mucache declare that,

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

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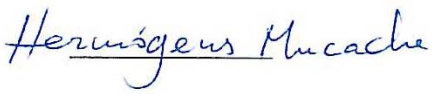
Signed: 

DEDICATION

I dedicate this work to my late father, Sansão Osias Mucache.

ABSTRACT

Trypanosoma congolense is a protozoan parasite transmitted by tsetse flies. It causes bovine trypanosomosis, the major disease for livestock in sub-Saharan Africa. Control methods include trypanocidal drugs and vector control, but none is fully satisfactory, due to resistance and environmental issues. A method that would have the greatest impact on controlling the disease is vaccination. However, development of a conventional vaccine has been hampered by the mechanism of antigenic variation, which allows the parasite to evade the host's immune system.

An alternative strategy in vaccine design is to target the bioactive compounds released by dead and dying trypanosomes. This approach is termed ''anti-disease'', and does not affect the survival of the parasite but targets the pathogenic factors released by the trypanosomes. The development of a successful anti-disease vaccine necessitates knowledge of all pathogenic factors involved in the disease process. Several macromolecules, primarily peptidases, have been implicated in the pathogenesis of trypanosomosis. Pyroglutamyl peptidase type I (PGP) was shown to be involved in abnormal degradation of thyrotropin- and gonadotropin-releasing hormones in rodents infected with *T. brucei*, but to date no data are available on the *T. congolense* PGP.

Molecular cloning and expression in *E. coli* of the coding sequence of *T. congolense* PGP, as well as the enzymatic characterisation of the recombinant protein, are reported here, completed by the development of reverse genetics tools for studies of gene function.

A 678 bp PCR fragment covering the complete open reading frame of PGP was cloned and sequenced. The deduced amino acid sequence showed 52% and 29% identity with the *T. brucei* and *Leishmania major* enzymes respectively. The catalytic residues Glu, Cys and His described in *Bacilus amyloliquefaciens* PGP are conserved in the *T. congolense* sequence. PGP was expressed in bacterial systems as a soluble active, 26 kDa enzyme. The recombinant enzyme showed activity specific for the fluorescent substrate pGlu-AMC, with a k_{cat}/K_m of 1.11 $s^{-1}\mu M$. PGP showed activity in the pH 6.5-10 range, with maximal activity at pH 9.0. The enzyme was strongly inhibited by sulfhydryl-blocking reagents such as iodoacetic acid and iodoacetamide with a k_{ass} of 125 M^{-1} s^{-1} and 177 M^{-1} s^{-1} respectively. Antibodies raised in chickens against the recombinant enzyme allowed the detection of native PGP in both procyclic and bloodstream *T.*

congolense developmental stages, and displayed complete inhibition of the enzyme *in vitro* at physiological concentrations. To get insight into the role of PGP in parasite biology and trypanosomosis progression, two types of vectors for reverse genetics studies were developed. For RNA interference, a 400 bp 3′ end segment of the PGP open reading frame was cloned into the plasmid p2T7^{Ti}, that will allow PGP gene down-regulation upon integration into the genome of an engineered tetracycline-inducible strain such as TRUM:29-13. For gene knock-out, several rounds of molecular engineering were carried-out in order to create two plasmid vectors, pGL1184-based (blasticidin resistance) and pGL1217-based (neomycin resistance), each bearing 200 bp-long regions at the 5′ and 3′ ends of the PGP open reading frame. In subsequent studies, taking advantage of the recent advances in culture and transformation of *T. congolense*, these plasmids will allow the creation of single and double knock-out mutants of PGP.

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My mother, my sister and my brother for their unconditional love, understanding and support.

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LIST OF ABBREVIATIONS

AAT	African animal trypanosomosis
ABTS	2,2-azino-di-[3-ethylbrnzthiazoline sulfonate]
AEBSF	4-(2-aminoethyl)benzenesulfonylfluoride
AMC	7-amino-4-methylcoumarin
Bis-Tris	2-bis(2-hydroxyethyl)amino-2-(hydroxymethyl)-1,3-propanediol
BLAST	basic local alignment search tool
bp	base pair(s)
Brij	polyoxyethylenlaurylether
BSA	bovine serum albumin
C-terminal	carboxy terminal
DNA	deoxyribonucleic acid
dsRNA	double-stranded RNA
DTT	dithiothreitol
EDTA	ethylenediaminetetra-acetic acid
ELISA	enzyme linked immunosorbent assay
E-64	*trans* -epoxysuccinyl-L-leucyl-amido(4-guanidino)butane
GnRH	gonadotropin releasing-hormone
GST	glutathione-S-transferase
g	relative centrifugal force
HRPO	horse radish peroxidase
IPTG	isopropyl-beta-D-thiogalactopyranoside
[I]	Inhibitor concentration
IgY	immunoglobulin Y
kDa	kiloDalton
k_{ass}	rate of complex association
k_{cat}	turnover number

K_i	Inhibition constant
K_m	Michaelis-Menten constant
KO	knock-out
k_{obs}	pseudo first-order inhibition constant
MES	acetate-2(N-morpholino)ethanesulphonic acid
min	minute(s)
N-terminal	amino terminal
nt	nucleotide(s)
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PEG	polyethylene glycol
pGlu-AMC	pyroglutamyl aminomethyl coumarin
pNA	para-nitroanilide
RNAi	RNA interference
RT	room temperature
[S]	substrate concentration
s	second(s)
SAP	shrimp alkaline phosphatase
SDS	sodium dodecyl sulfate
TBS	tris buffered saline
TEMED	N,N,N´,N´-tetramethyl ethylene diamine
TRH	thyrotropin releasing-hormone
Tris	2-amino-2-(hydroxymethyl)-1,3-propanediol
V_{max}	maximum velocity
VSG	variable surface glycoprotein

1. LITERATURE REVIEW

1.1. AFRICAN TRYPANOSOMOSIS

The trypanosomoses are diseases of humans and domestic animals caused by infection with parasitic protozoa of the genus *Trypanosoma.* The majority of animal diseases caused by trypanosomes occurs in the tropics, and in Africa several species are tsetse-transmitted and cause African trypanosomosis in domestic animals, known as "nagana" (Connor, 1995). African animal trypanosomosis (AAT) is one of the major constraints to agricultural development in the sub-humid and humid zones of Africa (Swallow, 2000). Tsetse flies are distributed in 37 sub-Saharan African countries covering about 9 million km^2, that corresponds to one-third of the continent (Mattioli et al., 2004). About 45 to 60 million cattle and tens of millions of small ruminants are at risk of trypanosome infection (Chadenga, 1994; Gilbert, 2001) and the total losses caused by the disease are estimated at US$ 4.75 billion per year (FAO, 2004).

1.1.1 Trypanosome morphology and transmission

Trypanosomes are protozoans belonging to the order *Kinetoplastida*, characterised by the presence of a kinetoplast, which contains the DNA of a single mitochondrion, and a flagellum (Uilenberg, 1998). They are elongated spindle-shaped parasites ranging from 8 to 39 μm in length. The flagellum arises at the posterior end of the parasite, stretches from a basal body in the flagellar pocket and runs to the anterior end of the body and is attached along its length to the pellicle to form an undulating membrane that may continue forward as a free flagellum, as shown in Fig. 1.1 (Urquhart et al., 1998).

The pathogenic trypanosome species important in domestic animals are found in three subgenera: *Dutonella*, *Nannomonas* and *Trypanozoon* (Table 1). The fourth salivarian subgenus, *Pycnomonas*, is represented only by *Trypanosoma suis*, which has little veterinary importance (Connor, 1995).

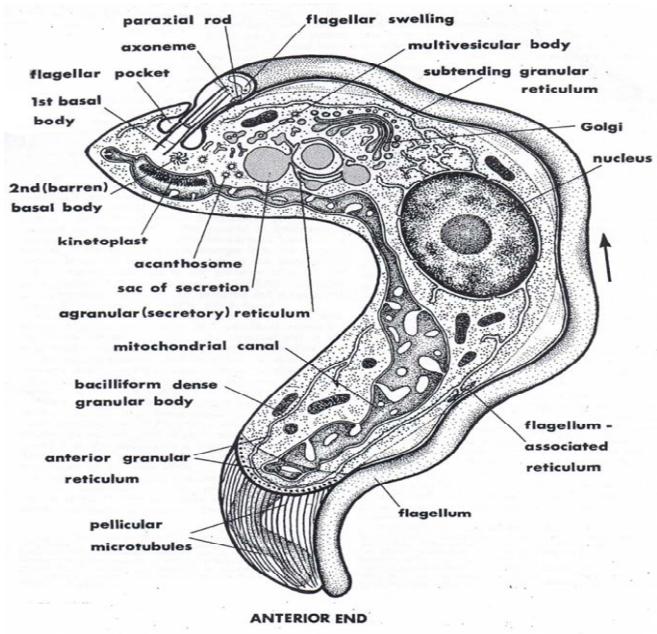

Fig. 1.1 General morphology of trypanosomes, (Vickerman, 1969).

Table 1.1 Salivarian trypanosomes pathogenic to livestock under field conditions (Connor, 1995)**.**

Subgenus	**Species**	**Host**					
		Cattle	**Goat**	**Sheep**	**Pig**	**Horse**	**Donkey**
Trypanozoon	*T. brucei*	+	++	++	+	+++	++
	T. evansi[1]	++	+	+	++	+++	++
	T. equiperdum[2]	_	_	_	_	+++	++
Nannomonas	*T. congolense*	+++	++	++	+	++	++
	T. simiae	_	+	+	+++	_	_
Dutonella	*T. vivax*	+++	++	++	_	++	+
Pycnomonas	*T. suis*[3]	_	_	_	++	_	_

[1]Mechanical transmission; [2]venereal transmission; [3]rarely encountered; (-) = not infective/pathogenic; (+) = mildly pathogenic; (++) = moderately pathogenic; (+++) = severely pathogenic

Typically, with the exception of *T. equiperdum*, *T. evansi* and sometimes *T. vivax* in South America, trypanosomes require two hosts to complete their life cycle: they multiply in the blood and body fluids of the mammalian host and when ingested by a haematophagus invertebrate vector, they undergo a cycle of maturation and development within this vector, as shown in Fig. 1.2 (Matthews et al., 2004). Based on the development cycle within the vector, trypanosomes can be classified into two broad sections of 'salivaria'(e.g. the African trypanosomes) and 'stercoraria' (e.g. the south American trypanosome *T. cruzi*), based on whether the infective metacyclic forms are in the saliva or faeces of the vector (Connor, 1995).

1.1.2 Trypanosoma life-cycle

Tsetse flies ingest trypanosomes from blood or lymph while feeding on an infected mammalian host (Fig. 1.2). After establishing an infection in the tsetse fly, trypanosomes invade the ectoperitrophic space and move into the proventriculous region (Aksoy, 2003). Thereafter they lose their variable glycoprotein surface coat (VSG), and in the case of *T. brucei* and *T. congolense*, transform to elongated procyclics which multiply. The dividing procyclics migrate to the proboscis and transform to epimastigotes and then move to the hypopharynx where they attach and complete their development to become coated metacyclics (Matthews et al., 2004). The infection occurs when the metacyclic trypanosomes are inoculated into the new vertebrate host when the tsetse fly feeds again. With *T. vivax* a similar process of cyclic development takes place, except that it occurs entirely within the proboscis, with no developmental stages occurring in the midgut or salivary glands of the fly. Infective trypanosomes are inoculated through the saliva when the fly bites an animal. At the site of inoculation the metacyclic trypanosomes multiply locally as the typical blood forms, producing in a few days a cutaneous inflammatory swelling called chancre. Thereafter they enter the bloodstream, multiply and parasitaemia becomes detectable in the peripheral blood (Urquhart et al., 1998). The period between the ingestion of trypomastigotes from the mammalian host and the change into metacyclic forms in the tsetse varies from one to three weeks (Uilenberg, 1998).

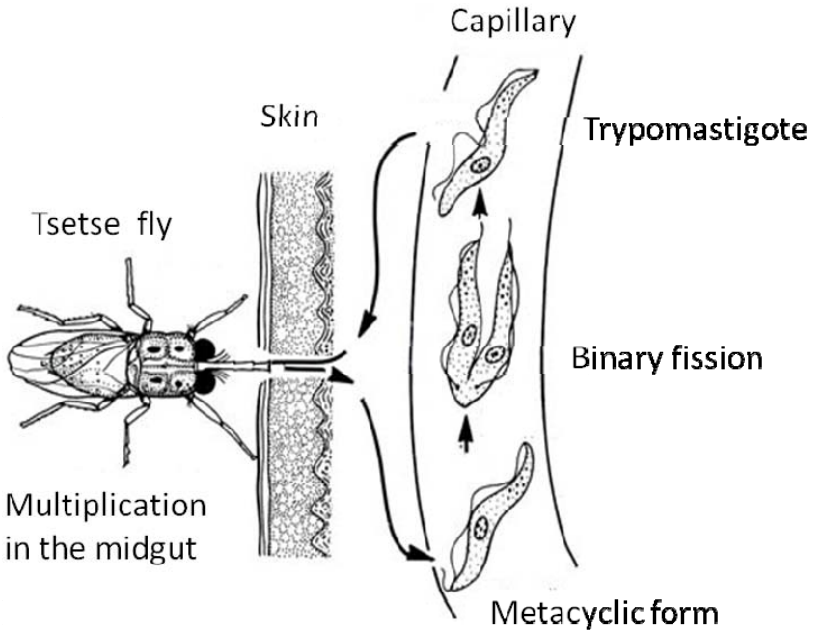

Fig. 1.2 Trypanosoma life cycle. The different developmental stages in the vertebrate and invertebrate host are shown(Gardiner et al., 1998).

1.1.3 Epidemiology of trypanosomosis

The epidemiology of AAT in tsetse infested areas is determined mainly by the distribution of the vector, the virulence of the parasite and the response of the host (Urquhart et al., 1998). The major determining factor for the distribution and epidemiology of bovine trypanosomosis in Africa is the availability of a suitable habitat for the vector, the tsetse fly (Van den Bossche, 2001). The current distribution of *Glossina* species in Africa overlap with cattle distribution and is shown in Fig. 1.3, where the risk of trypanosomosis makes it difficult to keep cattle and small ruminants in these areas. On the other hand, *Glossina*'s ability to transmit the infection to mammals depends on the fly's susceptibility to trypanosome infection. For example, *G. fuscipes* appears to be a better vector of *T. vivax* to cattle than *G. pallidipes*, which is a better transmitter of *T. congolense* than *G. swynnertoni* (Stephen, 1986).

Parasite biology also plays an important role in the epidemiology of trypanosomosis. Since the disease has generally a chronic course, animals may remain parasitaemic and survive for long periods, increasing the opportunities for disease spreading by tsetse flies. The chronic course of trypanosomosis occurs as a consequence of the way the parasite escapes the host immune system, a phenomenon called antigenic variation (Urquhart et al., 1998).

Host-related factors tend to complicate an understanding of trypanosomosis epidemiology. Wild animals and some west African taurine cattle are tolerant to infection with trypanosomes and thus, especially wild animals, form a major reservoir of infection, (Connor, 1995). The role of trypanotolerant cattle on disease transmission has not been investigated (McDermott and Coleman, 2001).

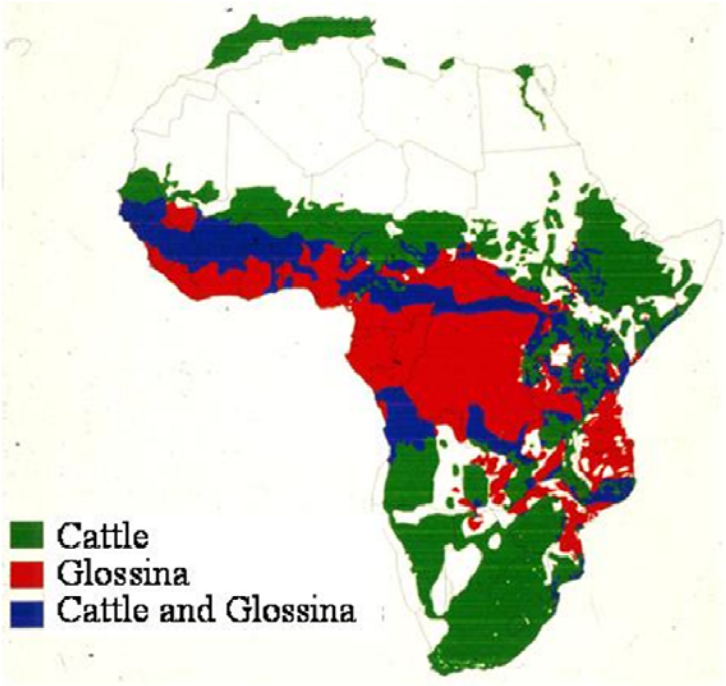

Fig. 1.3 Tsetse distribution in Africa. The cattle distribution is limited by the risk of trypanosomosis in tsetse infested areas (Uilenberg, 1998).

1.1.4 Pathogenesis

Many of the clinical and pathological features of trypanosomosis are common in all domestic animals, independent of the trypanosome species that is involved (Taylor and Authié, 2004). Anaemia, tissue damage and immunosupression dominate the pathology of the disease. Infection becomes established at the site of inoculation of metacyclic trypanosomes in the skin, causing an immune reaction by the host that results in a local visible swelling, the chancre; Enlargement of local draining lymph nodes occurs concurrently (Naessens, 2006).

Trypanosomosis, like many other infectious diseases, starts with fever. A few days after the first contact with trypanosomes, the host's immune system starts to produce specific antibodies against the surface proteins of the parasite. After a few days, almost all trypanosomes in the

blood are destroyed and the body temperature drops. However, a few parasites survive as they have been able to replace their surface protein by a different one, against which the circulating antibodies have no effect. These surviving trypanosomes are able to multiply and cause a new peak of parasitaemia and fever, until the host produces specific antibodies against the new surface proteins (Uilenberg, 1998). This process may continue for a long time, in most instances with a fatal result, and is known as antigenic variation (Wilson and Cunningham, 1972).

Anaemia observed in trypanosomosis is haemolytic in origin, occurring intravascularly in the acute phase and also extravascularly in the subacute and chronic stages of the disease. The cause of this anaemia has not been well defined but bone marrow depression, erythrophagocytosis, haemolysis of erythrocytes either by haemolytic factors or immunological means are possibly responsible (Biryomumaisho and Katunguka-Rwakishaya, 2007; Connor, 1995; Taylor and Authié, 2004; Uilenberg, 1998). Bone marrow depression might involve the secretion of nitric acid (Mabbott and Sternberg, 1995) and erythrophagocytosis is related to over activation of macrophages in the spleen and liver (Naessens, 2006). The pathogenesis of tissue lesions has not fully been elucidated, but it has been shown that biologically active products released by trypanosomes play an important role (Stijlemans et al., 2007).

Immunodepression is an important feature in AAT, and it occurs mainly in the chronic stage, but is also present in the acute phase of the disease (Uilenberg, 1998). It is also related to the production of harmful substances, such as tumour necrosis factor (TNF), by over-activated macrophages (Magez et al., 2004).

1.1.5 Clinical signs

Infection with *T. congolense* shows the most severe course in cattle (Radostis et al., 2006a). The pre-patent period is usually 1-3 weeks (Taylor and Authié, 2004) and the principal clinical signs in ruminants include anaemia, swelling of superficial lymph nodes, lethargy, progressive emaciation and fever during the parasitaemia peaks (Radostis et al., 2006a). Reproductive and endocrine disorders are a common occurrence in animal trypanosomosis and include irregular oestrus, abortion, neonatal death, infertility, and aberrations in adrenocortical and gonadal activity (Connor, 1995; Mutayoba et al., 1995).

1.1.6 Diagnosis

Definitive diagnosis of trypanosomosis requires the demonstration of parasites in blood or tissue fluids of infected animals by conventional parasitological methods (micro-haematocrit centrifugation technique, wet blood film and thin stained blood smear). These techniques lack sensitivity, but are widely used for rapid diagnostics in the field, because they are simple and less expensive when compared with molecular and serological techniques (Fernandez et al., 2009). Serological techniques used in trypanosome diagnosis include antigen- and antibody-ELISAs. Despite the lack of sensitivity (Desquesnes, 1996), these techniques have been shown to be useful in large scale epidemiological studies and allow automated analysis of the generated data (Eisler et al., 1998). An inhibition ELISA using the *T. congolense* heat shock protein 70 (hsp70) and Immunoglobulin Binding Protein (BiP) fusion protein was recently assayed. The hsp/BiP ELISA detected primary infections with *T. congolense* IL1180 and secondary infections with other *T. congolense* clones as well as field infections with *T. congolense* and *T. vivax* (Bossard et al., 2010).

Molecular diagnosis, based on the detection of trypanosomal DNA by polymerase chain reaction (PCR) provides a highly specific diagnosis and increased sensitivity (Desquesnes and Davila, 2002), based on, for example, the amplification of the internal transcribed spacer-1 region (ITS-1) for the differentiation of trypanosome species and subtypes (Cox et al., 2005; Njiru et al., 2005). This technique has the disadvantage of requiring specialised equipment and qualified personnel (Uilenberg, 1998), but it still remains the most sensitive and is recommended for the establishment of large scale epidemiological studies (Ramirez-Iglesias et al., 2011).

1.1.7 Control of trypanosomosis

The control of trypanosomosis currently relies on vector control, use of trypanocidal drugs (Lalmanach et al., 2002; Murray et al., 2004), and use of trypanotolerant cattle (d'Ieteren et al., 1998).

1.1.7.1 Trypanocidal drugs

The use of trypanocidal drugs is the primary control strategy of AAT in sub-Saharan Africa, and only three compounds, isometamidium chloride, homidium (bromide and chloride) and diminazene aceturate, are available in the market for the treatment of the disease in cattle (Holmes et al., 2004). The use of these drugs is limited by the development of drug resistance by

the parasite, reported in several regions of the continent and the drug's toxicity (Anene et al., 2001; Delespaux and de Koning, 2007). Commercial trypanocides currently in use for the control of trypanosomosis in domestic animals are listed in Table 1.2.

1.1.7.2 Vector control

The control of the tsetse vector is the most efficient method for controlling trypanosomosis (Seifert, 1995). However, as shown in Zanzibar, it can take several years and millions of dollars to eradicate tsetse from small areas (Rogers and Randolph, 2002). Vector control methods include the use of insecticides, release of sterile tsetse males (e.g. Zanzibar) and use of blue and black traps (containing substances that are attractive to flies, such as acetone or phenolic molecules) and screens impregnated with insecticides (Uilenberg, 1998).

1.1.7.3 Trypanotolerance

Vector control and trypanocidal drugs for trypanosomosis control, when properly implemented, have been shown to be cost-effective and sustainable at a local level. However, at a continent level, these methods are not significantly improving the control of the disease. Moreover, future prospects of a major break-through for new methods to control the disease, including vaccination, are not good (Murray et al., 2004).

Another approach for trypanosomosis control is the investigation and understanding of trypanotolerance mechanisms (Hill et al., 2005). Trypanotolerance is defined as the ability that some cattle breeds have to remain productive under trypanosome infection (Naessens, 2006) by limiting the harmful effects of the parasite, such as anaemia (Murray et al., 1983). This phenomenon is usually attributed to the *Bos taurus* breeds of cattle in West and Central Africa, particularly the N'Dama and West African Shorthorn (Murray et al., 2004). Under low to medium tsetse challenge, trypanotolerant taurine cattle exhibit lower parasitaemia, develop less severe anaemia and are more productive than susceptible zebu cattle (Authié, 1994).

The use of trypanotolerant cattle breeds has been practiced as a major option for sustainable livestock production in many West and central African countries. However, trypanotolerance is a feature of only a third of the cattle in tsetse-infested areas, and of no more than 10% (or 15 million) of the total cattle population in sub-Saharan Africa. These breeds are not widely used because of the innacurate belief that these animals are unproductive because of their relatively small size (Murray et al., 2004).

Table 1.2 Currently available trypanocidal drugs used in AAT (Holmes et al., 2004).

Drug	Trade names [a]	Activity/use	Animal	Use
Diminazene aceturate	Berenil®	*T. congolense*	Cattle	T
	Many others	*T. vivax*	Small ruminants	
		*T. brucei**	Dogs**	
		*T. evansi**	Equidae**	
Homidium chloride	Novidium®	*T. congolense*	Cattle	T/P[b]
Homidium bromide	Ethidium®	*T. vivax*	Small ruminants	
			Pigs	
			Equidae**	
Isometamidium chloride	Samorin®	*T. congolense*	Cattle	T
	Trypamidium®	*T. vivax*	Small ruminants	P
	Veridium®	*T. brucei*	Equidae	
		T. evansi	Camels	
Quinapyramine dimethyl sulfate	Trypacide sulfate®	*T. congolense*	Camels	T
Quinapyramine dimethyl sulfate: chloride (3:2 w/w)	Trypacide pro-salt®	*T. vivax*	Equidae	P
		T. brucei	Pigs	
		T. evansi	Dogs	
Suramin	Naganol®	*T. evansi*	Camels	T/P**
			Equidae	

T, therapeutic; P, prophylactic; [a]incomplete list; [b]prophylaxis observed in areas of low tsetse challenge;
*Limited activity; **Small therapeutic index

1.1.7.4 Vaccine and therapy development

A strategy that is expected to have a major impact on the control of trypanosomosis is vaccination (Borst and Cross, 1982; Donelson and Rice-Ficht, 1985). The major constraint for vaccine development is the ability of the parasite to evade the host immune response by antigenic variation and immunosuppression (Donelson, 2003).

Another approach in the investigation of a vaccine against trypanosomosis is to eliminate the pathogenicity of the parasite and not the infection *per se*. This type of vaccine is called "anti-disease", and it may not affect the parasite survival, but would neutralise the pathogenic factors, and thus, control the disease (Authié, 1994). In trypanosomosis, the mechanisms behind the development of lesions are often attributed to bioactive substances, such as peptidases, phospholipases, lipopolysaccharides, and free fatty acids, released into the host circulation by dead and dying parasites (Connor, 1995; Seifert, 1995; Tizard et al., 1978). These pathogenic factors are targeted in the anti-disease approach.

The study of peptidases in protozoan parasites in general, and in trypanosomatids in particular, has acquired considerable importance (Klemba and Goldberg, 2002) as they are important virulence factors, drug targets and vaccine candidates in parasitic infections (McKerrow et al., 1999; Vermelho et al., 2007). Cysteine peptidases are important for growth and survival of kinetoplastid parasites (Caffrey and Steverding, 2009) and have been implicated in a variety of biological events, including invasion of host cells, immune evasion, pathogenesis and virulence (Brooks et al., 2000).

Antibodies directed against cysteine peptidase antigens can have an inhibitory effect on the enzyme proteolytic activity and provide significant immunological protection, as seen in the parasite *Fasciola hepatica* (Dalton et al., 1996), *T. b. brucei* (Troeberg et al., 1997) and *T. congolense* (Huson et al., 2009). For such anti-disease vaccine strategies, a precise knowledge of parasitic factors involved in pathogenesis is needed (Sajid and McKerrow, 2002).

1.2 PARASITE PEPTIDASES

Peptidases are enzymes that cleave peptide bonds in proteins and polypeptides. Peptidases are classified into subclasses according to their catalytic mechanism: aspartic, cysteine, glutamic acid, metallo, serine and threonine peptidases (Barrett and Rawlings, 2004). More recently, a 7th new catalytic type was identified: the asparagine peptide lyases. Such peptidases perform self-cleavages when the nucleophile asparagine forms a succinimide ring, which happens after a second active site residue, an aspartate or glutamate, had been brought into close proximity (Rawlings et al., 2011). Metallo, glutamic acid and aspartyl peptidases use the activated oxygen of water as a nucleophile and active residues with (metallo) and without (aspartyl) the aid of a metal cation to form a non transient bond with the peptidase. Serine, threonine and cysteine

peptidases form a covalent bond with the substrate through the catalytic oxygen of serine and threonine and sulfur of an essential cysteine respectively (Vermelho et al., 2007). *Trypanosomatidae* peptidases with potential use as drug targets are listed in Table 1.3. Before the cleavage of peptide bonds, the peptidases must specifically bind the protein or peptide substrate in its active site. The pockets of the enzyme which binds the substrate residues on the N-terminal side (Fig. 1.4) are designated S_1, S_2, S_3, etc., while the sites on the C-terminal side are designated S_1' S_2', S_3', etc. The substrate residues on the N-terminal side of the scissile bond are designated P_1, P_2, P_3, etc, those on the C-terminal side are designated P_1', P_2', P_3', etc (Schechter and Berger, 1968). The present study will focus on cysteine peptidases.

Table 1.3 *Trypanosomatidae* peptidases investigated as drug targets (Vermelho et al., 2007)

Trypanosomatidae	**Catalytic type/peptidase/new name[a]**	**Reference**
T. cruzi	Cysteine/cruzipain/*Tcr*CATL	(Cazzulo et al., 1989)
	Serine/oligopeptidase B	(Ashall et al., 1990)
T. congolense	Cysteine/congopain/*Tco*CATL	(Authié, 1994)
T. b. rhodesiense	Cysteine/rhodesain/*Tbr*CATL	(Caffrey et al., 2001)
T. b.brucei	Cysteine/brucipain/*Tbb*CATL	(Caffrey et al., 2000)
	Cysteine/trypanopain/*Tbb*CATL	(Troeberg et al., 1996)
Leishmania mexicana	Cysteine/CPA/*Lme*CATL1	(Mottram et al., 1992)
	Cysteine CPB/*Lme*CATL2	(Mottram et al., 1997)
	Cysteine/CPC/*Lme*CATL3	(Bart et al., 1995)

[a]Caffrey, C.R. and Steverding, D. (2009). Kinetoplastid papain-like cysteine peptidases. Molecular and Biochemical Parasitology, 167, 12-19.

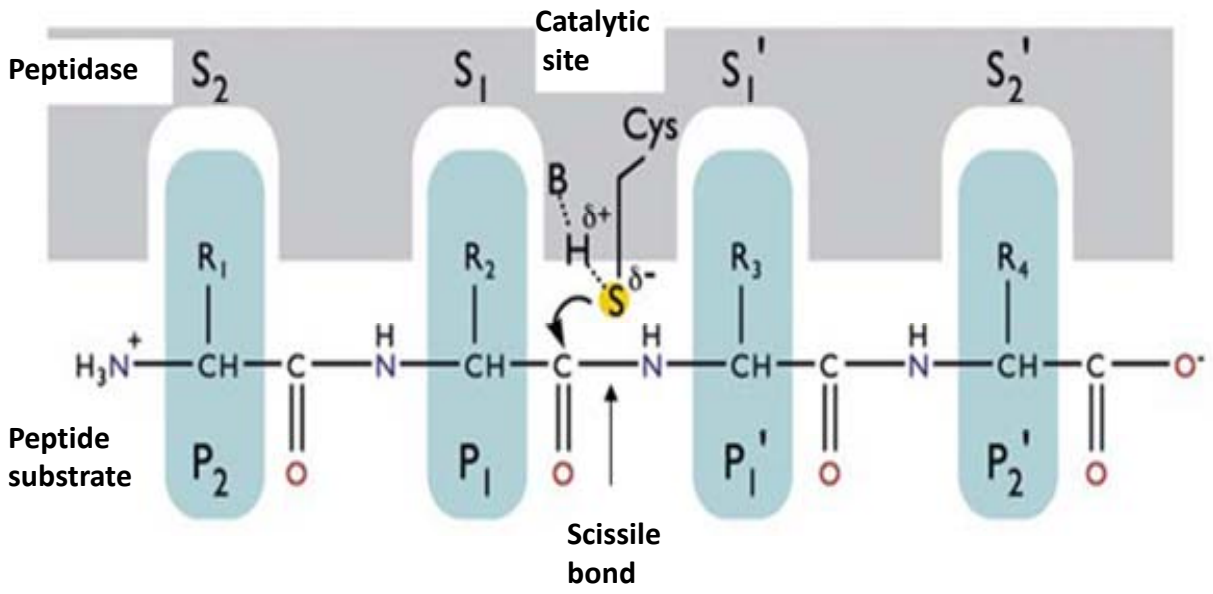

Fig. 1.3 Schematic diagram showing the binding sites of a peptidase and its substrate. Binding sites are designated S1, S2, S3,…and P1, P2, P3…in the enzyme and substrate respectively. The peptide substrate is hydrolysed in the scissile bond through nucleophilic attack by the sulfur of the catalytic residue Cys from the peptidase. The His acts as proton donor, enhancing the nucleophilic properties of sulfhydryl group. Key: R, amino acid side chain; B, catalytic base (Atkinson *et al*., 2009).

1.2.1 Cysteine peptidases

Cysteine peptidases possess a catalytic cysteine residue that is essential for the hydrolysis of protein or peptide substrates through nucleophilic attack. The proximity of an active site histidine, which acts as proton donor/general base, enhances the nucleophilic properties of the active site cysteine sulfhydryl group (Fig. 1.4). The sulfhydryl group of the cysteine side chain and the imidazole of histidine give rise to a thiolate-imidazolium charged diad (Sajid and McKerrow, 2002). The negative sulfur atom makes a nucleophilic attack on the carbonyl carbon of the peptide bond, forming a tetrahedral intermediate, which is acylated forming an enzyme-substrate thiol ester. In this process the C-terminal portion of the substrate is released. The resulting enzyme-substrate is hydrolysed by water to form a second tetrahedral intermediate which is subsequently deacylated releasing the free enzyme and the N-terminal portion of the substrate (Barrett and Rawlings, 2004).

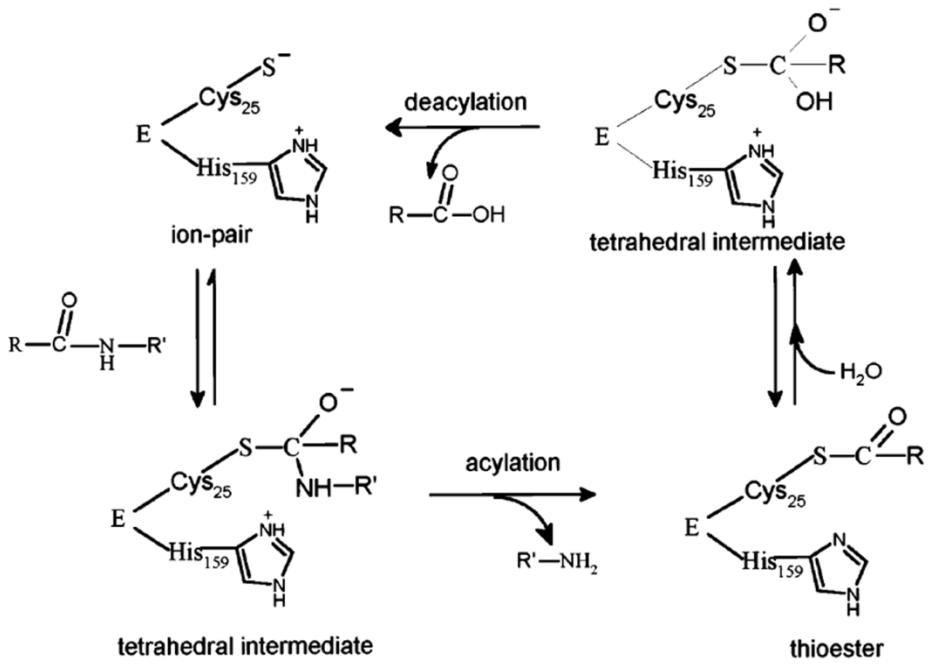

Fig. 1.4 Schematic represantation of the spatial proximity of the sulfur of active site cysteine and the chemical base in a cysteine peptidase. The sulfhydryl of cysteine and the imidazole group of histidine give rise to a thiolate-imidazolium charged diad (Sajid and McKerrow, 2002).

Cysteine peptidases are classified into clans, which are differentiated into families based on the sequence similarities, possession of inserted peptide loops, and biochemical specificity to small peptide substrates (Barrett and Rawlings, 2004). Cysteine peptidases of parasitic organisms are found in six clans: CA, CD, CE, CH, CF and CO. The majority of known parasite peptidases are distributed in clan CA (Atkinson et al., 2009), also known as papain-like peptidases, which includes the family C1 (cathepsin B and cathepsin L-like) and C2 (calpain-like) (Sajid and McKerrow, 2002). Family C1 contains parasite peptidases such as cruzipain or *Tcr*CATL in *T. cruzi*, the falcipains (*Pfa*CATL from *Plasmodium falciparum*), CPA, CPB and CPC or *Lme*CATL1, 2, 3 from *L. mexicana* and congopain or *Tco*CATL from *T. congolense* (Barrett and Rawlings, 2004; Rosenthal, 2004). Authié *et al*. (1993) showed that susceptibility to trypanosomosis in cattle is correlated to the ability of cattle to produce a greater immune response against *Tco*CATL after infection with *T. congolense*. *Tco*CATL is the major cysteine protease of *T. congolense* and it plays a role in the pathogenesis of anaemia and immunosuppression in trypanosome-infected animals. Cattle immunised with *Tco*CATL and subsequently challenged with *T. congolense* maintained or gained weight, and showed a faster tendency to recover from anaemia when compared with non-immunised cattle (Authié et al.,

2001b). However, this protective effect is still limited, and other anti-disease vaccine candidates are needed to enhance efficacy (Antoine-Moussiaux et al., 2009).

Peptidases of clan CD are associated with tightly defined substrate specificity, and they are readily distinguished from all other clans in terms of amino acid sequence, tertiary structure fold, substrates and function (Mottram et al., 2003). Parasite peptidases in clans CO, CE and CH are mostly uncharacterised. Clan CF contains peptidases with pyroglutamyl peptidase I activity, which removes N-terminal pyroglutamyl residues of neuropeptides (Atkinson et al., 2009). This peptidase, specifically of *T. congolense*, is the main focus of the present study.

1.2.2 Pyroglutamyl peptidases

Several parasite cysteine peptidases have been described as potential targets for drug and vaccine development (Lalmanach et al., 2002). Pyroglutamyl-peptidase [also referred to as pyrrolidonecarboxylate peptidase (Tsuru et al., 1978), pyroglutamate aminopeptidase (O'Connor and O'Cuinn, 1985), pyrrolidonyl peptidase, pyrrolidonecarboxylate peptidase (Kwiatkowska et al., 1974; Sullivan et al., 1977), 5-oxoprolyl peptidase, pyroglutamate aminopeptidase (O'Connor and O'Cuinn, 1985; Taylor and Dixon, 1978) hydrolyses N-terminal pyroglutamate (pGlu) residues from peptides (Doolittle and Armentrout, 1968). Such an activity is classed as omega peptidase activity (EC 3.4.19) rather than aminopeptidase activity because the substrate contains no free N-terminal amino group (Barrett and Rawlings, 2004). Two classes of pyroglutamyl peptidases (PGP) are recognised: the cysteine peptidase pyroglutamyl-peptidase I (EC 3.4.19.3 MEROPS clan CF, family C15) and the metallopeptidase pyroglutamyl-peptidase II (EC 3.4.19.6 MEROPS clan M01, family M1) (Barrett and Rawlings, 2004). The enzyme has been described in mammals (Cummins and O'Connor 1996), bacteria (Ito et al., 2001; Ogasahara et al., 2001), reptiles (Prasad et al., 1982) and trypanosomes (Morty et al., 2006).

pGlu is a residue found at the amino terminal end of many proteins and bioactive compounds, and minimises their susceptibility to degradation by aminopeptidases (Awadé et al., 1994). The mechanism by which pGlu is placed at the amino terminus of proteins and peptides is still not fully understood, but it is thought to occur through a post-translational cyclisation of an amino terminal glutamyl or glutaminyl residue by the enzyme glutaminyl cyclase (Fischer and Spiess, 1987; Huang et al., 2005). Bioactive compounds that have this modification include thyrotropin

releasing hormone which stimulates the release of the thyroid-stimulating hormone in the anterior pituitary (Boler et al., 1969).

1.3 PYROGLUTAMYL PEPTIDASE TYPE I

1.3.1 Biochemical properties

In vitro, PGP displays a strict requirement for a thiol-reducing agent, such as dithiothreitol (DTT) or 2-mercaptoethanol, and consequently it is strongly inhibited by sulfhydryl-blocking reagents such as N-ethylmaleimide, *p*-chloromercuribenzoic acid (PCMB), and 2-iodoacetamide (Cummins and O'Connor, 1996; Mantle et al., 1991) although the enzyme has broad substrate specificity, the rate of hydrolysis of any given substrate depends on the nature of the amino acid residue (or other group) immediately carboxyl to the pGlu residue (Cummins and O'Connor, 1996). Natural substrates include thyrotropin and luteinising hormone releasing hormones, neurotensin and synthetic substrates such as pyroglutamyl-aminomethylcoumarin (pGlu-AMC), pyroglutamyl-*p*-nitroanalide (pGlu-p-Na), pyroglutamyl-beta-naphthylamide (pGlu-βNA), pGlu-Ala and pGlu-Val (Albert and Szewczuk, 1972; Browne and O'Cuinn, 1983; Cummins and O'Connor, 1996). The reaction catalysed by PGP is shown in Fig. 1.6.

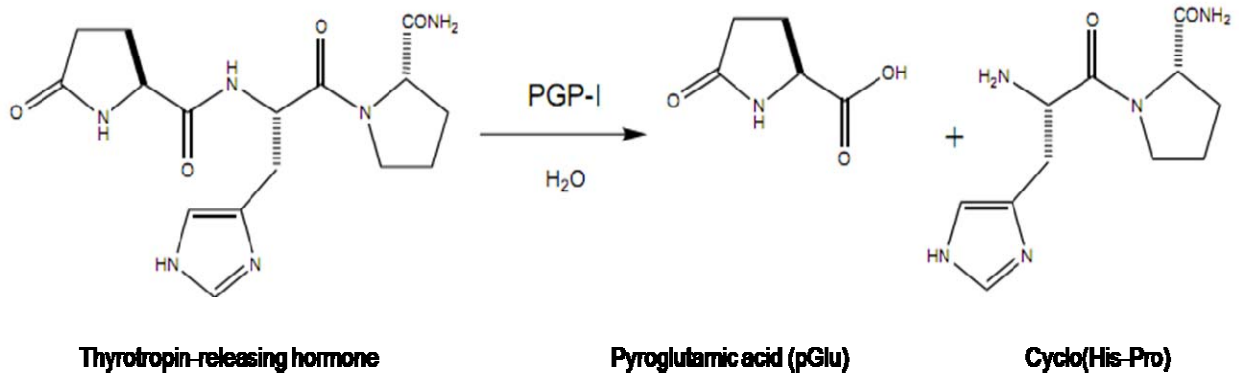

Fig. 1.5 The reaction catalysed by PGP. The choice of TRH as substrate illustrates how PGP cleaves pGlu of a wide variety of substrates (Odagaki *et al*., 2003).

The molecular mass of the PGP determined by denaturing SDS-PAGE is similar for bacterial and mammalian enzymes, ranging from 22 to 25 kDa. The mammalian enzymes appear to be monomeric, but sizes reported for the native bacterial enzyme range from 50 to 91 kDa (Dando et al., 2003). The three dimensional structure of PGP has been described for *Baccillus*

amyloliquefaciens (Odagaki et al., 1999), *Thermococcus litorallis* (Singleton et al., 1999) and *Pyrococcus furiosus* (Tanaka et al., 2001) and shows that the enzyme is a tetramer. In *B. amyloliquefaciens,* the PGP monomer (Fig. 1.7) folds into an alpha/beta globular domain with a hydrophobic core consisting of a twisted beta sheet surrounded by five alpha helices. Cys^{144}, His^{168} and Glu^{81} were identified as the catalytic triad residues (Odagaki et al., 1999).

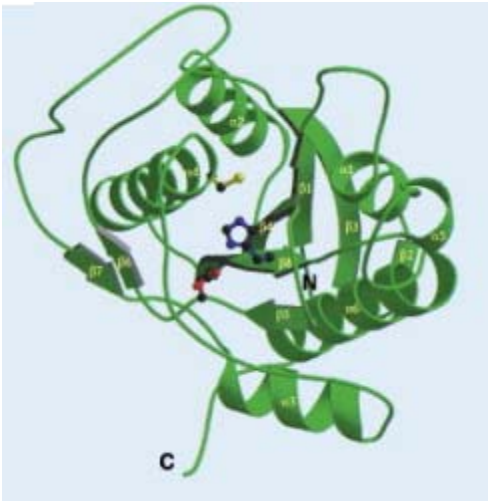

Fig. 1.6 Ribbon diagram of PGP monomer in *B. amyloliquefaciens*. Catalytic residues are shown in colors red (Glu), blue (His) and yellow (Cys). C, carboxy terminal; N, amino terminal (Odagaki *et al*., 2003).

1.3.2 Pyroglutamyl peptidase as a pathogenic factor

Reproductive disorders are a common feature in animal and human trypanosomosis (Seifert, 1995) and these disorders are associated with gonadal and endocrine abnormalities (Ng'wena et al., 1997). It has been proposed that trypanosome peptidases are involved in the abnormal degradation of some hormones, observed in trypanosome-infected animals (Hublart et al., 1990; Tetaert et al., 1993). More recently the involvement of pyroglutamyl peptidase I has been demonstrated in the abnormal degradation of thyrotropin- and gonadotropin- releasing hormones in *T. brucei* infected rats. The enzyme is entirely responsible for the reduced plasma half-life of thyrotropin-releasing hormone (TRH), and partially responsible for the reduced plasma half-life of gonadotropin-releasing hormone (GnRH) in rats (Morty et al., 2006).

Pyroglutamyl peptidase I activity was demonstrated in *Leishmania major* (Schaeffer et al., 2006a) but there are potential differences in the role of the enzyme in *L. major* when compared with the *T. brucei* enzyme. The data suggest that pyroglutamyl peptidase can be involved in regulating the action of L-pGlu-modified peptides required for differentiation of *L. major*.

In order to elucidate the precise role of a target protein, reverse genetics techniques are important tools to achieve this goal. By lowering or abolishing the expression level of a target protein and determining the effects in the parasite and or in the disease development, it is possible to understand the precise role of the protein in the parasite biology or disease pathology.

1.4 REVERSE GENETICS IN THE STUDY OF GENE FUNCTION

Typically, genetic analysis starts from the phenotype and proceeds to the genotype to understand the role of a certain gene in the organism. An altered phenotype (the result of naturally or randomly induced mutagenesis) provides information about the function of the responsible gene (Barbosa and Lin, 2004). Such a forward approach may be replaced by a reverse genetics approach, in which the target gene is altered first, and the resulting phenotype is studied (Baumeister, 2002; Hacking, 2008).

A reverse genetics approach includes techniques such as gene gene knock-out, gene silencing or RNAi (gene knock-down), insertional mutagenesis and targeting induced local lesions in genomes (TILLING) (Bhadauria et al., 2009). To elucidate the role of PGP on the *T. congolense* development and trypanosomosis pathogenesis, this study will make use of gene knock-out and RNAi techniques.

1.4.1 RNAi

Double–stranded RNA-mediated interference (RNAi) is a post-transcriptional gene-silencing mechanism in which the introduction of double-stranded RNA (dsRNA) triggers the degradation of homologous mRNA in the cytoplasm of a cell (Pauls and Esté, 2004). This phenomenon was first described in the nematode *Caenorhabditis elegans* (Fire et al., 1998) and plants (Hamilton and Baulcombe, 1999). In eukaryotic cells, RNAi has been described as a defense mechanism in response to dsRNA generated by viruses or parasitic transposons (Baulcombe, 2002; Sijen and Plasterk, 2003; Waterhouse et al., 2001). In the past few years, RNAi has become a powerful tool for the study of gene function and drug and vaccine targets validation (Jain, 2004; Pauls and Esté, 2004), as seen in *T. brucei*, where RNAi-depletion of mitotic kinesin-13 in a mouse model of infection completely prevented infection with the parasite (Chan et al., 2010). Reduction of the level of gene expression with RNAi is a fast and efficient method for determining whether the respective gene is essential for growth and viability and it also increases the understanding of complex biological processes (Motyka and Englund, 2004). RNAi is a highly conserved

mechanism throughout taxonomic groups (Maine, 2000) and has been shown to occur in a wide variety of organisms, including trypanosomes (Inoue et al., 2002; Ngo et al., 1998).

1.4.1.1 Mechanism of RNAi

The mechanism of RNAi can be divided into initiation and effector or execution stages (Fig 1.8). In the first stage the dsRNA is processed into small RNA molecules, known as small interfering RNAs (siRNA). In the execution stage, the siRNA is incorporated into effector protein complexes (AGO) that use the small RNA strand to identify the complementary mRNA, which is cleaved and degraded (Geley and Muller, 2004).

Two ribonucleases are involved in the RNAi pathway in African trypanosomes: dicer and slicer. The dicer enzyme from *T. brucei* contains one PAZ domain that binds RNA and two RNAse domains that degrade dsRNA to siRNA, which are fragments of 25 nucleotides containing 1-2 nt 3' overhangs. The RNAi is effected by the slicer enzyme, which is a member of the argonaute (AGO) family containing an RNAse H motif that cleaves mRNA (Balana-Fouce and Reguera, 2007). In *T. brucei*, the slicer contains two domains: PAZ and PIWI. The PIWI domain has RNAse H type predicted activity (Durand-Dubief and Bastin, 2003) and is the active site of the enzyme (Shi et al., 2004). In the effector stage, the siRNA is incorporated in the AGO complex, unwinds to form a single stranded RNA (ssRNA). The ssRNA molecules are then recognised by mRNA and are specifically degraded (Balana-Fouce and Reguera, 2007). This process involves specific base pairing between the antisense strand of the siRNA and the target mRNA as well as endonucleolytic cleavage of the mRNA strand across the middle of the siRNA strand (Elbashir et al., 2001b).

Fig. 1.7 Schematic representation of the RNAi system in trypanosomatids (Balana-Fouce and Reguera, 2007).

1.4.1.2 Genetic and molecular tools for RNAi studies

The accidental discovery of the RNAi phenomenon provided a powerful tool for gene function studies in eukaryotic cells. The success of the whole process is mainly affected by three factors: the selection of the best siRNA sequence, the efficiency of delivery of siRNA into the target cells and transient down-regulation of the targeted protein by siRNA (Pauls and Esté, 2004).

The choosing of the siRNA sequence can be done by searching in libraries of validated, commercially available siRNAs directed to some of the most commonly targeted genes. If the gene of interest was not previously targeted using siRNA, a novel siRNA must be designed. Although the efficiency of a specific siRNAi is not guaranteed, a number of observations have been made which can increase the probability of success in producing a siRNA. The principal factor to take into account is the gene target site developed (Duxbury and Whang, 2004). General guidelines recommend that the target site should be located at least 100-200 nt from the AUG initiation codon (Sui et al., 2002) and 50-100 nt from the termination codon should be avoided (Duxbury and Whang, 2004).

1.4.1.3 RNAi in trypanosomes

The RNAi phenomenon has already been identified in two trypanosomatids, namely *T. brucei* (Ngo et al., 1998) and *T. congolense* (Inoue et al., 2002). Two methods are currently used to induce RNAi in trypanosomes (Fig. 1.9). Both methods use cell lines in which chromosomally-

integrated plasmids modulate the synthesis of siRNA by addition of a transcriptional inducer (Drozdz et al., 2002). The first of such plasmids (Fig. 1.9a) is called a stem loop vector and it carries the target gene fragment in a head to head orientation downstream of a tetracycline-inducible RNA polymerase I promoter (Ngo et al., 1998). The stability of the construct is improved by the inclusion of a "stuffer" fragment of an unrelated sequence between the inverted segments (Shi et al., 2004). In the second plasmid (Fig. 1.9b), the target gene is located between opposing tetracycline-inducible T7 promoters. Examples of these type of vectors include pZJM (Wang et al., 2000) and p2T7^{Ti} (LaCount et al., 2000).

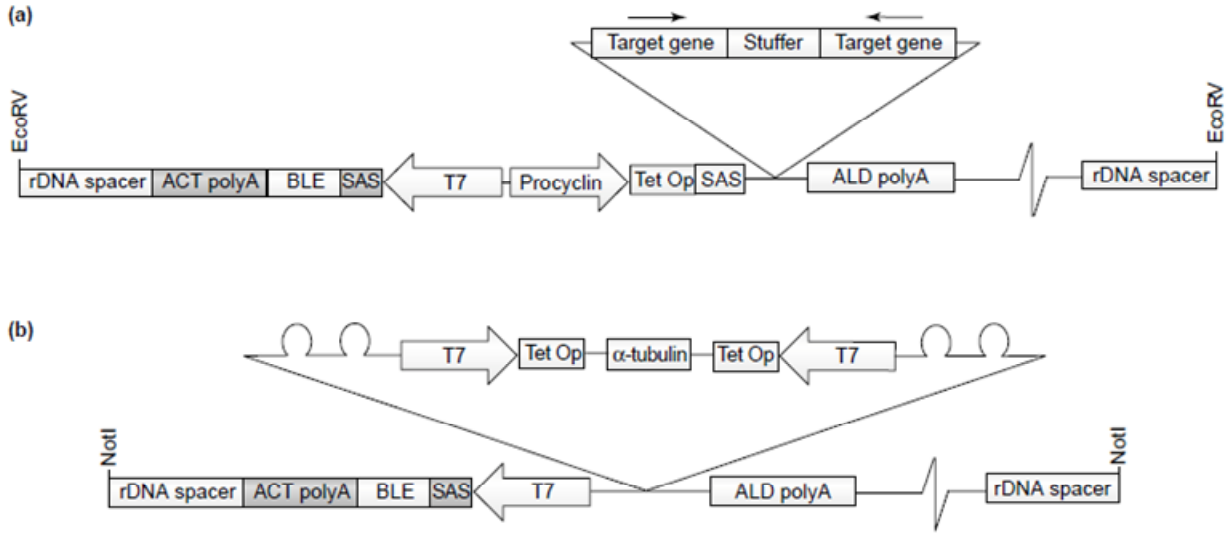

Fig. 1.8 Vectors for RNAi in trypanosomes. (a) Stem loop vector **(b)** The pZJM vector. The sequence of the target gene replaces the α-tubulin sequence. Key: Tetracycline-inducible procyclin promoter (procyclin arrow), the tetracycline operator (Tet op), dual T7 terminators (ΩΩ), tetracycline-inducible T7 promoter (T7 arrows), rDNA spacer (rDNA), actin poly(A) addition sequence (ACT polyA), phleomycin resistance gene (BLE), splice acceptor site (SAS), aldolase poly(A) addition sequence (ALD polyA) (Motyka and Englund, 2004).

1.4.2 Gene disruption/replacement (gene knock-out)

Gene knockout is an efficient and powerful method for dissecting gene function (Barbosa and Lin, 2004) and it relies on the biological property that similar sequences of chromosomal DNA can be swapped in the nucleus through homologous recombination (Hacking, 2008). The targeted gene knockout results from the homologous recombination between the target gene and an introduced DNA construct carrying its mutant allele. The inactivation of the endogenous gene occurs by disruption and replacement (Bhadauria et al., 2009; Mortensen, 1993).

For gene disruption constructs, the cassette consists of sequences from a target gene flanking a selectable marker gene. These constructs are linearised within the region of homology. The cassette is introduced into the homologous site interrupting the normal structure of the gene (Fig. 1.10a). In this case homologous recombination adds sequences to the target gene by a single crossover event (Mortensen, 1993). The position of the recombinant allele is dependent on the position of the linearisation site in the construct (Ledermann, 2000). In replacement constructs, homologous recombination is used to replace the endogenous gene with exogenous sequences. These constructs are linearised outside the region of homology and the mutation site (usually a positive selectable marker, such as neomycin) is flanked by regions homologous to the target gene. After homologous recombination by double crossover, the endogenous sequences are replaced by the construct sequences as shown in Fig. 1.10b (Mortensen, 1993). In the present study, we propose to make a disruption construct for the PGP gene knockout.

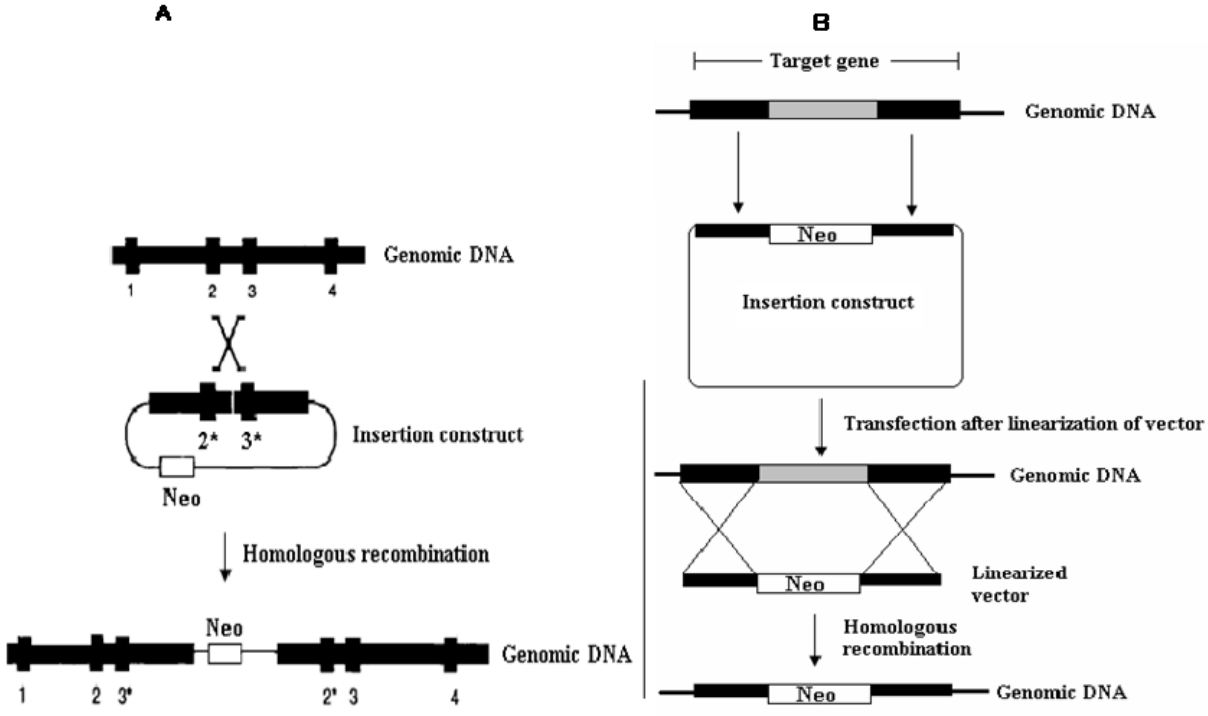

Fig. 1.9 Diagram of basic constructs for homologous recombination based knock-out. (A) Insertion constructs. Exogenous sequences inserted in the genome with consequent disruption of the normal gene structure **(B) Replacement constructs.** Sequences in the construct substitute the endogenous sequence. Neo, neomycin; 2* and 3*, exogenous homologous sequences (Mortensen, 1993).

1.5 Objectives of the study

The identification of pathogenic factors is a crucial step for drug and vaccine targets validation. Cysteine peptidases are among the molecules that play an important role in the pathogenesis of trypanosomosis. Pyroglutamyl peptidase I is one of several enzymes described to act as a pathogenic factor in trypanosomosis. This enzyme is responsible for abnormal levels of some hormones observed in *T. brucei* infected rodents. Preliminary data-base mining results have shown that indeed a gene showing 60% identity with *T. brucei* PGP is present in the *T. congolense* genome.

In this study, the objectives are: cloning and expression of the PGP open reading frame of *T. congolense*, its enzymatic characterisation (Chapter 2) and construction of RNAi and gene knockout construct plasmids for PGP enzyme of *T. congolense* (Chapter 3). In Chapter 4, the findings of the study are summarised and discussed.

2. RECOMBINANT EXPRESSION OF *T. CONGOLENSE* PYROGLUTAMYL PEPTIDASE TYPE I

2.1 INTRODUCTION

The trypanosomoses are diseases of humans and animals caused by infection with parasites of the genus *Trypanosoma* (Connor, 1994). In domestic animals, it is the major constraint to livestock development in the sub-humid and humid zones of Africa (Swallow, 2000). One prominent clinical feature of trypanosomosis is the reproductive disorders associated with gonadal and endocrine lesions (Ng'wena et al., 1997), resulting from the activity of biological products released by trypanosomes in infected animals (Tizard et al., 1978).

Pyroglutamyl peptidase I (PGP) is a soluble, intracellular enzyme (Cummins and O'Connor, 1998) that hydrolyses N-terminal pyroglutamate (pGlu) residues from peptides and proteins containing this modified amino acid (Cummins and O'Connor, 1996). This post-translational modification confers relative stability towards aminopeptidase degradation and is an important determinant of biological activity of peptides (Perlman et al., 1994). PGP has been described in a variety of organisms, such as bacteria, plants, animals (Awadé et al., 1994) and most recently trypanosomes (Morty et al., 2006) and leishmania (Schaeffer et al., 2006b). PGP has been shown to be involved in the abnormal degradation of thyrotropin- and gonadotropin-releasing hormones in *T. brucei* infected rodents (Schaeffer et al., 2006b), and thus, plays a role in the reproductive disorders associated with trypanosomosis (Ng'wena et al., 1997). Such disorders include irregular oestrus, abortion, neonatal death and infertility (Mutayoba et al., 1995; Taylor and Authié, 2004). A definitive role for PGP has not been attributed in mammalian species but has been suggested to participate in protein metabolism (Lauffart and Mantle, 1998). In bacteria it has been proposed to be important in intracellular protein metabolism, protein maturation and utilisation of peptides as nutrients (Lazdunski, 1989).

Under denaturing SDS-PAGE conditions, the molecular size of bacterial and mammalian PGPs ranges from 22 to 25 kDa (Dando et al., 2003). In the *Trypanosomatidae* family, PGP has an estimated size of 26 kDa in *T. brucei* (Morty et al., 2006) and 30 kDa in *L. major* (Schaeffer et al., 2006b). Sulfhydryl-blocking agents such as N-ethylmaleimide, p-chloromercuribenzoate

(PCMB) and 2-iodoacetamide inhibit PGP activity (Cummins and O'Connor, 1996). Despite being economically the most important trypanosome specie for livestock in Africa (Seifert, 1995), no data is currently available on *T. congolense* PGP. In this study, the cloning, expression and enzymatic characterisation of recombinant *T. congolense* PGP is described.

2.2 MATERIAL AND METHODS

2.2.1 Materials

Common laboratory chemicals and reagents used for buffer preparation were obtained from Merck Chemicals (Darmstadt, Germany) and Sigma-Aldrich (Munich, Germany). *Taq* polymerase, was obtained from Solis Biodyne (Tartu, Estonia). EcoRI, NotI [using the nomenclature of (Roberts et al., 2003)], FastRuler™ DNA ladder middle and high range, 10 mM dNTP mix, X-gal, pTZ57R-T, T4 DNA ligase, Transformaid® kit and GeneJET™ plasmid miniprep kit were purchased from Fermentas (Vilnius, Lithuania). E.Z.N.A® gel extraction kit was purchased from Peqlab (Erlangen, Germany). DNA Clean and Concentrator Kit was obtained from Zimo Research (Orange, CA, USA). Gluthathione agarose, HRPO rabbit anti-IgY-horseradish peroxidase (HRPO) conjugate, His-select® Ni-NTA resin and Freund's complete and incomplete adjuvants were purchased from Sigma-Aldrich (Munich, Germany). BCA™ assay kit was obtained from Pierce (Rockford, USA) and chicken anti-GST antibodies were an inhouse preparation. Thrombin restriction grade was obtained from Novagen (Darmstadt, Germany), AminoLink® coupling resin was purchased from Pierce (Rockford, USA) and Poly-prep® chromatography columns were obtained from BioRad® (Hercules, CA, USA).

2.2.2 Bioinformatics

The *T. brucei* PGP sequence (GenBank accession number DQ017472) was used to conduct a BLASTN search against the *T. congolense* reads database [http://www.genedb.org/genedb/tcongolense/ (last accessed 24-11-09)]. Nucleotide sequences were translated into the corresponding amino acid sequences by the on-line software Expasy Proteomics Server Translate tool (hosted at http://www.expasy.ch/tools/dna.html) and aligned using ClustalW (Chenna et al., 2003) with *T. brucei* and *L. major* (LmjF34.2000, www.genedb.org) sequences.

2.2.3 DNA visualisation

DNA samples were separated and visualised by 1% (w/v) agarose gel electrophoresis conducted at 80 V for 30 min. Gel images were captured under ultraviolet light using the BioRad VersaDoc system (Hercules, CA, USA).

2.2.4 Trypanosome culture

Procyclic forms of *T. congolense* (IL1180) were propagated in minimum essential medium (Sigma) supplemented with essential amino acids and prepared as follows: minimum essential medium (MEM) powder was mixed with basal medium (25 mM HEPES, 26 mM $NaHCO_3$, 0.1 mM hypoxanthine) supplemented with 10% (v/v) heat-inactivated foetal calf serum, 2 mM glutamine, 10 mM proline and 0.1% (v/v) haemin (Gray et al., 1984; Gray et al., 1985). After filter-sterilisation, the medium was stored at 4°C until use. Trypanosomes were cultured at 28°C until the stationary phase was reached, after which the suspension was centrifuged (12 000 *g*, RT, 10 min) and the pellet stored till further use.

2.2.5 *T. congolense* purification and isolation of genomic DNA

DEAE-cellulose (20 g) was suspended in PBS, pH 8.0 (80 ml). After the resin settled the fines were decanted and the resin resuspended. The process was repeated three times. The slurry was packed in a chromatography column (10 ml) and equilibrated with 1 column volume of PSG buffer [75 mM phosphate buffer pH 8.0, 65 mM NaCl, 1% (w/v) glucose]. Blood was collected from infected mice by cardiac puncture. The heparanised blood was added to the column, allowed to settle for 10 min by closing the column outlet. The parasites were collected in the eluant. The collected fractions were centrifuged (3000 *g,* 10 min, RT) and the parasite pellet stored at -80°C until used.

T. congolense cells were resuspended in 1 ml of PBS and centrifuged (3000 *g,* 10 min, RT). The resulting supernatant was discarded and the pellet resuspended in 150 µl of TELT buffer (50 mM Tris-HCl buffer pH 8.0, 62.5 mM EDTA, 2.5 M LiCl) and incubated at 37°C for 5 min. Phenol-chloroform (1:1) was added to the lysate and the emulsion centrifuged (14 000 *g*, 5 min, RT). The upper phase was collected, mixed with absolute ethanol (300 µl) and centrifuged (14 000 *g*, 5 min, RT). The supernatant was discarded, absolute ethanol added (1 ml) and the mixture centrifuged (14 000 *g*, 5 min, RT). The supernatant was removed and the pellet dried for 10 min

at 37°C. TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, 100 µl,) was used to resuspend the pellet, RNAse was added (1 µl) and the sample incubated at 37°C for 45 min. The isolated DNA was visualised on a 1% (w/v) agarose gel.

2.2.6 Amplification of the PGP ORF

The nucleotide sequence of *T. congolense* PGP was used to design the forward (GGA GAA TTC CAT ATG AGC TCA GTC AAG CCG ATT C) and reverse (CAT GCG GCC GCC CCG CCT TCC ATG GCA ATT TTT) primers, containing EcoRI/NdeI and NotI restriction sites (underlined), respectively. The PGP coding region was amplified by PCR using 50 ng of genomic *T. congolense* DNA as template in a Applied Biosystems thermocycler (GeneAmp PCR system 2400, Foster City, USA), using the following program: initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 45 s, 25 cycles and final extension at 72°C for 7 min. The PCR reaction volumes of 20 µl contained 1 µl of DNA, 0.25 µl of *Taq* polymerase (0.0625 U/µl), 0.5 µl of primers (0.25 mM), 2 µl 10x *Taq* buffer, 2 µl of 10x $MgCl_2$, 1 µl of dNTPs (0.5 mM) and 12.75 µl of distilled water. The PCR products were visualised on a 1% (w/v) agarose gel.

2.2.7 Cloning of PGP ORF into a T-vector

The amplified PGP-coding fragment was excised and purified from the agarose gel using the E.Z.N.A® gel extraction kit according to the manufacturer's instructions. Briefly, the excised DNA fragments were mixed with XP2 buffer and incubated at 65°C until complete dissolution of the agarose. The samples were applied to HiBind® columns and centrifuged (10 000 *g*, 1 min, RT). The columns were washed using SPW buffer and centrifuged (10 000 *g*, 1 min, RT). Elution buffer was added and DNA eluted by centrifugation as before. The purified fragment was ligated into pTZ57R-T in a reaction volume of 10 µl containing 0.5 µl of linearised vector, 3 µl DNA insert, 1 µl of 10x T4 DNA ligase buffer, 0.3 µl of T4 DNA ligase and 5.2 µl of sterile distilled water. The reaction mix was incubated overnight at 4°C. Competent *E.coli* JM 109 cells were prepared and transformed using the Transformaid® kit as follows: a freshly streaked bacterial culture (4 x 4 mm) was inoculated in C-medium (1.5 ml) and incubated at 37°C for 2 h. The cells were pelleted by centrifugation (13 000 *g*, 1 min, RT), resuspended in T-solution, incubated on ice for 5 min before centrifugation (13 000 *g*, 1 min, RT). The supernatant was discarded, the pellet resuspended in T-solution and incubated on ice for 5 min. The prepared

cells (50 µl) were mixed with ligation mix (3 µl), incubated on ice for 5 min and plated immediately on 2xYT agar plates (50 µg/ml ampicillin, 20 mg/ml X-gal, 100 mM IPTG). Recombinant white colonies were screened by PCR using a single colony as template.

2.2.8 Subcloning of PGP into expression vectors

Each positive colony confirmed by PCR was inoculated into 2 ml of 2xYT-ampicillin medium and incubated overnight at 37°C with shaking. Recombinant plasmids were isolated using the GeneJET™ plasmid miniprep kit as follows: 1.5 ml of culture was centrifuged (13 000 *g*, 5 min, RT) and the pelleted cells resuspended in resuspension solution (250 µl). The cell suspension was transferred to another tube and lysis (250 µl) as well as neutralisation solution (350 µl) added. The mixture was mixed and centrifuged (13 000 *g*, 5 min, RT). The supernatant was transferred to the GeneJET™ spin column and centrifuged (13 000 *g*, 1 min, RT). The flow-through was discarded, wash solution (500 µl) added and the column centrifuged (13 000 *g*, 1 min, RT). The latter three steps were repeated once. The column was transferred to a fresh 1.5 ml microcentrifuge tube, elution buffer added (50 µl) and the plasmid DNA eluted by centrifugation as before.

The recombinant T-vector sample was sequenced at the Segoli Sequencing Unit (ILRI, Nairobi, Kenya). EcoRI and NotI enzymes were used to restrict the recombinant T-vector in a 50 µl reaction volume containing 40 µl of purified miniprep plasmid DNA, 5 µl of 10x buffer Orange, 1 µl of each enzyme and 3 µl of sterile distilled water. After electrophoresis, the insert was excised and extracted from the gel using E.Z.N.A® extraction kit, purified and concentrated using DNA Clean and Concentrator Kit following the manufacturer's instructions. The fragment was ligated into pGEX-4T-1 and pET-28a(+) expression vectors, digested with EcoRI and NotI beforehand, in frame with the coding sequences of glutathione-S-transferase (GST) and a His-tag (Fig. 2.1). Ligations were made using a 10 µl reaction mix containing T4 DNA ligase (0,5 µl), 5x T4 ligase buffer (2 µl), insert DNA (3 µl), vector (0.5 µl) and sterile distilled water (4 µl). The mix was incubated at 4°C overnight. Competent *E. coli* BL21(DE3) and JM 109 cells were prepared using a Transformaid® kit, transformed with pET28a(+) and PGP insert and pGEX-4T-1 and PGP insert, respectively, and plated on 2xYT agar plates containing kanamycin (30 µg/ml) for pET28a(+) and PGP and ampicillin (50 µg/ml) Pgex-4T-1 and PGP. Colony PCR (described in Section 2.2.6) was used to screen recombinant colonies, using a single colony as template.

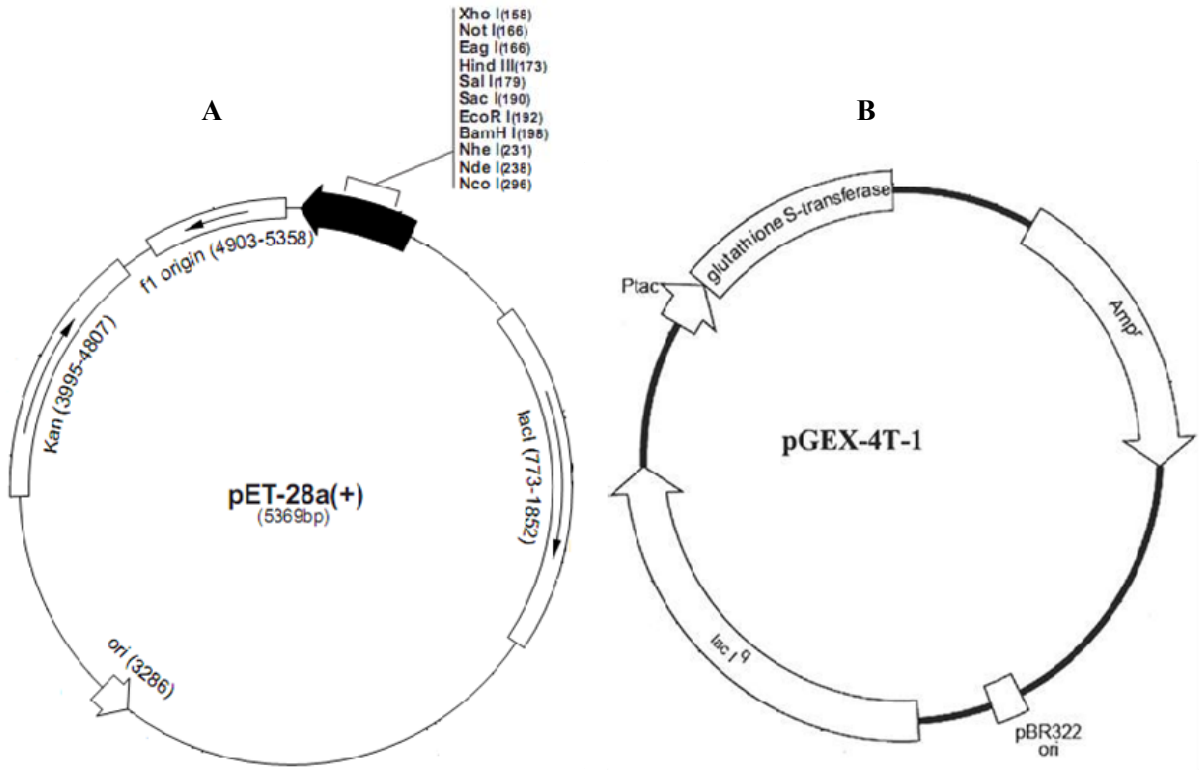

Fig. 2.1 Schematic maps of vectors used for PGP expression. (A) pET-28a(+) includes a T7 promoter, a T7 transcription start region, a His-tag coding sequence, a multiple cloning site, T7 terminator, *lac*I coding sequence, a pBR322 origin of replication, a kanamycin resistance coding sequence and a f1 origin of replication. **(B) pGEX-4T-1** includes a Ptac origin of replication, the glutathione-S-transferase coding sequence, ampicillin resistance coding sequence, a *Lac*I coding sequence and a thrombin cleavage site.

2.2.9 Expression of Pyroglutamyl Peptidase I

2.2.9.1 Recombinant expression in a pGEX vector

Recombinant and non-recombinant (control) colonies were grown overnight in 2xYT medium (100 ml) at 37°C with shaking. The overnight culture was transferred into 2xYT medium (900 ml) containing ampicillin (50 μg/ml). The culture was incubated at 37°C until an OD_{600} of 1.0 was reached. IPTG (0.1 mM) was added to induce expression and the culture was incubated for 4 h at 37°C. The culture was centrifuged (2000 *g*, 10 min, 4°C), the pellet resuspended in 20 ml of PBS-T [137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.76 mM KH_2PO_4, 0.1% (v/v) Tween-20] and lysozyme added (1 mg/ml). The suspension was incubated for 10 min at RT and stored at –20°C. After thawing, the cells were disrupted by sonication on ice for 4x30 s with 10 s intervals and centrifuged (5000 *g*, 10 min, 4°C). The supernatant was filtered through Whatman N^o 1 filter paper and protease inhibitors L-*trans*-epoxysuccinyl-leucylamido(5-guanidino)butane (E-64, 1 mM), leupeptin (1 μM), pepstatin A (1 μM) and EDTA (1 mM) added. To assess the expression

and solubility of the protein, the supernatant and pellet of recombinant and non-recombinant cells were analysed by 12% SDS-PAGE (section 2.2.11).

2.2.9.2 Recombinant expression in pET28a vector

Recombinant and non-recombinant (control) colonies were grown in 100 ml terrific broth overnight at 37°C with shaking. Cells (50 ml) were harvested by centrifugation (5000 g, 5 min, 4°C) and the pellet resuspended in 8 ml of binding buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0) and lysozyme (8 mg/ml) added. The cells were frozen, thawed and disrupted by sonication on ice using 4x30 s bursts at high intensity with 10 s cooling between each burst. The lysate was centrifuged (3000 *g*, 15 min, RT) to pellet the cellular debris. Protease inhibitors were added to the lysate as detailed in Section 2.2.9.1. To confirm the expression and solubility of the expressed protein, the supernatant and pellet of recombinant and non recombinant cells were analysed by 12% SDS-PAGE (Section 2.2.11).

2.2.10 Purification of recombinant Pyroglutamyl Peptidase I

The glutathione-agarose affinity purification system allows the purification of the protein of interest by the binding of GST-tagged protein to immobilised glutathione. The protein of interest is subsequently released by enzymatic cleavage with thrombin (Clark et al., 1977). The Ni-NTA system (Hochuli et al., 1987) takes advantage of the high affinity and selectivity of Ni-NTA agarose for recombinant fusion proteins that are tagged with six tandem histidine residues.

2.2.10.1 On-column cleavage of GST-PGP fusion protein

Lyophilised glutathione agarose was swollen in distilled water (14 ml per mg) overnight at 4°C. Swollen resin (1 ml) was placed in a Poly-Prep® chromatography column (BioRad) and equilibrated with 20 column volumes of PBS (20 ml).

The lysate (20 ml, Section 2.2.9.1) from recombinant cells was cycled over the glutathione agarose column (1 ml) overnight at 4°C. The resin was washed with 1% (v/v) PBS-Triton X-100 (15 ml), and equilibrated with 20 ml of thrombin cleavage buffer (20 mM Tris-HCl buffer, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$). Thrombin cleavage buffer (1 ml) was used to gently resuspend the resin into a 50% slurry to which thrombin was added (5 IU). The column was incubated overnight at RT with gentle mixing. Cleaved recombinant protein present in the buffer was directly collected from the column. The column was washed with 10 volumes of thrombin

cleavage buffer (20 mM Tris-HCl buffer, pH 8.4, 150 mM NaCl, 2.5 mM $CaCl_2$) and the collected fractions containing PGP monitored by measuring the A_{280} of the eluate. Bound GST and any uncleaved fusion protein were eluted from the resin with 10 mM reduced glutathione (10 ml). The column was regenerated by washing with 5 column volumes of 100 mM sodium borate buffer, 500 mM NaCl, pH 8.0, followed by 5 column volumes of distilled water, 5 column volumes of 100 mM sodium acetate buffer, 500 mM NaCl, pH 4.0, and 5 column volumes of distilled water. The resin was stored in PBS with 0.02% (w/v) NaN_3 at 4°C. The thrombin that was present in the recombinant sample was inactivated by the addition of AEBSF (1 mg/ml). To assess the presence of pure protein, the eluted fractions were analysed by SDS-PAGE (Laemmli, 1970) and western blot (Towbin et al., 1979), section 2.2.11.

2.2.10.2 Purification of PGP on His-select® Ni-NTA affinity system

The Ni-NTA agarose was resuspended by inverting and gently tapping the bottle repeatedly and 1.5 ml of the resin was transfered into a 10 ml Polyprep® column (BioRad). The supernatant was gently removed by aspiration when the resin was completely set. Sterile distilled water (6 ml) was added and the resin resuspended by alternative inverting and gentle tapping of the column. The supernatant was gently removed when the resin was completely settled and 6 ml of binding buffer was added and the resin resuspended. The resin was allowed to settle and the supernatant gently removed by aspiration. The lysate (8 ml, Section 2.2.9.2) was added onto the column and left to bind for one hour with gentle agitation. The resin was allowed to settle and the supernatant carefully aspirated. The resin was washed with wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, 20 mM imidazole, pH 7.0). This step was repeated three more times. The protein was eluted with 12 ml of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, pH 8.0).

2.2.11 Reducing SDS-PAGE and western blots

Protein samples were analysed by 12% reducing SDS-PAGE (Laemmli, 1970) as follows: samples (10 µl) were combined with an equal volume of 2x reducing treatment buffer [125 mM Tris-HCl buffer, pH 6.8, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol], incubated in a boiling water bath for 2 min and placed on ice until loaded onto a 12% gel, consisting of resolving (pH 8.8) and stacking (pH 6.8) Tris-glycine buffers. After loading the samples onto the gel, electrophoresis was conducted at 18 mA for 1.5 h. The gel was stained with Coomassie blue staining solution [0.2% (w/v) Coomassie blue, 7.5% (v/v) acetic acid, 50% (v/v)

ethanol] overnight, or silver stained (see below). Coomassie stained gels were destained with solution I [50% (v/v) methanol, 10% (v/v) acetic acid], overnight, followed by solution II [7% (v/v) acetic acid, 5% (v/v) methanol].

For silver staining gels (Blum et al., 1987) the procedure was as follows: all steps were carried on an orbital shaker, at room temperature in scrupulously clean glass containers. Following SDS-PAGE, the gel was soaked for 1 h or overnight in fixing solution [50% (v/v) methanol, 12% (v/v) acetic acid, 0.5% (v/v) formaldehyde], before incubation in 50% (v/v) ethanol (3x20 min), followed by soaking for 1 min in pre-treatment solution (4 mg/ml $Na_2S_2O_3.5H_20$). The gel was rinsed in distilled water (3x20 s) and soaked for 20 min in impregnation solution [0.2% (w/v) $AgNO_3$, 0.75% (v/v) 40% formaldehyde]. Following rinsing in distilled water (2x20 s), the gel was incubated in developing solution [60 g/l $NaCO_3$, 0.5% (v/v) 40% formaldehyde, 5 mM $Na_2S_2O_3.5H_20$] until the first protein bands became visible. Development was stopped by immersing the gel for 10 min in stopping solution [50% (v/v) methanol, 12% (v/v) acetic acid] and the gel was finally washed with 50% (v/v) methanol.

For western blots, SDS-PAGE gel, nitrocellulose and Whatman N^o 4 filter paper were immersed in blotting buffer (45 mM Tris-HCl, 173 mM glycine, 0.1% (w/v) SDS] and a sandwich consisting of four layers of blotting paper, Scotchbrite® pad, nitrocellulose, the gel, blotting paper and a further Scotchbrite® pad assembled. Blotting was conducted at 17 V in a semi-dry blotting apparatus (Sigma B2529) for 1 h. The nitrocellulose was transiently stained with Ponceau S [0.1% (w/v) Ponceau S, 1% (v/v) glacial acetic acid], and rinsed in distilled water until the protein bands become visible. The positions of the molecular weight bands were marked with a pencil and the membrane washed with distilled water containing 100 mM NaOH, before blocking for 1 h at RT with 5% (w/v) non fat milk in TBS (20 mM Tris-HCl buffer, 200 mM NaCl, pH 7.4). Following washing with TBS (3x5 min), the nitrocellulose was incubated with primary antibody (chicken anti-GST, chicken anti-PGP or mouse anti-Histag antibodies) diluted 1:10 000 in 0.5% (w/v) BSA-TBS (2 h, RT). The membrane was washed (2x5 min) using TBS and then incubated in HRPO-linked secondary antibody (rabbit anti-chicken or goat anti-mouse-HRPO antibodies, 5 ml) diluted 1:10 000 in 0.5% (w/v) BSA-TBS (1 h, RT), before immersing in chromogen/substrate solution [0.06% (m/v) 4-chloro-1-naphthol in 20% methanol, 0.0015% (v/v) H_2O_2]. Membranes were incubated in the dark until protein bands were clearly visible and washed in distilled water before digital imaging.

2.2.12 Protein quantification

The BCA™ Protein Assay Kit (BioRad) was used to determine protein concentration following the manufacturer's instructions. Briefly, protein standards (0-2 mg/ml) were mixed with BCA™ working reagent (sample to reagent ratio of 1:8), mixed in a microplate and incubated at 37°C for 30 minutes. Absorbance was read at a wavelength of 562 nm and a standard curve constructed (Fig. 2.2).

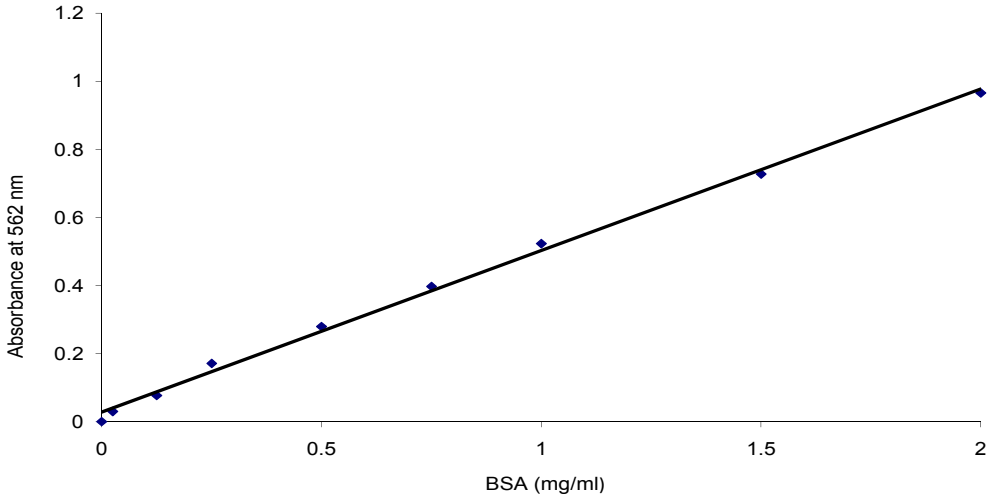

Fig. 2.2 Standard curve for BCA™ assay kit for protein quantification. Known concentrations of bovine serum albumin were used to construct a standard curve to determine the concentrations of samples of unknown concentrations. The equation of the trend line is y=0.4745x + 0.0281 with a correlation coefficient of 0.997

2.2.13 Raising of anti-PGP antibodies and isolation of antibodies from egg yolk

Antibodies against PGP were raised in chickens by intramuscular injections of 100 µg of purified PGP emulsified in Freund's complete adjuvant in a 1:1 (v/v) ratio in two sites in the breast muscles. Two further inoculations, in the same manner and dose, were administered fortnightly in Freund's incomplete adjuvant. Eggs were collected during and after the immunisation period and used to isolate anti-PGP antibodies (Goldring and Coetzer, 2003; Polson et al., 1980)

Egg yolks were separated from the egg white and carefully washed. The yolk sac was punctured and the yolk volume determined. Two volumes of 100 mM Na-phosphate buffer, 0.02% (w/v), NaN_3, pH 7.6, were added and mixed in thoroughly. Solid PEG (M_r 6000) was added (3.5% w/v) and dissolved by gentle stirring. The precipitated vitellin fraction was removed by centrifugation

(4 420 g, 30 min, RT), and the supernatant was filtered through absorbent cotton wool to remove the lipid fraction. PEG was added (8.5% w/v) and the solution was mixed and centrifuged (12 000 g, 10 min, RT). The supernatant was discarded and the pellet was dissolved in 100 mM Na-phosphate buffer, 0.02% (w/v) NaN_3, pH 7.6, in a volume equal to the volume obtained after filtration. The final concentration of PEG was brought to 12% (w/v), the solution stirred thoroughly and centrifuged (12 000 g, 10 min, RT). The supernatant fluid was discarded and the final pellet was dissolved in 1/6 of sodium phosphate buffer and stored at 4°C. The A_{280} of a 1:40 dilution of IgY in 100 mM Na-phosphate buffer, 0.1% (w/v) NaN_3, pH 7.6 was measured and the concentration calculated using $\varepsilon_{280}^{1\,mg/ml} = 1.25$ (Goldring and Coetzer, 2003). Antibody titres were assessed using an indirect- ELISA (Section 2.2.14) and further affinity purified on a column containing purified PGP (Section 2.2.15).

2.2.14 Evaluation of antibody production by indirect ELISA

The wells of NuncMaxisorp™ (Ebioscience, San Diego, USA) ELISA plates were coated with PGP at a concentration of 1 µg/ml in PBS (100 µl, overnight at 4°C). Non specific binding was prevented by blocking with 0.5% (w/v) BSA-PBS (200 µl, 1 h at 37°C), and the plates were washed three times with 0.1% (v/v) Tween-20 in PBS (PBS-Tween). IgY (10 µg/ml) from eggs collected at weekly intervals was diluted in 0.5% (w/v) BSA-PBS was added to the wells and incubated (100 µl, 2 h at 37°C). The plates were washed, the HRPO-linked secondary antibody (rabbit anti-chicken IgY), at a 1:15 000 dilution in 0.5% (w/v) BSA-PBS added and incubated (100 µl/well, 1 h at 37°C). Plates were washed as before and chromogen/substrate solution [0.05% (w/v) ABTS and 0.0015% (v/v) H_2O_2 in 150 mM citrate-phosphate buffer, pH 5.0] added (100 µl) and the color allowed to develop in the dark (15 min). The enzyme reaction was stopped by addition of 50 µl of stopping buffer [citrate-phosphate buffer, pH 5.0, 0.1% (m/v) NaN_3] and the A_{405} of duplicate samples measured.

2.2.15 Affinity purification of IgY

Purified PGP was coupled to AminoLink® (Pierce, Rockford, USA) Coupling Resin following the manufacturer's instructions. Briefly, the resin was equilibrated with coupling buffer (100 mM sodium phosphate buffer, 150 mM NaCl, pH 7.2), and PGP (3 mg diluted in coupling buffer) was added to the column. Cyanoborohydride solution (5 M, 40 µl) was added and the mixture incubated by end-over-end mixing for 6 h, at RT. The flow-through (1 ml) was kept for SDS-

PAGE analysis and the resin washed with quenching buffer (1 M Tris-HCl buffer, pH 7.4). Cyanoborohydride was added and incubated by end-over-end mixing for 30 min. The resin was washed with 1 M NaCl and stored at 4 °C in TBS containing 0.05% (w/v) sodium azide.

The isolated IgY was filtered through a Whatman N^o 1 filter paper and cycled through the affinity column, firstly overnight in the reverse direction and then in the forward direction for two hours for optimal binding. The column was washed with 20 ml of PBS, pH 7.2 to remove unbound antibodies. Bound IgY was eluted with 8 ml 100 mM glycine-HCl, pH 2.5 and 1 ml fractions collected in microfuge tubes containing 50 µl of 1 mM Tris, pH 9.0. The A_{280} of the samples was monitored, the IgY-containing fractions pooled and dialysed against 100 mM Na-phosphate buffer, 0.1% NaN_3 (w/v), pH 7.2. The specificity of purified antibodies was confirmed by western blot as described in Section 2.2.11.

2.2.16 Isolation of soluble proteins from *T. congolense* parasites

Pellet of both blood-purified and cultured trypanosomes (Section 2.2.4) collected by centrifugation (3000 *g*, 10 min, RT) were resuspended in 1 ml (per 1×10^9 parasites) of 50 mM Tris-HCl buffer, pH 8.0. Protease inhibitors E-64 (1 mM), leupeptin (1µM), pepstatin A (1 µM) and EDTA (1 mM) were added and the mixture frozen in liquid nitrogen. Cells were thawed at 37°C and refrozen. The freeze and thaw cycle was repeated 5× before centrifugation (12 000 *g*, 5 min, RT) to collect the supernatant that contains soluble proteins.

2.2.17 Enzymatic characterisation of PGP

A standard curve for the quantification of the 7-amino-4-methylcoumarin (AMC) product (0.005-15 µM) was prepared in assay buffer (Fig. 2.3). The linear regression of the calibration curve was used to quantify the fluorescence product. Enzyme (1.5 ng) diluted in Brij-35 [0.1%(v/v)] was incubated with assay buffer (100 mM Tris-HCl buffer, 10 mM DTT, pH 8.0) at 37°C for 10 min. Substrates (0-100 µM) were added and the fluorescence measured (excitation 360 nm; emission 460 nm) for 10 cycles in a FLUOStar Optima spectophotomter (BMG Labtech, Germany).

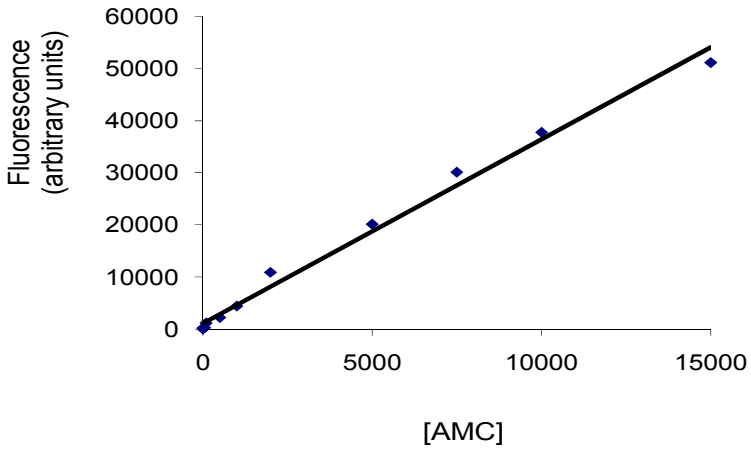

Fig. 2.3 Standard curve relating amount of AMC to fluorescence. The equation of the trend line is y=3.5349x + 1085.1, with a correlation coefficient of 0.991.

The pH optimum for recombinant PGP was determined by incubating 1.5 ng of PGP [diluted in 0.1% (v/v)Brij-35] in constant ionic-strength AMT buffers (Ellis and Morrison, 1982) (100 mM Tris-HCl, 50 mM acetic acid, 50 mM Mes, pH 4-10) at 37°C for 10 min. pGlu-AMC (20 µM, 25 µl) was added and the fluorescence measured (excitation 360 nm; emission 460 nm).

2.2.18 Inhibition profiles

The effect of inhibitors on PGP activity was investigated as follows: PGP (1.5 ng) diluted in 0.1% (v/v) Brij-35 was incubated with assay buffer (50 µl, 100 mM Tris-HCl buffer, 10 mM DTT, pH 8.0) and E-64 (10 and 100 µM), iodoacetamide (10 and 100 µM), iodoacetic acid (10 and 100 µM), AEBSF (100 and 1000 µM), pepstatin A (1 and 10 µM) and 1,10-phenanthroline (100 and 1000 µM), in separate experiments. The mix was incubated at 37°C for 10 min and pGlu-AMC added (20 µM, 25 µl) and the fluorescence measured (excitation 360 nm; emission 460 nm). Residual activity was measured as a percentage of the control activity (100%). The apparent rate of association constant (k_{ass}) was measured for iodoacetamide and iodoacetic acid under second-order conditions. Enzyme (10 nM) diluted in 0.1% (v/v) Brij-35 was activated in assay buffer (100 mM Tris-HCl buffer, 10 mM DTT, pH 8.0), inhibitor added (10 nM) and the mix assayed against pGlu-AMC. The observed association rate constant (k_{obs}) is obtained from

the slope of a plot of the natural log of residual activity against time. The constant k_{ass} is calculated from the following equation (Salvesen and Nagase, 2001):

$$k_{ass} = \frac{k_{obs}}{[I]} \times 1 + \frac{[S]}{K_m}$$

Where k_{ass} is the true association constant, k_{obs} is the association rate constant, K_m is the Michaelis constant, [*S*] is the substrate concentration, and [*I*] is the inhibitor concentration.

2.2.19 Effect of antibodies on the PGP activity in trypanosomal lysates

Inhibition of pyroglutamyl peptidase I activity in trypanosomal procyclic lysate by IgY was assayed using antibodies generated in chickens against recombinant PGP. Trypanosomal lysate was combined with assay buffer (100 mM Tris-HCl buffer, 10 mM DTT, pH 8.0), antibody (125, 250, 500 or 1000 $\mu g.ml^{-1}$) and incubated at 37°C for 10 min. Enzyme activity against pGlu-AMC was measured and inhibition expressed as a percentage of the activity in the presence of non-immune antibody at the same concentrations.

2.3 RESULTS

2.3.1 Bioinformatics

BLAST searches in the *T. congolense* database using the *T. brucei* PGP sequence (GenBank accession number DQ017472) as query identified a 678 bp open reading frame, encoding a protein of 224 amino acids. Subsequent analysis of the sequence from the cloned *T. congolense* PGP ORF shows four nucleotide base-pair differences when compared with the database sequence: G→ A (position 119), T→ C (position 350), T→ C (position 673) and A→ C (position 374), causing conservative Lys^{40}→Arg and Val^{117}→Ala amino acid substitutions, since Val and Ala are hydrophobic amino acids, and Lys and Arg are basic amino acids. The conserved active-site residues observed in other homolog sequences are also present in that of *T. congolense* and include Glu^{104}, Cys^{169} and His^{193} (Fig. 2.4). The deduced amino acid sequence of *T. congolense* PGP was 52%, 29% and 10% identical to *T. brucei*, *L. major* and *B. amyloliquefaciens* enzymes respectively. Complete sequence alignment of PGP is available in Appendix I.

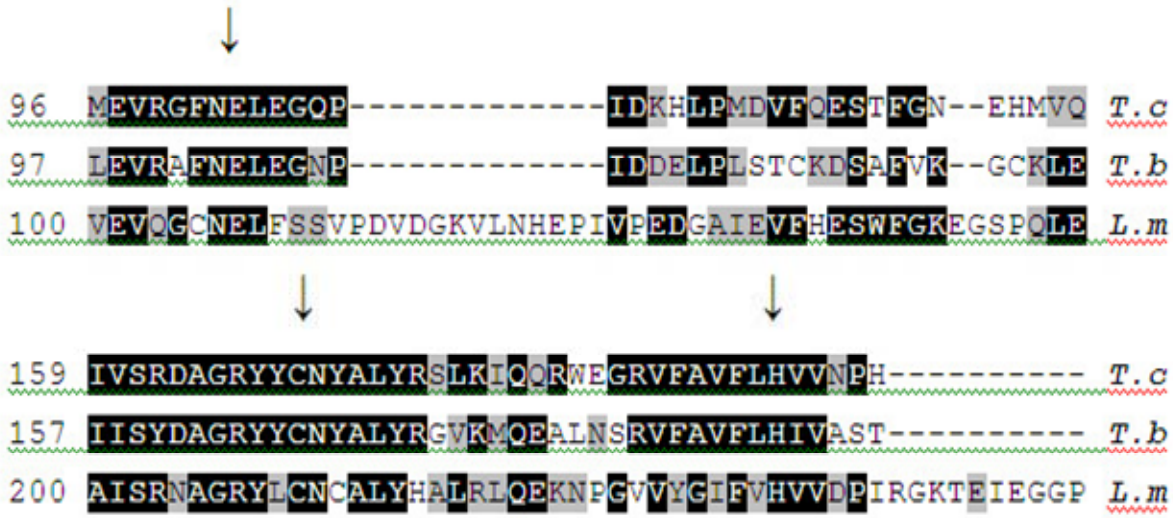

Fig. 2.4 Partial amino acids sequence alignment of PGP of different trypanosomatids. Sequences of *T. congolense* (*T.c*), *T. brucei* (*T.b*) and *Leishmania major* (*L.m*) were aligned using ClustalW software (Chenna *et al*., 2003). Arrows indicate the positions of catalytic triad residues (Glu, Cys, His). White font on a black background (E) indicates identities, white background (E) show differences and grey background (E) indicates similarities between the sequences.

2.3.2 Cloning and sub cloning of *T. congolense* PGP

Genomic DNA (Fig. 2.5A) of *T. congolense* was used as a template for PCR using primers designed from the PGP ORF. The obtained PCR product, of about 700 bp is shown in Fig. 2.5B. The observed fragment length is consistent with the size of 678 bp predicted for the PGP ORF, to which was added the restriction sites born by the primers. The PCR product was then excised and purified from the gel (Fig. 2.5C), ligated into the pTZ57R-T vector, and used to transform *E. coli* cells.

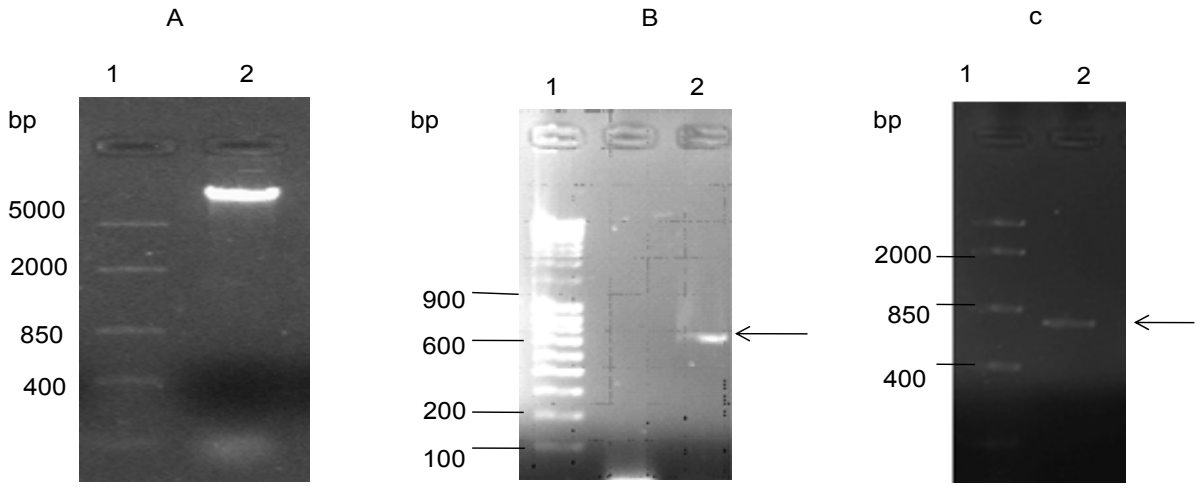

Fig. 2.5 Agarose gel analysis of genomic *T. congolense* DNA and amplification of the PGP ORF. (A) Isolated DNA. Lane 1, FastRuler™ DNA middle range ladder; lane 2, purified genomic DNA. **(B) Amplified fragment.** Lane 1, DNA molecular weight marker; lane 2, PCR product amplified by PGP primers; **(C) Gel purified fragment.** Lane 1, FastRuller™ DNA ladder middle range; lane 2, Purified PGP fragment from agarose gel. Arrows indicate the position of the PGP fragment.

The recombinant nature of the white colonies was confirmed by colony PCR using PGP-specific primers. Products are shown in Fig. 2.6 (lanes 4, 8, 11, 13, 14, 15, 17 and 18), with an approximate size of 700 bp, as expected.

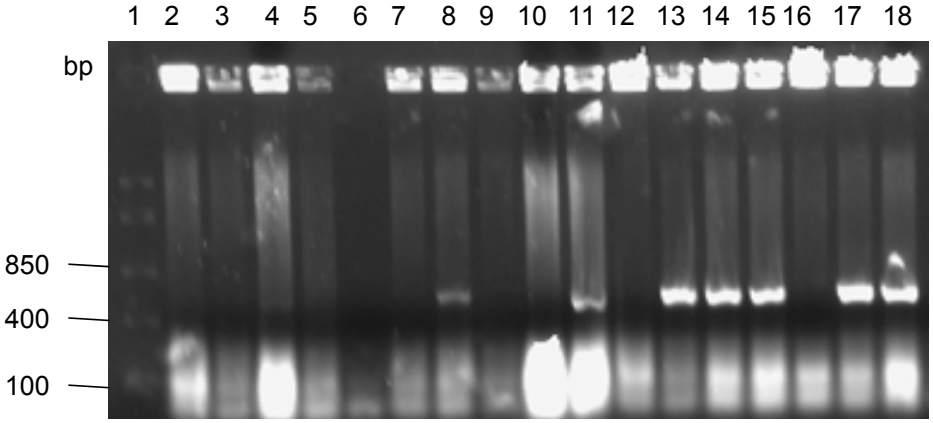

Fig. 2.6 Agarose gel analysis of colony PCR for screening of recombinant pTZ57R-T vector after transformation. Lane 1, FastRuler™ DNA ladder middle range; lanes 2-18, PCR products amplified using PGP specific primers using single colonies as template.

Recombinant T-vector plasmids were isolated (Fig. 2.7A). As shown in Fig 2.7B, double restriction of recombinant T-vector with EcoRI and NotI released fragments of estimated size of

2800 bp and 700 bp, corresponding to the pTZ57R-T vector and PGP ORF fragments respectively. The size of the linearised plasmid (lane 5) is estimated to be 3500 bp.

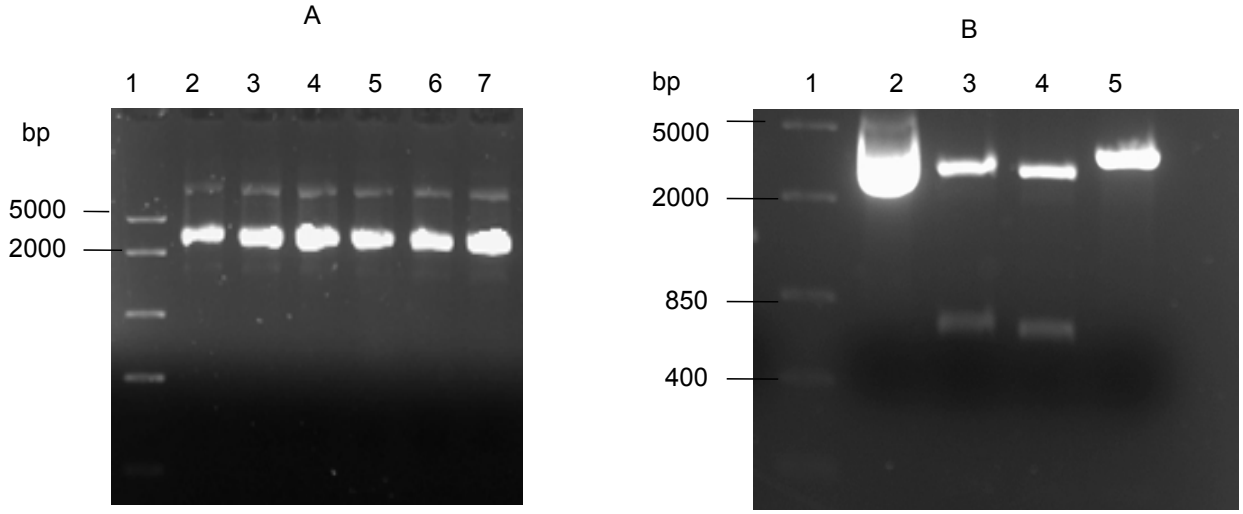

Fig. 2.7 Agarose gel analysis of isolated miniprep T-vector and restriction digest. (A) Plasmid minipreps of T-vector. Lane 1, FastRuler™ DNA ladder middle range; lanes 2-7, isolated plasmid miniprep; **(B) Restriction digest of recombinant T-vector.** Lane 1, FastRuler™ DNA ladder middle range; lane 2, Non digested plasmid; lanes 3 and 4, EcoRI and NotI restricted plasmid; lane 5, linearised plasmid (EcoRI). Arrow indicates the position of released PGP fragment after EcoRI and NotI restriction of T-vector.

The fragment of interest (678 bp) shown in Fig. 2.7B lanes 3 and 4 was recovered and purified from the gel, concentrated and visualised on an agarose gel (Fig. 2.8), before being sub-cloned into bacterial expression vectors [pGEX-4T-1 and pET28a(+)]. Recombinant colonies were screened by colony PCR using PGP- and vector-specific primers (pET 28a). The products obtained are shown in Fig. 2.9.

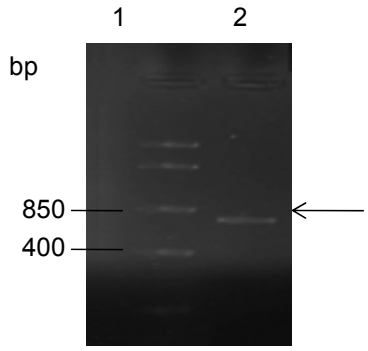

Fig. 2.8 Agarose gel analysis of restricted and gel purified PGP-coding fragment. Lane 1: FastRuler™ DNA middle range ladder; Lane 2: Purified PGP fragment (1 µl). Arrow indicates the PGP fragment position in the gel.

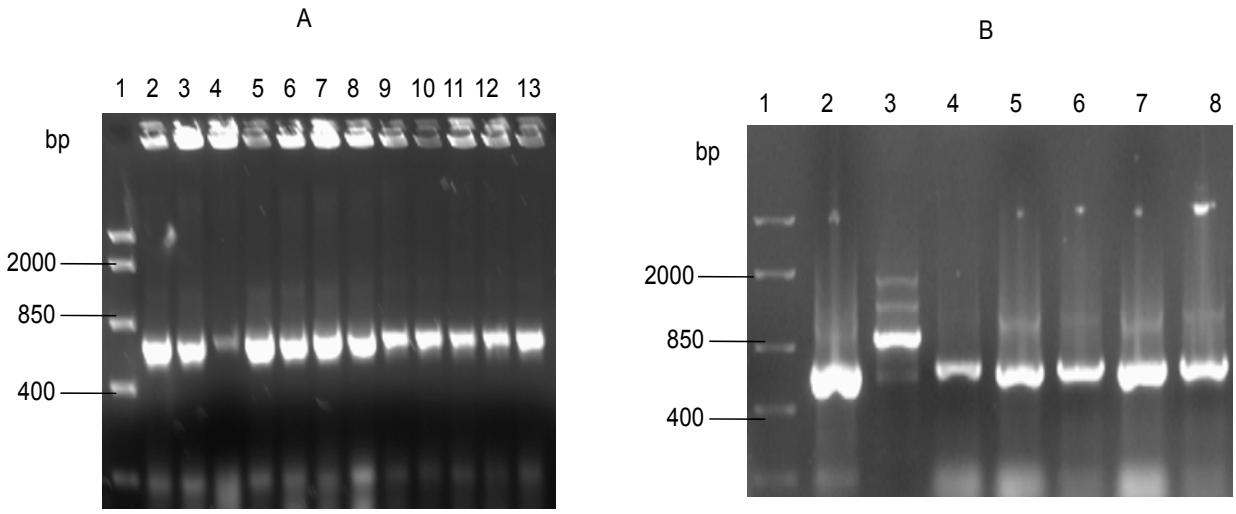

Fig. 2.9 Agarose gel analysis of colony PCR for screening of PGP ORF recombinants in expression vectors. (A) pGEX-4T-1-PGP-transformed colonies. PCR was performed in 20 µl reaction volumes. Lane 1, FastRuler™ DNA ladder middle range; lanes 2-13, PCR products using PGP specific primers **(B) pET28a(+)-PGP transformed colonies.** Lane 1, FastRuler™ middle range DNA ladder; lane 2, PCR product using pET primers; lanes 3-11, PCR products using PGP specific primers.

All the screened colonies for both vectors were recombinant, as shown by the presene of a PCR product at 700 bp using PGP primers. Lane 2 of Fig. 2.9B shows the PCR product as a band of approximately 900 bp, that corresponds to the PGP fragment plus the cloning region of the pET 28a(+) vector, amplified by the vector-specific primers.

2.3.3 Expression and purification of PGP of *T. congolense*

The PGP ORF of *T. congolense* encoding a 25.3 kDa protein was cloned in two bacterial expression vectors, pGEX-4T-1 and pET 28a(+). *E. coli* JM109 cells carrying recombinant pGEX-4T-1 were grown in 2xYT and the expressed fusion protein, GST-PGP, is shown in Fig. 2.10., with an apparent size of approximately 50 kDa, consistent with the 51 kDa fusion protein expected. PGP was released from the glutathione affinity-column by thrombin cleavage and GST eluted by reduced glutathione. The purified protein showed the expected size of 26 kDa, while the apparent molecular weight of free GST is slightly smaller, at 25 kDa, as expected (Fig. 2.11A).

BL21(DE3) *E. coli* cells carrying recombinant pET 28a-PGP were grown and the 31 kDa His-tagged fusion protein expressed in terrific broth medium (Fig. 2.10, lane 6). PGP was purified using the Ni-NTA system and showed a size of approximately 31 kDa, as expected since it

includes a cloning/expression/tag additional region amounting to approximately 6 kDa (Fig. 2.11B).

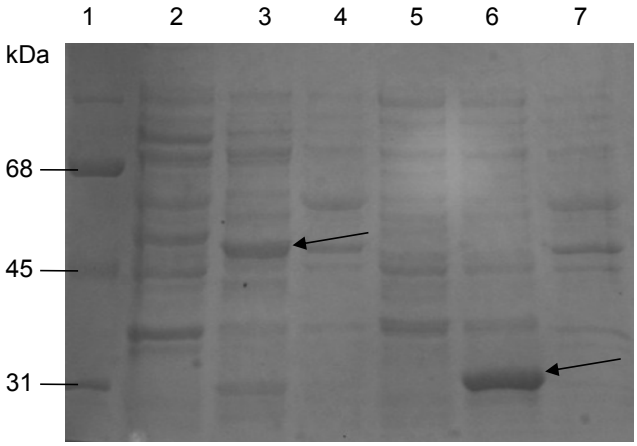

Fig. 2.10 Analysis of PGP expression in E. coli using SDS-PAGE. Samples were separated on a 12.5% gel and stained with Comassie brilliant blue. Lane 1, low molecular weight markers; lane 2, non-recombinant JM 109 cell culture; lane 3, soluble fraction of recombinant JM 109 cell culture; lane 4, insoluble fraction of recombinant *E.coli* JM 109 culture; lane 5, non-recombinant *E. coli* BL21(DE3) culture; lane 6, soluble fraction of recombinant BL21(DE3) culture; lane 7, insoluble fraction of recombinant *E. coli* JM 109 culture; Arrows indicate the recombinant fusion proteins.

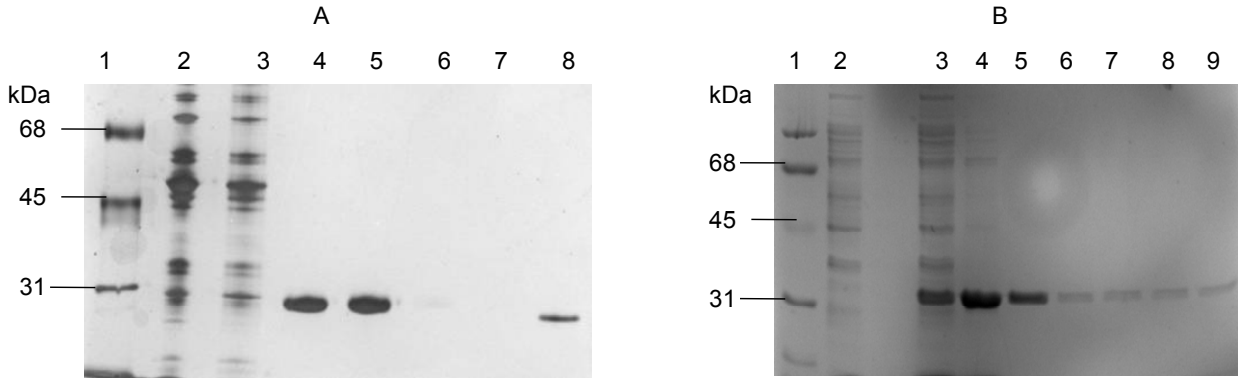

Fig. 2.11 Reducing SDS-PAGE analysis of PGP purification. (A) Glutathione affinity chromatography purification of PGP. Lane 1, molecular weight markers; lane 2, crude recombinant soluble *E coli* JM 109 lysate; lane 3, unbound lysate; lanes 3-7, eluted PGP fractions; lane 8, eluted GST. (**B) Ni-NTA purification system of PGP.** Lane 1, molecular weight markers; lane 2, unbound lysate; lane 3, crude recombinant soluble *E. coli* BL21(DE3) lysate; lanes 4-9, purified PGP-His tagged fractions.

Western blotting was used to confirm the successful expression of PGP in *E. coli* and subsequent purification of *T. congolense* PGP fusion proteins. Crude lysates and purified proteins were probed with anti-GST and anti-poly-His-tag antibodies (Fig 2.12).

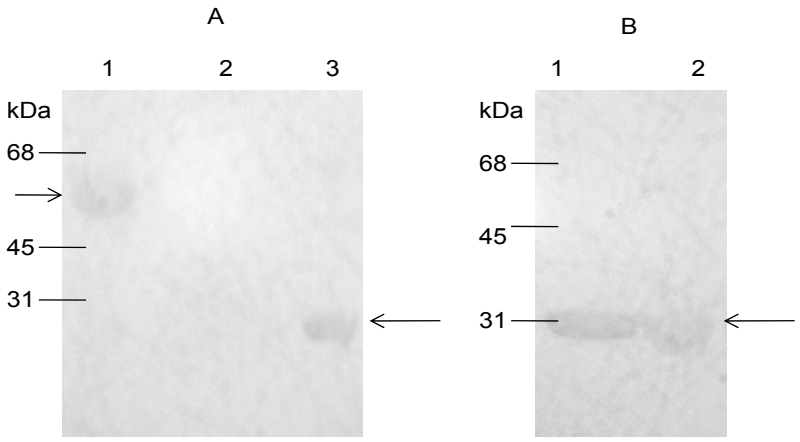

Fig. 2.12 Western blotting analysis of expressed and purified PGP fusion proteins. (A) Blot probed with chicken anti-GST antibodies. Lane 1, pGEX expressed crude lysate; lane 2, purified PGP; lane 3, eluted GST. **(B) Blot probed with mouse anti-His tag antibodies and goat anti-mouse IgG.** Lane 1, pET expressed crude lysate; lane 2, purified PGP-His tag fusion protein. Following incubation with rabbit anti-IgY conjugate (A) or goat anti-mouse IgG HRPO conjugate (B) blots were developed with 4-chloro-1-naphthol/H_2O_2. Arrows indicate the position of fusion and isolated PGP proteins.

Fig. 2.12 panel A shows that the anti-GST antibodies recognised the fusion protein of 51 kDa (lane 1) and free GST eluted from the column (lane 3) but did not recognise purified PGP (lane 2). In panel B, anti-His-tag antibodies recognised the fusion protein in the crude lysate (lane 1) and PGP-His-tag after purification (lane 2).

Recombinant purified PGP (GST-free) was used to immunise chickens. The production of chicken anti-PGP IgY antibodies over time was assessed by indirect-ELISA, and absorbance readings are shown in Fig. 2.13.

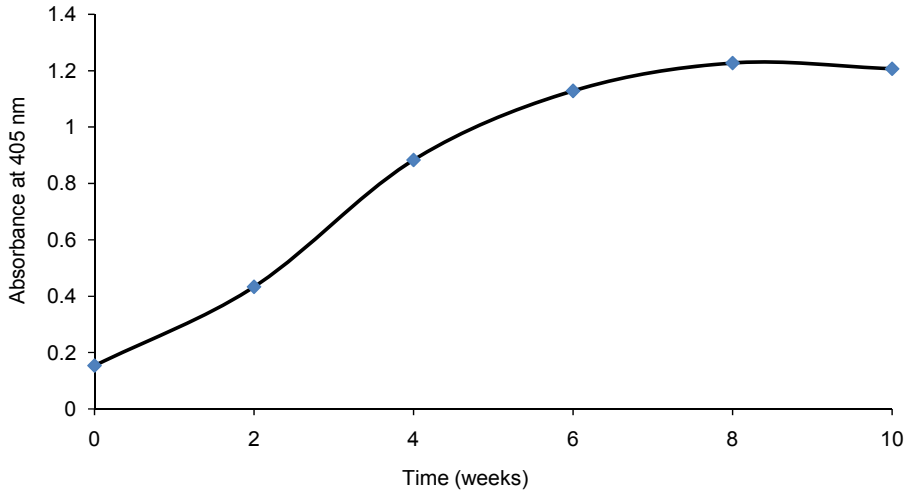

Fig. 2.13 ELISA evaluation of anti-PGP antibodies production in chickens. Plates were coated with recombinant PGP (1 µg/ml) and incubated with IgY (10 µg/ml) isolated at time points after the first immunisation and compared to non-immune control collected before immunisation at week 0. Rabbit anti-chicken HRPO-conjugate was used as detection antibody followed by ABTS/H_2O_2. The absorbance readings at 405 nm represent the average of duplicate experiments.

Recombinant PGP was coupled to an AminoLink® coupling resin (Pierce). The coupling efficiency was assessed by comparing the concentrations of the protein solution before and after coupling procedure (3 mg/ml and 0.012 mg/ml respectively) and reducing SDS-PAGE analysis (Fig. 2.14). Results shown in lanes 2 and 3, confirm the concentration values obtained, indicating that most of the protein was successfully coupled to the resin. The PGP resin was used to affinity purify the anti-PGP IgY. The affinity purified antibodies were subsequently used to verify the presence of native PGP in *T. congolense* lysates by western blot (Fig. 2.15) and to conduct inhibition assays of PGP activity in trypanosome lysates.

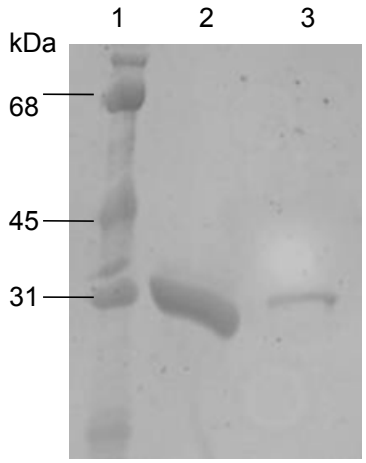

Fig. 2.14 SDS-PAGE analysis of coupling efficiency of PGP to AminoLink® coupling resin. Lane 1, molecular weight marker; lane 2, PGP before coupling; lane 3, PGP in supernatant after coupling

As shown in Fig. 2.15, PGP is present in procyclic (lane 2) and bloodstream (lane 3) developmental stages, and corresponds in size to recombinant protein shown in lane 1.

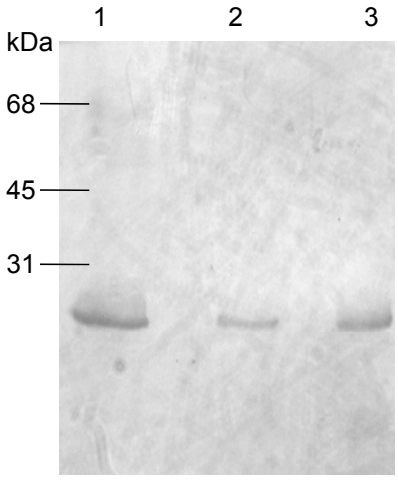

Fig. 2.15 Western blotting analysis of *T. congolense* lysates probed with chicken anti-PGP antibodies. Lane 1, Recombinant PGP. lane 2, *T. congolense* bloodstream forms lysate; lane 3, *T. congolense* procyclic lysate; Proteins were separated on a 12% SDS-PAGE gel, electroblotted to nitrocellulose and incubated with chicken anti-PGP antibodies followed by rabbit anti-chicken HRPO conjugate and 4-chloro-1-naphthol/H_2O_2 chromogenic substrate. The position of PGP is indicated by an arrow in each lane.

2.3.4 Enzymatic characterisation of recombinant PGP

T. congolense PGP was recombinantly expressed and purified as a soluble active protein in *E. coli*. Recombinant PGP exhibited activity against pGlu-AMC, but no hydrolysis was detected against Glu-AMC and Asp-AMC synthetic substrates (Table 2.1).

The results of the efficiency analysis of pGlu-AMC hydrolysis by recombinant PGP are shown in Fig. 2.16 and Table 2.1. Michaels constant (K_m) characterize the enzyme's affinity for a substrate. A low K_m value means that the enzyme has a high affinity for the substrate, i.e. that small amount of substrate is enough to run the reaction at half its maximum speed, $V_{max}/2$, which represents the number of substrate molecules that are converted into product by a molecule of enzyme in a unit of time. K_m value of 142 µM was determined by Lineweaver-Burk plot. This value is comparable to those obtained for PGP against synthetic L-pGlu substrates in *T. brucei* (Morty et al., 2006) and human (Dando et al., 2003) at 28 µM and 50 µM, respectively The catalytic efficiency of PGP was evaluated through determining the k_{cat}/K_m, ratio. The 1.11 s^{-1}. μM^{-1} value obtained is comparable to 1.7 s^{-1}. μM^{-1} value reported for the *T. brucei* enzyme (Morty et al., 2006).The pH activity profile of recombinant PGP against pGlu-AMC over a pH range of 4.0 to 10 is shown in Fig. 2.17. PGP activity is observed from pH 6.5- to pH 10, with the maximal activity at pH 9.0.

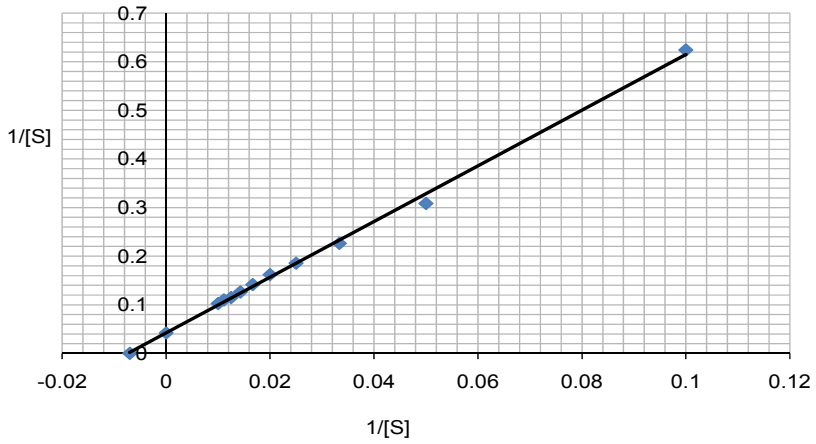

Fig. 2.16 Lineweaver-Burk plots for the hydrolysis of pGlu-AMC by recombinant PGP. Enzyme (1.5 ng) was assayed in the presence of assay buffer (100 mM Tris-HCl 10mM DTT, pH 8.0) against pGlu-AMC. Graph was plotted using Excel Software 2007 (Microsoft©, Redmond, WA, USA). The equation of the trend line is y=5.78+0.042, with a correlation coefficient of 0.997.

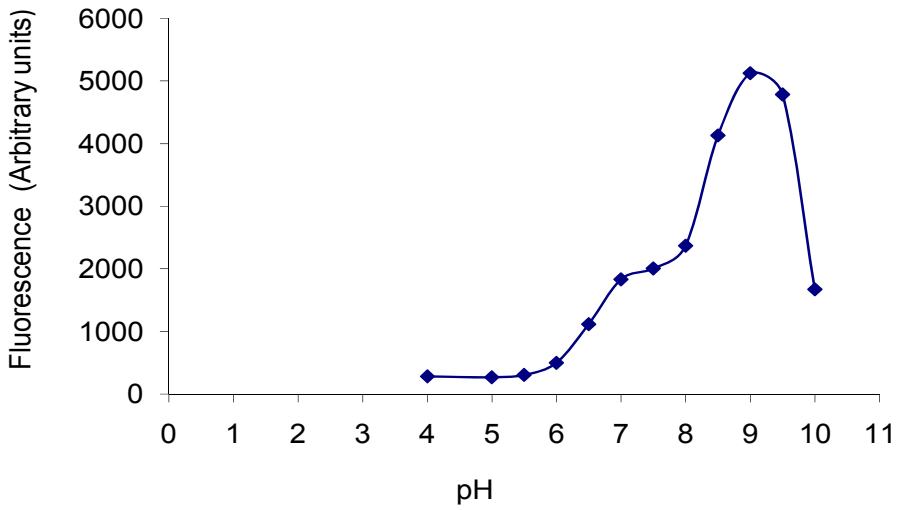

Fig. 2.17 pH activity profile of recombinant PGP. Enzyme activity was measured over pH 6.0-10 range in 100 mM Tris-HCl, 50 mM acetic acid and 50 mM Mes using pGlu-AMC as the substrate.

Table 2.1 Catalytic activity of recombinant PGP

	K_m (µM)	V_{max} (µM. s^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (s^{-1}. μM^{-1})
pGlu-AMC	142	23.8	158.6	1.11

No hydrolysis detected against Glu-AMC and Asp-AMC

The effects of different inhibitors on PGP activity are shown in Table 2.2. As expected for cysteine peptidases, sulfhydryl-blocking agents such as iodoacetamide and iodoacetic acid completely inhibited PGP activity. No significant inhibitory effect was observed for the other inhibitors, including E-64, a typical cysteine peptidase inhibitor, the chelating agent 1,10-phenanthroline, and AEBSF, a serine protease inhibitor.

Table 2.2 Effect of diferent inhibitors on recombinant PGP activity. Enzyme was activated in assay buffer (100 mM Tris-HCl, 10 mM DTT, pH 8.0) in the presence of inhibitor at 37°C for 10 min and subsequently assayed against pGlu-AMC.

Inhibitor	Concentration (µM)	Relative activity (%)
Iodoacetamide	10	**0**
	100	**0**
Iodoacetate	10	**0**
	100	**0**
E-64	10	**88**
	100	**85**
Pepstatin A	1	**96**
	10	**95**
AEBSF	100	**97**
	1000	**91**
1,10 phenanthroline	100	**96**
	1000	**89**

Second-order rate constants for the sulfhydryl-blocking reagents iodoacetic acid and iodoacetamide were measured and are shown in Table 2.3. It is manifest that both inhibitors have equivalent an effect on PGP activity.

Table 2.3 Inhibition constants of iodoacetic acid and iodoacetamide on recombinant PGP of *T. congolense*. Enzyme was activated in assay buffer (100 mM Tris-HCl, 10mM DTT, pH 8.0) in the presence of inhibitor and assayed against pGlu-AMC. Initial inhibition rates were used to calculate the second-order rate constants.

Inhibitors	k_{ass} ($M^{-1}.s^{-1}$)
Iodoacetic acid	**125 ±29**
Iodoacetamide	**177 ±31**

2.3.5 Effect of antibodies on PGP activity in a trypanosome lysate

The neutralising effect of anti-PGP antibodies on PGP activity present in a trypanosomal lysate is shown in the Fig. 2.18. At the lowest antibody concentration (125 µg/ml) the inhibitory effect is nearly absent (4.8%). As antibody concentration is increased, higher inhibition is observed, with the maximal inhibition reaching 96% at an antibody concentration of 1000 µg/ml, a physiological concentration.

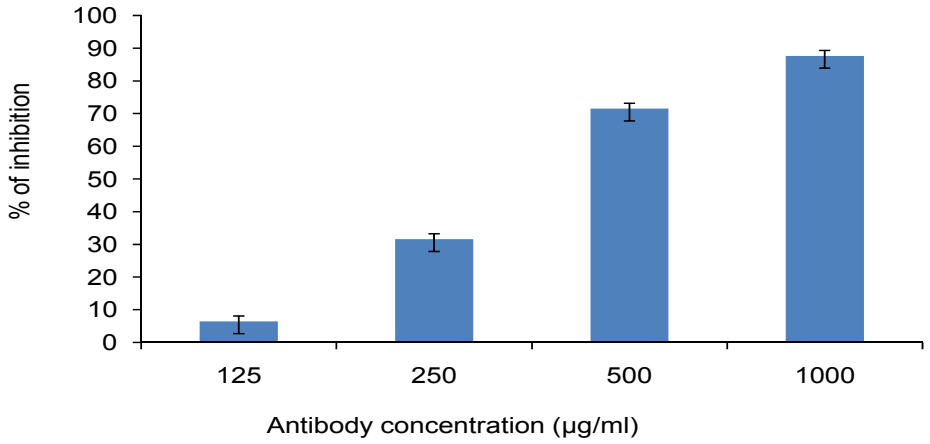

Fig. 2.18 Effect of chicken anti-PGP on the activity of PGP in trypanosomal lysates. Antibodies at different concentrations were combined with trypanosome lysate, assay buffer (100 mM Tris-Cl, 10 mM DTT, pH 8.0) and incubated at 37°C for 10 min. Inhibition was expressed as a percentage of the activity against pGlu-AMC in the presence of non-immune antibody (0% inhibition) at the same concentrations.

2.4 DISCUSSION

Parasite peptidases and other bioactive substances released by dead or dying parasites are often implicated in the development of lesions in trypanosome infected mammals (Tizard et al., 1978). Morty *et al*., (2006) demonstrated that trypanosome pyroglutamyl peptidase type I (PGP), a cysteine peptidase, is entirely responsible for the reduced plasma half-life of thyrotropin releasing hormone (TRH), and partially responsible for the reduced plasma half-life of gonadotropin releasing hormone (GnRH) in *T. brucei* infected rodents. In this study PGP of *T. congolense*, the most common trypanosome in livestock, was cloned, expressed and characterized.

The *T. congolense* PGP ORF sequence was obtained from searching on the *T.congolense* database using *T. brucei* PGP sequence as the BLAST query. Both sequences were aligned with that of *Leishmania major*, another member of the *Trypanosomatidae* family. Comparing the three PGP amino acid sequences, *T. congolense* PGP showed higher amino acid identity with *T. brucei*, PGP than with the *L. major* homologue. Despite the differences, the catalytic triad residues of the active site (Cys-Hist-Glu) described in bacterial and mammalian enzymes are fully conserved among the three *Kinetoplastidae*.

PGP of *T. congolense* was recombinantly expressed as a fusion protein using pGEX and pET expression vectors, in both cases as a soluble and active enzyme. The yield was approximately 2 and 6 mg/l of culture, for pGEX and pET respectively. Target proteins are expressed as fusion proteins to facilitate further purification of the protein of interest (Murby et al., 1996). The pGEX-4T-1 vector expresses fusion proteins with a glutathione-S-transferase-tag (GST), which can be proteolytically removed by thrombin hydrolysis of the bond Arg-Gly residues in the sequence Leu-Val-Pro-Arg-Gly-Ser (Smith and Johnson, 1988). This property allows the recombinant protein to be cleaved of the carrier protein directly on the glutathione column, which avoids further purification steps to separate the carrier from the recombinant protein. A better yield of PGP was obtained with pET28a(+) than with pGEX. For the former, N-terminal polyhistidine tag was not removed and the expressed PGP showed full activity, consistent with the histidine-tagged PGP of *L. major* (Schaeffer et al., 2006b).

The observed molecular weight of 26 kDa for recombinant *T. congolense* PGP purified on a glutathione agarose column was consistent with the 25.3 kDa size deduced from the amino acid sequence. The N-terminal histidine-tagged PGP purified by Ni-agarose chromatography migrated at a molecular mass of approximately 31 kDa on reducing SDS-PAGE. The extra 6 kDa can be explained by the his-tag and additional sequences located in the multiple cloning site and before the stop codon. Since GST and PGP have an estimated size of 26 kDa, the successful purification after thrombin cleavage, and the absence of contaminating GST, was confirmed by western blotting using anti-GST antibodies: the anti-GST antibodies did not detect GST in the purified PGP fraction. The 26 kDa size of *T. congolense* PGP is comparable to those of other organisms such as *T. brucei* (26 kDa) (Morty et al., 2006), *L. major* (32 kDa) (Schaeffer et al., 2006b), *B. amyloliquefaciens* (23 kDa) (Yoshimoto et al., 1993), *Thermococcus litoralis* (24 kDa) (Singleton and Littlechild, 2001) and *Pseudomonas florescens* (22 kDa) (Gonzales and

Robert-Baudouy, 1994). A western blotting with anti-histidine antibodies confirmed the presence of the histidine-tag on the Ni-agarose purified PGP.

The recombinant PGP migrated at the same molecular weight as the native PGP in lysates of both bloodstream and procyclic lifecycle forms of trypanosomes in a western blot probed with anti-PGP antibodies. This is an interesting finding, which shows that not only is PGP indeed expressed in live *T. congolense* parasites, but also in both procyclic and bloodstream developmental stages, as reported by Morty *et al*. (2006) in *T. brucei*. It suggests that the enzyme may play a role in the development and/or virulence of the parasite, as seen for *L. major* whereby the enzyme is important involved in the regulation of the action of L-pGlu-modified peptides required for *L. major* differentiation (Schaeffer et al., 2006b).

Recombinant PGP showed high activity against the fluorogenic substrate pGlu-AMC, while no activity was detected against either Glu-AMC or Asp-AMC. These findings confirm the high specificity of the enzyme for a pGlu residue in the P_1 position, as reported for PGP of other organisms. The kinetic constants for pGlu-AMC (K_m = 142 µM and k_{cat}/K_m = 1.11 $s^{-1}.\mu M^{-1}$) are in agreement with values found in other organisms, including *T. brucei* recombinant enzyme (Morty et al., 2006).

Since pGlu is the N-terminal residue of certain hormones, such as thyrotropin releasing hormone (TRH), gonadotropin-releasing hormone (GnRH) and luteinizing hormone releasing hormone (LH-RH), *T. congolense* PGP activity *in vivo* could have important implications for the understanding of the pathogenesis of endocrinal and reproductive lesions observed in trypanosome infected hosts. As a matter of fact, *T. congolense* infected animals show a pathology consistent with low circulating levels of TRH, such as declined plasma thyroxin levels (Mutayoba et al., 1988). Similarly, decreased testosterone and LH secretion were described in *T. congolense* infection (Mutayoba et al., 1995; Soudan et al., 1992), consistent with low levels of GnRH reaching the pituitary gland. Already in 1992, Huet *et al*. Hypothesised that such low levels of GnRH reaching pituitary gland might be due to degradation by proteases released by the trypanosomes. Interestingly, PGP is not the only protease involved in the cleavage of hormones in trypanosome-infected animals, as reported by Morty *et al*. (2006): Oligopeptidase B, a serine protease, is responsible for reduced levels of atrial natriuretic factor (ANF) (Morty et al., 2005).

Recombinant PGP of *T. congolense* showed activity against pGlu-AMC over a broad range of pH, from 6.5 to 10, with higher activity at pH 9.0. Alkaline pH optima were also reported for the *T. brucei* enzyme at pH 8.0 (Morty et al., 2006) and those of *L. mexicana* at pH 8.0 (Schaeffer et al., 2006b), human between pH 7.0 and 8.5 (Dando et al., 2003), bovine at pH 8.5 (Cummins and O'Connor, 1996) and *B. amyloliquefaciens* at pH 8.0 (Fujiwara and Tsuru, 1978). This is not a phenomenon exclusive to PGPs, since significant activity and stability at alkaline pH was reported for cysteine peptidases of other classes such as rhodesain of *T. rhodesiense* and cruzain from *T cruzi*, both belonging to Clan CA and family C1. These findings suggest a possible extracellular function (Caffrey et al., 2001).

When exposed to several inhibitors, the enzyme was found to be completely inhibited only by the sulfhydryl-blocking reagents, iodoacetate and iodoacetamide, which demonstrates that PGP is a thiol-dependent enzyme. However, E-64 a typical cysteine peptidase inhibitor, did not significantly inhibit *T. congolense* PGP activity (the enzyme remained 85% active at 100 μM of inhibitor), which is consistent with findings for *T. brucei* (Morty et al., 2006), *L. major* (Schaeffer et al., 2006b) and human (Dando et al., 2003), whereby the enzyme conserves more than 80% of activity at the tested concentrations of E-64. The absence of inhibition of PGP by E-64 is interesting since the active sites of PGP (Cys-Hist-Glu) and papain (Cys-His-Asp) (Matsumoto et al., 1999)are very similar. To our knowledge, no molecular explanation has so far been provided, and it may require PGP crystallisation studies. Second-order rate constants for iodoacetate and iodoacetamide were 155 M^{-1} s^{-1} and 197 M^{-1} s^{-1} respectively. These values are comparable to those obtained by Dando *et al*. (2003) in human (169 M^{-1} s^{-1} and 340 M^{-1} s^{-1} for iodoacetate and iodoacetamide respectively) and by Morty *et al*. (2006) in *T. brucei* (200±17 M^{-1} s^{-1} and 214 ±16 M^{-1} s^{-1} for iodoacetate and iodoacetamide respectively). We were not able to assay the active-site-directed inhibitor pyroglutamyl-diazomethyl ketone (pGlu-DMK) against PGP. pGlu-DMK is reported to inhibit PGP in a very potent, specific and irreversible manner. Studies in mice revealed that it inactivates PGP activity in all tissues at a dose of 0.1 mg/kg (Wilk et al., 1985) as well as in the plasma of trypanosome infected rodents (Morty et al., 2006).

Antibodies raised in chickens using recombinant PGP were able to significantly inhibit the hydrolysis of pGlu-AMC in trypanosome lysates at physiological concentration (0.25-1 mg/ml). PGP activity inhibition by specific antibodies was reported in *P. fluorescens*, where PGP activity was totally abrogated by antibodies at a concentration of 1 mg/ml (Gonzales and Robert-

Baudouy, 1994). Therefore, the antibodies developed in this study are potentially important tools for future studies on the inhibition of the catalytic activity of the enzyme against natural substrates *in vitro* and *in vivo*, and thus could contribute significantly for a better understanding of the role of the PGP in the biology of the parasite and in the pathogenesis of trypanosomosis.

In conclusion, this study confirmed the presence of PGP in bloodstream and procyclic life-stages of *T. congolense.* Recombinant PGP showed activity at physiological pH and its substrate specificity strongly suggest that the enzyme might play an important role in the pathogenesis of reproductive and endocrine disorders, which are common features in African animal trypanosomosis. Such findings are especially important because efficient economic animal production also depends on the efficient functioning of the reproductive functions. Finally, the ability of ant-PGP antibodies to totally inhibit the enzyme activity at physiological concentrations provides valuable in the context of the development of an “anti-disease” vaccine.

3. CONSTRUCTION OF TARGETING VECTORS FOR REVERSE GENETICS STUDIES ON PYROGLUTAMYL PEPTIDASE TYPE I OF *T. CONGOLENSE*

3.1 INTRODUCTION

For a better understanding of the disease process of African animal trypanosomosis, a precise knowledge of the host-parasite interactions and the parasitic factors involved in pathogenesis is needed (Antoine-Moussiaux et al., 2009). In trypanosomes, peptidases that are shown to be involved in the pathogenesis of the disease include congopain (Authié, 1994) and oligopeptidase B (Morty et al., 1999). A less conventional peptidase, the pyroglutamyl peptidase type I (PGP) was more recently described in *T. brucei* as a potential pathogenic factor. *T brucei* PGP was shown to be responsible for the abnormal levels of thyrotropin releasing hormone (TRH) and gonadotropin releasing hormone (GnRH) in infected rodents. Such abnormalities can be directly linked to reproductive lesions observed in naturally infected cattle (Morty et al., 2006). As no data were available on the PGP of *T. congolense*, arguably the most pathogenic parasite in livestock (Seifert, 1995). The enzyme was characterised the enzyme in Chapter 2. In order to gain insight into the role of PGP in the parasite as well as in host-parasite interactions, it was decided to take the reverse genetics approach. The development of the necessary molecular tools for use in reverse genetics is reported in this Chapter.

Reverse genetics tools, primarily RNA interference (RNAi) and gene knockout (KO) are powerful techniques to elucidate gene functions and by extension to study host-pathogen interactions, virulence and pathogenicity in host models (Bhadauria et al., 2009). By reducing (or deleting, in the case of double KO) the level of gene expression, it is possible through the observation of the resulting phenotype *in vitro* or *in vivo* to deduce or verify the functions of genes involved in parasite biology or in its interactions with the host. In addition, in the case of drug or vaccine research, indications can be obtained pertaining to the importance of the gene product in the parasite, and thus the validity of the target (Owino et al., 2008).

RNAi is a technique that triggers post-transcriptional silencing of gene expression by introducing double-stranded RNA into host cells (Vanhecke and Janitz, 2005). RNAi has been identified in

various organisms, including trypanosomes (Kolev et al., 2011; Ngo et al., 1998). Numerous genes were silenced through RNAi in *T. brucei*, including, as far as peptidase are concerned cathepsin B and L (Abdulla et al., 2008) and oligopeptidase B (Kangethe et al., 2011). Fewer studies have been conducted in *T. congolense,* essentially for tubulin (Inoue et al., 2002) and sialidases (Coustou et al., 2011). In higher eukaryotes, RNAi is generally induced by introducing short interfering RNA (siRNA), a class of double-stranded RNA molecules 20-25 nucleotides in length, into cultured cells (Elbashir et al., 2001a; Elbashir et al., 2001b). In trypanosomes, however, RNAi can be achieved by integrating specific vectors into the trypanosome genome and expressing dsRNA in an inducible manner.

The most commonly used system makes use of the $p2T7^{Ti}$ plasmid (LaCount et al., 2000), developed in *T. brucei*, in which the target sequence is located between two opposing tetracycline-inducible T7 promoters, generating uon induction two complementary transcripts able to produce a double-stranded RNA (Fig. 3.1). RNAi inducible strains were created, comprising genes coding for the bacteriophage T7 RNA polymerase (*T7RNP*) and bacterial tetracycline repressor protein (Tet^R), through insertion into the genome of plasmids pLWE13 and pLWE29 respectively (Ferrari et al., 1995). In such strains, denominated 29-13, *T7RNP* and Tet^R provide tight regulation of the two tetracycline operators (O^{tet}), located between the target promoters, of the third plasmid $p2T7^{Ti}$, so that in the absence of tetracycline, Tet^R binds the two O^{tet} sequences and blocks transcription from both promoters. This allows for a transient or permanent transcription of double-stranded interfering RNA of the target cloned in $p2T7^{Ti}$, by addition of tetracycline *in vitro* or *in vivo* sequence (Wirtz et al., 1999). The plasmid $p2T7^{Ti}$ was initially constructed for RNAi studies involving α-tubulin in *T. brucei* (LaCount et al., 2000). Inoue *et al*. (2002) modified the *T. brucei* plasmids pLEW13 and pLEW29 to create a tetracycline-inducible RNAi system in *T. congolense*, the *T. congolense* strain TRUM18:29-13. In this strain, RNAi can be induced by transfection with recombinant $p2T7^{Ti}$, in which the *T. brucei* rRNA spacer region was replaced with a modified recombinant $p2T7^{Ti}$ (Fig. 3.1): the *T. brucei* rRNA spacer region containing NotI site and the rRNA promoter preceding the bleomycin resistance gene were replaced by a 0.66-kb PCR fragment bearing the *T. congolense* rRNA promoter (Downey and Donelson, 1999b).

Although RNAi in parasites grown in culture can provide important insights into the role of a specific gene product in parasite replication and viability, its role in the pathogenesis of the

disease can only be validated *in vivo* (Abdulla et al., 2008) by determining whether RNAi-mediated down-regulation of the targeted gene or protein affects the progression of the disease.

Gene knock-out, based on the phenomenon of homologous recombination, also constitutes a powerful method for modifying and studying gene function (Capecchi, 1989) by gene knockout. Since trypanosomatids are diploid, a complete gene knockout requires two rounds of homologous recombination, usually using two constructs containing two different selectable markers (Clayton, 1999). In gene disruption constructs, the cassette consists of sequences from the target gene flanking a selectable marker gene, generally a gene of resistance to a given antibiotic. These constructs are linearised within the homology region and when the homologous recombination occurs in the parasite, the selectable marker sequence to disrupt the normal structure of the gene. In this case, homologous recombination adds sequences to the target gene by a single crossover event, as shown in Fig. 1.8A (Ledermann, 2000; Mortensen, 1993).

The objective of this part of the study was to construct reverse genetic tools, namely RNAi plasmid p2T7^{Ti}-RNAi, and gene knock-out disruption plasmids pGL1184-KO5-KO3 and pGL1217-KO5-KO3, to generate transgenic parasites whose PGP expression could be down-regulated. Those could be subsequently used to study the function of *T. congolense* PGP role in the parasite, and its potential role in host-parasite interactions..

3.2 MATERIALS AND METHODS

3.2.1 Materials

Common laboratory chemicals and reagents used for buffer preparation were obtained from Merck Chemicals (Darmstadt, Germany) and Sigma-Aldrich (Munich, Germany). *Taq* polymerase was obtained from Solis Biodyne (Tartu, Estonia). EcoRI, NotI, XbaI, SAP, enzyme and buffer, FastRuler™ DNA ladder middle and high range, 10 mM dNTP mix, pTZ57R-T, T4 DNA ligase, 10x DNA ligase buffer, Transformaid® kit and GeneJET™ plasmid miniprep kit were purchased from Fermentas (Vilnius, Lithuania). E.Z.N.A® gel extraction kit was purchased from Peqlab (Erlangen, Germany). DNA Clean and Concentrator Kit were obtained from Zimo Research (Orange, CA, USA). PureYield™ Plasmid Midiprep System was purchased from Promega (Madison, WI, USA). p2T7^{Ti} was provided by Prof. John Donelson (University of Iowa, USA), pGL1217 and pGL1184 plasmid vectors were provided by Prof. Jeremy Mottram (University of Glasgow, Scotland).

3.2.2 RNAi construct

3.2.2.1 DNA amplification

For RNAi experiments, primers to amplify the 3′ end of the PGP ORF, of approximately 400 bp, (Appendix II) were: forward Fw-PGPRNAi, CAT TCTAGA TTGCTGCGGAGGTGCG and reverse Rv-PGPRNAi, CAT TCTAGA GGCCGCCCCGCCTTCCATGG, both containing an XbaI restriction site (underlined).

A 25 cycles PCR was carried out using the previously sequenced recombinant T-vector-PGP as template with the following program: initial denaturation at 94°C for 5 min, denaturation at 94°C for 45 s, annealing at 55°C for 45 s, extension at 72°C for 45 s and final extension at 72°C for 7 min. The PCR was carried out in a 20 µl reaction containing 1 µl DNA template (50 ng), 0.25 µl *Taq* polymerase (0.0625 U/µl), 0.5 µl of each Fw-RNAi-PGP and Rv-RNAi-PGP primers, (0.25 mM), 2 µl 10x *Taq* buffer, 2 µl 25 mM $MgCl_2$, 1 µl of dNTPs (0.5 mM) and 12.75 µl distilled water. The PCR products were visualised in 1% (w/w) agarose gel. The resulting fragment was named PGP-RNAi.

3.2.3.2 Cloning of PGP-RNAi into a T-vector

The PCR products were excised and purified from agarose gel using E.Z.N.A® gel extraction kit following the manufacturer's instructions. Briefly, the excised DNA bands were mixed with XP2 buffer and incubated in a water bath at 65°C until complete dissolution of the agarose. The samples were applied to HiBind® columns and centrifuged (10 000 *g*, 1 min, RT). The columns were washed using SPW buffer, centrifuged (10 000 *g*, 1 min) and dried. Elution buffer was added and the DNA eluted by centrifugation (10 000 *g*, 1 min, RT). The purified PGP-RNAi was ligated into the pTZ57R-T vector in a reaction volume of 10 µl, containing 0.5 µl linearised vector, 3 µl insert DNA, 1 µl 10x T4 DNA ligase buffer, 0.3 µl T4 DNA ligase and 5.2 µl sterile distilled water. The reaction was incubated overnight at 4°C.

Competent *E. coli* cell strain JM 109 were prepared and transformed using the Transformaid® kit, following the manufacturer's instructions. Briefly, a freshly streaked solid bacterial culture (4x4 mm of agar slab) was used to inoculate C-medium (1.5 ml) and incubated at 37°C for 2 h. The cells were pelleted by centrifugation (13 000 *g*, 1 min, RT), resuspended in T-solution and

incubated on ice for 5 min before centrifugation (13 000 *g*, 1 min, RT). The supernatant was discarded, the pellet resuspended in T-solution (120 µl) and incubated on ice for 5 min. The prepared cells (50 µl) were mixed with each ligation mix (up to 5 µl) and incubated on ice for 5 min before being plated immediately on 2xYT agar plates (50 µg/ml ampicillin, 20 mg/ml X-gal, 100 mM IPTG). Screening for recombinants was carried out by PCR using single white colonies as template as described in Section 3.2.2.1.

Positive colonies confirmed by PCR were used to inoculate 2 ml of 2xYT-ampicilin medium and incubated overnight at 37°C with shaking. The recombinant plasmids were isolated using the GeneJET™ plasmid miniprep kit, following the manufacturer's instructions. Briefly, 1.5 ml of culture was centrifuged (13 000 *g*, 5 min, RT) in a 1.5 ml microcentrifuge and the pelleted cells resuspended in resuspension solution (250 µl). The cell suspension was transferred to a new tube, lysis (250 µl), then neutralisation (350 µl) solutions were added. The mixture was centrifuged (13 000 *g*, 5 min, RT) and the supernatant transferred to a GeneJET™ spin column and centrifuged (13 000 *g*, 1 min, RT). The flow-through was discarded, wash solution (500 µl) added and the column centrifuged as before. This washing procedure was repeated once. The spin column was then transferred to a new 1.5 ml microcentrifuge tube, elution buffer added (50 µl) and after 2 min of incubation at RT the plasmid DNA eluted by centrifugation as before.

3.2.3.3 Subcloning of the PGP-RNAi fragment in the RNAi vector

The recombinant PGP-RNAi-T-vector was restricted using XbaI restriction enzyme for 1h at 37°C, fragments separated on a 1.2% agarose gel, and the released PGP-RNAi fragment excised and purified from gel using the E.Z.N.A® extraction kit, as described above.

The RNAi vector p2T7^{Ti} (Fig. 3.1) was restricted as follows: p2T7^{Ti} plasmid (20 µl), 10x XbaI buffer (3 µl), XbaI restriction enzyme (1 µl) and distilled water (6 µl) were incubated overnight at RT. After visualisation on an agarose gel, the plasmid was purified from the gel using the E.Z.N.A® extraction kit and dephosphorylated using shrimp alkaline phosphatase (SAP, 1 µl) at 37°C for 1 h. SAP was inactivated at 65°C for 15 min before the dephosphorylated plasmid was purified from the mix using a DNA clean and concentrator kit.

The XbaI-restricted PGP-RNAi fragment (6.75 µl) was ligated to the purified and dephosphorylated XbaI-restricted p2T7^{Ti} plasmid (1 µl) in a 10 µl reaction volume containing

0.25 µl T4 DNA ligase and 2 µl 5x T4 ligase buffer. The reaction was incubated at 4°C overnight. Competent *E.coli* JM 109cells were prepared and transformed using a Transformaid® kit, as described in Section 3.2.3.2.

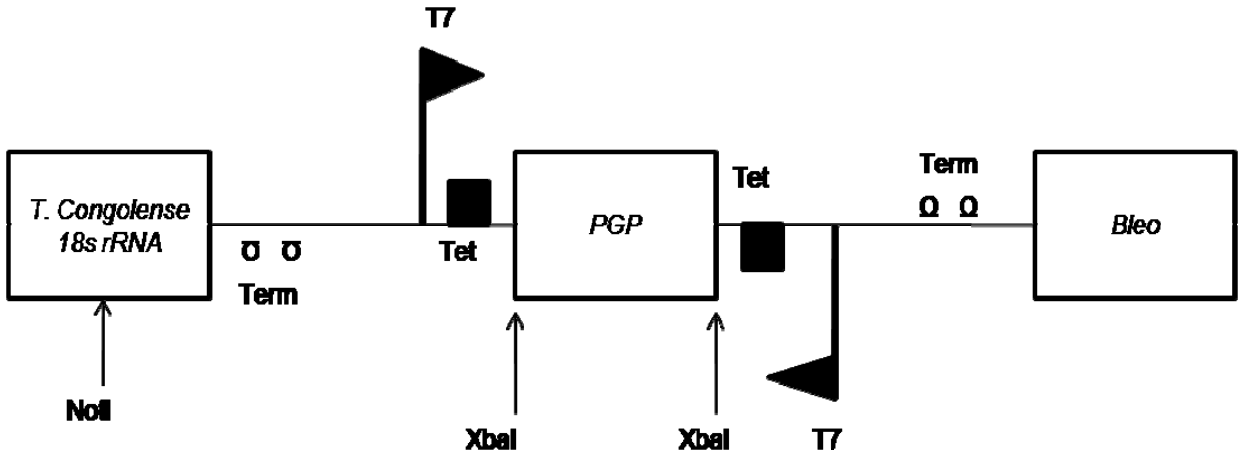

Fig. 3.1 Schematic diagram of the RNAi plasmid p2T7^{Ti}. T7, promoter for T7 RNA polymerase; term, two adjacent termination signals for T7 RNA polymerase; Tet, tetracycline operator; Bleo, bleomycin resistance gene; PGP, pyroglutamyl peptidase I;

Colony PCR, as described in Section 3.2.2.1, was used to screen for recombinant colonies, using both insert- and vector-specific primers. Colonies positive by PCR were used to inoculate 50 ml of 2xYT-ampicilin medium and incubated overnight at 37°C with shaking. The recombinant plasmids were isolated using the PureYield™ Plasmid Midiprep System, following the manufacturer's instructions. Briefly, cells were pelleted by centrifugation (10 000 *g*, 10 min, RT), resuspended in resuspension solution (2 ml), before adding lysis (2 ml) and neutralisation (3 ml) solution. The lysate was poured into a blue PureYield™ Clearing Column, incubated for 2 min at RT and centrifuged (1500 *g*, 5 min, RT) using a 50 ml Falcon® tubes. The lysate was placed in a PureYield™ binding column in 50 ml tubes, and centrifuged (1500 *g*, 1 min, RT). The column was washed with the endotoxin removal solution (5 ml) and the column wash solution (20 ml), before elution (600 µl). Restriction enzyme (NotI) was used to linearise the recombinant p2T7^{Ti}-PGP-RNAi, in a 600 µl reaction volume containing 538.5 µl purified midiprep DNA, 60 µl 10x buffer, 2 µl NotI enzyme (at 10 U/ µl) and the mix was incubated overnight at RT, in a 2 ml microcentrifuge tube. To precipitate the DNA, 1/10 volume of 3 M sodium acetate buffer, pH 5.2 was added (60 µl) and mixed, followed by two volumes of ice-cold at 96% (v/v). The mixture was placed on ice for 30 min before centrifugation (12 000 *g*, 15

min, 4°C). The supernatant was carefully removed and 70 % (v/v) ethanol added (1 ml) and the mixture centrifuged (12 000 *g*, 5 min, 4°C). The supernatant was decanted and the tube air dried in an extractor hood. The dried pellet was then dissolved in 50 µl of sterile distilled water and stored.

3.2.3 Gene knockout constructs

3.2.3.1 DNA amplification

For knockout experiments, regions of approximately 200 bp at the 5′ end and 3′ end of the PGP coding sequence (Appendix III) were amplified by PCR using recombinant T-vector-PGP as template. The primers KO5-Fw CATGCGGCCGCATGAGCTCAGTCAAGCCGA (NotI site underlined) and KO5-Rv CATTCTAGAGCGGTCCACTAACTGGCAAGC (XbaI site underlined) were designed to amplify the 5′ region. The primers KO3-Fw CATGGGCCCTGTCTCGTGATGCTGGGCGGTA (ApaI site underlined) and KO3-Rv CATGGGCCCGGCCGCCCCGCCTTCCATGGC (ApaI site underlined) were designed to amplify the 3′ region. The PCR program described Section 3.2.2.1 was used and the resulting products were named KO5 (5′ region) and KO3 (3′ region).

3.2.3.2 Cloning of KO5 and KO3 into T-vector

The methodology was essentially the same as the one followed for p2T7^{Ti}-PGP-RNAi. PCR products KO5 and KO3 were gel purified as described in Section 2.3.4 and ligated into the pTZ57R-T vector in a reaction volume of 10 µl, containing 0.5 µl linearised vector, 4 µl insert DNA, 1 µl 10x T4 DNA ligase buffer, 0.3 µl T4 DNA ligase and 4.2 µl sterile distilled water before incubation overnight at 4°C. Competent *E.coli*, JM 109 cells were prepared and transformed using the Transformaid® kit, as described in Section 2.3.4. Transformants were screened using colony PCR (Section 3.3.3) using insert-specific primers and recombinant plasmid isolated as described in Section 3.3.4. Recombinant T-vectors were digested with restriction enzymes NotI/XbaI and ApaI to release KO5 and KO3 respectively, which were gel-purified as described before.

3.2.3.3 Subcloning of KO5 and KO3

Knockout pGL1184 and pGL1217 vectors, bearing resistance for blasticidin and neomycin respectively (Fig. 3.2) were prepared by overnight digestion at RT with NotI and XbaI [plasmid (20 µl), 10x tango buffers (6 µl), NotI and XbaI restriction enzymes (1 µl each) and distilled water (2 µl)]. After visualisation on an agarose gel, plasmids were purified from the gel, dephosphorylated and purified as described in Section 2.3.5.

KO5 was cloned into plasmid pGL1184 and pGL1217 using the NotI/XbaI sites to generate pGL1184-KO5and pGL1217-KO5 respectively. Recombinants were screened by PCR as described before using KO5-specific primers. Plasmids pGL1184-KO5and pGL1217-KO5 were isolated by miniprep as described before and digested overnight at RT with ApaI in a reaction mix containing plasmid (20 µl), 10x ApaI buffer (3 µl), ApaI restriction enzyme (1 µl) and distilled water (6 µl). After visualisation, restricted plasmids pGL1184-KO5 and pGL1217-KO5 were purified from the gel, dephosphorylated and used to clone the KO3 fragment using the ApaI site, to generate the final constructs pGL1185-KO5-KO3 and pGL-1217-KO5-KO3 (Fig. 3.2). Positive colonies were confirmed by colony PCR using insert-specific primers and the respective recombinant plasmids isolated by midipreps as described in Section 3.2.3.1.

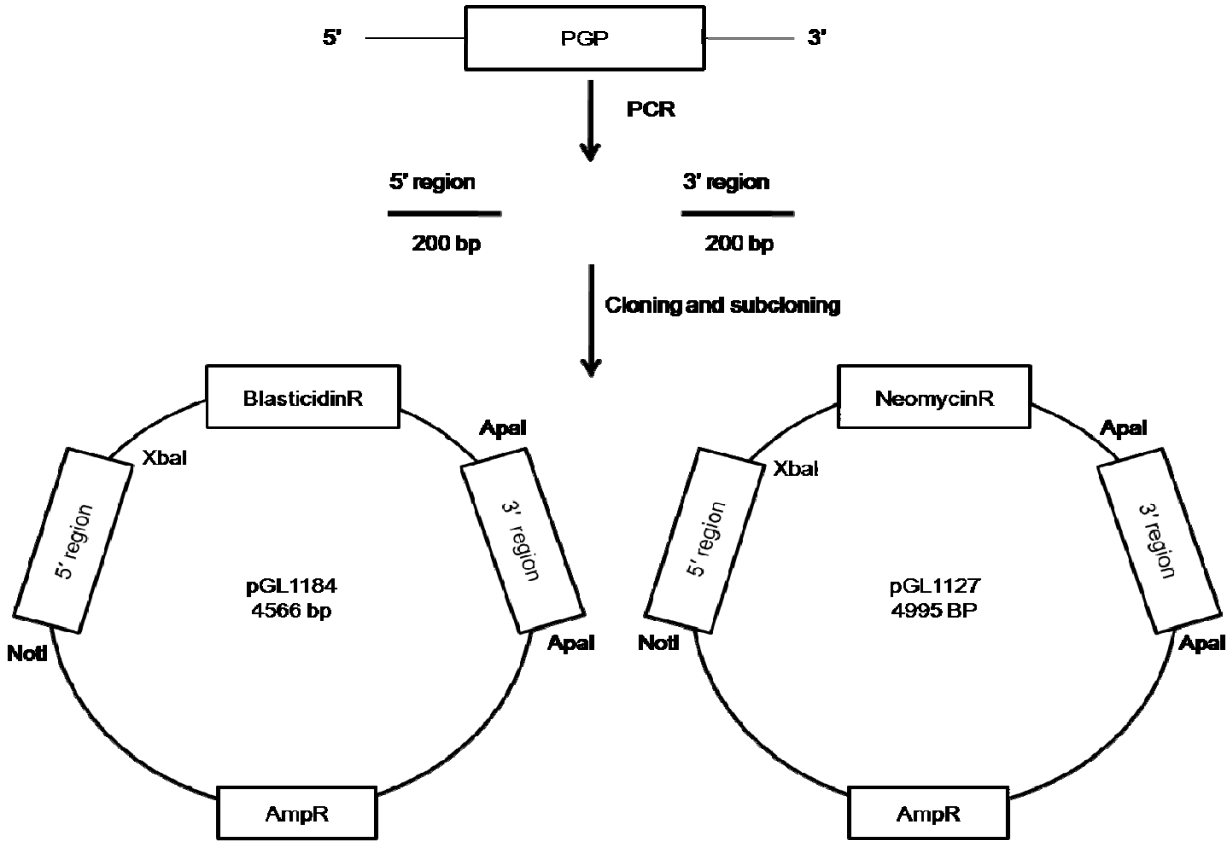

Fig. 3.2 Schematic representation of PGP knock-out plasmid constructs. PGP flanking regions at 5' and 3' were amplified by PCR and subcloned into both pGL knockout vectors..

3.3 RESULTS

In order to develop reverse genetics tools to gain insight into the role of PGP in the parasite, and a better understanding of the host-parasite interactions and the potential role of the enzyme in trypanosomosis pathology, RNAi and KO plasmids were constructed.

3.3.1 RNAi construct

3.3.1.1 DNA amplification

Primers were designed to amplify a 402 bp fragment, representing the 3′ region of the PGP ORF. A PCR was carried out using recombinant T-vector-PGP as template and the obtained products are shown in Fig. 3.3. The apparent size of the amplified PGP-RNAi is approximately 400 bp (lanes 3-7) as expected. The PCR product amplified by the PGP full length ORF primers is shown in the same Fig. (lane 2), as a 670 bp long fragment.

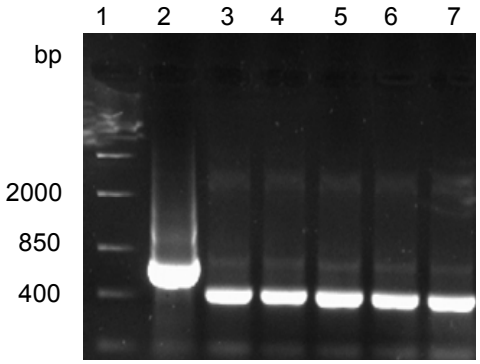

Fig. 3.3 Agarose gel analysis of PCR products using PGP-RNAi specific primers. Lane 1, FastRuler™ DNA ladder middle range; Lane 2, PGP ORF full length; Lanes 3-7, amplified PGP-RNAi.

3.3.1.2 Cloning and subcloning of PGP-RNAi into p2T7^{Ti}

Cloning of PGP-RNAi into the T-vector was successful, as confirmed by the colony PCR products (Fig. 3.4) of expected size of approximately 400 bp, using Fw-RNAi and Rv-RNAi primers. The recombinant T-vector was subsequently isolated and restricted with XbaI, releasing the 402 bp PGP-RNAi fragment, which was purified from the gel and subcloned into p2T7^{Ti} using the XbaI site. Transformants were screened by colony PCR using Fw-RNAi/Rv-RNAi (Fig. 3.5A) and p2T7^{Ti} (3.5B) primers. As shown, sixteen of the nineteen colonies were positive to PCR, giving products of around 400 bp and 900 bp for Fw-RNAi/Rv-RNAi and p2T7^{Ti} primers, respectively. Thus, subcloning of PGP-RNAi into p2T7^{Ti} vector was successful.

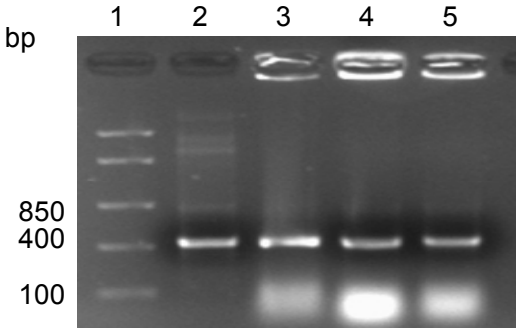

Fig. 3.4 Agarose gel analysis of colony PCR for screening of T-vector-PGP-RNAi recombinants. Lane 1, FastRuler™ DNA ladder middle range; Lanes 2-5, PCR products amplified using PGP-RNAi specific primers.

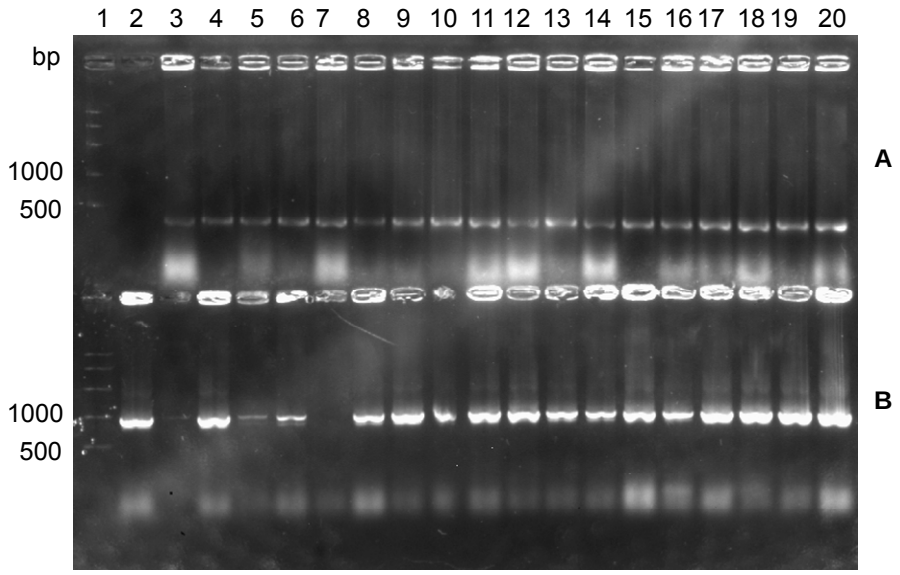

Fig. 3.5 Agarose gel analysis for screening recombinants PGP-RNAi-p2T7^{Ti}. Lane 1, FastRuler™ DNA ladder high range; Lanes 2-20, PCR products using **(A)** PGP-RNAi specific primers and **(B)** p2T7^{Ti} primers.

The recombinant p2T7^{Ti}PGP-RNAi plasmid from a selected clone and checked by linearisation using NotI restriction enzyme (Fig. 3.6, lane 3). A larger quantity of plasmid was then prepared, restricted by NotI and subsequently precipitated in ethanol for long term storage (Fig. 3.6, lane 4). This linearised plasmid is to be used in further studies, outside the scope of this dissertation, to transfect *T. congolense* RNAi-inducible strains such as TRUM183:29-13. The DNA was stored at -20 °C for further use.

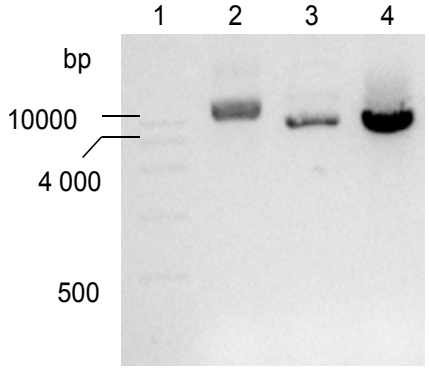

Fig. 3.6 Agarose gel analysis of isolated recombinant plasmid p2T7^{Ti} vector (inverted display). Lane 1, FastRuler™ DNA ladder high range; Lane 2, Uncut p2T7^{Ti}; Lanes 3 and 4, NotI-linearised p2T7^{Ti}.

3.3.2 Gene knock-out constructs

3.3.2.1 DNA amplification

Primers were designed to amplify the 5′ and 3′ regions (Appendix III) of the PGP ORF of *T. congolense* for cloning into knockout plasmid vectors. The coding sequences at 5′ (KO5) and 3′ (KO3) were amplified by PCR using the recombinant T-vector-PGP previously sequenced as template. The PCR products of KO5-Fw/KO5-Rv and KO3-Fw/KO3-Rv (Lanes 3-8) are shown in Fig. 3.7. The amplified fragments have the expected size of approximately 200 bp for both regions.

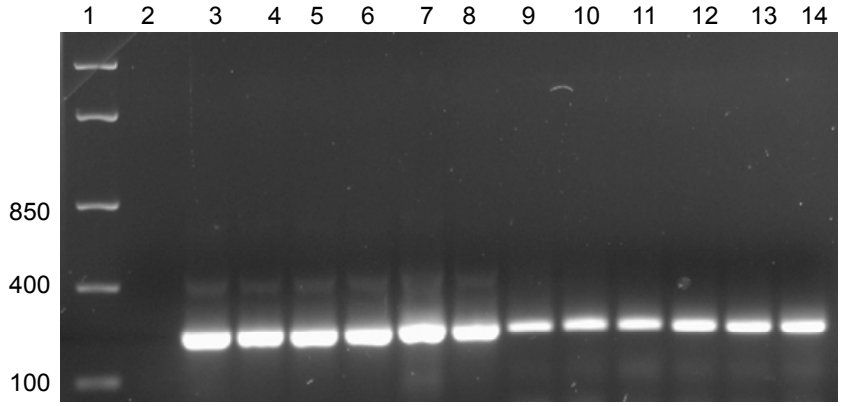

Fig. 3.7 Agarose gel analysis of PCR products. Lane 1, FastRuler™ DNA ladder middle range; Lanes 3-8, 5′ region amplified by PCR using the KO5 primers; Lanes 9-14, 5′ region amplified by PCR using the KO3 primers.

3.3.2.2 Cloning and subcloning of KO5 and KO3

The amplified KO5 and KO3 PCR products were purified from the gel, cloned into a T-vector. Recombinants were screened by colony PCR using insert primers. Plasmids from recombinant cells were isolated and restricted with NotI/XbaI (KO5-T-vector) or ApaI (KO3-T-vector) to release KO5 and KO3 from the T-vector (2886 bp) (Fig. 3.8). The uncut plasmid (Fig. 3.8A, lane 2 and B, lane 1) is in the supercoloid form and migrates faster than the restricted forms due to its compacted and knotted-up form as a smaller bundle, allowing it to move easily through the mesh of the gel. Some multimeric forms are equally visible at higher molecular weights, emanating from the rolling circle mode of plasmid replication (Khan, 1997).

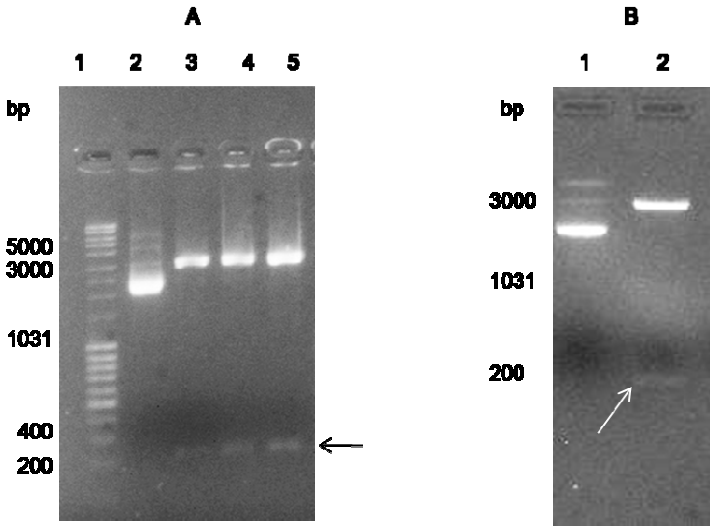

Fig. 3.8 Agarose gel analysis of restricted recombinant T-vector. (A) Restricted KO5-T-vector. Lane 1, MassRuler™ DNA ladder; Lane 2, Uncut recombinant T-vector; Lanes 3-5, XbaI/NotI restricted vector; **(B) Restricted KO3-T-vector**. Lane 1, Uncut recombinant T-vector; Lane 2, ApaI restricted T-vector. Arrows indicate the position of released fragments.

After gel purification, KO5 and KO3 inserts were subcloned into pGL1184 and pGL1217 plasmid vectors using NotI/XbaI and ApaI sites respectively, to generate pGL1184-KO5 and pGL1217-KO3. Recombinants were confirmed by colony PCR using KO5-Fw/KO5-Rv and KO3-Fw/KO3-Rv primers, giving products of roughly 200 bp, as expected (Fig. 3.9).

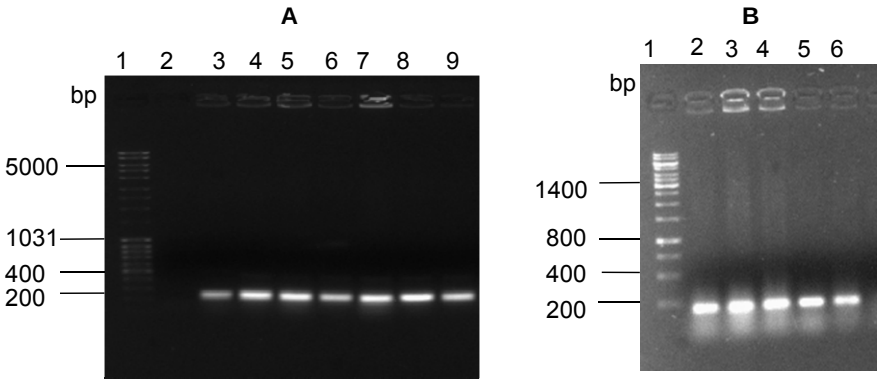

Fig. 3.9 Agarose gel analysis of PCR products from colony PCR screening of recombinants. (A) pGL1184-KO5. Lane 1, MassRuler™ DNA ladder; Lanes 2-9, PCR products of KO5 primers; **(B) pGL1217-KO3.** Lane 1, Fermentas 200 bp DNA ladder; Lanes 2-6, PCR products of KO3 primers.

Isolated pGL1184-KO5and pGL1217-KO3plasmids were restricted again with ApaI and NotI/XbaI restriction enzymes, respectively (Fig. 3.10). The restricted pGL1184-KO5 migrated at the expected size (4770 bp), which comprises the 4566 bp of the plasmid itself plus the 204 bp

of the KO5 insert (Fig 3.10A). The faint band with an approximate size of 400 bp is visible in lane 3 of Fig. 3.10A (Arrow) originates from a *T. brucei* fragment previously cloned using the ApaI site in a distinct study, with no consequence in the current experiment. Plasmid pGL1217-KO3 was expected to have a size (4995 bp of the plasmid plus 200 bp of the KO3 insert), which is confirmed by the size of the band on lane 3 of Fig 3.10B that is visible slightly above the 5000 bp position.

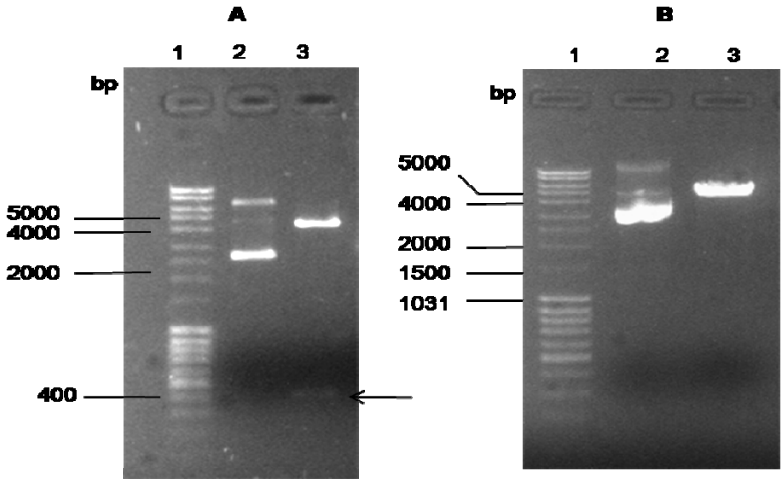

Fig. 3.10 Agarose gel analysis of knock-out plasmid restriction. (A) pGL1184-KO5. Lane 1, MassRuler™ DNA ladder; Lane 2, uncut plasmid; Lane 3, ApaI restricted plasmid. **(B) pGL1217-KO3.** Lane 1, MassRuler™ DNA ladder; Lane 2, uncut plasmid; Lane 3, NotI and XbaI restricted plasmid.

KO5 and KO3 were subcloned into pGL1217-KO3 and pGL1184-KO5respectively to generate the final pGL1184-KO5-KO3 and pGL1217-KO3-KO5 constructs. Recombinant *E.coli* clones were screened by colony PCR using insert-specific primers and the respective products of the expected size of approximately 200 bp are shown in Fig. 3.11.

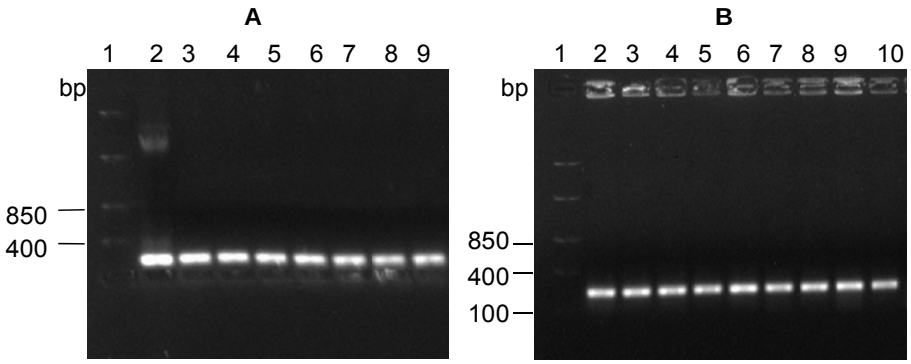

Fig. 3.11 Agarose gel analysis of PCR products from colony PCR screening of recombinants. (A) pGL1184-KO5-KO3. Lane 1, FastRuler™ DNA ladder middle range; Lanes 2-9, PCR products of KO3 primers; **(B) pGL1217-KO3-KO5**. Lane 1, FastRuler™ DNA ladder middle range; Lanes 2-10, PCR products of KO5 primers.

Final plasmid constructs pGL1184-KO5-KO3 and -pGL1217-KO3-KO5 were isolated (shown in Fig. 3.12) and stored to be used in future knock-out studies. The recent development in the *in vitro* cultivation and efficient transfection of *T. congolense* opened the door to such high-throughput investigations (Coustou et al., 2010).

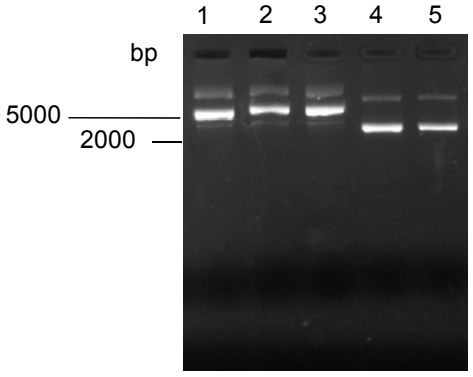

Fig. 3.12 Agarosis gel analysis of plasmid constructs minipreps. Lanes 1-3, KO3-pGL1184-KO3 plasmid minipreps; Lanes 4 and 5, KO3-pGL12-KO5 plasmid minipreps.

3.4 DISCUSSION

Pyroglutamyl peptidase type I (PGP) is an enzyme that has been described in mammals (Szewczuk and Kwiatkowska, 1970), bacteria (Doolittle and Armentrout, 1968) and *Trypanosomatidae* (Morty et al., 2006; Schaeffer et al., 2006a), but there is at present no data about *T. congolense* PGP function in trypanosome biology or its role in the pathogenesis of trypanosomosis in ruminants.

Reverse genetics approaches facilitated by the development and optimisation of gene manipulation strategies and transfection protocols (Coustou et al., 2010) have played a critical role in the characterisation of gene function in *Trypanosomatidae* (Owino et al., 2008; Teixeira and Da Rocha, 2003). The objective of this part of the study was to make reverse genetics constructs, namely plasmids for (PGP) RNAi and gene knockout, to be used in future studies aiming the elucidation of the role of PGP in disease process.

The plasmid p2T7^{Ti} (LaCount et al., 2002), which uses a dual T7 promoter to express dsRNA was used to make a construct to induce RNAi of the *PGP* gene of *T. congolense*, the most important pathogenic agent in cattle trypanosomosis (Radostis et al., 2006b). Since PGP has not been targeted using RNAi before, the choice of the target site in the gene sequence is critical (Duxbury and Whang, 2004). The designed primers amplified a 402 bp fragment (PGP-RNAi) from genomic DNA, corresponding to the 3′ region of the PGP ORF of *T. congolense*. This was designed to conform to the general recommendation that the target site should be located at least 100-200 nucleotides from the initiation codon (Sui et al., 2002). PGP-RNAi was cloned in a T-vector and subsequently subcloned into p2T7^{Ti}. After transformation, recombinants cells were screened by PCR and the results confirmed the high efficiency of insertion, with almost all the screened colonies giving products at the expected sizes for both specific and vector primers. The recombinant p2T7^{Ti}-PGP-RNAi construct was isolated, linearised, precipitated and stored. The p2T7^{Ti}-based vectors allow further integration of the construct into the genome of host cells expressing T7 polymerase and tetracycline repressor, thus expressing the dsRNA in a inducible manner. Another feature that makes this methodology very useful is that it is applicable in both bloodstream (Morris et al., 2001) and procyclic (Motyka and Englund, 2004) forms of the parasite. The p2T7^{Ti}-PGP-RNAi construct prepared in the present study can now be used to transfect *T. congolense* TRUM183:29-13 cells to assess PGP protein knockdown on parasite development and ultimately on disease progression.

Gene knockout is another approach used in reverse genetics experiments. It takes advantage of homologous recombination that occurs in cells to alter and test gene function. This strategy uses two different selectable markers as trypanosomes are diploid organisms lacking sexual cycle, hence both alleles of the gene needs to be altered for a functional knock-out through two transfection events (Cruz et al., 1993). In this study, disruption constructs were made for the *PGP* gene of *T. congolense*. Coding flanks at the 5′ and 3′ ends of PGP ORF were both successful amplified by PCR, cloned into T-vector, subcloned into pGL1217 and pGL1184 plasmid vectors, containing neomycin and blasticidin resistance genes respectively. The process requires identification of multiple compatible restriction sites for ligation reactions and vector linearisation, and several cloning steps that make the process tedious and time-consuming (Clayton, 1999). This is to be compared to RNAi contructs based on dual T7 promoters, as used in the present study, that require only a single cloning step and single transfection event. However, RNAi generally does not result in a complete knockdown of the desired gene (Motyka and Englund, 2004), unlike knock-out. Still, it is the technique of choice when dealing with essential genes that cannot be removed without impairing the survival of the parasite. A corollary is that RNAi is usually the first step before proceeding to the gene knock-out approach, to assess whether the target gene is vital or not.

A system of gene knock-out of two alleles of a gene using a single replacement construct was reported more recently (Nascimento et al., 2006; Ommen et al., 2009) and could prove to be a time-saving approach. However, it still needs to become better established regarding parasite species, transfection efficiency and cultivation techniques (Ommen et al., 2009). Regarding to PGP, gene knock-out was successfully used in *Leishmania major* (Schaeffer et al., 2006a). PGP null mutants were equally infective to macrophages and mice when compared with wild type parasites, suggesting no role for PGP in the virulence of leishmania. Taking into account that *L. major* and *T. congolense* enzymes are only 29% identical (Chapter 1), and the radically different biology of the two parasites, one being intracellular and the other a free-living form, it would not be surprising that PGP have different roles in the respective parasites, as shown with the *T. brucei* PGP. Gene KO of oligopeptidase B was recently described (Kangethe et al., 2011) using the same pGL1217 and pGL1184 plasmid vectors to generate null mutants, but no altered phenotype was detected.

In conclusion, this study reported the construction of different plasmids for reverse genetics studies, namely RNAi and gene knockout of the *T.congolense* PGP gene. Recombinant p2T7^{Ti}-PGP-RNAiand pGL1217-KO3-KO5 and pGL1184-KO5-KO3 plasmids can be now used for RNAi and knockout experiments. These constructs provide an important "ready to use" set of tools for future studies on the elucidation of *T. congolense* PGP function *in vivo* and *in vitro*. This could be done by assessing eventual changes in the morphology, replication and viability of PGP-deficient clones *in vitro*. *In vivo* assessment of the role of PGP in trypanosomosis pathogenesis could be done by inducing RNAi in susceptible animals following infection with engineered strain of *T. congolense*, initially by measuring physiological and pathological parameters, but also levels of hormones that are substrates for PGP (thyrotropin-releasing hormone and gonadotropin-releasing hormone).

4. GENERAL DISCUSSION

Trypanosoma congolense is the main causative agent of African animal trypanosomosis (AAT), the major constraint to livestock production in Africa, with estimated losses up to US $ 5 bilion per annum (Van den Bossche and Delespaux, 2011). Reproductive disorders are prominent clinical features in trypanosomosis and are associated with gonadal lesions and endocrine disorders (Ng'wena et al., 1997). Control measures include the use of trypanotolerant cattle, control of the tsetse vector and use of trypanocidal drugs (McDermott and Coleman, 2001), but the strategy expected to have a significant impact on controlling the disease is vaccination (Borst and Cross, 1982). Indeed, an investment-cost ratio study conducted by Kristjansson *et al.* (1999) has found that the benefit/cost ratio of developing a vaccine for animal African trypanosomosis ranges from 34:1 to 77:1. However, the development of a vaccine has been hindered by the capacity of the parasite to escape the host immune response through the mechanism of antigenic variation (Donelson, 2003; Donelson and Rice-Ficht, 1985). Thus, an alternative approach to vaccine development for trypanosomosis is the anti-disease strategy, in which antibodies are directed against bioactive substances released by trypanosomes and involved in the pathogenesis of the disease. This anti-disease vaccine approach might not affect parasite survival but would neutralise the pathogenic factors, thus controlling pathology. This was demonstrated in cattle immunised with congopain (*Tco*CATL), the major cysteine peptidase of *T. congolense* (Authié et al., 2001a), although the observed protection remained limited, rendering necessary the identification of new targets (Antoine-Moussiaux et al., 2009).

Pyroglutamyl peptidase type I (PGP) is a cysteine peptidase recently identified as a novel pathogenic factor, being entirely responsible for the abnormal levels of thyrotropin-releasing hormone (TRH) and partially responsible for abnormal levels and gonadotropin-releasing hormone (GnRH) in rodents infected by *T. brucei* (Morty et al., 2006). In the present study, the focus was on the PGP of *T. congolense*, the main causative agent of African animal trypanosomosis, for which no experimental data about was available. In the first part of the study, *T. congolense* PGP was cloned, expressed, purified and characterised while reverse

genetics vectors for RNAi and gene knockout were constructed and are described in the second part of the study.

The PGP open reading frame of *T. congolense* was obtained from BLAST searches on the database [http://www.genedb.org/genedb/tcongolense/ (last accessed 24-11-09)] using the *T. brucei* PGP nucleotide sequence as the query. PGP open reading frames and deduced amino acid sequences from the two trypanosome species share more than 50% identity. Subsequent analysis of the *T. congolense* PGP sequence confirmed the presence of typical cysteine, histidine and glutamate catalytic triad residues observed in PGPs of other organisms.

PGP was successfully subcloned in the bacterial expression vectors pGEX-4T-1 and pET28a and the enzyme recombinantly expressed at a high yield as an active and soluble fusion protein. Purification of PGP was successfully achieved using glutathione (pGEX) and Ni-NTA (pET) affinity chromatography and the enzyme showed an apparent molecular weight of 26 kDa by reducing SDS-PAGE, which is consistent with the size reported for the *T. brucei* enzyme (Morty et al., 2006) and conforms to the predicted molecular weight. The purified recombinant protein was used to raise anti-PGP antibodies in chickens. Immunised chickens showed the highest titres of IgY against PGP at weeks 6-8 post immunisation. These antibodies recognised not only the recombinant protein, but also the native PGP in *T. congolense* lysates, and that in both bloodstream and procyclic forms, at the same apparent molecular weight on a western blotting. The occurrence of PGP in the procyclic stage, also described in T. brucei (Morty et al., 2006) is a significant finding and suggests that the enzyme might play a role in the differentiation of the parasite, as reported in *Leishmania major* (Schaeffer et al., 2006a). In actual fact, only slight cysteine peptidase activity has been observed previously in procyclic and epimastigote forms of *T. congolense* (Downey and Donelson, 1999a). The PGP activity in the trypanosomal lysate was completely inhibited by anti-PGP antibodies generated against the recombinant enzyme. This is an important finding, as not only does it confirms the normal folding of the recombinant enzyme, used to generate antibodies, but also suggests the possible effectiveness of recombinant PGP as an immunogen in the context of an anti-disease vaccine. In addition, these antibodies provide a useful tool for PGP activity inhibition studies by antibodies in plasma of PGP-immunised and/or

trypanosome-infected hosts and also for assessing the successe of PGP RNAi and PGP gene knock-out studies (see below).

Glu-AMC which contains the corresponding free amino acid and the related Asp-AMC substrate were both not hydrolysed by recombinant PGP. By contrast, the enzyme exhibited strong activity against pGlu-AMC, confirming the high specificity for the pGlu residue in the P_1 position reported for PGPs of other organisms (Cummins and O'Connor, 1996; Dando et al., 2003; Morty et al., 2006). K_m values obtained for pGlu-AMC is comparable to those reported in others organisms, and the k_{cat}/K_m is very similar to the value obtained in *T. brucei* by Morty *et al.* (2006). In this study it was not possible to assay PGP activity against other known synthetic substrates such as pGlu-*p*-nitroanalide and pGlu-β-Na as well as against natural substrates such as TRH (pGlu-His-Pro-NH_2) and GnRH (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), due to the difficulties encountered in procuring the compounds.

PGP activity against natural substrates containing susceptible bonds *in vivo* might have significant implications in our understanding of the host-parasite interactions, and the pathogenesis of trypanosomosis in *T. congolense* infection, where circulating levels of testosterone and thyroxin are reduced (Mutayoba et al., 1988). Although the role of PGP in these endocrine abnormalities observed in *T. congolense* is still speculative, it is important to mention that low circulating levels of thyroxin and testosterone can be directly related to TRH and GnRH levels respectively (Al-Qarawi et al., 2001; Soudan et al., 1992), which are natural substrates for PGP (Cummins and O'Connor, 1998; Morty et al., 2006).

Recombinant PGP of *T. congolense* was irreversibly inhibited by thiol-reducing agents such as iodoacetamide and iodoacetate, which is consistent with the enzyme being a cysteine peptidase. The K_{ass} values showed that the enzyme is more sensitive to iodoacetamide and these values for thiol-reactive agents were comparable to those obtained for the *T. brucei* (Morty et al., 2006) and human (Dando et al., 2003) enzymes. E-64, a typical cysteine peptidase inhibitor, did not inhibit PGP, also reported in the case of *T. brucei* (Morty et al., 2006) and mammalian (Cummins and O'Connor, 1996; Dando et al., 2003) PGP.

PGP exhibited optimal activity at an alkaline pH of 9.0. This is consistent with reports on other PGPs, namely those of *Trypanosomatidae* (Morty et al., 2006; Schaeffer et al., 2006a), as well as

mammals (Cummins and O'Connor, 1996; Dando et al., 2003) and bacteria (Fujiwara and Tsuru, 1978). At the host physiological pH, recombinant PGP of *T. congolense* still showed substantial catalytic activity. This is a significant finding since it strongly suggests a possible role for the native trypanosomal enzyme *in vivo*. This idea is reinforced by the fact that PGP activity is not inhibited by general plasma peptidase inhibitors, such as α_2-macroglobulin and quininogens (Morty et al., 2006).

In the second part of this study, plasmid constructs for RNAi and gene knockout experiments were designed for a better approach in the study of gene function and drug target validation(Pauls and Esté, 2004). RNAi is a technique that involves triggering of post-transcriptional silencing of gene expression by introducing a double-stranded RNA into the cells (Vanhecke and Janitz, 2005). RNAi has been a major technical breakthrough in the study of many parasites. It has led to an increased ability to analyse the basic biology of such organisms and opened the way for large scale identification of genes involved in specific functions (Beverley, 2003). A typical example is the cytosolic peroxiredoxin (TbCPX). This is a peroxidase that was shown to be involved in protecting *T. cruzi* (Wilkinson et al., 2002; Wilkinson et al., 2000) and *T. brucei* (Wilkinson et al., 2003) from external oxidants. The RNAi induced silencing of TbCPX in *T. brucei* increased 16-fold the susceptibility of bloodstream forms to killing by H_2O_2 (Wilkinson et al., 2003).

To induce RNAi in trypanosomes, genetically modified cell strains which contain chromosomally-integrated plasmids coding for T7-polymerase and tetracycline repressorare used. Such plasmids allow the synthesis of dsRNA by addition of tetracycline as transcriptional inducer (Drozdz et al., 2002). In this study, the $p2T7^{Ti}$ (LaCount et al., 2002) plasmid vector, which uses a dual T7 promoter to express dsRNA, was used. This methodology is very useful because it has a single cloning step and its applicable in procyclic (Motyka and Englund, 2004) and bloodstream (Morris et al., 2001) forms of the parasite. A 402 bp 3′ region of PGP was successfully amplified and subcloned into the $p2T7^{Ti}$ plasmid vector. Recombinant plasmid can now be used to transfect *T. congolense* cell line TRUM183:29-13 cells and PGP protein knockdown levels assessed by western blotting, using the anti-PGP antibodies that were successfully raised in chickens in the present study, before moving on to parasite viability studies, and further *in vivo* assessment of pathogenicity..

Gene knockout is another reverse genetics approach that takes advantage of homologous recombination in cells to alter gene structure and study gene function. In order to disrupt PGP of *T. congolense*, the 3′ and 5′ coding flanks of PGP were amplified by PCR and successfully subcloned into knockout plasmid vectors pGL1184 and pGL1217. This approach uses two different constructs because, firstly, trypanosomes are diploid organisms and both alleles need to be disrupted for an effective functional testing (Cruz et al., 1991). Secondly, disruption of one allele may be straight-forward, but the disruption of the second allele is infrequent if the second disruption construct is homologous to the first (Enloe et al., 2000). Although time-consuming since it needs several cloning steps, this conventional approach has proved very effective in a general manner in *T. cruzi* (Xu et al., 2009) and for PGP in *L. major* (Schaeffer et al., 2006a). However, a simplified strategy to knockout genes in trypanosomes is still needed and would vastly improve the characterisation of genes coding for proteins with unknown or unconfirmed functions. The recombinant plasmid vectors pGL1184-KO5-KO3 and pGL1217-KO3-KO5 are now ready to be used to transfect trypanosomes, disrupt the PGP gene and eventually assess the resulting effects on the parasite biology and host-parasite interactions. From there, a definitive role for PGP *in vivo* may be elucidated, as well as its validation as a drug target or vaccine candidate.

The present study constitutes the first report of the cloning, expression, purification and enzymatic characterisation of the recombinant *T. congolense* PGP enzyme.. The data confirmed the occurrence of PGP in *T. congolense*, and its biochemical properties are, in general, in agreement with the reports for other recombinant PGPs in related parasites. However, more work needs to be done at different levels. It was not in the scope of the present study to undertake the purification and enzymatic characterisation of the *T. congolense* native PGP, but in future studies this can be undertaken by purifying the native enzyme from trypanosome lysates by immuno-affinity, using the anti-PGP antibodies that were raised in chickens in the present study. Such antibodies should still be used for inhibitory assays with both native and recombinant enzymes. It would be interesting to assess the inhibitory effect of plasma peptidase inhibitors such as quininogens and α_2-macroglobulin on PGP activity, or absence thereof, as seen in *T. brucei*, in which the enzyme was shown to remain 100% active. Activity assays against other synthetic and natural substrates described in the literature could also be carried out, to form the basis of drug design where an inhibitory group is attached to a peptide that mimics a substrate. Ultimately,

experiments in animal models are crucial for the validation of PGP as a pathogenic factor in trypanosomosis. These experiments should include the classic approach of immunisation of animals with the recombinant PGP, subsequent challenge with wild type *T. congolense* strain and assessment of physiological, clinical and immunological parameters. The same experiment should be done using clones in which PGP levels are diminished or completely abolished through RNAi and gene knockout techniques using the constructs prepared in the present study. This experiment should provide sufficient data to eventually define a precise role for PGP in the pathogenesis of trypanosomosis, specifically in the observed endocrine lesions.

In the context of an anti-disease vaccine, the demonstration of PGP activity in *T. congolense* is an important achievement, since a precise knowledge of the host-parasite interactions is needed to design an efficient therapy strategy. However, this finding reinforces the necessity for careful identification of all pathogenic factors that contribute to the pathology of trypanosomosis for an eventual inclusion in a multi-component anti-disease vaccine able to protect susceptible hosts.

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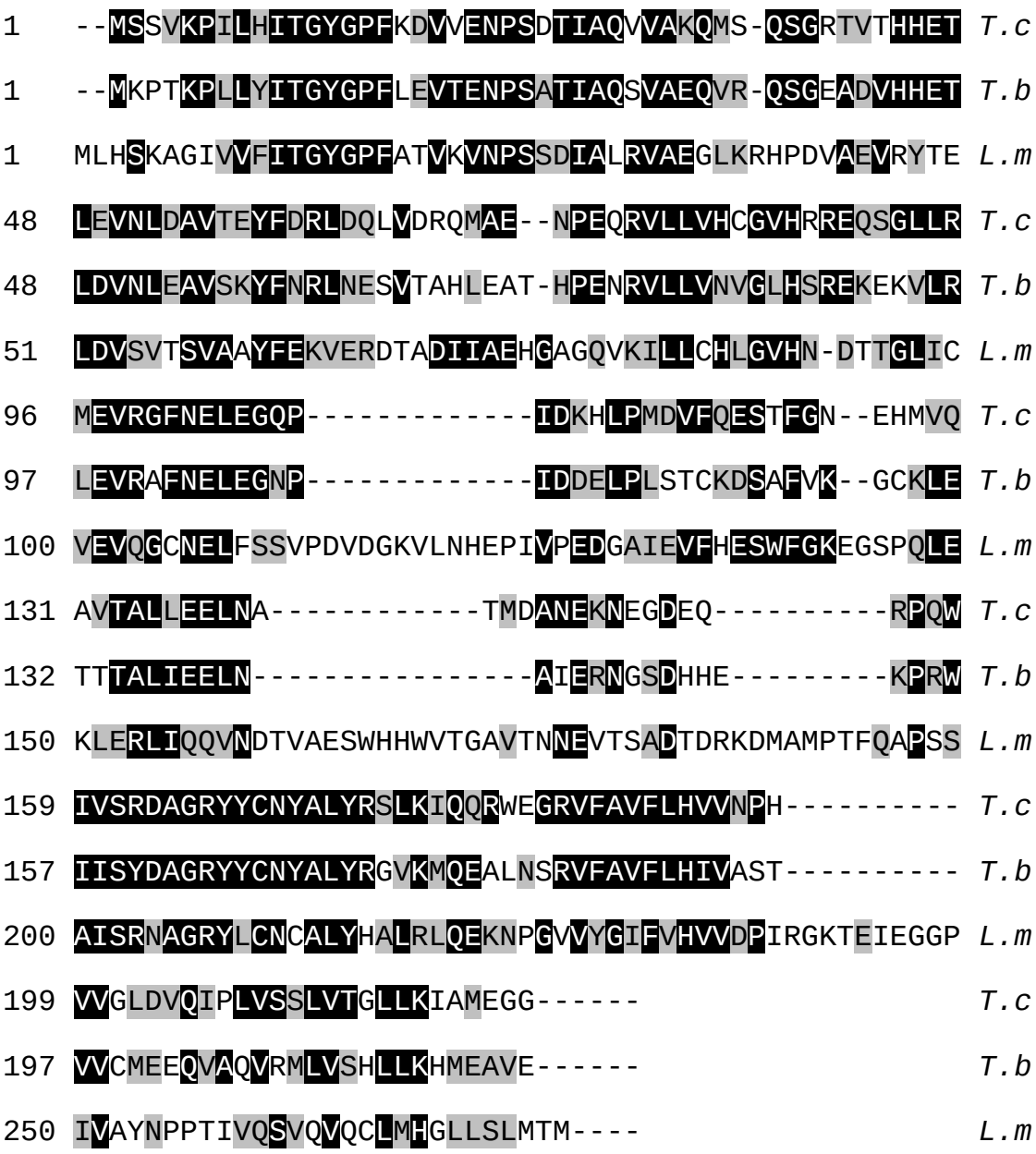
APPENDIX 1:

PGP AMINO ACID SEQUENCE MULTIPLE ALIGNMENT



APPENDIX 2:

TARGETED SITE FOR RNAi ON PGP OF *T. CONGOLENSE*

5′ATGAGCTCAGTCAAGCCGATTCTTCACATAACTGGCTACGGTCCTTTCAAAGATGT
AGTGGAGAACCCGAGCGACACTATTGCGCAGGTGGTGGCAAAGCAAATGAGTCAGA
GTGGGAAAACCGTGACGCACCACGAAACTTTGGAAGTCAATTTGGACGCCGTCACC
GAGTACTTTGACAGGCTTGACCAGTTAGTGGACCGCCAAATGGCTGAGAACCCCGA
ACAGCGTGTTCTGCTCGTGCACTGCGGCGTACATCGCCGTGAACAAAGCGGGTTGCT
GCGGATGGAGGTGCGTGGTTTTAACGAGTTGGAGGGACAACCAATTGATAAACATT
TACCAATGGACGCCTTCCAAGAGAGTACGTTTGGAAATGAGCACATGGTTCAAGCT
GTCACCGCACTCCTAGAAGAACTCAATGCAACGATGGACGCGAATGAGAAAAACGA
AGGTGATGAACAAAGGCCACAGTGGATCGTGTCTCGTGATGCTGGGCGGTATTACT
GCAACTATGCCCTTTACAGGAGCTTGAAGATACAACAGAGATGGGAGGGGCGTGTC
TTTGCAGTGTTTTTGCACGTTGTCAACCCCCATGTAGTCGGGTTGGACGTGCAAATTC
CCCTGGTGAGCTCCCTTGTGACTGGACTGCTAAAAATTGCCATGGAAGGCGGGGCG
GCC**3′**

APPENDIX 3:

PGP 5′- AND 3′- REGIONS USED FOR GENE KNOCK-OUT OF PGP

5′ATGAGCTCAGTCAAGCCGATTCTTCACATAACTGGCTACGGTCCTTTCAAAGATGT
AGTGGAGAACCCGAGCGACACTATTGCGCAGGTGGTGGCAAAGCAAATGAGTCAGA
GTGGGAAAACCGTGACGCACCACGAAACTTTGGAAGTCAATTTGGACGCCGTCACC
GAGTACTTTGACAGGCTTGACCAGTTAGTGGACCGCCAAATGGCTGAGAACCCCGA
ACAGCGTGTTCTGCTCGTGCACTGCGGCGTACATCGCCGTGAACAAAGCGGGTTGCT
GCGGATGGAGGTGCGTGGTTTTAACGAGTTGGAGGGACAACCAATTGATAAACATT
TACCAATGGACGCCTTCCAAGAGAGTACGTTTGGAAATGAGCACATGGTTCAAGCT
GTCACCGCACTCCTAGAAGAACTCAATGCAACGATGGACGCGAATGAGAAAAACGA
AGGTGATGAACAAAGGCCACAGTGGATCGTGTCTCGTGATGCTGGGCGGTATTACT
GCAACTATGCCCTTTACAGGAGCTTGAAGATACAACAGAGATGGGAGGGGCGTGTC
TTTGCAGTGTTTTTGCACGTTGTCAACCCCCATGTAGTCGGGTTGGACGTGCAAATTC
CCCTGGTGAGCTCCCTTGTGACTGGACTGCTAAAAATTGCCATGGAAGGCGGGGCG
GCC **3′**