Molecular characterization of the lectin-trypsin complex from the midgut of the tsetse fly, *Glossina fuscipes fuscipes* (Newstead) and its role in determining the susceptibility to trypanosome infection

By

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DECLARATION

I, Laila Uweso Abubakar, hereby decare that this thesis is my original work and has not been presented for a degree award in any other university

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To Abdulaziz, Warda and my late son Ali, for making a difference into my life.

ABSTRACT

A lectin with trypsin activity has been purified from the midguts of tsetse fly, *Glossina fuscipes fuscipes* (Newstead), using a combination of anion-exchange and D(+) glucosamine-affinity chromatography. The molecule (native M_r ~65, 700) had two non-covalently linked subunits, M_r ~28, 800 and M_r ~35, 700. The native molecule was found to be capable of inducing differentiation of bloodstream-form trypanosomes into procyclic (midgut forms), *in vitro*. This induction was specifically inhibited by D-glucosamine.

Screening the *G. fuscipes* midgut cDNA library with antibody raised against native lectin-trypsin complex revealed a 933 bp cDNA that encoded a 274 amino acids polypeptide. The sequence, designated *Glossina* proteolytic lectin (Gpl), contained the catalytic domain of serine protease belonging to the trypsin family. The expressed Gpl (M_r ~ 32, 500± 2828 da) exhibited D-glucosamine binding and agglutination activity against bloodstream trypanosomes and rabbit RBCs. In addition, Gpl exhibited trypsin activity of 6.025 units x 10^{-4}.

A 458bp trypsin cDNA was further isolated using degenerate primers and PCR techniques. Screening the cDNA expression library with this probe revealed a 952 bp *Glossina fuscipes* serine protease (GfSP) gene. The sequence encoded a 269 amino acids protein that contained several potential phosphorylation sites. The expressed protein (M_r ~ 26, 807 ±2748 da) had trypsin activity of 4.390 units x 10^{-4} and exhibited remarkable lysis activity against bloodstream trypanosomes.

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Abbreviations

BCA	Bicinchocinic acid
BSA	Bovine serum albumin
bp	Base pair
cDNA	Complimentary deoxyribonucleic acid
Chromozym-TRY	Carbobenzoxy-val-gly-arg-4-nitrianilide acetate
DNA	Deoxyribonucleic acid
DEAE-	Diethylaminoethyl-
h	Hour
ICIPE	International Centre of Insect Physiology and Ecology
Kb	Kilobase(s)
M	Molar
Mg	Milligram
$MgCl_2$	Magnisium chloride
Min	Minutes
mM	Millimolar
µ	Micro
µg	Microgram
µl	Microlitre
µM	Micromolar
NaCl	Sodium chloride

ng	Nanogram (s)
nm	Nanometres
OD	Optical density
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PEG	Polyethylene glycol
RBCs	Red blood cells
RLOs	Rickettsiae-like organisms
Rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
Sec	Second (s)
TBS	Tris buffered saline
Tris	Tris-(hydroxy methyl) amino methane
VATs	Variant antigen types

SUMMARY

Tsetse are obligate intermediate hosts for the protozoan parasite, *Trypanosoma*, the causative agent of trypanosomosis in humans and animals. Successful transmission of trypanosome infection in the tsetse vector involves transformation of bloodstream-form trypanosomes into procyclic (midgut) forms. This process is mediated by a wide variety of factors, all of which are intrinsic to the tsetse, trypanosome and the host blood. Of these factors, lectins, trypsin-like molecules and lysins have received the most attention. More recently, a lectin in association with trypsin activity from *Glossina longipennis* was discovered. This molecule was immunologically detected within members of *Glossina* species but not in other haematophagous insects. This suggested that it may have an important role to play in the interactions between tsetse and the trypanosome and hence the vectorial capacity of tsetse flies. The present study was undertaken to gain insight into the role played by this molecule in the establishment of trypanosome infection. In this study, a lectin-trypsin molecule was isolated from *Glossina fuscipes fuscipes* (Newstead) and its involvement in triggering transformation and establishment of the parasites in tsetse was demonstrated. As a further step towards understanding the function of the molecule, the lectin and trypsin genes were cloned and characterized.

Lectin-trypsin complex was purified from the midguts of *Glossina fuscipes fuscipes* by a combination of anion-exchange and D(+) glucosamine-affinity chromatography. The molecule (native M_r ~65, 700) had two non-covalently

linked subunits, M_r ~28, 800 and M_r ~35, 700. This molecular weight compares favourably with a similar molecule previously reported from the midgut of the tsetse fly, *Glossina longipennis*. The role of lectin-trypsin complex in transformation of bloodstream trypanosomes into procyclic (midgut) forms was assessed *in vitro*. Loss of variant surface glycoprotein (VSG) and the synthesis of an invariant coat composed of procyclins, were used to confirm the transformation process. Using indirect immunoflourescence assays, transformation of the parasites was followed by induction of procyclin synthesis using specific antibodies to the protein. This induction was specifically inhibited by D-glucosamine. The rate of transformation by lectin-trypsin complex was not significantly different from the transformation rate observed when the parasites were incubated with the *in vitro* trigger, cis-aconitate (t-test, $p > 0.05$). However, high concentrations of the lectin-trypsin molecule in the incubation assays led to high mortality of the parasites.

In an attempt to identify the lectin-trypsin gene, a *G. f. fuscipes* midgut cDNA expression library was constructed and screened with polyclonal antibodies raised against the lectin-trypsin complex. A 933 bp cDNA that encoded a 274 amino acids polypeptide was isolated. Interestingly, the sequence, designated *Glossina* proteolytic lectin (Gpl), contained the catalytic domain of serine protease belonging to the trypsin family. Moreover, the presence of aspartate in the specificity pocket suggested that the clone was a typical trypsin. Expression of the gene in a bacterial expression system yielded a protein with molecular weight of ~ 32, 500± 2828 Da. The recombinant Gpl

exhibited D-glucosamine binding and agglutination activity against bloodstream trypanosomes and rabbit RBCs. In addition, Gpl exhibited trypsin activity of 6.025 units x 10^{-4}. Using immunoflourescence assays, the recombinant protein was found to be capable of inducing differentiation of bloodstream-form trypanosomes into procyclic forms *in vitro*. Although it was initially concluded that this was a property of the lectin-trypsin complex, the later findings suggest that Gpl (which is closer to the α subunit of the complex) can by itself stimulate the transformation process.

A 458bp trypsin cDNA was further identified using degenerate primers that were designed based on highly conserved regions found in *Anopheles gambiae*, *Aedis aegypti* and *Drosophila melanogaster* trypsins. Sequence analysis of the clone against databases showed similarity to a serine protease fragment of the blowfly, *Lucilia cuprina* with an expectation of 2 x 10^{-62}, 73% identities and 84% positives. Using this clone as a probe to screen the cDNA expression library, a full-length gene was isolated. The cDNA encoded a mature protein of 269 amino acids with a predicted molecular weight of ~ 29 245 Da. A comparison of the amino acid sequence with the protein database using BLASTP showed that it displays similarity to various serine proteases from insects. Multiple sequence alignment showed that the cDNA, aptly called *Glossina fuscipes* serine protease (GfSP), possess the catalytic triad, His, Asp and Ser, and the highly conserved regions surrounding the His and Ser residues that are typical of serine proteases. In addition, the GfSP sequence contained several potential phosphorylation sites. Expression of the gene in a bacterial expression system yielded a protein with

molecular weight of ~ 26, 807±2748 Da. The recombinant GfSP had trypsin activity of 4.390 units x 10^{-4}. In contrast to GpI, this protein neither showed agglutination activity nor could it induce transformation of parasites. However, it exhibited remarkable lysis activity against bloodstream trypanosomes and rabbit RBCs.

Although several factors have been implicated in the successful development of trypanosomes in tsetse, the results of this study clearly demonstrate the important role played by lectin-trypsin complex in the transformation process. On the other hand, higher concentrations of lectin-trypsin molecule led to high mortality of the parasites. These results provide evidence for the possible involvement of the midgut lectin-trypsin molecule in both clearance and development of parasites in tsetse.

CHAPTER ONE

1.0 INTRODUCTION AND LITERATURE REVIEW

1.1 General Introduction

Trypanosomosis, which occurs across more than a third of Africa, is arguably the most important livestock and human vector-borne disease on the continent. This disease, commonly known as sleeping sickness in humans and Nagana in cattle, is caused by a group of parasitic protozoa called trypanosomes. Being digenetic, these parasites require both the vertebrate and the tsetse hosts to complete their life cycle. Tsetse (Diptera: Glossinidae), the vectors of trypanosomosis occur only in Africa, inhabiting an area of over 10 million square km (ILRAD, 1990). The risk of trypanosomosis in much of this area precludes farmers from keeping cattle and other ruminants. This fact is a major contribution to Africa's low livestock productivity. The impact of trypanosomosis is even greater because the tsetse-inhabited areas are potentially the most agriculturally productive in Africa. Nagana is a wasting disease responsible for an estimated US $5 billion annual losses in meat production. This economic deprivation is exacerbated by losses in milk production, tractive power and other secondary products. In addition, 50 million people are currently exposed to the risk of contracting human trypanosomosis.

Initial attempts to control tsetse flies and trypanosomosis included eradication of wildlife to remove the reservoir hosts of both flies and trypanosomes and widespread bush clearance to destroy the fly's breeding habitats (Jordan, 1985). Such efforts are

however, not popular since they pose a serious threat to biodiversity. An alternative approach to vector management relies heavily on aerial spraying and insecticidal pour-ons. Although effective initially, their rigorous use invariably results in the development of resistance. Moreover, the high cost of developing new pesticides coupled with the adverse effects of their residues in the biosphere renders the approach unacceptable as a long-term solution (Holmes, 1997).

A logical extension to this control method involved mass trapping of tsetse to levels below economic injury. The bi-conical trap was developed and successfully used in the early 1970s against riverine species such as *G. palpalis palpalis* (Allsopp, 1984). The technique has been made more efficient by the incorporation of odour baits (CO_2, acetone, butanone, 1-octen-3-ol, cow urine) and visual cues (Vale, 1980; Owaga, 1984; 1985; Brightwell, 1987; Owaga *et al.*; 1988). More recently, cheaper and simpler targets consisting of insecticide-treated cloth screens baited with synthetic odours, have been developed. In view of the relatively low cost, the odour –baited, insecticide – impregnated traps or targets have proved to be a sustainable method of control and their positive impact has been enormous. Numerous trials have demonstrated that a low density of such traps/targets can achieve tsetse control in a cost-effective manner thus offering advantages over previously used control methods. Despite their suitability for community-based tsetse control programmes, these traps and targets require regular supervision to combat damage and theft, and their effectiveness varies between species and geographic subspecies of *Glossina*.

An additional environmentally acceptable method for eradicating tsetse consist of an integrated campaign using insecticide-treated screens or traps followed by massive release of sterile males (Bauer *et al.*, 1992b). The recent successful eradication of *G. austeni* from the island of Zanzibar has demonstrated the feasibility and applicability of this technology in area-wide vector control programmes (Saleh *et al.* 1999; Vreysen *et al.* 2000). Sterile insect technique (SIT) involves sustained, systematic releases of sterile male insects to fertilize wild females among the wild population, which are then unable to produce progeny.

Another common strategy for the management of trypanosomosis involves parasite control. This approach depends on trypanocidal drugs like dimenazine, isometamidium and homidium for both treatment and prophylaxis. Although highly effective when prescribed regimes are strictly followed, the drugs are expensive and the infrastructure needed for their effective administration is lacking in many African countries. As a result, under-dosing occurs thereby increasing the risk of resistance to the few effective drugs (Peregrine, 1994). On the other hand, the possibility of developing a vaccine against trypanosomosis has been frustrated by the ability of the parasites to vary their surface antigens and thus evade the host's immune response (Vickerman, 1978; Jacobs-Lorena and Lemos, 1995). Thus, a conventional vaccine that primes an animal's immune system against only one or a few antigens will not be broadly effective against trypanosomosis.

Genetic resistance to trypanosomosis, commonly referred to as trypanotolerance, is known to occur in certain breeds of domestic livestock and species

of wildlife (Murray *et al.*, 1982). In West Africa, trypanotolerance has been identified in taurine cattle breeds, namely, the N'Dama and West African Shorthorn (Murray *et al.*, 1991). Recent studies in Kenya have shown that the Orma Boran and Maasai Zebu possess a degree of natural resistance to trypanosomosis (Njogu *et al.*, 1985a; Ismael *et al.*, 1985; Mwangi *et al.*, 1998). Keeping these trypanotolerant livestock in tsetse-affected areas presents a method of control, which is likely to be sustainable. However, keeping them has not been fully embraced by farmers due to the perception that their relatively small size results in poor milk and meat yields.

All these problems have spurred new interest in the search for alternative tsetse management strategies, especially those that closely integrate a sound knowledge of the vector biology and ecology as well as the inter relationship between vectors, parasites and host animals. One such approach that could form part of an integrated system involves genetic manipulation of the tsetse vector. This could provide a means for altering the vectorial capacity of the vector in such a way as to have a profound and long lasting effect on disease transmission. However, for such an approach to be feasible, an understanding of the mechanisms involved in the interaction between tsetse and trypanosome is required.

1.2 Literature Review

1.2.1 Tsetse: The Invertebrate Vectors of Trypanosomosis

Tsetse flies are haematophagous arthropods belonging to the order *Diptera*, family *Glossinidae*. Only one genus, *Glossina*, is included in this family, which contains 30 living taxa, 22 species and 8 subspecies (Potts, 1973). Based on their morphological, ecological and behavioral characteristics, these species can broadly be classified into 3 groups. The *Fusca* group (forest dwellers) consist mainly of *G. brevipalpis* and *G. longipennis*. The *Morsitans* group (savanna dwellers) consists of *G. morsitans*, *G. pallidipes*, *G. swynnertoni* and *G. austeni*. The *Palpalis* group (riverine distribution) comprises of *G. fuscipes*, *G. palpalis* and *G. tachinoides*.

In contrast to most insects, tsetse flies are larviparous, with the female giving birth to a single larva at intervals (Engelman, 1970). Being obligatory blood-feeders (Buxton, 1955), tsetse flies rely on vertebrate blood for their exclusive protein diet. Consequently, they are able to transmit trypanosomes. The ability of tsetse to transmit trypanosomes is of great economic importance when man and his domesticated animals intrude into the cycle, resulting in trypanosomosis.

1.2.2 Trypanosomes

All parasitic trypanosomes belong to the order *Kinetoplastida*, family *Trypanosomatidae,* which consists of 9 genera. These include *Crithidia*, *Blastocrithidia*, *Herpetomonas*, *Phytomonas*, *Edotrypanum*, *Rhynchodomonas, Leptomonas*,

Leishmania and *Trypanosoma* (Molyneux and Ashford, 1983). Trypanosomes are digenetic and thus require both the vertebrate and invertebrate hosts (Hoare, 1972). Based on their developmental cycle and mode of transmission, the genus *Trypanosoma* can be further divided into stercorarian and salivarian. In stercorarian trypanosomes, the infective stages appear in the faeces of the vector and transmission is by contamination. In salivarian trypanosomes on the other hand, the infective forms appear in the saliva of the tsetse and transmission is by innoculation. The salivarian trypanosomes are the causative agents of African trypanosomosis.

Four subgenera have been identified within the salivarian species. They are *Trypanozoon* (*T. brucei* spp, *T. evansi*), *Nannomonas* (*T. congolense*, *T. simie*, *T. vanhoofi*), *Dutonella* (*T. vivax*, *T. uniforme*) and *Pycnomonas* (*T.suis*) (Logan-Henfrey *et al.*, 1992). With the exception of the subgenus *Dutonella* whose life cycle is confined to the mouthparts, all salivarian trypanosomes possess a midgut stage in the tsetse prior to maturation either in the mouthparts or salivary glands.

In Sub-saharan Africa, *T. brucei*, *T. congolense*, *T. vivax*, *T. simie* species are the principal pathogens of livestock, while *T. evansi* affects camels, horses and buffalo (Logan-Henfrey *et al.*, 1992; Croft, 1997). The main agent of chronic sleeping sickness present in west and North-central Africa is *T. b. gambiense*, which is transmitted primarily by the *Morsitans* group. On the other hand, the more acute sleeping sickness in East and Central Africa is caused by *T. b. rhodesiense*, which is transmitted mainly by the *Palpalis* group (Vickerman, 1985; Seed and Hall, 1992; Jordan, 1993).

1.2.3 Tsetse-Trypanosome Interactions

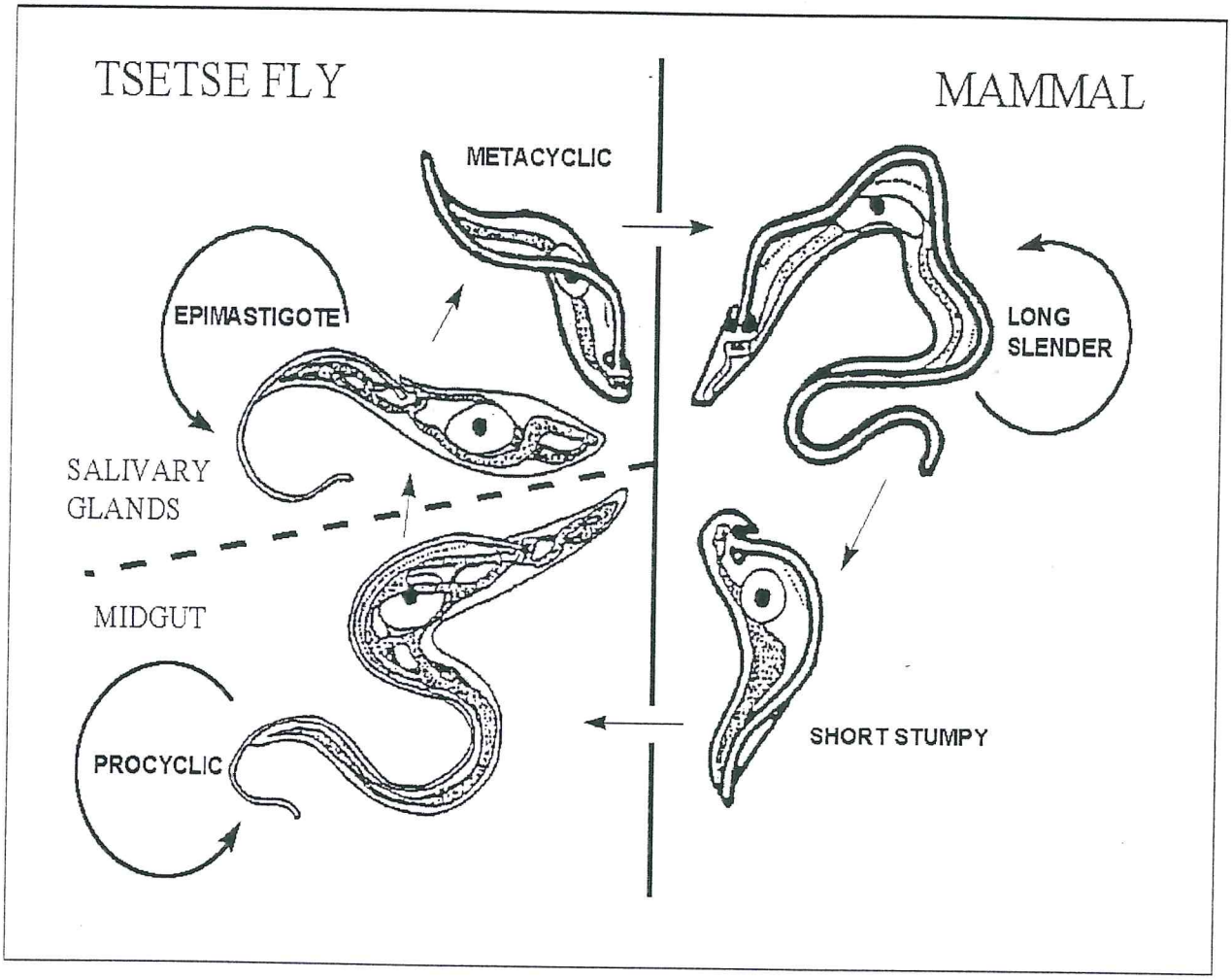
The transition from vertebrate hosts to insect vector presents some of the most formidable barriers that arthropod-borne parasites must overcome during their life cycles. Within the mammalian hosts, the trypanosomes reside extracellularly in the blood and extravascular tissues as long slender and short stumpy bloodstream forms (Schopf and Mansfield, 1998). Each trypanosome carries a large number of genes coding for antigenically different variant surface glycoproteins (VSGs) on their surface coat (Cross, 1990; Kamper and Barbet, 1992). At a given time, only one of these genes located in an active expression site is transcribed and translated. The resulting VSG stimulates the formation of antibodies by the host, leading to the elimination of this variant by uptake in macrophages or by complement-mediated lysis (Mansfield, 1995). However, small numbers of trypanosomes expressing new VSG surface coats arise from within the initial variant antigenic type (VAT) and are not affected by the antibody response; giving rise to another wave of parasitemia. The new VATs trigger another VSG-specific antibody response and the cycle of immune selection and antigenic variation progresses, with the parasites avoiding immune elimination. Ultimately, the host immune system is overwhelmed and death occurs.

In the insect vector however, the parasites have to contend with both cellular and acellular (midgut epithelium and peritrophic matrix) barriers and the onslaught by a wide array of midgut factors (Maudlin, 1991). To successfully cope with this alteration in habitat, the parasites undergo a series of adaptations and transform into procyclic (midgut) forms (Logan-Henfrey *et al.*, 1992). Transformation of trypanosomes from

bloodstream to procyclic forms in the tsetse vector is accompanied by loss of the surface coat, which is correlated with loss of infectivity for mammalian hosts (Ghiotto *et al.*, 1979). Furthermore, there is a simultaneous cessation of variable surface glycoprotein (VSG) synthesis, and induction of procyclin synthesis. The most striking changes during this process however, occur in the mitochondrion. The mitochondrial canals proliferate and the cristae become more numerous, with subsequent activation of mitochondrial enzymes and the cytochrome electron-transport system (Vickerman, 1985; Hecker *et al.*, 1973). These changes are an expression of a shift from glycolysis to complete glucose oxidation and a concurrent preference to proline metabolism (Ghiotto *et al.*, 1979; Tielans and Hellemond, 1998).

Once established in the midgut, trypanosomes migrate forward to the proventriculus and the mouthparts, where they differentiate into epimastigotes and eventually colonize the salivary glands (*T. brucei*) or proboscis (*T. congolense*) (Maudlin, 1985; Maudlin and Welburn, 1987; Abbelle *et al.*, 1999). Here, they undergo maturation to the infective metacyclic forms and can be transmitted to the next host during blood feeding by the fly. The remaining immature trypanosome population in the ectoperitrophic space is reported to undergo self-regulation by apoptotic cell death (Welburn and Maudlin, 1997; Welburn *et al.*, 1997).

Scheme 1 The life cycle of the *Trypanosoma brucei* group of parasites


TSETSE FLY
MAMMAL
METACYCLIC
EPIMASTIGOTE
LONG
SLENDER
SALIVARY
GLANDS
MIDGUT
PROCYCLIC
SHORT STUMPY

The complex life cycle of the African trypanosome has been positively correlated to the infection rates in tsetse. For example, in *T. vivax*, the developmental cycle is restricted in the tsetse cibarial region and proboscis. As a result, most of the *T. vivax* infections mature while the gut-adapted trypanosome infections are arrested in the midgut (Moloo and Kutuza, 1988; Moloo *et al.*, 1992). On the other hand, different *Glossina* species have been reported to differ in their susceptibilities to *Trypanozoon* and *Nannomonas* infections. The *Morsitans* group tsetse is more susceptible compared to the *Palpalis* group (Harley and Wilson, 1968; Moloo and Kutuza, 1988; Moloo *et al.*, 1992; 1998; Reifenberg *et al.*, 1997). Furthermore, mixed infections have been reported to be more prevalent within the susceptible *Morsitans*-group. The observed differences in infection rates could neither be attributed to the geographical origin of the vectors nor to the origin of the trypanosome stock (Moloo *et al.*, 1992; Reifenberg *et al.*, 1997). Nevertheless, it has been postulated that these differences in infection rates may be a reflection of differences in the gut environment of the different tsetse. Consequently, the gut-adapted *Trypanozoon* and *Nannomonas* parasites can become established more readily and undergo full cyclical development in some tsetse, while arrested in the gut of other tsetse species (Maudlin, 1991).

1.2.4 Molecular basis of tsetse- trypanosome interaction

Tsetse flies are in general refractory to parasite transmission. Indeed, it has been reported that irrespective of the trypanosome species involved, infection rates in the various tsetse groups have remained unusually low (Harley and Wilson, 1968; Elce,

1971; 1974; Harley, 1971). Moreover, these infection rates have been found to be significantly higher in males than in females (Hoof, 1947; Harley, 1971; Distelmans *et al.*, 1982), in young teneral flies than in old non-teneral flies (Wijers, 1958; Harmsen, 1973; Distelmans *et al.*, 1982) and in *Morsitans*-group than in the *Palpalis*-group (Moloo *et al.*, 1992; 1998; Reifenberg *et al.*, 1997). The underlying molecular basis for this refractoriness is still poorly understood.

Vector insects are known to display both humoral (non-cellular) and cell-mediated (hemocytes) immunity in order to counteract environmental pathogens (Ratcliffe *et al.*, 1982; Azzolina *et al.*, 1985). The African mosquito *Anopheles gambie*, for example, possesses an efficient innate defence system reminiscent of vertebrate innate immunity (Hoffman *et al.*, 1999). mRNA levels of antibacterial peptide defensin, Gram–negative binding protein, a chitinase-like domain-containing protein, serine protease, a lectin-like protein and nitric oxide synthase increase in the midguts of *Anopheles gambie* 24 h post-infection with *Plasmodium berghei* (Dimopoulos *et al.*, 1997, 1998; Richman *et al.*, 1997). The effect of these reactions on parasite survival remains to be fully evaluated, although clear indications exist that some reactions are functionally important. Tsetse flies, on the other hand, have been shown to possess antimicrobial activity (Kaaya *et al.*, 1986; 1987; Hao *et al.*, 2001) and prophenoloxidase cascade (Nigam *et al.*, 1997) in the hemolymph. Apart from phagocytosis and limited aggregation of hemocytes in response to bacterial infections, it appears that cellular defenses are of limited importance in *Glossina*. Croft *et al.* (1982) observed an antitrypanosomal factor in the hemolymph of *G. m. morsitans* and reported a significant

reduction in motility of *T. b. brucei* incubated *in vitro* with hemolymph of teneral and non-teneral *G. m. morsitans*. However, several authors have reported hemocoelic infections of tsetse by *T. brucei* (Mshelbwala, 1972; Otieno, 1973; Kaaya *et al.*, 1986b) and the trypanosomes appear quite healthy and active in the tsetse hemolymph. Furthermore, 2-3% of *G. m. morsitans* support development and maturation of bloodstream form of *T. brucei* when inoculated into their hemocoel. Recent studies have demonstrated that the presence of trypanosomes either in the hemolymph or in the gut early in the infection process does not induce transcription of antimicrobial peptides, attacin and defensin, significantly (Hao *et al.*, 2001). However, after parasite establishment in the gut, both antimicrobial genes are expressed at high levels in the fat body, apparently, not affecting the viability of the parasites in the midgut. Unlike other insect immune systems, the antimicrobial peptide gene diptericin is constitutively expressed in both fat body and gut tissue of normal and immune stimulated tsetse flies, possibly reflecting tsetse immune responses to the multiple Gram-negative symbionts it naturally harbours (Hao *et al.*, 2001).

Although the hemolymph and fat body are at the center of immune response in vector insects, there is increasing evidence that effector molecules expressed in the tsetse midgut play a crucial role in tsetse-trypanosome interaction. The ingestion of a bloodmeal initiates numerous structural, biochemical and developmental changes in the tsetse midgut. These include the formation of a bilayered peritrophic membrane (Peters, 1992; Lehane and Msangi, 1991), secretion of proteases (Imbuga *et al.* 1992), lectins (trypanoagglutinins) (Maudlin, 1991; Stiles *et al.*, 1990), trypanolysins (Stiles *et*

al., 1990; Osir *et al.*, 1999) and maybe other yet unknown factors. The presence of these factors which reach peak levels after 48-72 hours (Stiles *et al.*, 1990; Onyango, 1993; Abubakar *et al.*, 1995; Osir *et al.*, 1999), create a hostile environment for trypanosomes ingested during an infective bloodmeal. Consequently, the majority of the ingested parasites, including the stumpy bloodstream forms that are believed to be preadapted for differentiation (Vickerman, 1985) are eliminated. The few surviving trypanosomes either circumnavigate or penetrate the peritrophic membrane to enter the ectoperitrophic space where they transform and establish themselves into procyclic (midgut) forms (Maudlin, 1985; Maudlin and Welburn, 1987). Midgut infections in tsetse, therefore, are dependent on the ability of the midgut factors to either lyse the parasites or induce their transformation. Accordingly, this has been linked to the quantity of the midgut factors present in susceptible and refractory flies.

In addition to these factors, the type of host blood at the time of an infective feed also plays an important role in determining the infection prevalence in tsetse (Moloo, 1981; Mihok *et al.*; 1993). For example, rat and goat blood have been reported to support transformation of bloodstream *T. b. brucei* into procyclic (midgut) forms, whereas buffalo and eland blood showed intermediate and least capacity, respectively (Nguu *et al.*, 1996). Furthermore, fresh buffalo and waterbuck sera have been shown to contain cytolytic factors that cause lysis of bloodstream trypanosomes (Mulla and Rickman, 1988; Reduth *et al.*, 1992). Such molecules may have a direct contribution to the low infection prevalence in tsetse feeding on these host bloods.

1.2.5 Role of peritrophic membrane in trypanosome establishment

The distention of the midgut caused by ingested bloodmeal in insects signal the formation of a chitinous sac-like structure called the peritrophic membrane (Peters, 1992). It is made of layers of chitin microfibrils on which other proteins, glycoproteins and proteoglycans accumulate to form a sheet-like structure that encases the entire food bolus (Richards and Richards, 1977). With the exception of the *Anopheles stephensi* mosquito, which exclusively contains N-acetyl galactosamine (Berner *et al.*, 1983), most peritrophic membranes are rich in N-acetyl glucosamine.

Two types of peritrophic membranes have been reported in insects (Miller and Lehane, 1993). Type I peritrophic membranes are discontinously secreted from a diffuse secretory area over the length of the midgut. In contrast, Type II peritrophic membranes are continuously produced from a localized area within the proventriculus and thus form a permanent barrier between ingested parasites and the midgut epithelium (Miller and Lehane, 1993). Thus, the higher infection rates in tenerals compared to non-tenerals tsetse flies have been attributed to the development of the peritrophic membrane (Lehane and Msangi, 1991).

In arthropods, the peritrophic membrane appears to serve several functions. It acts as a permeability barrier, allowing the end products of blood digestion to be filtered and freed of undigested particulate components. In addition, it may serve as a protective barrier against blood-borne pathogens or against abrasive particulate matter that might damage the midgut epithelium (Tellam, 1996; Shahabudin, 1998). Indeed, the refractoriness of the *Anopheles atroparvus* mosquito to *Plasmodium falciparum*

parasite has been thought to be due to the inability of the parasite ookinetes to penetrate the fully developed peritrophic membrane of *An. atroparvus*. Conversely, early maturation of *P. berghei ookinetes* before the peritrophic membrane is fully developed renders the *An. atroparvus* mosquito susceptible to this parasite. The barrier-like role of the peritrophic membrane is however, a matter of conjecture. Plasmodium and Leishmania parasites have been reported to possess chitinases, which enable them to traverse the peritrophic membrane barrier (Huber *et al.*, 1991; Shlein *et al.*, 1991; Shahabudin *et al.*, 1993; 1995; Shahabudin, 1998). On the other hand, the African trypanosomes bypass the tsetse peritrophic membrane by escaping through the hindgut where the peritrophic membrane is disrupted, before migrating and multiplying in the ectoperitrophic space. From here, some parasites reach the cardia where they penetrate the immature peritrophic membrane at the site of secretion and hence re-enter the endoperitrophic space and subsequently migrate to salivary glands or proboscis. Similarly, salivarian trypanosomes (*T.b. rhodesiense* and *T. congolense*) have been reported to be capable of active penetration of the peritrophic membrane and gut epithelium cells in the central two-thirds of the tsetse midgut (Evans and Ellis, 1983). This suggests that a fully formed peritrophic membrane may not necessarily be a potential barrier to trypanosome penetration.

1.2.6 Role of midgut proteases in trypanosome establishment

The ingestion of bloodmeal by haematophagous arthropods stimulates the release of a number of digestive proteases (Gooding, 1973; 1974; 1975; Briegel and Lea, 1975; Borovsky, 1985; 1986). In *Glossina* species, at least six proteolytic enzymes have been shown to be involved in the digestion of the bloodmeal (Gooding and Rolseth, 1976; Cheeseman and Gooding, 1985). These include trypsins, trypsin-like enzymes, chymotrypsin, chymotrypsin-like enzymes, carboxypeptidases and aminopeptidases (Cheeseman and Gooding, 1985). The most prominent of these are the serine proteases, trypsins and chymotrypsins (Applebaum, 1985). Recent studies have shown that the trypsin and chymotrypsin genes are expressed constitutively in tsetse, and the regulation of expression occurs at the post-transcriptional level (Yan *et al.*, 2001).

Apart from bloodmeal digestion, midgut proteases play an important role in parasite survival. For example, in *A. aegypti* mosquitoes, the midgut proteases destroy asexual stage parasites and early developing ookinetes of *P. gallinaceum* (Gass, 1977; Gass and Yeates, 1979). The subsequent maturation and transformation of these parasites protects them from the protease activity (Yeates and Steiger, 1981). Serine proteases have also been implicated in activation of the pro-phenoloxidase cascade and subsequent melanization of malaria parasites in refractory strain of *A. gambiae* (Paskewitz *et al.*, 1989; Ashida *et al.*, 1990). In addition, Shahabudin *et al.* (1995) observed that unique specificity of *in vitro* inhibition of mosquito midgut trypsin activity correlated with *in vivo* inhibition of malaria parasite infectivity. Previously, in *Aedes*

aegypti, midgut trypsin was shown to activate the *Plasmodium*-produced chitinase, which the parasite uses to traverse the peritrophic matrix barrier (Shahabudin *et al.*, 1993; 1996). More recent studies in *Anopheles gambie* revealed that the *Plasmodium*-refractory traits expressed higher levels of a serine protease gene (AgSp24D) than susceptible mosquitoes (Han *et al.*, 1997). Likewise, in the sandflies *P. papatasi*, a specific component of trypsin-like activity has been shown to prevent the survival of *Leishmania donovani* while modulation of this component by *Leishmania major* enables it to thrive (Borovsky and Schlein, 1987).

In *Glossina* species, trypsins are released in the posterior midgut and show peak activity between 48-72 hours post-bloodmeal (Abbelle and Declair, 1991; Stiles *et al.*, 1991; Onyango, 1993). These high levels of midgut trypsin activity have been shown to lyse bloodstream form trypanosomes (Imbuga *et al.*, 1992a). Interestingly, bloodstream trypanosomes and trypanosome membranes were also capable of inhibiting the midgut trypsin activity, thus avoiding lysis in susceptible fly species (Imbuga *et al.*, 1992 b). It has been shown that tsetse flies that receive parasites in their first bloodmeal, are more susceptible than those, which receive an infective meal as non-teneral adults (Welburn *et al.*, 1989). Compared to non-teneral flies, teneral midguts were found to have significantly less trypsin activity, which may in turn contribute to their higher susceptibility to parasite infection. On the other hand, optimal trypsin activity in the midgut has been implicated in transformation of bloodstream form trypanosomes to procyclic forms (Yabu and Takayanagi, 1988; Imbuga *et al.*, 1992a). This process is crucial for successful establishment of infection in tsetse. The development of the

procyclic form in the tsetse midgut is marked by the synthesis of a new glycoprotein coat, procyclin, which is highly hydrophilic, is glycosylated and contains an unusual glutamic acid-proline dipeptide (EP) repeats (Roditi *et al.*, 1987; Mowatt and Clayton, 1987). Further studies showed that neither trypsin nor proteinase K could cleave the EP repeats, suggesting that the surface disposition of procyclic forms is important in protecting them from proteases (Richardson *et al.*, 1988). Indeed under *in vivo* conditions, the procyclic trypanosomes thrive and replicates in an environment of high trypsin activity. Perhaps transformation of bloodstream-form trypanosomes to procyclic-form is a means by which the parasites adapts and avoid lysis from trypsin activity prevalent in the tsetse midgut.

1.2.7 Role of midgut lectins in trypanosome establishment

Lectins or agglutinins are proteins or glycoproteins of non-immune origin, which bind carbohydrates reversibly and with high specificity (Lis and Sharon, 1998). Their sources are multifarious in nature, ranging from viruses, parasites and bacteria to plants, vertebrates and invertebrates (Jacobson and Doyle, 1996). Being polyvalent, lectins can bind membrane carbohydrates, glycoproteins or glycolipids causing crosslinking of the cells and subsequent precipitation, a phenomenon commonly referred to as cell agglutination. The specificity of a lectin is usually defined in terms of the monosaccharide(s) or simple oligosaccharides that inhibit the lectin induced agglutination (or precipitation) reactions. Under some conditions, sugar specific enzymes with multiple combining sites agglutinate cells and/or precipitate

glycoconjugates, and therefore act as lectins (Goldstein *et al.*, 1980). On the other hand, toxins are not lectins since they bear only one sugar-binding site and thus do not agglutinate cells or precipitate glycoconjugates (Goldstein *et al.*, 1980).

In insects and other invertebrates, lectins (agglutinins) are speculated to be involved in defensive mechanisms that contribute to cellular and humoral immunity (Gupta, 1979; Ratcliffe *et al.*, 1985; Boman and Hutmark, 1987; Natori, 1990). In the fleshfly, *Sarcophaga peregrina*, a hemolymph lectin triggered by an injury of the cuticle was shown to facilitate recognition and phagocytosis of foreign bodies by macrophages (Takahashi *et al.*, 1986). Later, Lackie and Vasta (1988), Pendland *et al.* (1988), Drif and Brehelin (1989; 1993) and Wheeler *et al.* (1993) demonstrated the involvement of insect agglutinins in nodulation, encapsulation and opsonic activation of fungal blastospores and rabbit erythrocytes. Several other lectins that specifically bind bacterial lipopolysaccharide thus activating the prophenol oxidase humoral response have now been characterized (Jamori and Natori, 1991; Chen *et al.* 1995; Yu *et al.*, 1999).

There is growing evidence that lectin-carbohydrate interactions can mediate the infection of parasites to their insect vectors, and their disruption can affect subsequent establishment of the parasite within the vector. For example, Pereira *et al.* (1981) reported the presence of lectins of distinct carbohydrate specificities in the crop, midgut and hemolymph of the triatomine bug, *Rhodnius prolixus*, and that each lectin was highly specific in interacting with developmental stages of *Trypanosoma cruzi*. Similarly, *Triatoma infestans*, refractory to infection with *Trypanosoma rangeli* produces

an array of tissue and hemolymph lectins, whereas only hemolymph agglutinins are produced by the susceptible insect, *Rhodnius prolixus* (Gregoria and Ratcliffe, 1991). Ibrahim *et al.* (1984) first reported the presence of a glucosamine-binding lectin in tsetse. Evidence for its possible involvement in the establishment of trypanosomes within the vector came from observations that high midgut infections resulted when glucosamine was incorporated into an infective blood meal (Maudlin and Welburn, 1987). This observation led to the conclusion that the normal function of the lectins was to prevent the establishment of the parasites in the fly. The presence of glucosamine in the infective feed relieved the inhibition and, thus, facilitated the establishment of infection. On the other hand, the lectins were also needed for the parasites to differentiate (Maudlin, 1991). Consequently, maintaining flies on a glucosamine-containing diet throughout their life led to the complete inhibition of lectin activity with a concomitant reduction in the number of parasites that established themselves within the fly (Maudlin and Welburn, 1988a; b). Therefore, it appears that in trypanosome-tsetse fly interactions, vector lectins play a dual role; lectins released into the midgut not only lyse trypanosomes and prevent midgut infections but also provide a signal for the differentiation of the established trypanosomes to the procyclic form.

1.2.8 Tsetse endosymbionts

To compensate for their vitamins and nutrient- deficient blood meal diet, most haematophagous insects harbor intestinal symbiotic flora. For example, the triatomine bug, *Rhodnius prolixus*, maintains a symbiotic association with the actinomycete

bacteria *Rhodococcus rhodnii*, which provide the nutrients essential for their growth and development (Dasch *et al.*, 1984). Similarly, tsetse flies harbor three phylogenetically distinct bacterial endosymbionts, which are transmitted maternally to progeny (Beard *et al.*, 1993). *Wigglesworthia glossinidia* is the primary endosymbiont of tsetse, having a defined history of co-evolution with the tsetse host and reside within specialized cells called mycetocytes (Aksoy, 1995). *Wolbachia* spp. on the other hand, have been identified in gonadal and somatic tissues of various insects (Min and Benzer, 1997; Dobson *et al.*, 1999). Similarly, in *Glossina austeni*, *Wolbachia* have been detected in various somatic tissues in addition to the reproductive tissues (Cheng *et al.*, 2000). *Wolbachia* strains are known to be parasitic in insects and enhance their own selfish transmission through sex-ratio distortion and reproductive incompatibility. Cytoplasmic incompatibility is expressed when an infected male mates with an uninfected female resulting in abortive karyogamy. However, infected females can mate productively with both infected and uninfected males (Stouthamer *et al.*, 1993). *Sodalis glossinidae*, hitherto known as rickettsia-like organisms (RLOs), are the secondary endosymbionts of tsetse, residing both inter- and intra-cellularly in a wide range of tsetse tissues including the midgut, hemolymph and fat body (Dale and Maudlin, 1999). Both *Sodalis* and *Wigglesworthia* are considered to have a mutualistic relationship with the tsetse host and their elimination through the application of antibiotics has been reported to induce sterility in laboratory-reared flies (Nogge, 1976).

Susceptibility of *Glossina* spp. to pathogenic trypanosomes has been shown to be a maternally inherited trait (Maudlin and Dukes, 1985) and correlated to the levels of

lectin in the tsetse midgut. This pattern of susceptibility inheritance was found to be associated with the presence of the secondary tsetse endosymbionts, *Sodalis glossinidia* (Maudlin and Ellis, 1985; Welburn and Maudlin, 1988; 1991). During the larval-pupal stage, these endosymbionts have been shown to release an endochitinase, which hydrolyses the chitin associated with the peritrophic membrane. This leads to accumulation of D+ glucosamine sugar, a potent inhibitor of tsetse midgut lectins. Susceptible flies have been shown to have higher numbers of *Sodalis glossinidia* and accordingly, high levels of D+ glucosamine. This results in low lectin activities, which allows the establishment of midgut infection. In contrast, refractory flies have fewer *Sodalis*, and therefore low levels of D+ glucosamine. Subsequently, trypanosomes entering such midguts will encounter high lectin activity resulting in high mortality (Maudlin and Welburn, 1988; Maudlin, 1991). Moloo and Shaw (1989), however, found no causal association between the presence of these endosymbionts within the midgut cells of *G. m. centralis* and susceptibility to *T. congolense* infection. The relationship between the *sodalis* and susceptibility to trypanosome infection was later reported to be quantitative, and not an all-or-nothing effect (Welburn and Maudlin, 1991).

1.2.9 Lectin-trypsin complex

In tsetse flies, the roles of midgut trypsins and lectins appear to be closely related. They are both stimulated by bloodmeal (Abubakar *et al.*, 1995; Van den Abbelle and Decleir, 1991) and inhibited by D-glucosamine (Ibrahim *et al.*, 1984; Osir *et al.*, 1993). Furthermore, midgut lectins like the trypsins have been implicated in the

maturation and lysis of the trypanosomes (Maudlin, 1991; Imbuga *et al.*, 1992a). From the functional perspective, the discovery of a lectin in association with trypsin activity from *Glossina longipennis* by Osir *et al.* (1995) has therefore been intriguing. This molecule, aptly called the lectin-trypsin complex, has been shown to be a glycoprotein (M_r ~ 61 000 Da), comprising of two non-covalently linked subunits. The α subunit (M_r~27 000) had trypsin activity while the β subunit (M_r~33 000) had glycosyl residues (Osir *et al.*, 1995). The native protein was capable of agglutinating both bloodstream-form and procyclic trypanosomes. This activity was inhibited specifically by D-glucosamine. Interestingly, this molecule appeared to be present only within members of *Glossina* species but was absent in other haematophagous insects (Osir *et al.* 1995).

Transformation of bloodstream trypanosomes into procyclic (midgut) forms within the midgut of tsetse appears to be the most crucial step in the establishment of primary midgut infections. Both midgut lectins and trypsins have been postulated to influence this process. Whether trypsin activity alone is sufficient signal for parasites to be committed to transform, or whether lectin activity in the midgut has to come into play, is still not clearly understood. The recent discovery of a bifunctional molecule with both trypsin and lectin activities has augmented the vital role both play in regulating the outcome of trypanosome transmission.

1.2.10 Genetic manipulation of vectorial capacity in tsetse

Despite the central role arthropod vectors play in disease control, relatively little is known about their molecular biology. Modern molecular approaches can now reveal considerable information about gene regulation and expression in vectors. Such knowledge is essential for developing effective control strategies for vector-borne diseases.

Transgenic strategies have become widely used to induce resistance to virus infection in plants and animal host cells. The use of P-element transposon to generate transgenic flies has virtually revolutionized studies of the molecular biology of *Drosophila melanogaster* (Engels, 1989). Olson *et al.* (1996) have also generated mosquitoes that are refractory to dengue-2-virus. When the recombinant Sindbis virus expressing dengue-2-antisense viral message was introduced into female *Aedis aegypti*, replication of the virus was greatly inhibited in the salivary glands. More recently, a stable germline transformation of the malaria mosquito, *Anopheles stephensi* has been accomplished (Catteruccia *et al.*, 2000). Similarly, by using transgenic approaches, a vitellogenin promoter was used to drive the expression of antipathogen peptide gene, *Defensin A*, in the fat body of *Aedes aegypti* mosquitoes (Kokoza *et al.*, 2000). In the Chagas disease vector, *Rhodnius prolixus*, the presence of the endosybiont, *Rhodococcus rhodnii*, has been successfully exploited to express cecropin A, a peptide lethal to the parasite *T. cruzi* (Durvasula *et al.*, 1997). In this system, transformation of the endosymbiont was initially achieved using shuttle

plasmids that contain dual replication origins for the *Rhodococci* and for *E.coli* and a thiostrepton resistance marker (Beard *et al.*, 1992). The coprophagic behavior of the bug was effectively utilized to spread the transgene-carrying symbiont. These studies have shown that it is possible to introduce genetically modified endosymbionts into aposymbiotic bugs (insects that have been raised under sterile conditions) and get stable expression of the marker in insects that have been reconstituted with these bacteria. Furthermore, it has been shown that insects containing genetically modified endosymbionts complete normal development (Durvasula *et al.*, 1997).

Tsetse flies naturally harbor a number of endosymbionts that can be exploited to block the transmission of trypanosomes using the symbiont-based insect transformation approach. Lately, it has been possible to express foreign genes in the transformed tsetse gut endosymbiont, *Sodalis* (Beard *et al.*, 1993). The interuterine tsetse progeny can successfully acquire these *in vitro* manipulated recombinant *Sodalis* when microinjected into the female parent (Cheng and Aksoy, 1999). Administration of streptozotocin, a bacteriocidal analog of N-acetylglucosamine, in the bloodmeal of tsetse has led to reduction in the *Sodalis* numbers in the midgut (Dale & Welburn, 2000). Further experimentation with such dietary analogs could result in the establishment of *Sodalis*-free tsetse flies that can then be reconstituted with the recombinant symbionts, to increase the efficacy of this approach. Since *Sodalis* are found in the midgut, in close proximity to where trypanosomes differentiate and replicate, the expression and secretion of anti-trypanosomal gene products in the recombinant symbionts *in vivo* could distrupt parasite differentiation or establishment in

the gut. Recent characterization of *Wolbachia* in somatic tissues of tsetse has on the other hand, opened up the possibility of expressing antiparasitic gene products directly into this bacterium. The reproductive advantage of infected females over their uninfected counterparts, commonly referred to as cytoplasmic incompatibility, would allow the transformed *Wolbachia* to spread into natural tsetse populations.

1.3 Significance of the study

Establishment of infection in tsetse is dependent on the successful transformation of the parasites within the tsetse midgut. Consequently, elucidation of the factors involved in influencing the process has been the subject of many recent investigations (Overath *et al.*, 1983; Frevert *et al.*, 1986; Maudlin, 1991; Vassella *et al.*, 2000). Studies on monomorphic culture forms of *T. brucei* have shown that treatment of the parasites with trypsin provided the signal for transformation *in vitro* (Yabu and Takayanagi, 1988). Subsequently, a correlation was established between trypsin activity and the ability of tsetse midgut homogenates to induce the transformation of bloodstream trypanosomes (Imbuga *et al.*, 1992a). Whether the removal of the surface coat by trypsin activity alone is a sufficient signal for parasites to be committed to transform, or whether other factors within the midgut also come into play has remained a matter of speculation. More recently, a lectin in association with trypsin activity was identified from *Glossina longipennis* (Osir *et al.*, 1995). Although the ability of this molecule to agglutinate bloodstream and procyclic form trypanosomes has been demonstrated (Osir *et al.*, 1995), its involvement in transformation and establishment of

the parasites in tsetse has not yet been ascertained. The present study will therefore evaluate the role of the lectin-trypsin complex in differentiation of bloodstream trypanosomes to procyclic forms.

Tsetse flies are in general refractory to parasite transmission, although little is known about the molecular basis for the refractoriness. In laboratory infections, transmission rates vary between 1 and 20%, depending on fly species and parasite strain (Moloo and Kutuza, 1988; Moloo *et al.*, 1992), whereas in the field, infections with *T. brucei* spp. trypanosomes is typically detected in less than 1-5% of the fly population (Lehane *et al.*, 2000). Molecular characterization of factors that play a part in determining the success or failure of parasite infections will provide insight on the molecular and biochemical nature of the tsetse-trypanosome interactions. Tsetse species in the various subgenera of *Glossina* exhibit very different vector competence for the transmission of pathogenic trypanosomes. While the genetic basis of vector competence in tsetse is largely unknown, *G. morsitans* subgroup flies have been shown to be highly susceptible, whereas *G. austeni* and *G. fuscipes* flies are increasingly refractory for the transmission of the *T. brucei* complex parasites (Moloo and Kutuza, 1988a; 1988b). The isolation and characterization of lectin-trypsin gene(s) may therefore avail us with molecular markers for identification of potentially susceptible and refractory fly species.

Given the difficulties associated with the tsetse vector management, a completely novel approach that closely integrates a sound knowledge of vector biology and ecology, as well as the inter-relationships between the vectors, parasites and host

animals, is required. One such approach that could form part of an integrated system involves genetic alteration of the tsetse vector in a way that hinders their capacity to transmit disease. This study therefore provides the foundation for any future work involving genetic manipulation of tsetse. It has been proposed that refractoriness to trypanosome infection can be induced through the expression of anti-parasite molecules in the midgut. However, the availability of tsetse genes with well-characterized properties with respect to their species, tissue and stage specific expression is limited. Likewise, our knowledge about factors, which could act either antagonistically or synergistically to effect development of trypanosomes in tsetse, is restricted as well. The current study therefore, constitutes a crucial step towards understanding the molecular characteristics of trypanosome effective genes in tsetse.

1.4 Aims and Objectives

The overall aim of this study is to carry out molecular characterization of the lectin-trypsin complex from the midgut of the tsetse fly, *Glossina fuscipes fuscipes* (Newstead) and evaluate its role in vector management.

The specific objectives are:-

a. To purify the lectin-trypsin complex
b. To ascertain the role of lectin-trypsin complex on transformation of trypanosomes
c. To isolate the cDNA for lectin-trypsin gene(s)
d. To sequence the lectin-trypsin gene(s)
e. To study the expression and properties of the lectin-trypsin gene(s)
f. To find the relationship between the expression of the lectin-trypsin complex gene and the susceptibility of the *Glossina.*

CHAPTER TWO

2.0 MATERIALS AND METHODS

2.1 Reagents and Chemicals

The reagents and chemicals used in this study were of analytical grade and were obtained from BDH, Analar (UK); Aldrich (Dorset, England); Sigma (MO, USA); Serva (Heidelberg, Germany); Pharmacia (Upsalla, Sweden); Bio-Rad (Richmond, CA, USA); BRL (MD, USA); May and Baker (England); Boeringer Mannheim GmbH (Germany); Merck (Germany); Difco laboratories (MI, USA); BRL-Life Technologies Inc. (Gaithersburg, MD, USA); Promega Corp. (Madison, WI, USA.); Clontech (CA, USA) and Stratagene (La Jolla, CA, USA).

2.2 Experimental animals, insects and parasites

The Animal Rearing and Quarantine Unit (ARQU) of ICIPE supplied the male teneral tsetse flies (*Glossina fuscipes fuscipes*). The flies were maintained on a 12 h: 12 h light: dark photoperiod at 25° C and a relative humidity of 75-80%. Male Wistar rats used for feeding the flies and maintaining the parasites, and the New Zealand white rabbits used for raising polyclonal antibodies were supplied by ARQU.

The parasites, pleomorphic bloodstream-form *Trypanosoma brucei brucei*, used in these studies were derived from ILTaT 1.4 (Miller and Turner, 1981). Procyclic culture forms (PCF) were established from the above bloodstream-form by transformation at 27° C. Monoclonal antibodies (mAbs) specific for the major procyclin surface molecule ('EP' forms containing glutamic acid proline repeats) (Richardson *et al.*, 1988) were

obtained from the International Livestock Research Institute (ILRI) and Department of biochemistry and Microbiology, University of Victoria, Canada.

2.3 Preparation of midgut homogenates, parasites and erythrocytes

Tsetse flies (24 h after emergence) were fed on clean rat blood and then starved for 72 h. After immobilising the flies by brief chilling at 4° C, their midguts were dissected and washed several times in phosphate-buffered saline (PBS: 50 mM NaH_2PO_4, 150 mM NaCl, pH 8.0) to remove haemolymph and lipids and then resuspended in ice-cold PBS. The midguts were then homogenised briefly using Virtis homogenizer (Gardiner, USA) and centrifuged (10 000 x g, 4° C, 30 min: eppendorf Centrifuge 5415 C, Bremen, Germany). The resultant supernatant was filtered through a 0.22 μm pore filter (Nalge, Rochester, NY, USA), extensively dialysed against 20 mM Tris-HCl, pH 8.0 and stored at -20° C.

Parasites were obtained by infecting male Wistar rats with pleomorphic bloodstream *Trypanosoma brucei brucei*. Parasitised blood was collected from the rats by cardiac puncture just before peak parasitaemia using heparin as anticoagulant. Trypanosomes were harvested using diethyl aminoethyl cellulose (DE52; Whatman, Ltd, Kent, England) and their numbers estimated using a haematocytometer fitted with improved Neubauer ruling (Lanham and Godfrey, 1970).

Rabbit blood was collected and centrifuged (234 x g, 10 min: SIGMA 3K10 refrigerated centrifuge, Osterode, Germany) to isolate the red blood cells (RBC). After removal of the plasma and the 'buffy coat', the pellet containing the RBCs was washed

five times in phosphate buffered saline (PBS: 50 mM KH_2PO_4, 150 mM NaCl, pH 7.4) by centrifugation (1500 rpm, 10 min, 27° C).

2.4 Purification of the lectin-trypsin complex

A two-step column chromatography procedure, involving ion exchange and affinity chromatography, was used to purify the lectin-trypsin complex from crude tsetse midgut homogenate.

2.4.1 Ion-exchange chromatography

In the first step, diethyl aminoethyl Sepharose CL-6B (DEAE-Sepharose CL-6B; Pharmacia, Uppsala, Sweden) was used as an anion exchanger. The resin, pre-soaked in 20 mM Tris-HCl, pH 8.0 was packed in a Pharmacia XK column (1.6 x 20 cm). The flow rate was adjusted to 10 ml/h. Crude midgut homogenate (~85-100 mg) was loaded onto a DEAE-Sepharose CL-6B column that had been equilibrated in 20 mM Tris-HCl, pH 8.0. Bound proteins were eluted using a 0 - 0.5 M NaCl gradient in 20 mM Tris-HCl, pH 8.0. The gradient was created using a gradient maker G-1 (Pharmacia, Uppsala, Sweden). Fractions (1.5 ml) were collected and absorbance values measured at 280 nm (A_{280}). The collected fractions were assayed for trypsin activity and their ability to agglutinate bloodstream *T. b. brucei*. Fractions with respective activities were pooled and concentrated using polyethylene glycol (PEG).

2.4.2 Affinity chromatography

Affinity column chromatography was used as a second step to further purify the lectin-trypsin molecule. In this case, D(+)glucosamine (D-GlcN, Sigma, St. Louis, MO,

corresponding activities were pooled, concentrated using PEG and dialysed against BIS at 4° C.

The purity of the pooled samples was assessed by polyacrylamide gel electrophoresis (PAGE) using non-denaturing and denaturing conditions. Protein concentration of the samples was estimated by the bicinchonic method using bovine serum albumin as a standard (Pierce, Rockford, IL. USA).

2.5 Trypsin assay

Trypsin activity in the samples was assayed using a chromogenic substrate, carbo-benzoxy-val-gly-arg-4-nitrilide acetate (Chromozym-TRY, Boeringer-Mannheim, FRG) as described by Imbuga *et al.* (1992b). The reaction mixture (950 µl of 100 mM Tris-HCl, pH 8.0; 10 µl eluted fractions) was equilibrated at 30° C for 10 minutes. The reaction was initiated by addition of Chromozym-TRY (40 µmol.) while maintaining the total assay volume at 1.0 ml. Change in absorbance at 410 nm was monitored using a DU 640B spectrophotometer (Beckman, CA, USA). The change in molar extinction coefficient at 410 nm (ε_{410} =8800; Erlanger *et al.*, 1961) was used to determine the amount of substrate hydrolysed; 1 unit trypsin was considered as the amount of enzyme required to hydrolyse 1 µmol. Chromozym-TRY/ min. at 30° C.

Enzyme activity = ((change in A_{410}/ min)/ ε_{410})) * (V/v) * dilution factor

Where v = volume of Chromozym-TRY used in the assay

V = total volume of the assay

USA) was covalently coupled to epoxy-activated Sepharose 6B (Sigma) using a procedure modified by Pharmacia (1986) from the methods of Sundberg and Porath (1974) and Vretblad (1976).

Epoxy-activated Sepharose 6B (~1.0 g) was swollen for 15 minutes and thoroughly washed with 100 ml distilled water on a G1 sintered glass filter (Corning, Straffordshire, UK). The gel suspension (~ 3 ml) was mixed with D(+) glucosamine (35 mg/ ml) pre-dissolved in coupling solution [0.1 M carbonate/ bicarbonate buffer (pH 9): dimethylformamide (50:50)] and incubated in a water bath with a shaker (37° C, 16 h). Excess ligand was washed away with coupling solution, followed by distilled water, carbonate/bicarbonate buffer (0.1 M, pH 8.0) and acetate buffer (0.1 M, pH 4.0). Soaking the gel in 1 M ethanolamine overnight at 45° C in a water bath with a shaker blocked the remaining reactive groups. The coupled gel was finally washed with coupling solution followed by alternate washes with 0.1 M acetate buffer pH 4.0, containing 0.5 M NaCl and 0.1 M borate buffer (pH 8.0) containing 0.5 M NaCl.

The coupled resin was packed in a 10 ml Bio-Rad Econo-Pac column (Bio-Rad, Richmond, CA, USA) and the column equilibrated with buffered insect saline (BIS; 10 mM Tris-HCl, pH 7.9, 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$) at a flow rate of 10 ml/ h. Ion exchange sample that agglutinated trypanosomes (~4.0 mg protein) was loaded on to the affinity chromatography column and unbound proteins were eluted in BIS. Fractions (1.5 ml) were collected using a fraction collector (Bio-Rad model 2128) and absorbance values measured at 280 nm (A_{280}). Bound proteins were eluted from the column in BIS containing 0.2 M D-glucosamine (GlcN). Fractions were assayed for trypsin activity and their abilities to agglutinate bloodstream parasites. Fractions with

2.6 Agglutination assay

Agglutination activity was assayed in flat-bottomed and round-bottomed microtitre plastic wells (Nunc, Denmark) for parasites and erythrocytes, respectively. Two-fold serial dilutions of the samples were made with PBS and mixed with an equal volume of freshly isolated parasites (~5 x 10^6 parasites/ ml) or 2% rabbit red blood cells (10^7 cells/ ml). The plates were then incubated for 2 hours at 27° C and agglutination scored using an inverted microscope (Leitz Dialux, Germany) (Abubakar *et al.*, 1995). Agglutination titres were expressed as the reciprocals of the highest dilutions where complete agglutination of the parasites or RBCs was observed. The controls consisted of parasites or RBCs with buffer. The assays were carried out in triplicate.

2.7 Polyacrylamide gel electrophoresis

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the samples was performed as described by Laemmli (1970). The gradients (4-20% polyacrylamide) were cast using a gradient maker (BRL, Guithersburg,MA, USA), while the stacking gel consisted of 3.13% polyacrylamide. Samples were dissolved in equal volume of loading buffer (0.13 M Tris-HCL pH 6.8, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1% ß-mercaptoethanol) and boiled (100° C, 5 min). The denatured samples were centrifuged (10,000 rpm, 5 min) and the supernatant loaded on to the gel. Running buffer consisted of 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS, pH 8.3). Electrophoresis was carried out at room temperature at a constant current of 25 mA.

Non-denaturing PAGE (native-PAGE) was carried out under the same conditions as SDS-PAGE but SDS and ß-mercaptoethanol was omitted from the buffers. The samples

were also not heated. Electrophoresis was carried out at 4° C with a constant voltage of 100 V. Pharmacia protein standards were used for molecular weight estimation. The molecular weight of the lectin-trypsin complex was estimated from the plots of $\log_{10}$ Molecular weights versus the relative migration of the standards. Gels were stained with 0.6% Coomasie Brilliant Blue R250 (w/v) in 50% methanol (v/v) and 9.2 % acetic acid (v/v) for 2 hours and destained with several changes of destain solution 1 (50% methanol: 9.2% acetic acid: 48.2% distilled water (v/v)) followed by destain solution 2 (5% methanol: 7.2% acetic acid: 87.5% distilled water (v/v)). De-stained gels were preserved in 7% acetic acid.

2.8 Silver staining

Gels were also stained for protein by the modified silver staining method of Wray *et al.* (1981). Proteins were fixed by soaking the gels in fixative (50% methanol, 10% acetic acid (v/v)) for 2 minutes, washed twice in 50% methanol for 10 minutes each and rinsed in distilled water for 5 minutes. The fixed gel was further washed twice in 50% methanol for 10 min each and subsequently incubated in 2.5% aqueous glutaraldehyde for 30 minutes. The gel was then rinsed five times in distilled water for 5 minutes each and washed twice in 50% methanol for 10 minutes each. Silver stain solution was prepared by adding solution A (0.8g $AgNO_3$ in 2.5 ml water) to solution B (1.0 ml of 2 M NaOH, 1.6 ml conc. NH_4OH, to a final volume of 100 ml). The gel was incubated in the silver stain solution for 15 minutes and rinsed 3 times in distilled water for 5 minutes each. Finally, the gel was soaked in the developer (2.5 ml of 1% citric acid, 125 µl of 37-

41% formaldehyde, diluted to 250 ml) until the desired protein band intensity was achieved. Soaking the gel in 5% acetic acid halted the colour development.

2.9 Transformation studies

2.9.1 Role of purified lectin-trypsin complex on parasite transformation

Using specific antibodies against procyclin, the role of the lectin-trypsin complex in inducing transformation was assessed by immunofluorescence (Tolson *et al.*, 1989). Bloodstream-form trypanosomes (~5 x 10^6 parasites/ ml) were incubated with purified lectin-trypsin complex (~4.5 μg) in modified minimum essential medium (PCF medium, GIBCO BRL) at 27 °C. At 4 hour intervals, 0.5 ml aliquots were withdrawn from the incubation mixture, centrifuged and washed once in PBS. Smears were prepared for flourescence microscopy by centrifugation (2000 x g, 5 min) in a cytocentrifuge (Cytospin, Shandon-Southern Products, Astmoor, UK). The controls consisted of bloodstream trypanosomes incubated in PCF medium or PBS.

In a parallel experiment, bloodstream trypanosomes (~5 x 10^6 parasites/ ml) were incubated at 27 °C in PCF medium containing 6 mM cis- aconitate.

2.9.2 Indirect immunofluorescence

Cytospin preparations of trypanosomes on slides were fixed in 100% methanol (-20° C, 20 min) and air-dried. The cells were then blocked with 5% bovine serum albumin (BSA) (27 °C, 20 min), and incubated with anti-procyclin mAbs (27°C, 45 min). Immunofluorescence was performed on transformed parasites with fluorescein-conjugated goat anti-mouse Ig (Sigma, St. Louis, MO) as the secondary antibody

(Tolson *et al.*, 1989). Flourescence was observed using a Zeiss Axiophot 1 microscope equipped with epifluorescence and a 100x oil emmersion fluorescence objective. DNA counterstaining with DNA-binding dye 4'6'-diamidino-2-phenylindole (1 μg/ ml) (DAPI; Molecular Probes, Eugene, OR) was used to observe untransformed parasites.

2.9.3 Effect of inhibitors of lectin-trypsin complex on transformation

The effect of lectin-trypsin inhibitors, on transformation-stimulating capacity of lectin-trypsin complex was investigated. Lectin-trypsin complex (~4.5 μg) was mixed with 100 mM D-glucosamine or 1.0 μg/ ml soybean trypsin inhibitor (STI; Pierce, Illinois, USA). After incubation (4 °C, 30 min), bloodstream parasites were added and its effect on transformation of the trypanosomes was assessed as described in section 2.9.1 and 2.9.2.

2.10 Data analysis

Statistical analyses were performed using the Statistical Analysis System (SAS Institute, 1985). For transformation studies, four groups, each of 100 trypanosomes was used to estimate the mean number of transformed (procyclic-form) trypanosomes. The rate of transformation was analysed by fitting the mean observations into a non-linear model $y = A\ (1\text{-}e^{-bt})$ where b is the rate of transformation and A is the asymptotic value. The rates of transformation were tested for significance using the Students *t-test*. In all cases, rejection of null hypothesis was based on 0.05% probability level.

2.11 Immunological and Molecular studies

2.11.1 Preparation of polyclonal antibodies

A male New Zealand white rabbit was used to raise antibodies against the purified lectin-trypsin complex according to the method described by Osir *et al.* (1989). The rabbit was bled from the marginal ear vein to obtain the preimmune serum. Purified lectin-trypsin complex (~1.0 mg protein) was emulsified in an equal volume of Freund's complete adjuvant (FIC) and injected subcutaneously at several sites of the rabbit's hind thigh. A booster injection (~0.5 mg protein emulsified in Freund's incomplete adjuvant) was given on the other hind thigh on the 8^{th} and 24^{th} day. The animal was bled one week later through the main ear artery.

To obtain antiserum, the fresh blood was allowed to stand for 2 hours at room temperature and then kept overnight at 4° C. The serum was then separated from the clot by centrifugation (234 x g, 20 min: SIGMA 3K10 refrigerated centrifuge, Osterode, Germany) and stored at -20° C.

Crude immunoglobulin mixture was isolated from the serum by ammonium sulphate precipitation. The serum was diluted in equal volume of phosphate buffered saline (PBS, pH 7.2), precipitated in ammonium sulphate (50% saturation) and centrifuged (9800 x g, 4° C, 15 min; Beckman Avanti J-25 centrifuge). The immunoglobulin precipitate was dissolved in PBS (Ig mixture; ~2 mg protein/ ml), dialysed against several changes of PBS buffer and stored in small aliquots (200 µl) at -70° C.

2.11.2 Isolation of RNA from tsetse midgut

Male teneral *G. fuscipes fuscipes* (24 h after emergence) were fed twice on rabbit blood and subsequently starved for 72 h to eliminate vertebrate mRNA contaminants. Total RNA was extracted from the midguts of these flies using an RNA extraction kit (Promega Corp., Madison, WI, U.S.A). The midguts (20) were homogenised in 1 ml pre-chilled denaturing solution (26 mM sodium citrate, pH 4.0, 0.5% n-lauryl sarcosine, 0.125 M β-mercaptoethanol, 4 M guanidine thiocyanate) using a Virtis homogenizer (Gardiner, USA). To extract the RNA, 100 µl of 2 M sodium acetate (pH 4.0) was added to the homogenate and mixed thoroughly by inversion, followed by I ml of phenol: chloroform: isoamyl alcohol (125: 24: 1, pH 4.7), and the contents chilled on ice for 15 minutes. The suspension was then transferred to sterile microcentrifuge tubes and centrifuged (14 000 x g, 4° C, 10 min: eppendorf Centrifuge 5415 C, Bremen, Germany). The top aqueous phase containing RNA was carefully removed and transferred to a fresh tube and an equal volume of isopropanol added. The suspension was mixed by inversion and RNA precipitated by incubating at –70° C for 30 minutes or -20° C overnight. RNA pellet was collected by centrifugation (14 000 g, 4° C, 10 min) and resuspended in 200µl of denaturing solution. The RNA was re-precipitated by adding an equal volume of isopropanol and incubated (–70° C, 30 min). Finally, the RNA pellet was washed twice with 2.5 volumes of pre-chilled 70% ethanol, air-dried in RNase-free environment, and stored at –70° C.

The purity and integrity of the isolated RNA was monitored by agarose gel electrophoresis.

2.11.3 cDNA Synthesis from total RNA

cDNA was prepared from the total RNA using a SMART™ cDNA library construction kit protocol (Clontech, CA, USA).

2.11.3.1 Synthesis of first-strand complementary DNA (cDNA)

The first-strand cDNA was generated using the powerscript ™ reverse transcriptase, a point mutant of Moloney murine leukemia virus (MMLV) reverse transcriptase (RT), which lacks RNase H activity but contains both polymerase and terminal transferase activities. A modified oligo (dT) primer (CDSIII/3' PCR primer) and oligo(G) primer (SMART IV Oligonucleotide) were used. Total RNA (~ 1.0 µg) was mixed with 1.0 µM of each primer and incubated (72° C, 2 min). The contents were then cooled on ice and centrifuged briefly before addition of 1 x first-strand buffer (250 mM Tris, pH 8.3, 30 mM $MgCl_2$, 375 mM KCl), 2.0 mM dithiothreitol (DTT), 0.25 mM dNTPs and 1.0 µl Powerscript RT. The contents (10 µl) were gently mixed, spinned briefly and incubated (42° C, 1 h). Placing the tube containing the reaction mixture on ice terminated the first-strand synthesis. The resultant single-stranded (ss) cDNA was used in PCR reaction to generate double stranded cDNA or stored at -20° C.

2.11.3.2 Generation of double-strand cDNA by Polymerase Chain Reaction

The isolated single-stranded (ss) cDNA was amplified by long-distance polymerase chain reaction (LD-PCR) to generate full-length double-stranded (ds) cDNA (Barnes *et al.*, 1994; Cheng *et al.*, 1994; Chenchik *et al.*, 1998). The reaction mixture (100 µl) contained 1 x Advantage 2 PCR buffer, 0.25 mM dNTPs, 200 pmol of each

primer, 1 x Advantage 2 Polymerase Mix and 2 µl of the isolated first-strand cDNA as template. PCR was performed on a preheated (95° C) PTC-100™ (MJ Research Inc.) under the following cycling program: initial denaturation at 95° C for 20 seconds, followed by 24 cycles of denaturation at 95° C for 5 seconds and annealing at 68° C for 6 minutes. The PCR cDNA product was analyzed by agarose/ EtBr gel electrophoresis.

The PCR cDNA product was subsequently treated with Proteinase K to inactivate DNA polymerase activity. Amplified ds cDNA (2-3 µg) was mixed with 0.8 µg of proteinase K and incubated (45° C, 20 min). The digest was diluted twice with deionized water and cDNA extracted with an equal volume of phenol:chloroform: isoamyl alcohol. The mixture was centrifuged (14 000 x g, 5 min: eppendorf Centrifuge 5415C) to separate the phases, and the top (aqueous) layer was moved to a clean 0.5 ml microfuge tube. cDNA was precipitated with 3 M sodium acetate, glycogen (20 µg/µl) and 95% ethanol at room temperature and immediately centrifuged (14·000 x g, 20 min, 27° C: eppendorf Centrifuge 5415C). The cDNA pellet was washed with 80% ethanol, air-dried (~10 min) and resuspended in nuclease-free deionized water.

2.11.4 Construction of midgut cDNA expression library

cDNA expression library was constructed from the resultant cDNA following the SMART™ cDNA library construction kit protocol (Clontech, CA, USA).

2.11.4.1 Sfi 1 –restriction enzyme digestion

Once the quality of the cDNA was verified by agarose gel electrophoresis, asymmetrical Sfi1 restriction enzyme sites were incorporated at the 5' and 3' cDNA

ends. The isolated cDNA was digested (50° C, 4 h) with 2 units of Sfi 1 restriction enzyme, 1 x Sfi buffer, 1 x BSA, in a total reaction volume of 100µl. 1% xylene cyanol dye was then added to the reaction mixture and mixed.

2.11.4.2 cDNA size fractionation

Sfi1-digested cDNA was then size fractionated using CHROMA SPIN-400 columns (Clontech, CA, USA). The columns were equilibrated with the column buffer (700 µl) at a flow rate of ~ 1 drop/ 40-60 seconds. ~ 100 µl of Sfi 1-digested cDNA containing xylene cyanol dye was applied to the column and allowed to absorb. cDNA was eluted using 600 µl of column buffer and fractions (~35 µl) were collected. The collected fractions were monitored by 1.1% agarose/ EtBr electrophoresis and peak fractions determined by visualizing the intensity of the cDNA bands under UV. The first 3-4 fractions containing cDNA were pooled and precipitated in 1/10 volumes of sodium acetate (3 M; pH 4.8), 0.25 µg/µl of glycogen and 2.5 volumes of 95% ethanol at -20° C overnight. The Sfi 1-digested cDNA pellet was recovered by centrifugation (14 000 x g, 20 min, 27° C eppendorf Centrifuge 5415C), air-dried (~ 10 min) and resuspended in 7 µl of nuclease-free deionized water.

2.11.4.3 Ligation of cDNA to λTriplEx2 Vector

Sfi-digested and dephosphorylated λTriplEx2 vector was provided in the kit. Three parallel ligation reactions were set up to determine the optimal ratio of cDNA to vector required. Various concentrations of the above prepared cDNA (0.5, 1.0 and 1.5 µl) was each mixed in 100 ng/µl vector, 1x ligation buffer, 1.0 mM ATP, 40 units T4 DNA

ligase and the volume made up to 5.0 µl with nuclease-free deionized water. The ligation mixtures were incubated at 16° C overnight. Control reaction containing 10 ng control insert (Sfi 1 A & B) was also carried out.

2.11.4.4 λ-phage Packaging

λ-phage packaging was performed separately for each ligation mixture using Gigapack® III Gold packaging extract according to the manufacture's instructions (Stratagene, La Jolla, USA). Typically, 4 µl of ligation reaction was gently mixed with packaging extract and incubated at room temperature for 2 hours. 500 µl of SM buffer (SM buffer; 5.8g/ l NaCl, 2.0 g/ l $MgSO_4$, 0.01% (w/v) gelatin, 50 mM Tris-HCl, pH 7.5) was added to the incubation mixture followed by 20 µl of chloroform. The contents were mixed gently and spinned briefly to sediment the debris. Finally, the supernatant containing the phage was removed and titered. The efficiency of the Gigapack® III Gold packaging extract was also tested using ~0.2 µg wild-type lambda control DNA (λ*c*1857 *Sam*7) provided.

2.11.4.5 Titering the library

A primary bacterial stock plate was prepared by streaking *E. coli* XL1-Blue glycerol stock onto the LB agar plate (LB agar: 10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 10 g/l NaCl, pH 7.0, 1% agar) (Difco Laboratories, Detroit, MI, USA) containing tetracycline (15 µg/ml) and incubated at 37° C overnight. A working stock plate was then prepared by streaking a single colony from the primary stock plate onto another LB/ tetracycline agar plate supplemented with 10 mM $MgSO_4$. The plate was incubated at

37° C overnight and a single isolated colony picked to inoculate 15 ml of LB/ $MgSO_4$ / 0.2% (w/v) maltose broth. Overnight culture was grown at 37° C with shaking (~140 rpm) to an OD_{600} of 2.0. The cells were then centrifuged (3 000 x g, 5 min) and the pellet resuspended in 7.5 ml of 10 mM $MgSO_4$. Appropriate dilutions (1:5 to 1:20) of each packaged λ lysate were prepared in 1 x lambda dilution buffer (100 mM NaCl, 10 mM $MgSO_4$, 35 mM Tris-HCl, pH7.5, 0.01% gelatin). 1 µl of each dilution was then added to 200 µl of the XL1-Blue overnight culture, and the phage allowed to adsorb to the cells at 37° C for 15 minutes. 2 ml of LB/ $MgSO_4$ top agar (45° C) was added and plated immediately on pre-warmed LB/ $MgSO_4$ agar plates. Viral plaques were monitored for 6-18 hours at 37° C and the titre in plaque forming units per milliliter (pfu/ ml) calculated.

$$\text{pfu/ ml} = \frac{\text{number of plaques x dilution factor x } 10^3 \text{ µl/ ml}}{\text{µl of diluted phage plated}}$$

2.11.4.6 Library Amplification

Seven LB/ $MgSO_4$ agar plates of 530 mm were used to amplify the library. Overnight bacterial culture of XL1-Blue was prepared by inoculating 15 ml of LB/ $MgSO_4$/ maltose broth with a single isolated colony from the primary working plate and incubated with shaking (37° C, ~140 rpm). At an OD_{600} of 2.0, the culture (1500 µl) was aliquoted in 12-ml tubes and incubated with 200µl of 1/20 dilution phage lysate at 37° C for 15 minutes. 13.5 ml of LB/ $MgSO_4$ (45° C) top agar was then added and immediately poured onto the LB/ $MgSO_4$ agar plates and incubated for 6-18 hours at 37° C. When the plaques became confluent, 36 ml of 1 x lambda dilution buffer was added to each

plate and stored at 4° C overnight. Plates were then removed and incubated at room temperature on a platform shaker (~ 50 rpm) for 1 hour before pouring and pooling the λ- phage lysate. Cell debris was removed from the amplified-library lysate by adding 10 ml of chloroform. After vortexing for 2 minutes, the lysate was centrifuged (7 970 x g, 10 min: GSA rotor; Sorval RC-5C centrifuge) and supernatant collected. The titer of the amplified library was determined as described in section 2.9.4.5. Amplified library was mixed with glycerol and stored in 1-ml aliquots at -70° C.

2.11.4.7 Conversion of λTriplEx2 library to a pTriplEx2 plasmid form

λTriplEx2 multiple cloning site (MCS) is located within an embedded plasmid, which is flanked by *loxP* sites at the λ junctions. Transduction of λTriplEx2 lysate into *E. coli* BM25.8 promotes Cre recombinase-mediated release and circularization of the pTriplEx2 at the *loxP* sites (Scheme 2). A working stock plate was prepared by streaking *E. coli* BM25.8 glycerol stock onto LB/ $MgSO_4$ agar plate containing kanamycin (50 µg/ ml) and chloramphenicol (34 µg/ ml). An isolated colony was then picked from the plate and inoculated into 10 ml LB medium to form an overnight BM25.8 bacterial culture. The culture was incubated at 31° C with shaking (~150 rpm) until the OD_{600} reached 1.1-1.4 before adding $MgCl_2$ (10 mM final concentration). An aliquot of λTriplEx2 library lysate was diluted with 1x lambda dilution buffer and the phage allowed to elute at 4° C overnight. An equivalent amount of the overnight BM25.8 cell culture was then added and the suspension incubated at 31° C for 30 minutes without shaking. One volume of LB broth was added and the mixture incubated for an additional 1 hour with shaking (31° C, ~225 rpm). Isolated colonies of pTriplEx2 were obtained by

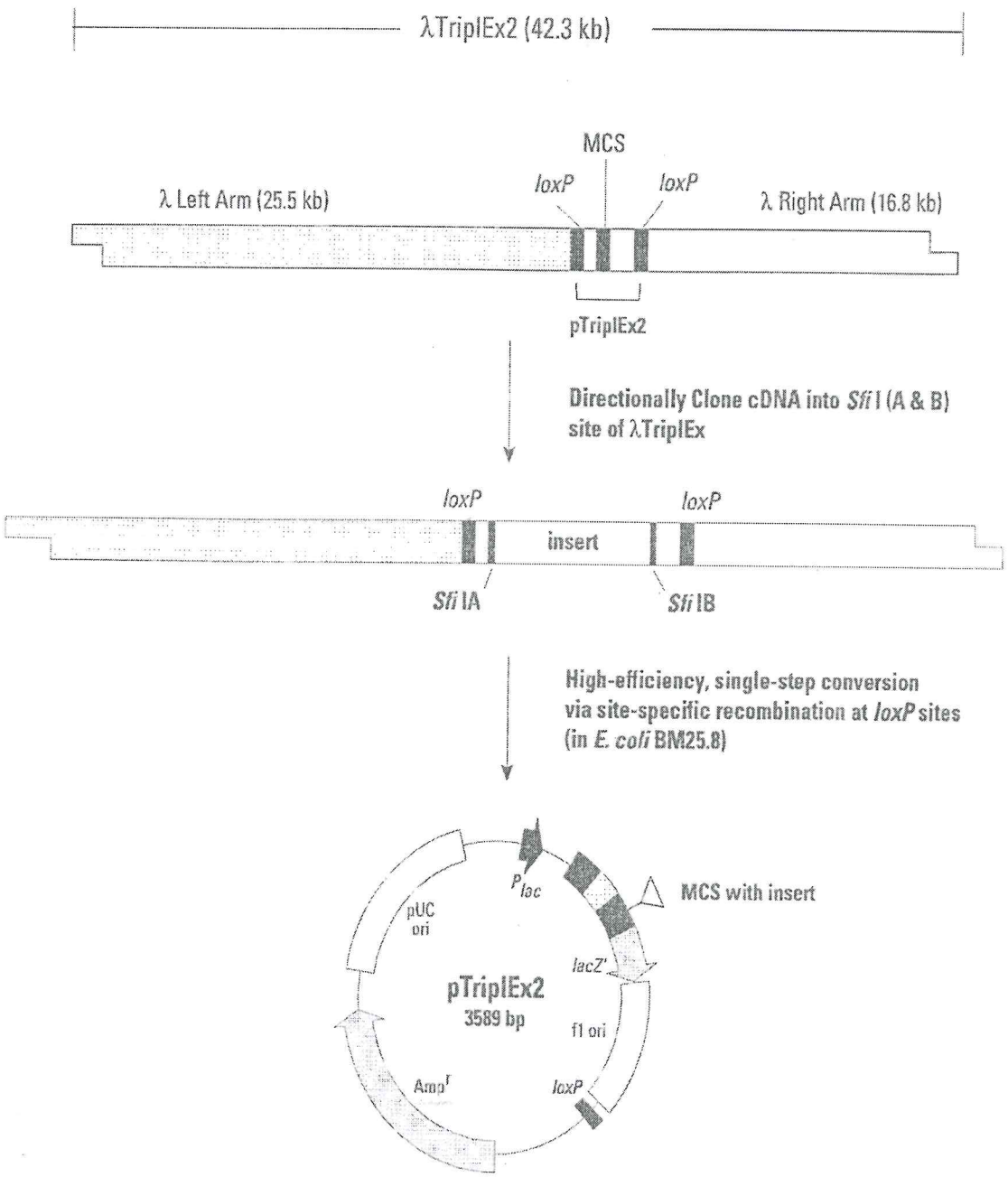
spreading ~1- 10 µl of the infected cell suspension on LB plates containing ampicillin (125 µg/ml).

2.11.5 Screening of the midgut cDNA library with antibody against lectin-trypsin complex

The plasmid form (pTriplEx2) cDNA library was screened with the polyclonal antibodies against the lectin-trypsin complex according to Sambrook *et al.* (1989). Briefly, the serum was preadsorbed with *E. coli* lysate at 1:10 dilution to reduce nonspecific binding to bacterial proteins. The preadsorbed serum was further diluted to a working dilution of 1:500. The library (in *E.coli* strain BM25.8) was plated on an agar plate containing ampicillin and ~ 4,000–5,000 colonies were transferred to nitrocellulose (Hybond C, Amersham, Cleveland, OH). The filters were blocked with 5% non-fat dry milk and incubated overnight with midgut lectin-trypsin antibody. After washing, the filters were incubated with goat anti-rabbit conjugated alkaline phosphatase secondary antibody (Bio-Rad, CA, USA) and positive clones were visualized by incubating with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) substrates (Sigma, MO, USA).

Scheme 2. Conversion of a recombinant λTriplEx2 to the corresponding pTriplEx2.

The λTriplEx2 multiple cloning site (MCS) is located within an embedded plasmid, which is flanked by *lox*P sites at the λ junctions. Transduction of a λTriplEx2 lysate into *E. coli* strain BM25.8 promotes Cre recombinase-mediated release and circularization of pTriplEx2 at the *lox*P sites. pTriplEx2 carries the *bla* gene for ampicillin resistance and the pUC ori for autonomous replication in *E. coli*. The MCS provides several unique restriction sites flanking the *Sfi* I A & B sites to facilitate the subcloning and analysis of inserts. In addition, every cDNA inserted into the MCS is expressed in all three reading frames (SMART™ cDNA Library Construction Kit User Manual, 2001).


λTriplEx2 (42.3 kb)
MCS
loxP
loxP
λ Left Arm (25.5 kb)
λ Right Arm (16.8 kb)
pTriplEx2
Directionally Clone cDNA into Sfi I (A & B) site of λTriplEx
loxP
loxP
insert
Sfi IA
Sfi IB
High-efficiency, single-step conversion via site-specific recombination at loxP sites (in E. coli BM25.8)
P lac
MCS with insert
pUC ori
lacZ'
pTriplEx2
3589 bp
f1 ori
Amp^r
loxP

2.11.6 Small scale plasmid purification (mini-prep) by alkaline lysis

Plasmids from positive clones were extracted using QIAprep Spin Miniprep kit (Qiagen, GmbH) following the instructions from the supplier. Briefly, 5 ml overnight cultures of *E.coli* carrying the plasmid were prepared by inoculating a single positive colony in L B medium (LB medium: 10 g/l Bacto-tryptone, 5 g/l Bacto-yeast extract, 10 g/l NaCl, pH 7.0) containing ampicillin (125 µg/ml final concentration). Bacterial cells were then pelleted by centrifugation (10,000 x g, 5 min: eppendorf Centrifuge 5415 C) and resuspended in 250 µl of buffer P1 containing RNase A. 250 µl of buffer P2 was added to the cell suspension and mixed gently by inversion. The lysate was neutralized by addition of 350 µl of Buffer N3 and mixed thoroughly by inversion. To separate the plasmid DNA from chromosomal DNA, the solution was centrifuged (12 000 x g, 10 min) and supernatant applied on QIAprep column membrane. Bound plasmid DNA was washed with buffer PB (500 µl), followed by buffer PE (750 µl) and finally eluted in nuclease-free water (50 µl).

2.11.7 Large scale plasmid purification (Maxi-prep) by alkaline lysis

Large-scale plasmid DNA was prepared by the method adapted from Birnboim and Dolly (1979). A single colony carrying the positive plasmid was inoculated in 250 ml LB medium with ampicillin (125 µg/ml final concentration) and incubated overnight with vigorous shaking (37° C, ~200 rpm). Bacterial cells were harvested by centrifugation (5 856 x g, 4° C, 10 min: GSA rotor; Sorval RC-5C centrifuge). The pellet was resuspended in 10 ml solution 1 (Solution 1; 50 mm glucose, 10 mM EDTA, 25 mM Tris-HCl, pH 8.0) and incubated at room temperature for 5 minutes. Twenty ml of freshly

prepared solution 2 (Solution 2; 0.2 N NaOH, 1% SDS) was added and the contents mixed gently by swirling and incubated (10 min, 4° C). The lysed mixture was neutralized by adding 15 ml of ice-cold solution 3 (Solution 3; 3 M potassium acetate, 11.5% glacial acetic acid), mixed and incubated (10 min, 4° C). The bacterial debris was then pelleted by centrifugation (16 266 x g, 20 min, 4° C: Sorval RC-5C centrifuge). The supernatant was carefully transferred to a fresh tube and 0.6 volumes of isopropanol added to precipitate the nucleic acids. The contents were mixed by inversion and allowed to stand for 15 minutes at room temperature. Nucleic acids were pelleted by centrifugation (16 266 x g, 30 min, room temp: Sorval RC-5C centrifuge). The supernatant was discarded, and pellet washed in 70% ethanol and the DNA subsequently dissolved in T.E buffer (T.E buffer; 10 mM Tris-HCl, 1 mM EDTA, pH 8.0).

The isolated plasmid DNA was purified by equilibrium centrifugation using cesium chloride (CsCl) - ethidium bromide (EtBr) gradient. 10 g CsCl and 200 µl EtBr (10 mg/ ml) were dissolved in 10 ml of isolated DNA solution and transferred into quick-seal ultracentrifuge tubes (Beckman). Pairs of tubes were balanced within 0.01 g before they were heat-sealed and centrifuged (356 230 x g = 55 000 rpm, 16-22 h, 20° C: 65Ti rotor; Beckman L8-70M ultracentrifuge). Two bands could be visualized and the lower band (supercoiled plasmid DNA) was collected using an 18-gauge needle and syringe into a fresh tube. An equal volume of CsCl-saturated isopropanol or water-saturated butanol was mixed with the plasmid DNA solution and the layers left to settle at room temperature. The upper layer containing ethidium bromide was collected and discarded. This procedure was repeated until all the ethidium bromide had been extracted. The plasmid DNA was precipitated by adding three volumes of 70% ethanol and the

contents left to incubate at room temperature for 30 minutes. Precipitated DNA was pelleted by centrifugation (27 216 x g, 15 min, 4° C: Beckman Avanti J-25 centrifuge) and finally dissolved in 200-500 µl TE buffer. DNA concentration was calculated from absorbance at 260nm multiplied by 50.

2.11.8 Transformation of *E.coli*

The selected positive plasmid clones (in *E.coli* strain BM25.8) were purified and transformed into JM109 *E. coli* strain. Frozen JM109 High efficiency competent cells were removed from –70° C storage and thawed on ice immediately before the transformation procedure. Plasmid DNA (~5 µl) was added to the cells (100 µl) and incubated on ice for 30 minutes. Uptake of the DNA was facilitated by a heat shock at 42° C for 45-50 seconds followed by 2 minutes on ice. 500 µl of NZY+ broth [10 g/l NZ amine (casein hydrolysate), 5 g/l yeast extract, 5 g/l NaCl, 12.5 mM $MgCl_2$, 12.5 mM $MgSO_4$, 20 mM glucose] was then added and the cells incubated at 37° C for 1 hour with shaking. Approximately, one-fifth of the transformation mixture was spread on Agar/LB/Ampicillin plates and incubated overnight at 37° C. Plasmid DNA was extracted from the JM109 colonies as described (section 2.9.6) and subjected to automated sequencing on an ABI Prism 371 DNA.

2.11.9 Agarose gel electrophoresis

The integrity of isolated RNA was analyzed by electrophoresis in 1.4% (w/v) agarose gel (BRL-Life Technologies, Gaithersburg, MD, USA). RNA sample (~10 µl) was mixed in 2 µl of 6x loading buffer (60 mM sodium phosphate, pH 6.5; 30 % (v/v)

glycerol, 0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 1.2% SDS) and heated at 75° C for 5 minutes before loading on the gel. Electrophoresis was carried out in 10 mM sodium phosphate buffer, pH 6.8 containing 0.4 µg/ml ethidium bromide, at 3-7 V/cm with buffer recirculation (Pellé and Murphy, 1993). RNA markers (Promega) were similarly treated and run alongside the test RNA to verify the size of major ribosomal bands.

DNA samples were analyzed by electrophoresis in 1% (w/v) agarose gel containing 1x TAE buffer (TAE; 40 mM Tris-acetate, 2 mM EDTA, pH 8.0) with 0.4 µg/ml ethidium bromide (Sambrook *et al.*, 1989). DNA markers were run alongside the test DNA to aid in identifying the size of the separated DNA. The RNA and DNA were visualized under UV light.

2.11.10 Expression of lectin in *E. coli*

pTriplEx2 has the *E. coli* lac promoter and operator to provide regulated expression of inserts in *E. coli* hosts expressing the lac repressor (Scheme 2). Furthermore, the 5'untranslated region (UTR) from the *E. coli ompA* gene stabilizes the mRNA, thereby increasing expression. To generate the recombinant protein for functional assay, the positive clones (in *E. coli* strain JM109) were cultured in LB medium containing 125 µg/ml Ampicillin and over-expression of the protein induced using isopropylthio-β-D-galactosidase (IPTG, 1mM final concentration). The cells were centrifuged (4000 g, 10 min) and resuspended in buffered insect saline (BIS: 10 mM Tris-HCl, pH 7.9, 130 mM NaCl, 5 mM KCl and 1 mM $CaCl_2$). After brief sonication on

ice (Soniprep 150, MSE Scientific instruments, Sussex, England), the lysate was centrifuged (10,000 x g, 15 min, 4° C) and the resultant supernatant stored at 20° C.

The recombinant protein was purified from *E. coli* extracts by D-glucosamine affinity column chromatography as described (section 2.4.2). Eluted protein was analyzed by 4-20% SDS-PAGE and functional studies performed as previously described (section 2.5-2.8).

2.11.11 Immunoblotting

A modified Western blot procedure of Towbin *et al.* (1979) was used. Proteins were separated on SDS-PAGE and transferred to a nitrocellulose membrane (Hybond C, 0.2 µm, Amersham). The gel and membrane were sandwiched between sheets of Whatman 3 MM filter paper previously soaked in transfer buffer (48 mM Tris-HCl, pH 8.3, 29 mM glycine, 20% methanol (v/v), 0.037% SDS). Proteins were transferred to the membrane at a constant current of 0.8 mA per cm^2 for 2 h at 27° C using a Novablot 2117 Multiphore II electrophoresis transfer unit (Pharmacia, Uppsala, Sweden). Success of the transfer was ascertained by staining with Rouge Ponceau (0.5% (w/v) in 1% (v/v) glacial acetic acid). The membrane was destained in distilled water and rinsed briefly in Tris- buffered saline (TBS; 25 mM Tris, 137 mM NaCl, 3 mM KCl, pH 7.4) containing 0.3% (v/v) Tween-20. Blots were subsequently blocked in blocking solution (5% non-fat dry milk in TBS) for 2 hours at room temperature and incubated overnight with lectin-trypsin antibody (1: 500) at 4° C. Unbound antibody was removed by washing the blots twice with TBS/Tween for 10 minutes each. After washing, the filters were incubated with goat anti-rabbit conjugated alkaline phosphatase secondary

antibody (Bio-Rad, CA, USA) for at least I hour. Blots were washed again thrice with TBS/Tween, 10 minutes per wash and positive bands were visualized by incubating in the dark with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) substrates (Sigma, MO, USA). The membrane was immersed in distilled water to stop further colour development.

2.11.12 Degenerate oligonucleotide primer-PCR

Two degenerate primers were designed to amplify the tsetse trypsin gene. The primers were designed based on: *A. gambiae* mRNA for serine protease, *A.aegypti* mRNAs 3A1 & 5G1 for putative trypsins, *A. aegypti* gut-specific, bloodmeal-induced late trypsin precursor gene, *A. aegypti* late trypsin precursor gene and *D. melanogaster* serine protease precursor mRNA. Polymerase chain reaction was performed using the previously synthesised midgut cDNA as template. The PCR mixes (25 µl) contained 100 pmoles of each primer, 0.2 mM dNTPs, 2.5 mM $MgCl_2$, 1 unit Taq polymerase, 1 x reaction buffer and 20 ng template cDNA. The cycling programme was:

1. Initial DNA denaturation at 94° C for 2 minutes
2. Denaturation at 94° C for 1minute
3. Annealing of the primers to DNA template at 50° C
4. Extension at 72° C for 2 minutes
5. Repeat steps 2 to 4 for 40 cycles
6. Final extension at 72° C for 5 minutes

The PCR product (~5 µl) was analysed by electrophoresis on 1% agarose gel.

2.11.13 Cloning of PCR product in a TA vector (pGEM-T)

Using a shotgun strategy, the PCR product was cloned in pGEM-T vector (pGEM®-T Easy Vector Systems kit, Promega Corp., Madison, WI, U.S.A). Ligations were performed in a total volume of 10µl. PCR product (2 µl) was mixed in 1 x Rapid ligation buffer, 50 ng pGEM-T vector and 3 Weiss units of T4 DNA ligase and the volume made up to 10 µl with sterile water. Reactions were incubated at 4° C overnight. Control reactions containing control insert DNA or pGEM-T vector alone were also carried out.

Transformation of the ligation mixture was carried out using JM109 high efficiency competent cells. 100 µl cells were mixed with the ligation reaction and incubated on ice for 30 minutes. After 1 minute heat shock at 42° C, the cells were incubated in 500 µl NZY broth (10g/l NZ amine, 5g/l yeast extract, 5g/l NaCl) at 37° C for 1 hour. 100 µl of IPTG (isopropylthio-β-D-galactosidase; 100 mM) and 20 µl of X-Gal (5-bromo-4-chloro-3-indoyl-β-D-galactoside; 50 mg/ ml) was spread on LB-Ampicillin (125 µg/ ml) agar plates. Transformation culture was then applied on the plates and incubated at 37° C overnight. Recombinant clones were identified by blue: white screening of the colonies. Positive colonies were streaked on another LB-agar plate and subsequently inoculated into 250 ml LB medium for preparation of plasmid maxi-prep.

2.11.14 Probe labeling by random priming

A positive recombinant clone, which was homologous to serine protease trypsin family, was used to construct a trypsin cDNA probe. The DNA was labeled using DIG-

High Prime Labelling and Detection Kit (Boeringer Mannheim) according to the manufacturer's protocol. A mixture of random hexanucleotides that anneal on multiple sites along the length of DNA to be labeled was used. The primer-template complex formed acts as substrate for the klenow fragment of DNA polymerase 1, which lacks the 5'-3' exonuclease activity but catalyses the synthesis of new DNA by incorporating DIG-dUTP at the free 3' hydroxyl group of the primer (Feinberg and Vogelstein, 1983). 16 µl (~ 1µg) of DNA diluted in sterile distilled water was denatured by heating in a boiling water bath for 10 minutes and quickly chilled on ice. 4 µl of DIG-High prime mix [(1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP, 0.35 mM alkali-labile DIG-II-dUTP, 1 U/µl labeling-grade Klenow enzyme, 5x reaction buffer) in 50% (v/v) glycerol] was added to the denatured DNA, mixed and centrifuged briefly. The reaction mixture was then incubated at 37° C overnight. Heating the sample at 65° C for 10 minutes terminated the labeling reaction. The digoxigenin-labeled probe was stored at –20° C.

2.11.15 Screening of the midgut cDNA library with trypsin DIG-labeled probe

The pTriplEx2 library (in *E.coli* strain BM25.8) was plated on an agar plate containing ampicillin and ~ 5,000 colonies were transferred to nitrocellulose paper (Hybond N, Amersham, Cleveland, OH). The membrane disc was placed on filter paper (Whatman 3MM) and soaked with denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 15 minutes to denature the immobilized DNA. The membrane was blotted briefly and neutralized by placing on filter paper soaked in neutralization solution (1.0 M Tris-HCl, pH 7.5; 1.5 M NaCl) for 15 minutes. The membrane was blotted briefly and placed for 10 minutes onto a filter paper soaked with 2 x SSC buffer (0.3 mM NaCl, 30 mM sodium

citrate, pH 7.0). The transferred DNA was then fixed to the membrane by baking at 80° C for 30 minutes to 2 hour.

2.11.16 Hybridization and detection

The DIG-labeled DNA probe was used for colony hybridization following the DIG-Easy Hyb system procedure (Boeringer Mannheim). The DNA membrane filter was prehybridized in DIG Easy Hyb solution (10 ml/ 100 cm^2 filter) at 42° C for 1 hour to block non-specific nucleic acid binding sites. The DIG-labeled probe was denatured by boiling for 5 minutes at 95-100° C, rapidly cooled on ice and added to DIG Easy Hyb solution at a concentration of 10-15 ng/ ml. The filters were hybridized at 50° C overnight. Hybridized membranes were washed twice, 5 minutes each, in 2 x SSC (0.3 mM NaCl, 30 mM sodium citrate, pH 7.0) containing 0.1% SDS at room temperature and twice, 15 minutes each, in 0.5 x SSC, 0.1% SDS at 68° C with gentle agitation. Positive colonies were detected using 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) and nitroblue tetrazolium (NBT) colorimetric detection reagents according to DIG system User's manual (Boehringer- Mannheim). After hybridization and post hybridization washes, the membranes were equilibrated in washing buffer (100 mM Maleic acid, 150 mM NaCl, pH 7.5, 0.3% Tween 20) for 1 minute followed by blocking in blocking solution [1% (w/v) Blocking reagent dissolved in maleic acid buffer (100 mM Maleic acid, 150 mM NaCl; pH 7.5)] for 2 hours. The membranes were incubated for 30 minutes in anti-digoxigenin-AP antibody solution diluted in blocking solution (1: 5 000) to a working concentration of 150 mU/ml. The membranes were washed twice for 15 minutes each in washing buffer to remove unbound antibodies and then incubated in

detection buffer (100 mM NaCl, 100 mM Tris-HCl, pH 9.5). ~10 ml of freshly prepared colour substrate solution [45 µl NBT (75 mg/ml) and 35 µl BCIP (50 mg/ ml) in 10 ml detection buffer] was added to the membranes and incubated in the dark. Once the desired spots were detected, the membrane was washed with sterile distilled water to stop colour development.

2.11.17 Database search and sequence analysis

Sequence analysis against databases was performed using the BLAST search program in National Center for Biotechnology Information (NCBI, Bethesda, MD). Sequence alignment and statistical analysis of alignment were performed with MultAlign program (Corpet, 1988).

CHAPTER THREE

3.0 RESULTS AND DISCUSSION

3.1 Role of lectin-trypsin complex in transformation of trypanosomes

3.1.1 Purification of the lectin-trypsin complex

The lectin-trypsin complex was purified using a two-step procedure involving anion-exchange and affinity chromatography. Of the four peaks obtained by anion-exchange chromatography, peak III fractions (eluted at ~ 42% of NaCl gradient) gave the highest agglutination titre (256) and trypsin activity of 10.4 x 10^{-2} μmoles/ml/ minute (Fig. 1). The unbound fractions (peak 1) agglutinated the parasites with a titre of 8 and trypsin activity of 2.15 x 10^{-2} μmoles/ml/ minute. No agglutination was observed in samples from peaks II, IV and V.

The affinity chromatography elution profile showed two peaks (Fig. 2). Samples from bound fractions (peak II) agglutinated bloodstream trypanosomes at a titre of 64 with a corresponding trypsin activity of 1.78 x 10^{-2} μmoles/ml/ minute, while samples from unbound fractions (peak I) had an agglutination titre of 4 and trypsin activity of 0.184 x 10^{-2} μmoles/ml /minute.

Electrophoresis of the purified lectin-trypsin complex under non-denaturing conditions gave a single band with a native molecular weight of ~65, 700 ± 123 Da (n=3) (Fig. 3, lane 2). On SDS-PAGE, the agglutinin gave two prominent bands with molecular weights of ~28, 800 ± 277 Da (n = 3) and ~35, 700 ± 425 Da (n = 3) (Fig. 4, lane 3).

Fig. 1. Anion-exchange chromatography.

Fed tsetse crude midgut homogenate (85-100 mg protein) was loaded onto a DEAE-Sepharose CL-6B column. Unbound proteins (peak I) were eluted in 20 mM Tris-HCl, pH 8.0, while the bound fractions were eluted using a linear gradient 0 - 0.5 M NaCl (--). Fractions (1.5 ml) were collected and absorbance values measured at 280 nm. The highest agglutination activity was in peak III (arrow).

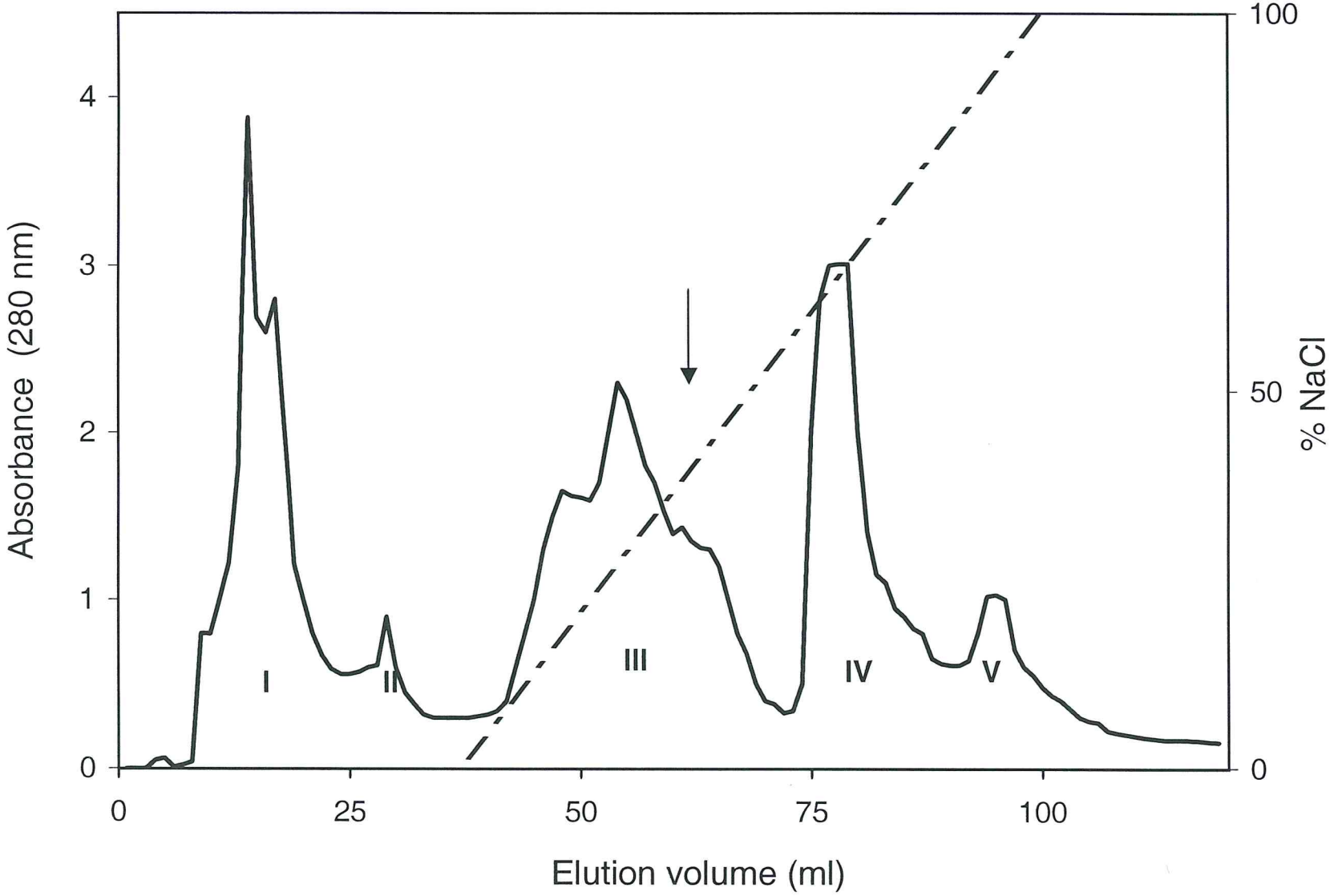

Absorbance (280 nm)
% NaCl
Elution volume (ml)
0
1
2
3
4
0
50
100
0
25
50
75
100
I
II
III
IV
V

Fig. 2. Affinity chromatography.

Peak III fractions from ion-exchange column were loaded onto an affinity column (D+) glucosamine-Sepharose. Bound proteins were eluted using 0.2 M D-GlcN (Peak II). Fractions (1.5 ml) were collected and assayed for both trypsin and lectin activities.

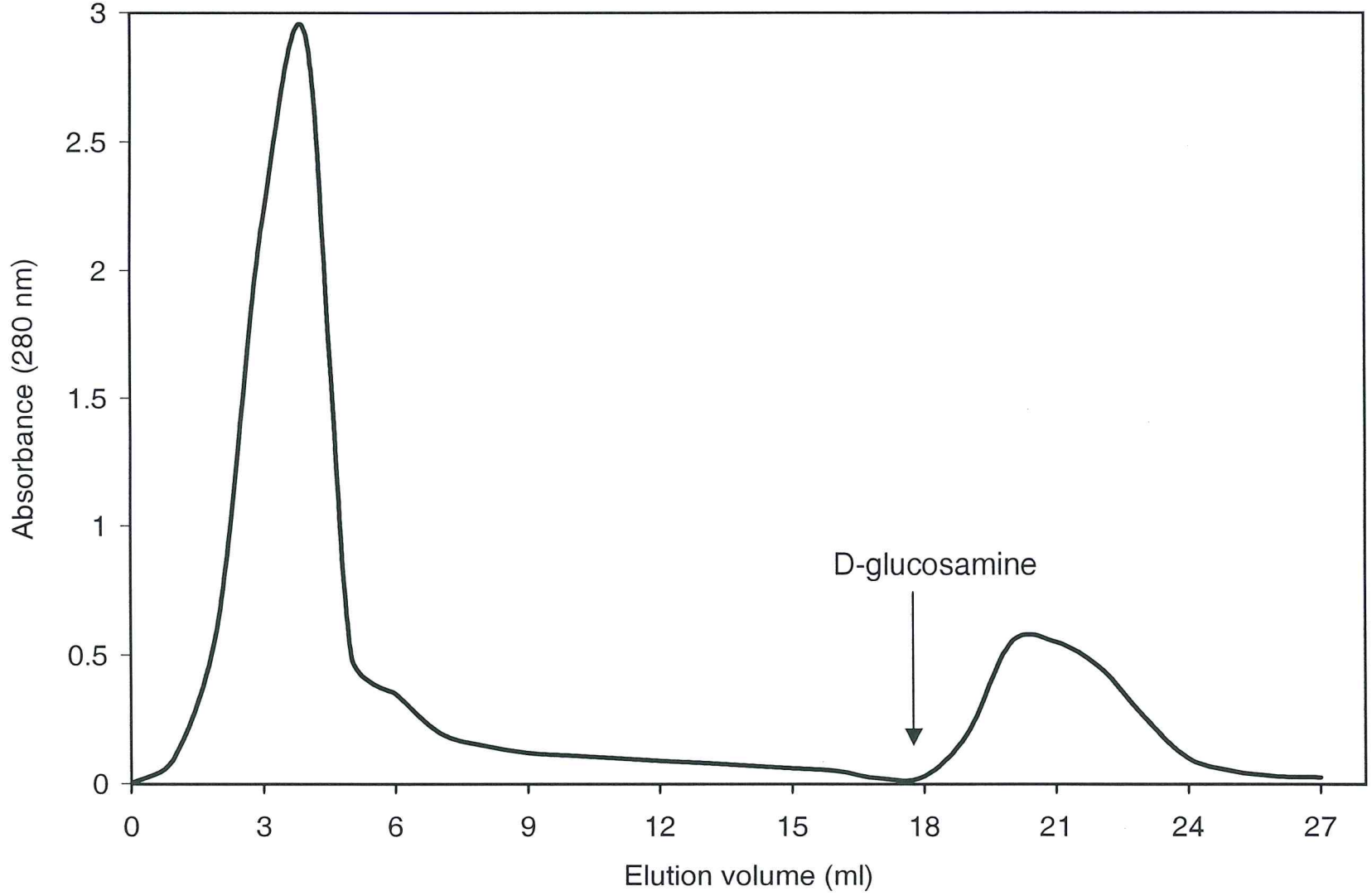
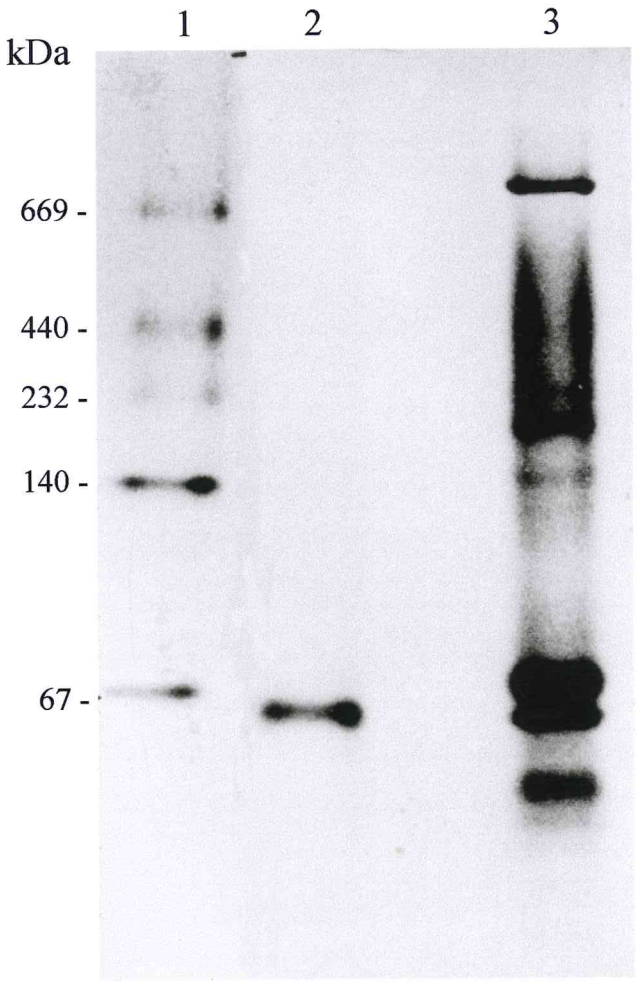

Absorbance (280 nm)
3
2.5
2
1.5
1
0.5
0
0
3
6
9
12
15
18
21
24
27
Elution volume (ml)
D-glucosamine

Fig. 3. Non-denaturing gel electrophoresis.

Protein samples were separated by non-denaturing gradient PAGE (4-15%).

Lane 1- High molecular weight standards (Pharmacia); 2- lectin-trypsin complex (~ 9 μg); 3- crude midgut extract (~ 50 μg).


1
2
3
kDa
669 -
440 -
232 -
140 -
67 -

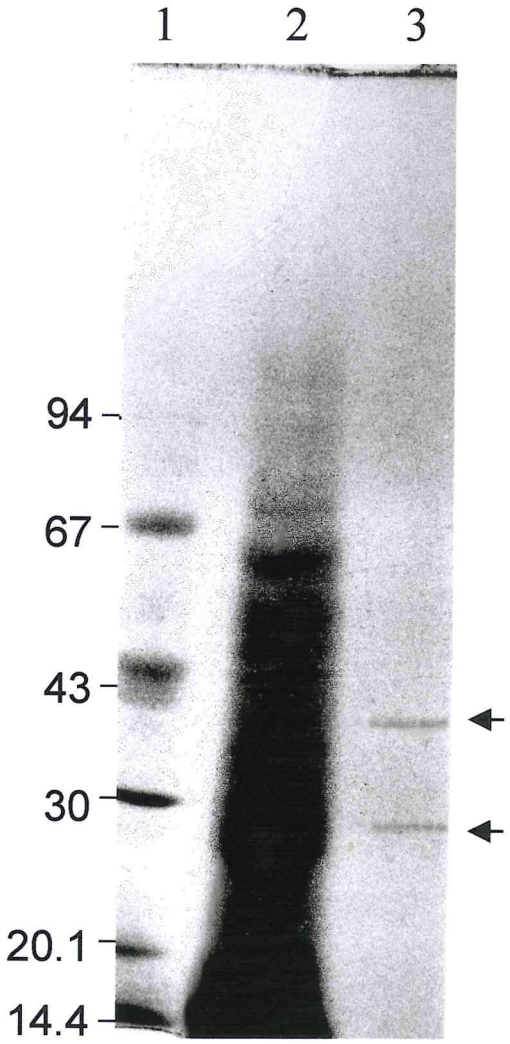

1
2
3
94
67
43
30
20.1
14.4

Fig. 4. Dissociating polyacrylamide gel electrophoresis.

Protein samples were separated by gradient SDS- PAGE (4-15%). Lane 1- Low molecular weight standards (Bio-Rad); 2- crude midgut extract (~40 μg); 3- lectin-trypsin complex (~4.5 μg).

3.1.2 Induction of trypanosome transformation by lectin-trypsin complex

The role of lectin-trypsin complex in transformation of bloodstream trypanosomes was assessed *in vitro*, using indirect immunoflourescence assay. Procyclic-forms surface protein, procyclin, was detectable after 4 h incubation of bloodstream-forms *T. brucei* with the purified lectin-trypsin protein at 27° C (Fig. 5 a). By 12 hours, there was increase in flourescence with ~60% of the trypanosomes expressing the procyclin protein (Fig. 5 b). No bloodstream-form trypanosomes were detectable in the incubation mixture after 24-30 hours. In comparison, bloodstream-form trypanosomes incubated in *cis*-aconitate supplemented procyclic medium started expressing procyclin only after 7 hours incubation. However, by 15 hours, the number of trypanosomes expressing procyclin was the same as those in the lectin-trypsin incubation mixture (Fig. 5 c). No transformation was observed with parasites incubated in PBS or PCF medium only (Fig. 5 d).

Based on the perfectly fitted logistic curve, the rate of transformation of the parasites induced by lectin-trypsin complex was 0.066 at the asymptotic value of 95.87% (Fig. 6). On the other hand, the rate of transformation induced by cis-aconitate was 0.0468 at the asymptotic value of 100% (Fig. 6). There was no significant difference between the rate of transformation observed when the parasites were incubated with lectin-trypsin complex or cis-aconitate (t-test, $p > 0.05$).

Fig. 5. Immunoflourescence microscopy of procyclic *T. brucei*.

Bloodstream-form *T. brucei* were incubated for various lengths of time with lectin-trypsin complex (~4.5 μg protein) and fixed in 100% methanol (-20° C, 20 min). Transformed parasites expressing procyclin were detected using monoclonal anti-procyclin antibody (EP) and flourescein isothiocyanate-coupled anti-mouse secondary antibody. Non-transformed bloodstream parasites were detected by DNA counterstaining using 1 μg ml^{-1} DNA-binding dye 4'6'-diamidino-2-phenylindole (DAPI).

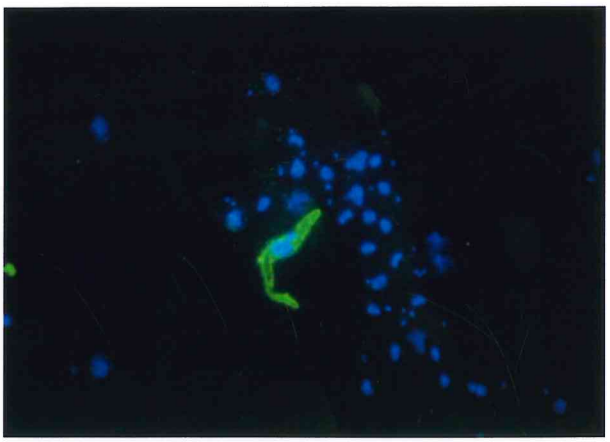
(a) After 4 hours incubation with lectin trypsin complex

(b) After 12 hours incubation with lectin trypsin complex

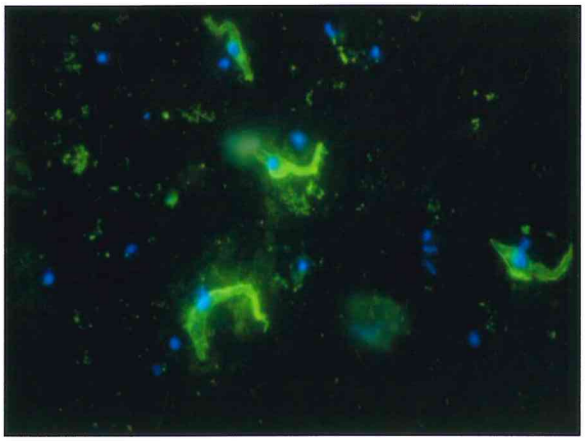
(c) After 15 hours incubation with *cis*-aconitate

(d) After 24 hours incubation (untreated parasites)

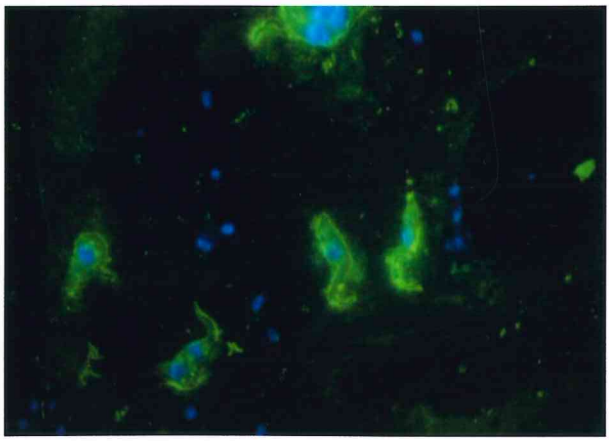
a



b



c



d

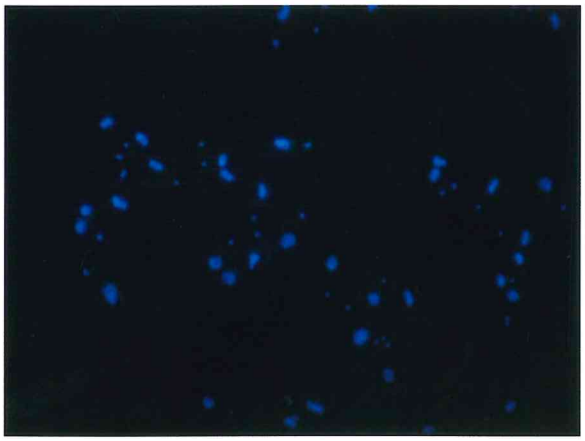


Fig. 6. Stimulation of trypanosome transformation by lectin-trypsin complex and *cis*-aconitate.

Bloodstream trypanosomes (~5 x 10^6 parasites/ ml) were incubated with either purified lectin-trypsin complex (~4.5 μg protein/ ml) or *cis*-aconitate (6 mM) in modified minimum essential medium at 27 °C. At 4 hour intervals, 0.5 ml aliquots were withdrawn from the incubation mixture and the transformed parasites assessed by flourescence microscopy.

Lectin-trypsin complex [~4.5 μg] (○) observed values

Lectin-trypsin complex (—) predicted values

cis-aconitate [~ 4 mM] (▲) observed values

cis-aconitate (---) predicted values

Trend (Predicted) represents mean values (n=4) fitted in non-linear model $y = A\ (1\text{-}e^{-bt})$.

Fig. 22. PCR screening of transformants using SP6 and T7 primers.

The positive clones 1, 2, 5, 6, 7, 8, 9,14 and 15 were screened by PCR using SP6 and T7 primers (pGEM-T multiple cloning site promoters) to determine the size of their inserts. The various size inserts were separated by electrophoresis using 1% agarose gel containing 0.3 µg/ ml ethidium bromide and visualized under UV

Clones 1, 2, 14 had ~100 bp insert (lanes 1, 2 and 8, respectively)

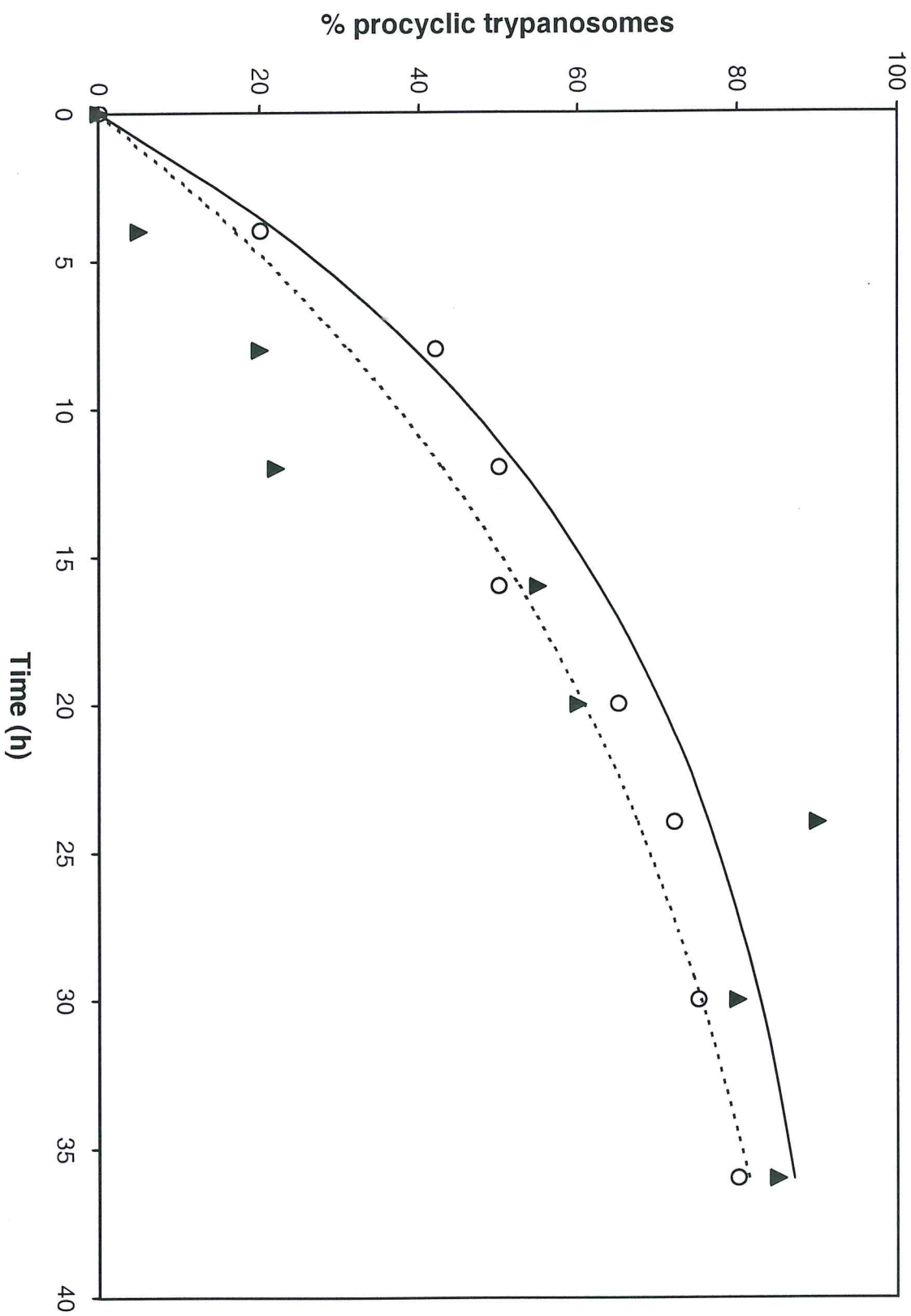
Clone 5 had ~780 bp insert (lane 3)

Clone 6 probably empty (lane 4)

Clone 7 had ~900 bp insert (lane 5)

Clones 8, 9 had ~530 bp insert (lanes 6 and 7, respectively)

Lane 10- 1 Kb ladder


% procyclic trypanosomes
0
20
40
60
80
100
Time (h)
0
5
10
15
20
25
30
35
40

When the concentration of lectin-trypsin in the assay was increased to ~100 μg protein/ ml, ~65% of the trypanosomes expressed procyclin after 8 hours incubation compared to ~ 42% observed when a lower concentration of lectin-trypsin (~ 4.5 μg protein/ ml) was used (Fig. 7). Similarly, the rate of transformation of the parasites increased to 0.122 at the asymptotic of 100%. This increase in transformation rate was significant (t-test; $p < 0.05$). The observed increase in transformation rate was however, accompanied by high parasite mortality such that no live parasites were observed in assays with higher lectin-trypsin concentrations after 16 hours (Fig. 7).

3.1.3 Effect of D-glucosamine on trypanosome transformation

The effect of D-glucosamine (D-GlcN) on the ability of lectin-trypsin complex to induce transformation of bloodstream trypanosomes was examined. The results showed that D-GlcN (~100 mM) significantly inhibited the lectin-trypsin induced transformation of the parasites. After 20 hours incubation, only ~18% of the trypanosomes expressed procyclin compared to ~72% observed in the absence of D-GlcN (Fig 8). Similarly, the rate of transformation in the presence of D-GlcN was 0.023 at the asymptotic value of 44.3% compared to the transformation rate of 0.066 and asymptotic value of 95.87% in the control. The decrease in transformation trend by D-GlcN was highly significant ($p < 0.001$).

Fig. 7. Effect of higher concentration of lectin-trypsin complex on trypanosome transformation

Bloodstream trypanosomes (~5 x 10^6 parasites/ ml) were incubated with higher concentration of lectin-trypsin complex (~100μg protein/ ml) in modified minimum essential medium at 27 °C. At 4 h intervals, 0.5 ml aliquots were withdrawn from the incubation mixture and the transformed parasites assessed by flourescence microscopy. Control consisted of bloodstream trypanosomes (~5 x 10^6 parasites/ ml) incubated with lectin-trypsin complex (~4.5μg protein/ ml)

Lectin-trypsin complex [~4.5 μg] (○) observed values

Lectin-trypsin complex (—) predicted values

Lectin-trypsin complex [~100 μg] (■) observed values

Lectin-trypsin complex [~100 μg] (---) predicted values

Trend (Predicted) represents mean values (n=4) fitted in non-linear model

$y = A (1-e^{-bt})$.

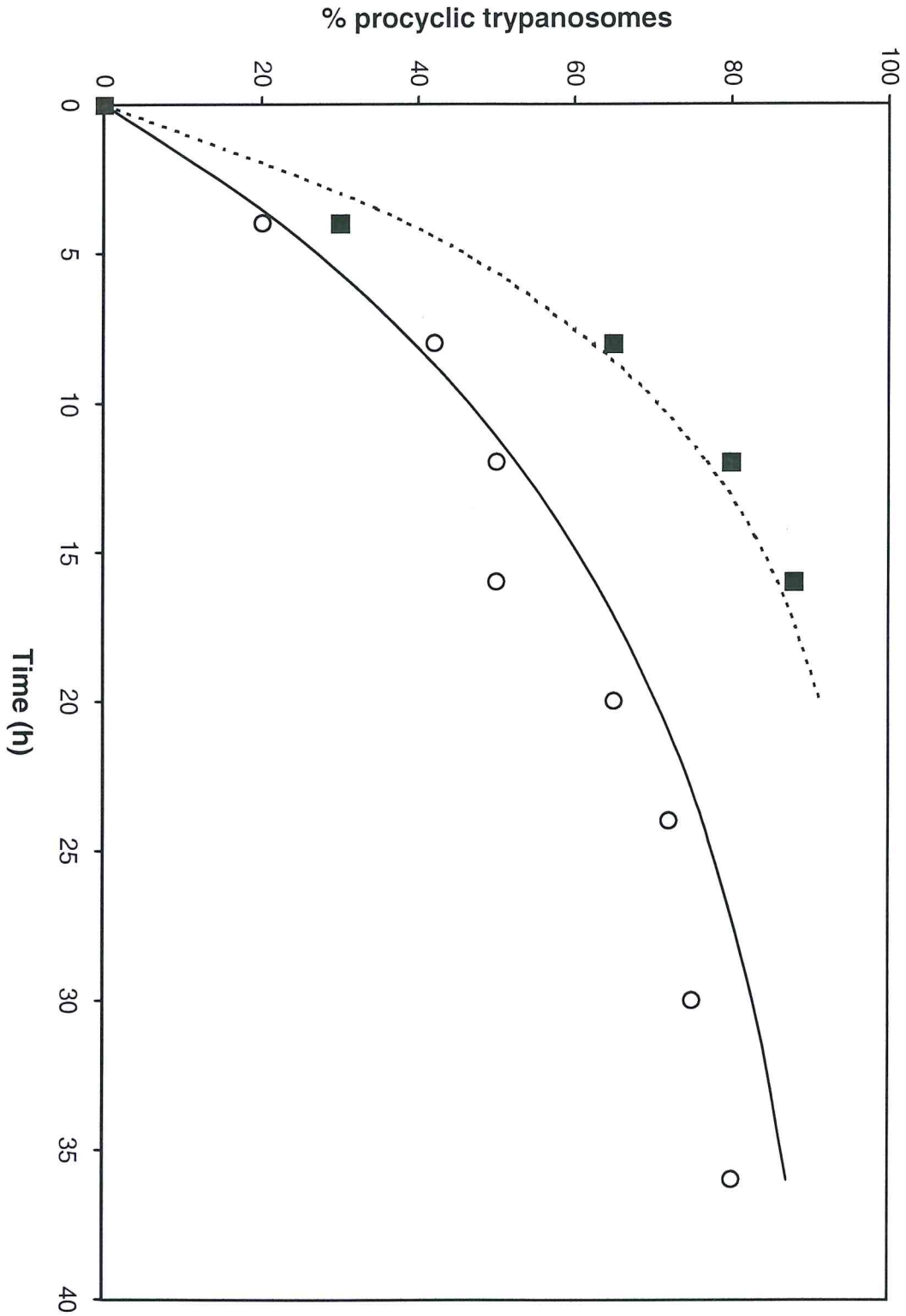

% procyclic trypanosomes
0
20
40
60
80
100
Time (h)
0
5
10
15
20
25
30
35
40

Fig. 8. Effect of D-glucosamine on trypanosome transformation.

Lectin-trypsin complex (~4.5 μg protein/ ml) was incubated with D-GlcN (50 mM) prior to addition of trypanosomes. Transformation of the parasites was assessed as described in Materials and Methods.

Lectin-trypsin complex only [~4.5 μg] (○) observed values

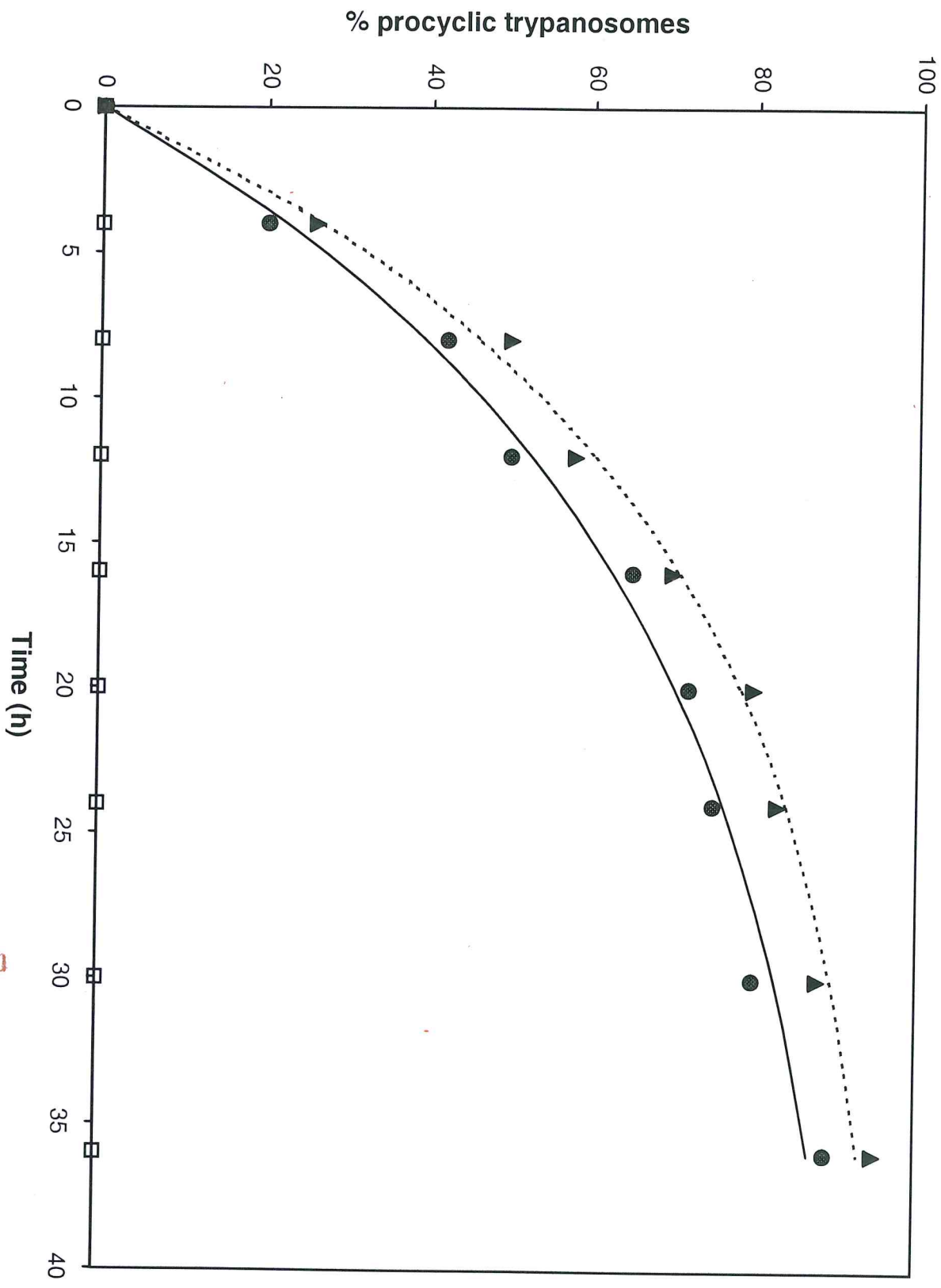
Lectin-trypsin complex only (—) predicted values

Lectin-trypsin complex [~4.5 μg] + 50 mM GlcN (•) observed values

Lectin-trypsin complex [~4.5 μg] + 50 mM GlcN (---) predicted values

Predicted trend represents mean values (n=4) fitted in non-linear model

$y = A\ (1\text{-}e^{-bt})$.


% procyclic trypanosomes
0
20
40
60
80
100
Time (h)
0
5
10
15
20
25
30
35
40

3.2 Discussion

The loss of variant surface glycoprotein (VSG) and the synthesis of an invariant coat composed of procyclins, characterize transformation of bloodstream-form salivarian trypanosomes to procyclic (midgut) forms. Procyclins are also known as procyclic acidic repetitive proteins (Mowart and Clayton, 1987). There are two distinct classes of procyclin that are characterized by their internal repeat motifs (Roditi and Clayton, 1999). EP procyclins contain up to 30 tandem repeats of glutamic acid-proline, and GPEET procyclin contains 6 pentapeptide repeats (gly-pro-glu-glu-thr). Both types of procyclin are co-induced when transformation is initiated (Vassella *et al.*, 2000). Subsequently, EP expression is maintained, whereas GPEET is repressed after 7-9 days. Experiments using procyclin knockout mutants have shown that the two types of procyclin have different roles, and that the EP form is important for survival in the fly (Ruepp *et al.*, 1997). Using specific antibodies against EP procyclin, the present study clearly demonstrated that the expression of EP procyclin was stimulated by *Glossina* lectin-trypsin complex. Inhibition of lectin-trypsin activity by D-GlcN led to repression of procyclin synthesis.

The first prerequisite for successful transmission of *T brucei* is that bloodstream forms must differentiate into procyclic forms in the midgut, become established, and proliferate. The majority of infections do not proceed beyond this stage, yet for the cycle to be completed, the parasites have to establish in the midgut and migrate to the salivary glands, where they differentiate further into epimastigote forms. Subsequently, they mature into metacyclic forms that are capable of initiating a fresh infection when transmitted to a new mammalian host. Several factors may influence the efficiency of

parasite transmission. The strains of trypanosome, the species of tsetse fly, the sex of the fly, the presence of endosybionts (Sodalis) in the midgut, and the type of host blood have all been implicated (Distelmans *et al.*, 1982; Moloo *et al.*, 1992; Maudlin and Welburn, 1988; Nguu *et al*, 1996). Initial evidence for the presence of a midgut factor that stimulates the transformation process in tsetse was obtained by incubating parasitised blood with crude midgut homogenates (Imbuga *et al.*, 1992a). Heating the midgut homogenates irreversibly destroyed its transformation-stimulating capacity, suggesting that the factors involved in the process are heat-labile and may therefore be protein in nature. Moreover, specific sugars such as glucosamine were shown to modulate either the establishment of infections by procyclic forms or the production of mature salivary gland forms, leading to the proposal that the tsetse fly factors are themselves lectins (Maudlin and Welburn, 1987; 1988a; b).

The transformation process observed in this study proceeded more efficiently at low lectin-trypsin concentrations. Thus, inhibition of the lectin-trypsin activity by D-glucosamine significantly blocked the transformation of bloodstream-form trypanosomes. While increasing the concentrations of the lectin-trypsin molecule resulted in significantly higher transformation rates, it also led to high mortality of the parasites. It is likely that the transformation process is stimulated between 0-24 h post-bloodmeal, before the lectin-trypsin activity reach peak levels (Abubakar *et al.*, 1995). This might explain the observation by Vickerman (1985) that most of the trypanosome transformation within the tsetse midgut is complete within the same period.

Although a reduction in temperature from 37 ^{o} to 27^{o} C has been reported to trigger transformation of *T. brucei* culture forms *in vitro* (Czichos *et al.*, 1986; Kaminsky

et al., 1988), the bloodstream trypanosomes used in the present study could not transform in the absence of the lectin-trypsin, even after 24 h incubation. Similarly, the TCA-cycle intermediates, citrate and cis-aconitate, have been reported to stimulate transformation of *T. brucei* at 27° C (Brun and Schönenberger, 1981; Overath *et al.*, 1986; Ziegelbauer *et al.*, 1990). While temperature naturally decreases during the transfer of trypanosomes from the mammalian blood into the insect vector, the role of cis-aconitate *in vivo* is less obvious. In the present study, no significant difference was observed between the rate of transformation induced by lectin-trypsin complex or *cis*-aconitate. Nevertheless, the reported stimulation of transformation by cis-aconitate is only effective at high concentrations (>3 mM) and cannot therefore be the physiological trigger. More recently, mild acidic stress was identified as an inducer of trypanosome transformation *in vitro* (Rolin *et al.*, 1998). In this case, preincubation of pleomorphic trypanosomes at pH 5.5 for 2 h at 37° C was shown to induce their differentiation to procyclic forms at 27° C, even in the absence of citrate or *cis* -aconitate. However, it is unclear whether acid stress has any physiological significance since the pH of the tsetse midgut is predominantly alkaline. While these factors appear to stimulate trypanosome transformation to procyclic forms *in vitro*, this process occurs much faster *in vivo* in the tsetse gut. The results of this study have now revealed that the efficient transforming activity associated with the midgut, is due to the lectin-trypsin complex.

There is growing evidence that lectin-carbohydrate interactions can mediate the infection of parasites to their insect vectors, and their disruption can affect subsequent establishment of the parasite within the vector. For example, in *Triatoma infestans*, lectin-like molecules produced in the intestinal extracts stimulate differentiation of *T.*

cruzi epimastigotes (Isola *et al.*, 1986). In addition, *Triatoma infestans* refractory to infection with *Trypanosoma rangeli* produces an array of tissue and hemolymph lectins, whereas the susceptible insect, *Rhodnius prolixus* produced only hemolymph agglutinins (Gregoria and Ratcliffe, 1991). In sandflies, midgut lectins have been proposed to play a role in susceptibility to *Leishmania* infections (Volf *et al.*, 1998). Similarly, in the mosquito *Aedes aegypti*, the addition of N-acetylglucosamine has been reported to increase the number of *Brugia pahangi* microfilarie successfully migrating through the midgut wall (Ham *et al.*, 1991). This suggested that endogenous lectins might be involved in some strains being refractory to nematode infection. Although the role of lectins and carbohydrate in mosquito-malaria interactions remains undefined, two stage- specific lectins with distinct characteristics have recently been identified in the serum of the mosquito, *An. Stephensi* (Chen and Billingsley, 1999). The mosquito mannan-binding lectin agglutinated bacteria, fungi and protozoan parasites including trypanosomes and *Plasmodium* -infected mouse erythrocytes (Chen and Billingsley, unpublished). On the other hand, in trypanosome-tsetse interactions, lectins have been proposed to play a dual role. Vector lectins released into the midgut not only provide a signal for differentiation of trypanosomes but also lyse trypanosomes and prevent the establishment of midgut infections (Maudlin and Welburn, 1987; Welburn *et al.*, 1989). Indeed, the significantly higher infection rates generally observed in the Morsitans-group compared to the Palpalis-group (Moloo *et al.*, 1992; 1998; Reifenberg *et al.*, 1997) have been attributed to higher midgut lectin output in the latter (Maudlin, 1991). Similarly in the present study, considerably high parasite mortality was observed with higher lectin-trypsin concentrations *in vitro*. These results reflect the importance of lectin-trypsin

complex output in determining the fate of the parasites in the tsetse gut, either transformation or death. It is possible that migration of the transformed trypanosomes to the ectoperitrophic space confers some protection from the destructive effect of the lectin-trypsin complex in the endoperitrophic space when the activities are elevated, 48-72 h post-bloodmeal.

Studies on monomorphic culture forms of *T. brucei* have shown that treatment of the parasites with trypsin provided the signal for transformation *in vitro* (Yabu and Takayanagi, 1988). Subsequently, a correlation was reported between trypsin activity and the ability of tsetse midgut homogenates to induce the transformation of bloodstream trypanosomes (Imbuga *et al.*, 1992). Whether the removal of the surface coat by trypsin activity alone is a sufficient signal for parasites to be committed to transform, or whether other factors within the midgut also come into play has remained a matter of conjecture. In the tsetse midgut, the roles of trypsins and lectins appear to be closely related. They are both stimulated by bloodmeal (Van Den Abbelle and Decleir, 1991; Abubakar *et al.*, 1995) and inhibited by D-glucosamine (Ibrahim *et al.*, 1984; Osir *et al.*, 1993). Furthermore, like the lectins, midgut trypsins have been implicated in the maturation and lysis of the trypanosomes (Imbuga *et al.*, 1992a, Welburn *et al.*, 1989). The presence of a bifunctional molecule (affinity purified lectin-trypsin complex) exhibiting both lectin and trypsin activities in tsetse is therefore not surprising.

The molecular weight of the isolated lectin-trypsin complex compares favourably with a similar molecule previously reported from the midgut of the tsetse fly, *G. longipennis* (Osir *et al.* 1995). This confirms that the molecule is prevalent in other

members of *Glossina* species. Initial isolation of the lectin-trypsin complex was carried out using anion-exchange chromatography (Osir *et al.*, 1995). The molecule was capable of agglutinating trypanosomes, an activity specifically inhibited by D-glucosamine. In this study, the glucosamine-binding property of the lectin-trypsin complex was exploited to purify the molecule by a combination of anion-exchange and glucosamine-affinity chromatography.

Although a decade has elapsed since the identification of the first procyclin protein (Richardson *et al.*, 1988) and encoding genes (Roditi *et al.*, 1987; Mowart and Clayton, 1987), their functions have remained unresolved. It has been hypothesized that the procyclins protect the parasites membranes from lytic enzymes. The fact that procyclins are largely resistant to proteases would confer obvious advantages in the midgut of the fly (Ferguson *et al.*, 1993; Richardson *et al.*, 1988). It has also been proposed that different domains of procyclins might be the targets for the tsetse factor(s) that might bind to either N-linked carbohydrate moieties or to sugar residues in the glycolipid anchor (Maudlin and Welburn, 1994). More recently, Pearson *et al.* (2000) reported that the procyclin molecules found on procyclic-forms are involved in the lectin-induction of a novel form of cell death in trypanosomes. To completely understand the process of midgut infection establishment, it is important that further investigations be carried out on the other factors that may act either antagonistically or synergistically with the lectin-trypsin complex in tsetse midgut.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 Screening of the midgut cDNA expression library with antibody against lectin-trypsin complex

4.1.1 Construction of midgut cDNA expression library

cDNA expression library was constructed in the λTriplEx2 phage vector from *G. fuscipes fuscipes* midgut total RNA. The isolated total RNA appeared as two bright bands (28S and 18S ribosomal RNA) on agarose gel (Fig. 9). Subsequently, cDNA was transcribed from the total RNA and reflected as a smear on the agarose gel (Fig. 10). Finally, a full-length (ds) midgut cDNA expression library containing ~ 1.0×10^7 independent clones was successfully generated in λTriplEx2. The resultant amplified library yielded a very high titer of $\geq 10^{10}$ plaques forming unit per millilitre (pfu/ ml).

4.1.2 Isolation of cDNA by immuno-screening

When the plasmid form (pTriplEx2) cDNA library was screened with polyclonal antibodies raised against the native lectin-trypsin complex, 12 positive colonies were generated. To identify the insert(s) with lectin activity, the positive clones (transformed in *E.coli* strain JM109) were expressed and their lysates assayed for agglutination activity. Lysates from three clones showed agglutination activity against bloodstream trypanosomes and RBCs (Table 1). Further screening of the clones using TriplEx2 screening primers revealed 3 different size clones (Fig. 11). When subjected to automated DNA sequencing, only one out of the three clones carried a full-length cDNA insert of ~ 933 base pair (bp), designated *Glossina* proteolytic lectin (Gpl).

Fig. 9. Analysis of RNA from the midguts of *G. f. fuscipes*.

Total RNA was extracted from twice-fed midguts of *G. fuscipes fuscipes* using the standard protocol of the RNAgents® System (Promega). 5 µg of the sample was resolved on a 1% denaturing agarose gel containing 0.3 µg/ml ethidium bromide and visualized under UV.

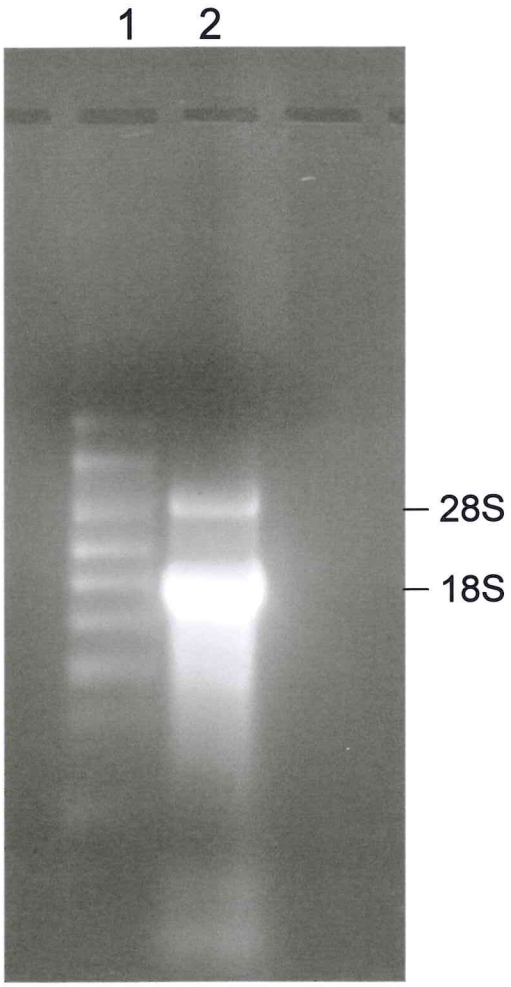

1
2
28S
18S

Fig. 10. Agarose gel electrophoresis of ds cDNA from the total RNA.

Total RNA was reverse transcribed using MMLV reverse transcriptase and double-stranded cDNA generated by Long-Distance Polymerase Chain Reaction (LD-PCR). Amplification reaction was analysed by 1% agarose gel electrophoresis and ethidium bromide staining.

Lane 1 - 10µl PCR product

Lane 2 - 1 Kb DNA marker (Boehringer)

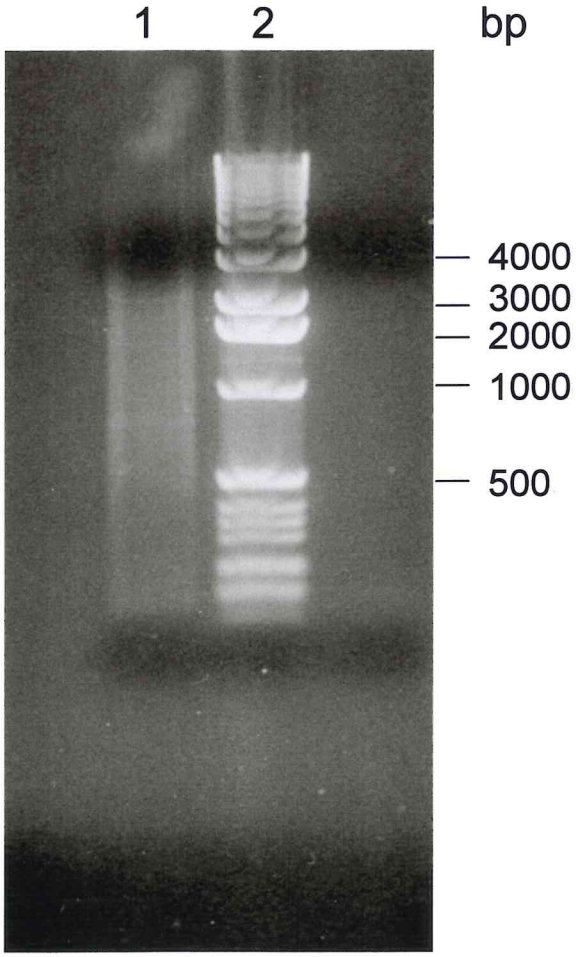

1
2
bp
4000
3000
2000
1000
500

Table 1. Agglutination activity of the positive-immunoscreened clones against bloodstream trypanosomes and rabbit red blood cells

Lysates (*E.coli* JM109)	Agglutination activity	
	Bloodstream parasites	RBCs
Clone 1	+	+
Clone 2	-	-
Clone 3	-	-
Clone 4	-	-
Clone 5	+	+
Clone 6	-	-
Clone 7	-	-
Clone 8	+	+
Clone 9	+	+
Clone 10	-	-
Clone 11	-	-
Clone 12	-	-

Doubling serial dilutions of *E.coli* lysate generated from immunoscreening were incubated (27° C, 1 h) with either bloodstream *T. b. brucei* or rabbit RBCs. Clones that showed agglutination activity were scored as +

Fig. 11. PCR screening of the positive clones using TriplEx2 screening primers

cDNA library was screened with the polyclonal antibodies against the lectin-trypsin complex and the positive clones assayed for lectin activity. Clones exhibiting lectin activity were further screened by PCR using TriplEx2 screening primers to determine their sizes. Plasmids from the positive clones were then extracted using QIAprep Spin Miniprep kit (Qiagen, GmbH)

PCR cycle used

94° C – 2 min

94° C – 1 min
55° C – 1 min
72° C – 2 min
} 35 cycles

72° C – 5 min

A- 1% agarose gel electrophoresis of amplified positive clones

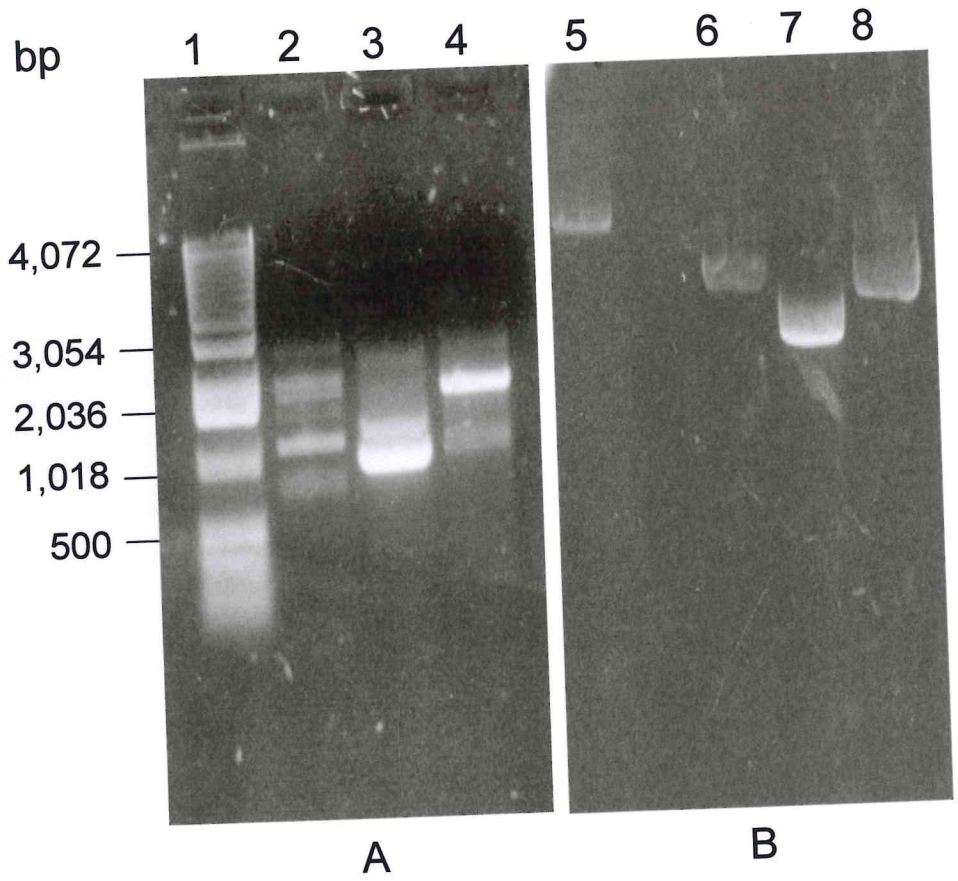
lane 1- 1kb ladder (Boehringer)

lanes 2, 3 and 4- PCR products of positive clones (10 µl)

B- 1% agarose gel electrophoresis of the purified plasmids

Lane 5 – λ 100 ng

Lanes 6, 7 and 8 – Purified plasmids from the positive clones


bp
1
2
3
4
5
6
7
8
4,072
3,054
2,036
1,018
500
A
B

4.1.3 Sequence homology of Glossina proteolytic lectin (Gpl)

The full sequence of the putative protein encoded by Gpl was used to search for similarity to other known genes at GENBANK using BLAST P (Altschul *et al.*, 1990) as well as the SWISS-PROT and TrEMBL Protein knowledge bases at the Expert Protein Analysis System (ExPASy) Molecular Biology Server. The highest similarity score was to chymotrypsin-like serine protease precursor from *Glossina morsitans morsitans* at 89% identities, 92% positives and an expectation of e-140. The second highest similarity score was to midgut-specific serine protease 1 from *Stomoxys calcitrans* at 58% identities, 69% positives with an expectation of 8e-81.

Multiple sequence alignment of the translated sequence of Gpl with four homologous insect serine proteases indicated that the active site residues, His-57, Asp-102 and Ser- 195 (using the bovine chymotrypsinogen A numbering system, Brown and Hartley, 1966) and the regions around these residues were highly conserved (Fig. 12, denoted as ▲). Moreover, the six cysteines residues that are thought to contribute to three disulphide bonds commonly found in invertebrate serine proteases are found at conserved sites (Fig. 12, denoted as ✱). The Asp-189 residue, which is diagnostic of tryptic specificity is similarly conserved (Fig. 12, denoted as ✚) (Stroud *et al.*, 1974). Likewise, the highly conserved N-terminal sequence GRI(V)NG was present from position 30 to 35.

Fig. 12. Sequence homology of Gpl.

The full sequence of Gpl cDNA were used to search similar sequences in GenBank using BLASTP, and multiple alignments were performed using MultAlign. Residues that are identical are shown in red. The residues that are in the catalytic triad (H,D,S) are indicated by '▲', the six conserved cysteine residues are indicated by '*' and the substrate specificity sites by '+.'

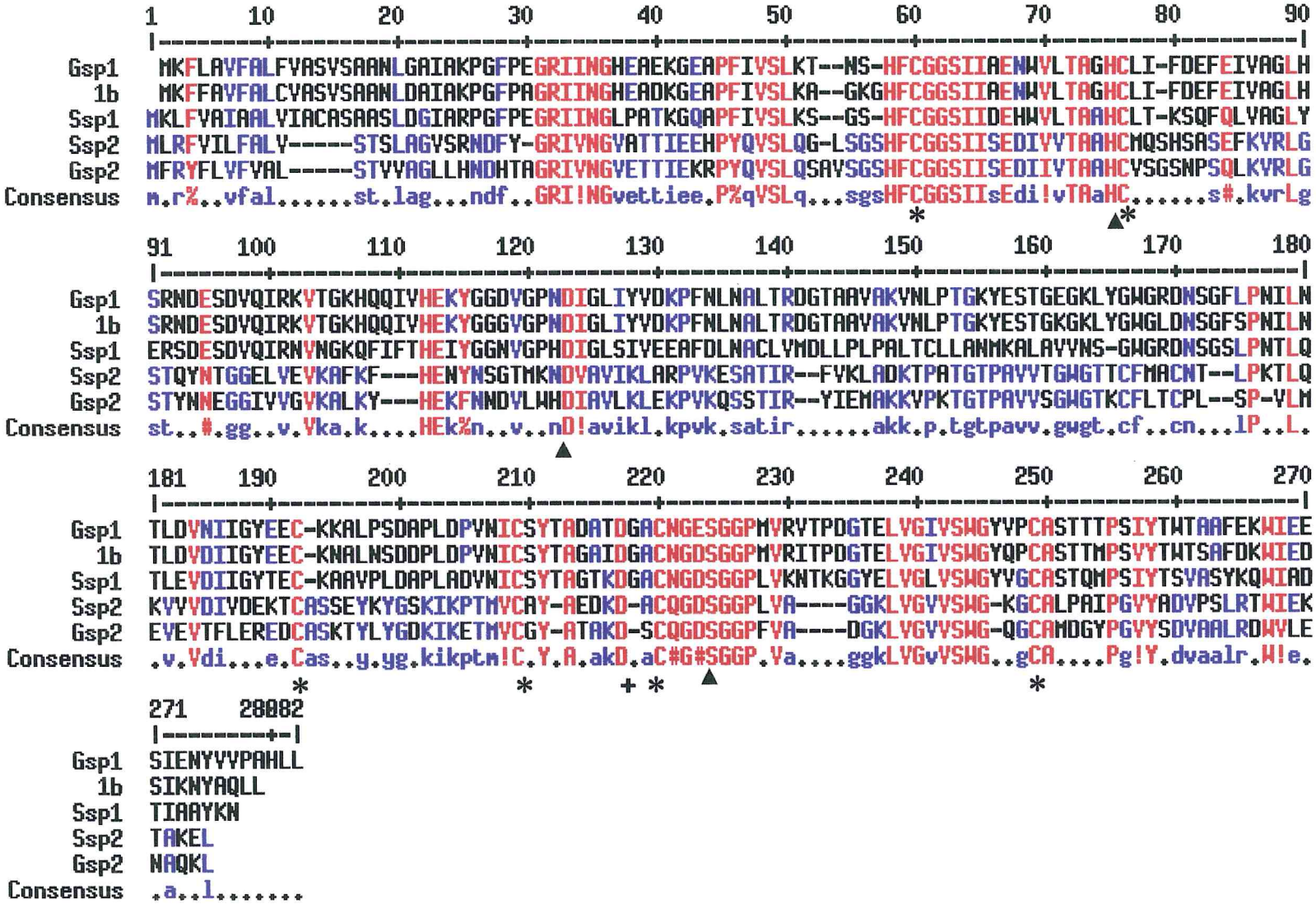
Gpl - *Glossina* proteolytic lectin (1b) (GenBank AF525314)

Gsp1- *Glossina* serine protease 1(GenBank AF252868)

Gsp2- *Glossina* serine protease 2 (GenBank AF252869)

Ssp1- *Stomoxys* serine protease 1(GenBank AF074955)

Ssp2- *Stomoxys* serine protease 2 (GenBank AF074956).


1 10 20 30 40 50 60 70 80 90
Gsp1 MKFLAVFALFVASVSAANLGAIAKPGFPEGRIINGHEAEKGEAPFIVSLKT--NS-HFCGGSIIAENWVLTAGHCLI-FDEFEIVAGLH
1b MKFFAVFALCVASVSAANLDAIAKPGFPAGRIINGHEADKGEAPFIVSLKA--GKGHFCGGSIIAENWVLTAGHCLI-FDEFEIVAGLH
Ssp1 MKLFVAIAALVIACASAASLDGIARPGFPEGRIINGLPATKGQAPFIVSLKS--GS-HFCGGSIIDEHWVLTAAHCLT-KSQFQLVAGLY
Ssp2 MLRFVILFALV-----STSLAGVSRNDFY-GRIVNGVATTIEEHPYQVSLQG-LSGSHFCGGSIISEDIVVTAAHCMQSHSASEFKVRLG
Gsp2 MFRYFLVFVAL-----STVVAGLLHNDHTAGRIVNGVETTIEKRPYQVSLQSAVSGSHFCGGSIISEDIIVTAAHCVSGSNPSQLKVRLG
Consensus m.r%..vfal.....st.lag...ndf..GRI!NGvettiee.P%qVSLq...sgsHFCGGSIIsEdi!vTAaHC.....s#.kvrLg
91 100 110 120 130 140 150 160 170 180
Gsp1 SRNDESDVQIRKVTGKHQQIVHEKYGGDVGPNDIGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGEGKLYGWGRDNSGFLPNILN
1b SRNDESDVQIRKVTGKHQQIVHEKYGGGVGPNDIGLIYVDKPFNLNALTRDGTAAVAKVNLPTGKYESTGKGKLYGWGLDNSGFSPNILN
Ssp1 ERSDESDVQIRNVNGKQFIFTHEIYGGNVGPHDIGLSIVEEAFDLNACLVMDLLPLPALTCLLANMKALAVVNS-GWGRDNSGSLPNTLQ
Ssp2 STQYNTGGELVEVKAFKF---HENYNSGTMKNDVAVIKLARPVKESATIR--FVKLADKTPATGTPAVVTGWGTTCFMACNT--LPKTLQ
Gsp2 STYNNEGGIVVGVKALKY---HEKFNNDVLWHDIAVLKLEKPVKQSSTIR--YIEMAKKVPKTGTPAVVSGWGTKCFLTCPL--SP-VLM
Consensus st..#.gg..v.Vka.k....HEk%n..v..nD!avikl.kpvk.satir......akk.p.tgtpavv.gwgt.cf..cn...lP..L.
181 190 200 210 220 230 240 250 260 270
Gsp1 TLDVNIIGYEEC-KKALPSDAPLDPVNICSYTADATDGACNGESGGPMVRVTPDGTELVGIVSWGYVPCASTTTPSIYTWTAAFEKWIEE
1b TLDVDIIGYEEC-KNALNSDDPLDPVNICSYTAGAIDGACNGDSGGPMVRITPDGTELVGIVSWGYQPCASTTMPSVYTWTSAFDKWIED
Ssp1 TLEVDIIGYTEC-KAAVPLDAPLADVNICSYTAGTKDGACNGDSGGPLVKNTKGGYELVGLVSWGYVGCASTQMPSIYTSVASYKQWIAD
Ssp2 KVVVDIVDEKTCASSEYKYGSKIKPTMVCAY-AEDKD-ACQGDSGGPLVA----GGKLVGVVSWG-KGCALPRIPGVYADVPSLRTWIEK
Gsp2 EVEVTFLEREDCASKTYLYGDKIKETMVCGY-ATAKD-SCQGDSGGPFVA----DGKLVGVVSWG-QGCAMDGYPGVYSDVAALRDWVLE
Consensus .v.Vdi...e.Cas..y.yg.kikptm!C.Y.A.akD.aC#G#SGGP.Va....ggkLVGvVSWG..gCA....Pg!Y.dvaalr.W!e.
271 28882
Gsp1 SIENYVVPAHLL
1b SIKNYAQLL
Ssp1 TIAAYKN
Ssp2 TAKEL
Gsp2 NAQKL
Consensus .a..l.......

4.1.4 Sequence analysis of GpI

The nucleotide sequence of GpI, together with the amino acid sequence derived from it (GenBank accession number AF525314) is shown in Fig. 13. The 933 bp cDNA contained thirty-five nucleotides upstream to the initiation ATG codon which represents the 5' untranslated region (UTR). The open reading frame (ORF) terminates with TGT, 823 bases downstream from the initiation codon. Therefore the insert codes for a polypeptide of 274 amino acids. There are forty-seven nucleotides after the stop codon, which represents the 3' UTR. The putative polyadenylation signal AATAAA is present within the 3' UTR. A poly A tail occurs fourteen nucleotides downstream from the signal. The calculated molecular weight of the predicted mature protein sequence is 29,179 da with a pI of 4.79.

When the protein sequence was scanned to identify functional domains using PrositeScan algorithm, several structural requirements that were pivotal to serine proteases were identified. A serine active site (GACNGD*S*GGPMV) belonging to the Serine proteases trypsin family was identified between amino acids 213 to 224 with a randomized probability of 7.319e-08. Similarly, a histidine active site belonging to the serine proteases, trypsin family was identified at amino acids 68 to 73 (LTAG*H*C) with a randomized probability of 2.601e-07. In addition, the sequence contained a potential adenosine and AMP deaminase site at amino acids 191-197.

Fig. 13. Sequence analysis of GpI

The nucleotide and deduced amino acid sequences of GpI cDNA, isolated from *G. fuscipes* midgut cDNA library is shown (GenBank accession number AF525314). The putative signal peptide sequence, and the possible polyadenylation signal are underlined. Serine and histidine active sites and their surrounding conserved residues are indicated in red.

933 residue sequence "Full-length Gpl (*Glossina* proteolytic lectin)" (CDS in uppercase)

```
   1                                                  M  K  F  F  A  V  F  A  L
   1 ggccattacggccggggagtacaatttcgatcatcATGAAGTTCTTTGCAGTGTTCGCTT
  10  C  V  A  S  V  S  A  A  N  L  D  A  I  A  K  P  G  F  P  A
  61 TATGTGTGGCTAGTGTGAGTGCGGCAAACTTGGATGCTATCGCCAAACCAGGTTTTCCGG
  30  G  R  I  I  N  G  H  E  A  D  K  G  E  A  P  F  I  V  S  L
 121 CAGGACGCATTATTAACGGACATGAGGCCGACAAAGGTGAAGCTCCTTTTATTGTGTCTT
  50  K  A  G  K  G  H  F  C  G  G  S  I  I  A  E  N  W  V  L  T
 181 TAAAGGCCGGTAAAGGTCATTTCTGCGGTGGTTCTATTATTGCTGAGAACTGGGTTTTGA
  70  A  G  H  C  L  I  F  D  E  F  E  I  V  A  G  L  H  S  R  N
 241 CTGCGGGTCACTGCTTGATCTTCGATGAATTCGAAATTGTAGCTGGATTACATTCGCGAA
  90  D  E  S  D  V  Q  I  R  K  V  T  G  K  H  Q  Q  I  V  H  E
 301 ACGATGAGTCTGACGTTCAAATTCGCAAGGTTACTGGTAAACATCAACAAATTGTCCATG
 110  K  Y  G  G  G  V  G  P  N  D  I  G  L  I  Y  V  D  K  P  F
 361 AAAAATATGGCGGTGGCGTTGGTCCCAACGATATTGGTCTCATTTACGTGGATAAACCAT
 130  N  L  N  A  L  T  R  D  G  T  A  A  V  A  K  V  N  L  P  T
 421 TCAATTTGAATGCCTTAACTCGTGACGGAACAGCTGCAGTAGCCAAGGTGAATTTGCCAA
 150  G  K  Y  E  S  T  G  K  G  K  L  Y  G  W  G  L  D  N  S  G
 481 CCGGCAAATATGAGTCTACTGGCAAGGGCAAATTGTATGGCTGGGGACTAGACAATTCCG
 170  F  S  P  N  I  L  N  T  L  D  V  D  I  I  G  Y  E  E  C  K
 541 GCTTCTCACCTAACATTCTGAACACTCTGGATGTAGACATTATTGGATACGAAGAATGCA
 190  N  A  L  N  S  D  D  P  L  D  P  V  N  I  C  S  Y  T  A  G
 601 AGAACGCTTTGAACAGCGATGATCCTTTAGATCCTGTCAATATCTGTTCCTACACAGCTG
 210  A  I  D  G  A  C  N  G  D  S  G  G  P  M  V  R  I  T  P  D
 661 GCGCTATTGATGGCGCCTGTAATGGCGATTCCGGTGGTCCAATGGTGCGTATCACACCTG
 230  G  T  E  L  V  G  I  V  S  W  G  Y  Q  P  C  A  S  T  T  M
 721 ACGGTACCGAATTAGTTGGCATTGTATCTTGGGGTTACCAACCTTGTGCCAGTACAACAA
 250  P  S  V  Y  T  W  T  S  A  F  D  K  W  I  E  D  S  I  K  N
 781 TGCCATCTGTTTATACTTGGACTTCTGCTTTCGACAAATGGATTGAAGACAGCATCAAGA
 270  Y  A  Q  L  L
 841 ACTATGCGCAACTTTTGTaacttactaccgttattgaatgtgaaaataaaagtatgcccc
 901 cccgaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Serine proteases, trypsin family, serine active site.
Site : 213 to 224 GACNGDSGGPMV. Identity.
Randomized probability: 7.319e-08

Serine proteases, trypsin family, histidine active site.
Site : 68 to 73 LTAGHC. Identity
Randomized probability: 2.601e-07

aataaa = Possible polyadenylation signal

M K F F A V F A L C V A S V S A A N L = The putative signal peptide sequence

4.1.5 Analysis of recombinant GpI

When the GpI cDNA was expressed in *E. coli*, a soluble protein was obtained. In immunoblotting experiments, the antibodies detected a protein band of M_r ~ 32,281 ± 3028 Da in the lysate (Fig. 14, lane 2). The recombinant protein successfully bound to D-glucosamine affinity column. Analysis of the purified recombinant lectin-trypsin by SDS-PAGE indicated a protein band of Mr ~ 32,500 ± 2828 Da (Fig.14, lane 3).

4.1.6 Agglutination and trypsin properties

The ability of the recombinant GpI to agglutinate rabbit RBCs and bloodstream *T.brucei brucei* was assessed. The red blood cells gave agglutination titre of 128 (Table 2; Fig. 15, lane C). Similarly, bloodstream-form trypanosomes gave agglutination titre of 512 (Table 2; Fig. 16). Addition of D-glucosamine to the assay strongly inhibited the agglutination activity (Fig. 15, lane F; Table 2). Likewise, when antibodies against the native lectin-trypsin were mixed with the recombinant protein, before addition of RBCs or trypanosomes, no agglutination was observed (Fig 15, lane D).

The substrate specificity of the recombinant molecule was assessed using a trypsin specific-chromogenic substrate, carbo-benzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY). Recombinant GpI lysate exhibited high trypsin activity (6.025 units x 10^{-4}) compared to the bacterial control lysate (0.303 units x 10^{-4}) (Table 2).

Fig. 14. SDS-PAGE and immunoblot of expressed Gpl.

A culture of *E.coli* transformed with pTriplEx2-Gpl was grown at 37° C. IPTG was added (0.1 mM final concentration) in early exponential growth phase to induce expression of recombinant Gpl. The culture was incubated (7 h, 37° C) and the resultant lysate centrifuged (10,000 g, 15 min, 4° C). Recombinant Gpl was purified from the lysate by D-glucosamine affinity column chromatography. The proteins separated by gradient SDS-PAGE (4-20%) and then transferred onto nitrocellulose paper. The blots were then reacted with antiserum.

A. Immunoblot: lane 1- purified Gpl1 (~4 µg)
lane 2- recombinant *E.coli* –Gpl lysate (~20 µg).

B. Silver-stained gel: lane 3- purified Gpl1 (~2 µg)
lane 4- recombinant *E.coli* lysate (~ 9 µg)
lane 5- low molecular weight standards (Pharmacia) 5 µl

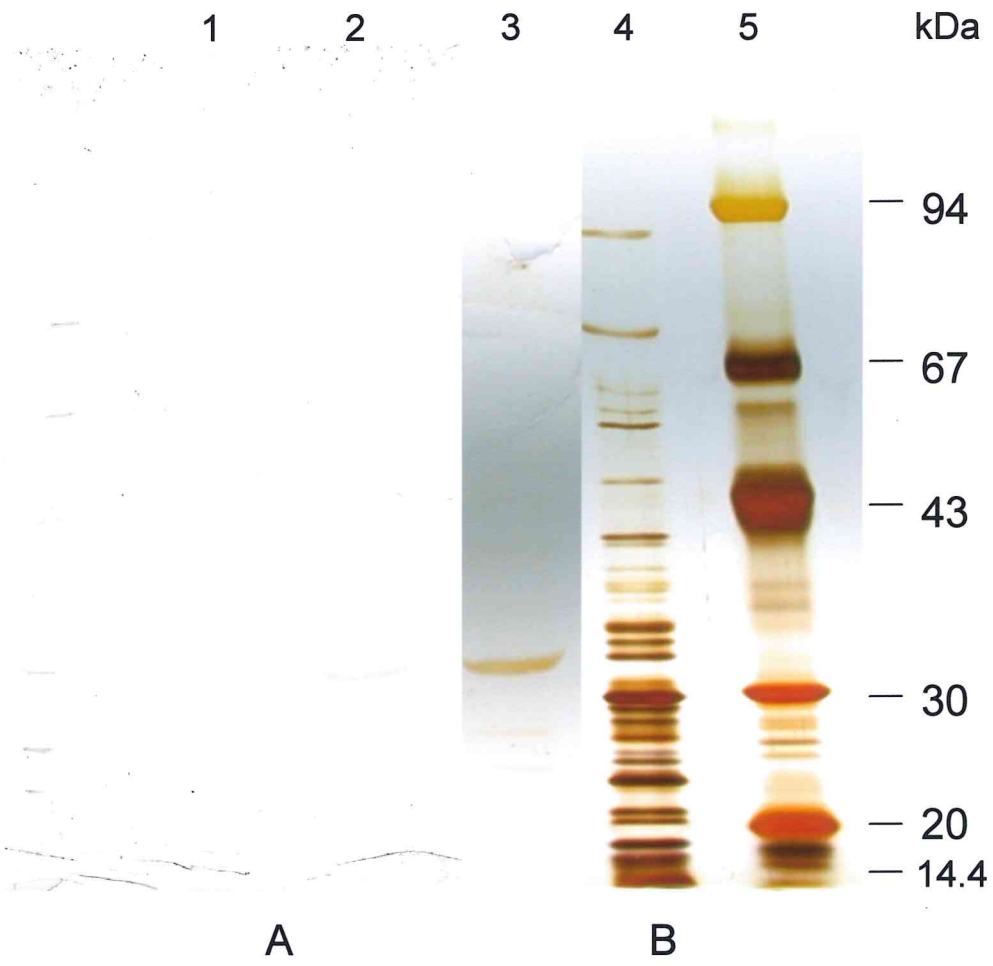

1
2
3
4
5
kDa
94
67
43
30
20
14.4
A
B

Table 2 Agglutination of bloodstream trypanosomes and RBCs

Lysate	Agglutination titres		Trypsin activity
	Bloodstream parasites	RBCs	(units x 10^{-4})
Control (PBS)	0	0	0
E.coli JM109 (control)	4	2	0.303
*E.*coli-Gpl (expressed)	512	128	6.025
Gpl (expressed) + glucosamine	0	0	-
Gpl (expressed) + antibodies	4	2	-

Doubling serial dilutions of expressed *E.coli* JM109 (transformed with pTriplEx2-Gpl) lysate were incubated (27° C, 1 h) with either bloodstream *T. b. brucei* or rabbit red blood cells (RBCs). Control consisted of non-transformed *E.coli* JM109 lysate. Titres are expressed as reciprocals of end point serial dilution that gave positive agglutination. The samples were also assayed for trypsin activity using Chromozym-TRY as substrate.

Fig. 15. Agglutination of rabbit RBCs by recombinant GpI

Doubling serial dilutions of expressed *E.coli* JM109 (transformed with pTriplEx2-GpI) lysate were incubated (27° C, 1 h) with rabbit RBCs. Control consisted of non-transformed *E.coli* JM109 lysate

Lane A - RBC + PBS buffer; lane B - RBC + non-transformed *E.coli* lysate; lane C - RBC + recombinant GpI; lane D - RBC + *E.coli* – GpI lysate + antibodies;
lane F - RBC + recombinant GpI lysate + glucosamine.

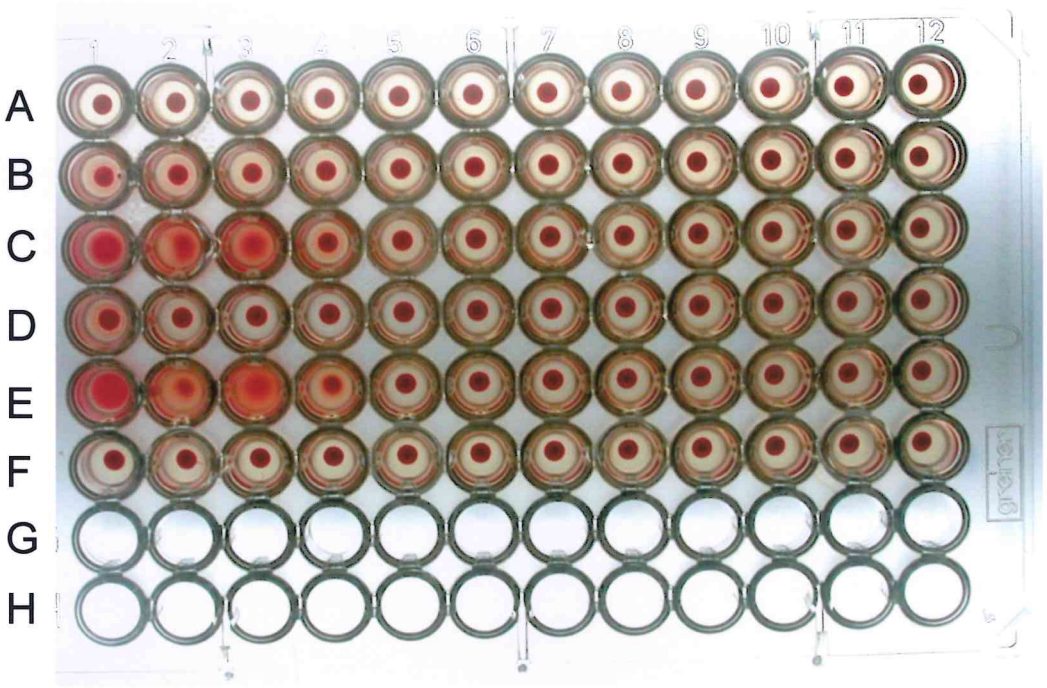

1
2
3
4
5
6
7
8
9
10
11
12
A
B
C
D
E
F
G
H
greiner

Fig. 16. Agglutination of bloodstream trypanosomes by GpI

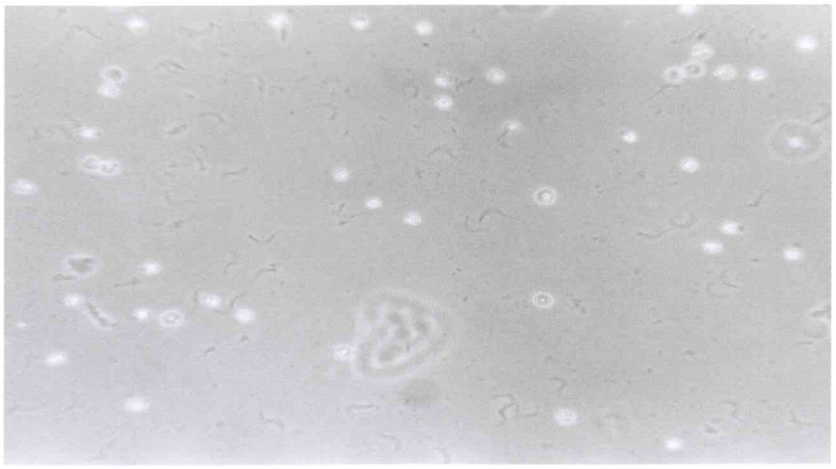
Two-fold serial dilutions of recombinant *E. coli* – GpI extracts were mixed with an equal volume of freshly isolated parasites (~5 x 10^6 parasites/ ml) and incubated (2 h, 27° C). Agglutination of parasites was assessed using an inverted microscope (Leitz Dialux, Germany)

A - PBS + Parasites (Control) x 40 magnification

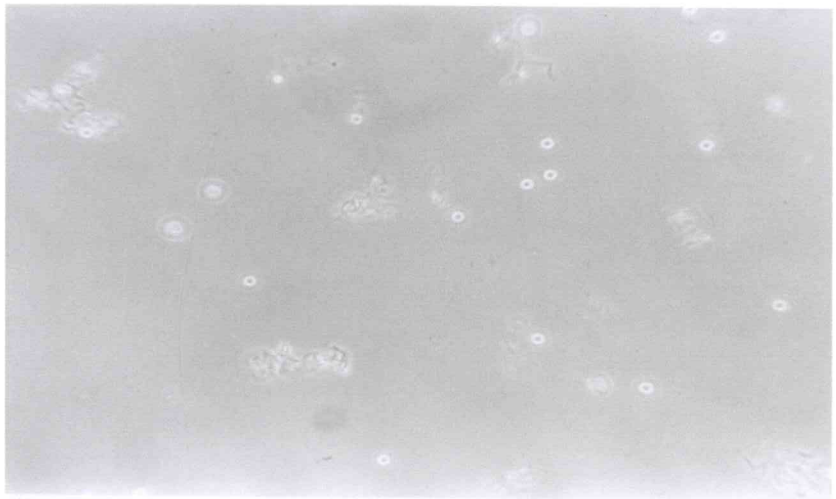
B - Recombinant GpI + parasites x 40 magnification

C - Recombinant GpI + parasites x 100 magnification

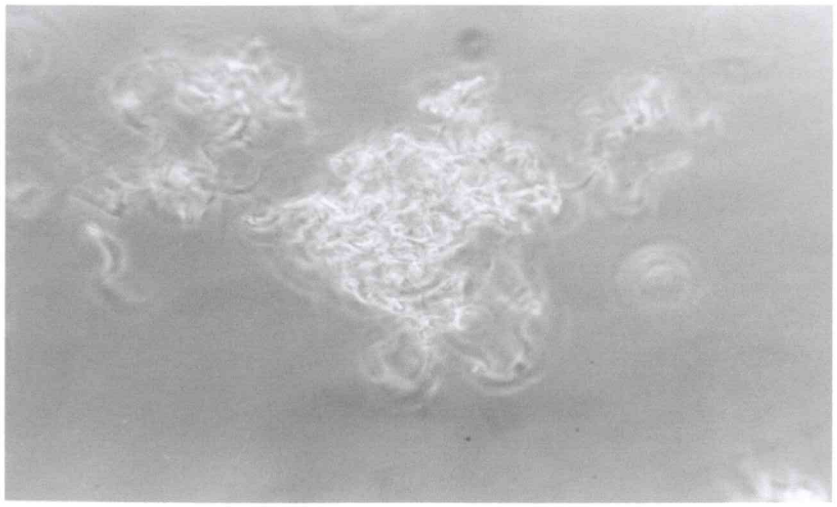
A



B



C



4.1.7 Induction of transformation

To test whether the proteolytic lectin may be involved in induction of transformation, the purified recombinant protein (~4.5 µg protein) was added to parasitized blood, and transformation activity assessed after incubation at 27° C. A gradual stimulation of transformation from bloodstream to procyclic (midgut) form was observed. After 4 hours, ~ 25% of the parasites were either in transition form or midgut forms (Fig. 17). No transformation was observed in the absence of the recombinant protein or native lectin-trypsin complex, even after 24 hours. The rate of transformation by the recombinant GpI was 0.0788 ± 0.0048 at the asymptotic value of 98.94% (Fig. 17). On the other hand, the rate of transformation by the native lectin-trypsin was 0.066 ± 0.0054 at the asymptotic value of 95.87%. There was no significant difference between the rate of transformation observed when the parasites were incubated with lectin-trypsin complex or recombinant GpI (t-test, $p > 0.0001$) (Fig. 17).

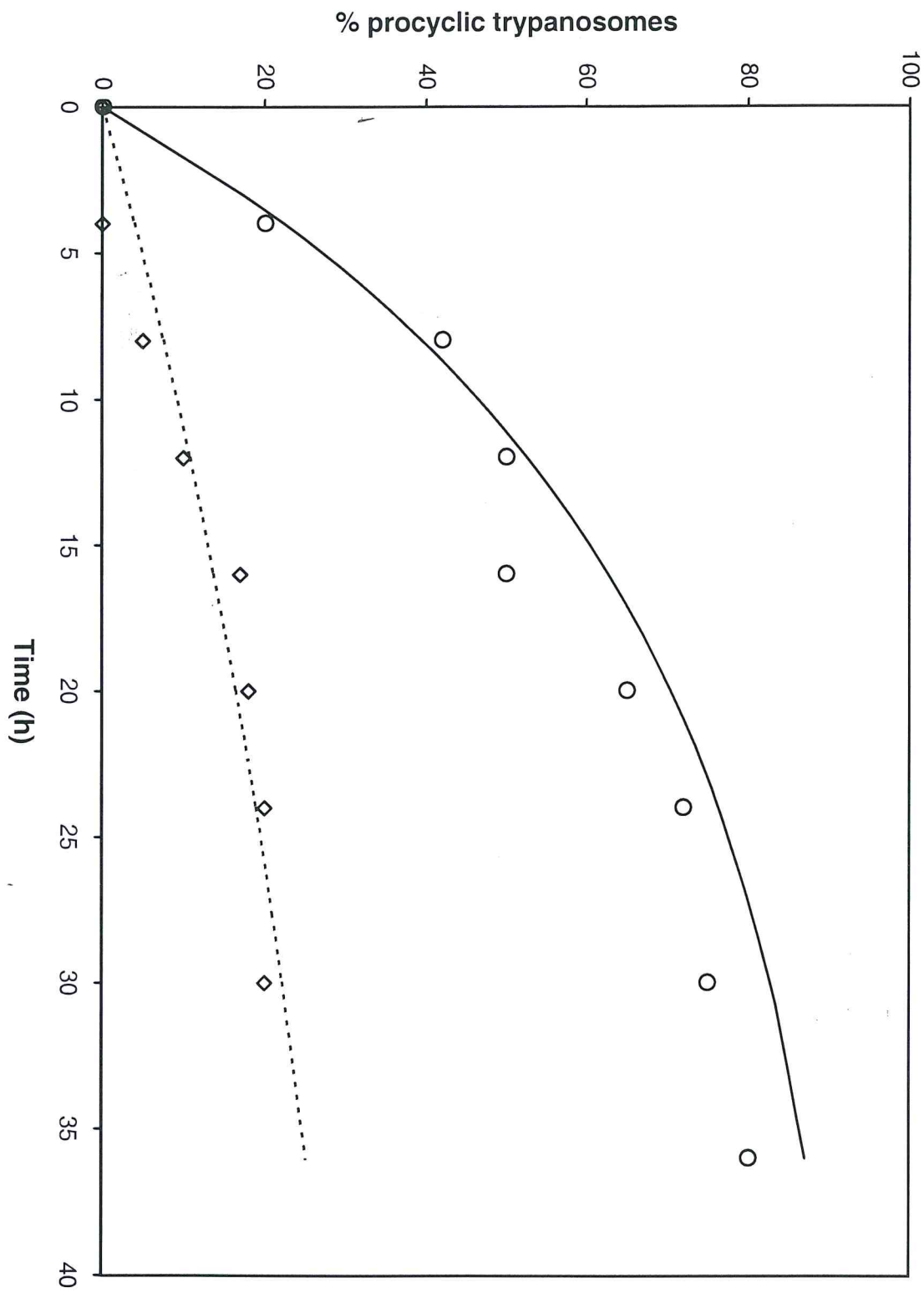

% procyclic trypanosomes
0
20
40
60
80
100
Time (h)
0
5
10
15
20
25
30
35
40

Fig. 17. Transformation of bloodstream trypanosomes by Gpl.

Parasitised blood (5 x 10^6 parasites/ ml) was mixed with the recombinant Gpl or native lectin trypsin complex and incubated (27° C). The control consisted of a mixture of parasitised blood and BIS, pH 7.9. Transformation of the parasites was assessed as described in Materials and Methods.

Lectin-trypsin complex [~4.5 µg] (●) observed values

Lectin-trypsin complex (—) predicted values

Recombinant Gpl [~ 4.5 µg] (▲) observed values

Recombinant Gpl (---) predicted values

Control (□) observed values

Trend (Predicted) represents mean values (n=4) fitted in non-linear model $y = A (1-e^{-bt})$.

Fig.18. PCR amplification of *G.f.f.* midgut cDNA using degenerate primers

Tsetse trypsin gene was amplified from once and twice-fed midgut cDNA using trypsin-degenerate 5' (trypsin-fwd) and 3' (trypsin-rev) primers. The PCR products were separated by electrophoresis using 1% agarose gel containing 0.3 µg/ ml ethidium bromide and visualized under UV

PCR cycle used

94° C – 2 min

94° C – 1 min
50° C – 1 min
72° C – 2 min
} 40 cycles

72° C – 5 min

Lane 1- 450 bp fragment amplified from once fed midgut cDNA template

Lane 2- 450 bp fragment amplified from twice-fed midgut cDNA template

Lane 3- 100 bp DNA ladder (Boehringer)

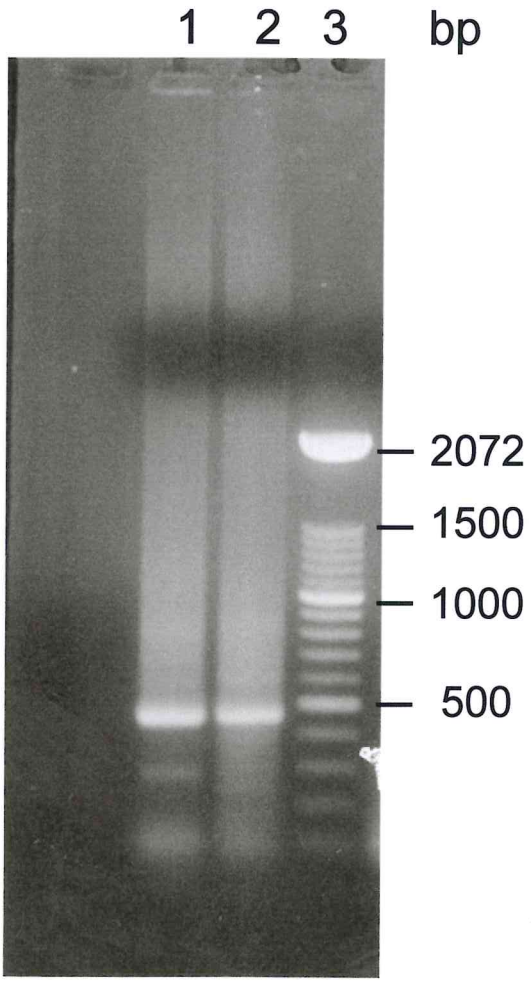

1
2
3
bp
2072
1500
1000
500

Fig. 19. Cloning and PCR screening of transformants in pGEM-T vector using SP6 and T7 primers.

The 450 bp PCR fragment was cloned into pGEM-T vector. The putative transformants were screened by PCR using SP6 and T7 primers (pGEM-T multiple cloning site promoters). The various size inserts were separated by electrophoresis using 1% agarose gel containing 0.3 µg/ ml ethidium bromide and visualized under UV

4.2 Screening of the midgut cDNA library with non-radioactive trypsin probe

4.2.1 PCR amplification and cloning of trypsin gene fragment

To identify other trypsin genes, the constructed cDNA expression library was further screened with non-radioactive trypsin probe. Degenerate primers designed from *A. gambiae* mRNA for serine protease, *A.aegypti* mRNAs 3A1 & 5G1 for putative trypsins, *A. aegypti* gut-specific, bloodmeal-induced late trypsin precursor gene, *A. aegypti* late trypsin precursor gene and *D. melanogaster* serine protease precursor mRNA were used to generate the tsetse midgut trypsin probe. The sequences of the primers were as follows; Trypsin Forward 5' -HTS RMD SSW GSH CAC TGY RC-3' and Trypsin Reverse 5' -GGG CCA CCG GAR TCW CC-3'. A strong band of ~450 bp was amplified (Fig. 18, lanes 1 and 2). When the PCR product was cloned in a TA vector (pGEM-T) using a shot-gun strategy and selected on Ampicillin plates containing IPTG and X-gal, several white colonies (putative transformants) were observed. Clones with expected size inserts were selected by PCR screening of the putative transformants using primers that anneal to the SP6 and T7 promoters flanking the multiple cloning site (MCS) of pGEM-T vectors (Fig. 19). Clones 1,2, 8, and 9 were selected and their plasmid DNA purified (Fig. 20). Upon sequencing, clone 1 revealed a 36 bp insert, clone 2 had 191bp insert, clone 8 had 458 bp insert and clone 9 had 303 bp insert. One of these (458 bp insert) showed similarity to the serine proteinase (fragment) of the blowfly, *Lucilia cuprina* with an expectation of 2×10^{-62}; 73% identities and 84% positives at the amino acid level respectively. This partial-length clone was used as a probe to isolate the full-length cDNA.

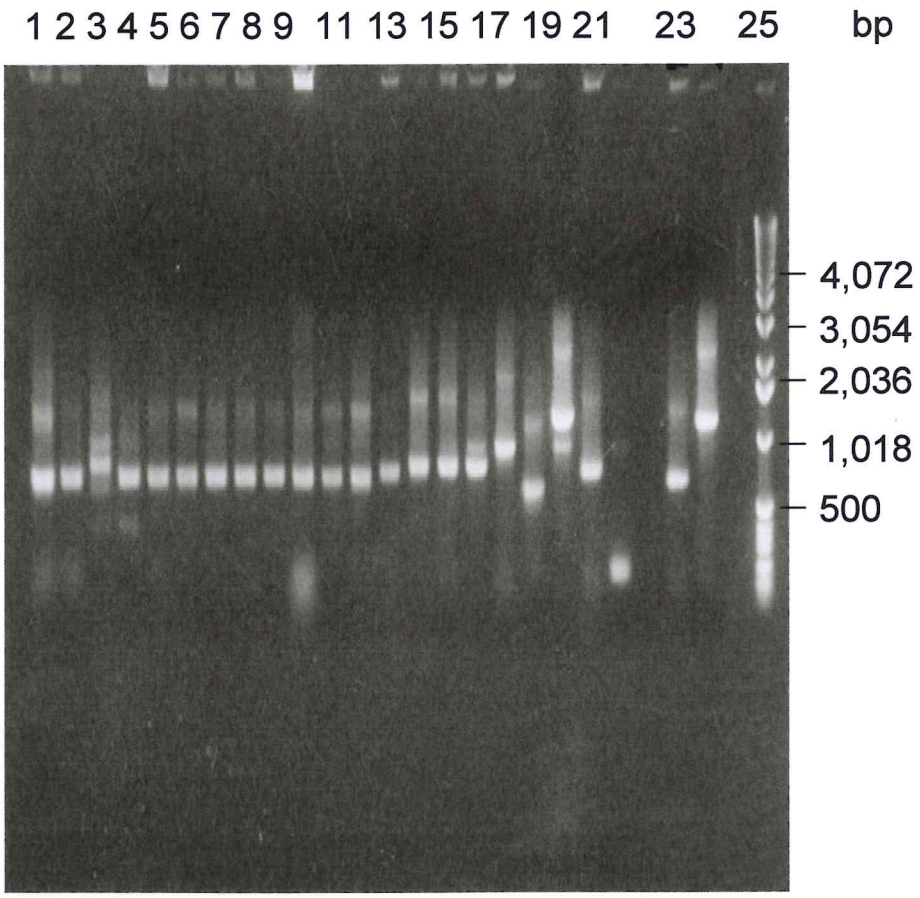

1 2 3 4 5 6 7 8 9 11 13 15 17 19 21 23 25 bp
4,072
3,054
2,036
1,018
500

Fig. 23. Sequence homology of GfSP.

The full sequence of GfSP cDNA was used to search similar sequences in GenBank using BLASTP, and multiple alignments were performed using MultAlign. Residues that are identical are shown in red. The residues that are in the catalytic triad (H,D,S) are indicated by '▲', the conserved cysteine residues are indicated by '✱' and the substrate specificity site by '◆'.

BOVTRYP- Bovine trypsin

SIMTRYP- Simulin trypsin

DROSTRYP- *Drosophila melanogaster* trypsin

SSP1- *Stomoxys calcitrans* trypsin-like serine protease

GSP1- *Glossina morsitans* trypsin-like serine protease

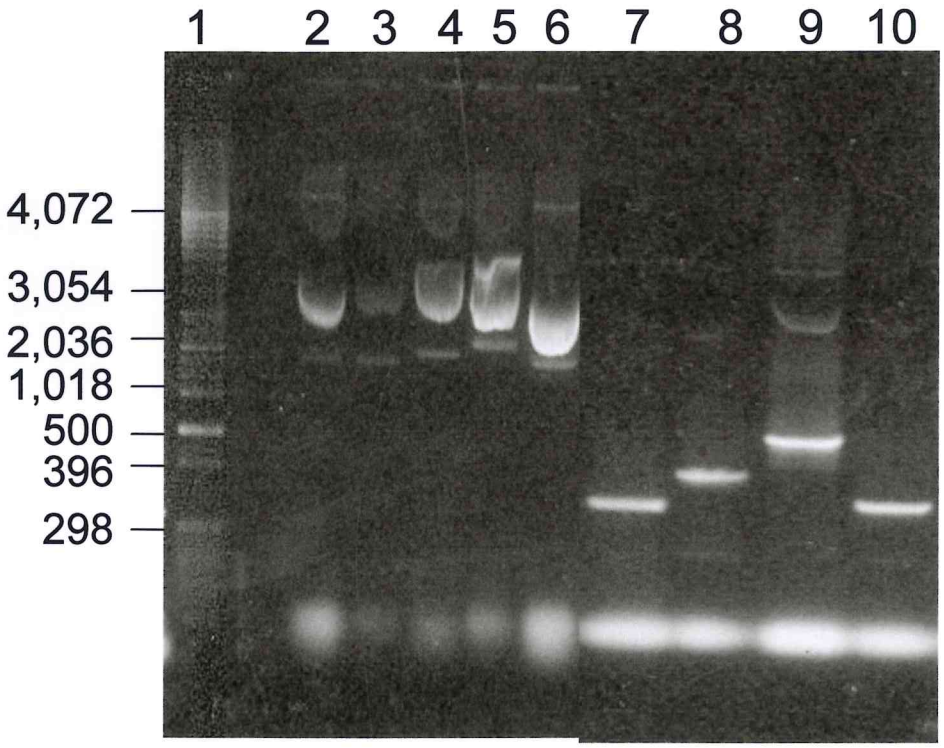
GffSP- *Glossina fuscipes fuscipes* serine protease (GfSP)

LUCHYM- *Lucilia cuprina* chymotrypsin

LITCHYM- *Litopenaeus vannamei* chymotypsin

HELCHYM- *Helicoverpa armigera* chymotrypsin

MANCHYM- *Manduca sexta* chymotrypsin


1
2
3
4
5
6
7
8
9
10
4,072
3,054
2,036
1,018
500
396
298

4.2.2 Isolation of full-length cDNA

When the plasmid form (pTriplEx2) cDNA expression library was screened with the cDNA probe, twenty positive colonies were generated. These positive colonies were further confirmed by PCR re-screening using trypsin nested-primers. Nine colonies yielded the expected single band of ~ 250 bp (Fig. 21). The sizes of the inserts were estimated by PCR using pTriplEx2-screening primers (Fig. 22). Three clones had ~100bp insert (Fig. 22; lanes 1, 2, 14), one clone had ~780 bp insert (Fig. 22, lane 3), one clone had ~900 bp insert (Fig. 22, lane 5), and two clones had ~530 bp inserts (Fig. 22, lanes 6,7). Four out of the nine cDNA clones with 530 bp, 530 bp, 780 bp and 900 bp inserts were purified and sequenced. The longest insert, designated *Glossina fuscipes* serine protease (GfSP), was found to carry a full-length cDNA.

4.2.3 Sequence homology of *Glossina fuscipes* Serine Protease (GfSP)

The putative protein sequence encoded by GfSP was used to search for similarity to other known genes at GENBANK using BLAST P (Altschul *et al.*, 1990) as well as the SWISS-PROT and TrEMBL Protein knowledge bases at the Expert Protein Analysis System (ExPASy) Molecular Biology Server. The highest similarity score was to a gene product from *Drosophila melanogaster* exhibiting 54% identities, 76% positives and an expectation of 8e-70 at the protein level. The second highest similarity score was to the larvai-specific serine protease from the Screwworm fly, *Chrysomya bezziana* with 69% identities, 80% positives and expectation of 7e-69. Another closely related serine protease was from first-instar larvae of the sheep blowfly, *Lucilia cuprina* at 75% identities, 87% positives and an expectation of 2e-67.

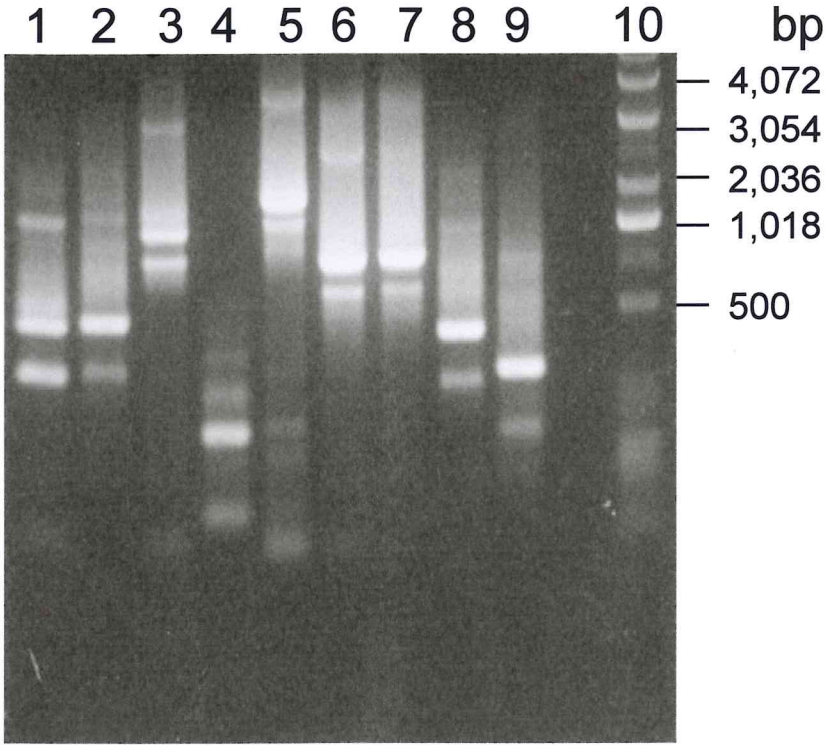

1 2 3 4 5 6 7 8 9 10
bp
4,072
3,054
2,036
1,018
500

Fig. 21. PCR screening of transformants using trypsin nested primers.

The twenty positive clones generated from screening the expression library with non-radioactive labeled cDNA probe were further screened by PCR using trypsin nested-primers. The various size inserts were separated by electrophoresis using 1% agarose gel containing 0.3 µg/ ml ethidium bromide and visualized under UV. Clones in lanes 1, 2, 5, 6, 7, 8, 9,14 and 15 gave the expected single band of ~250 bp.

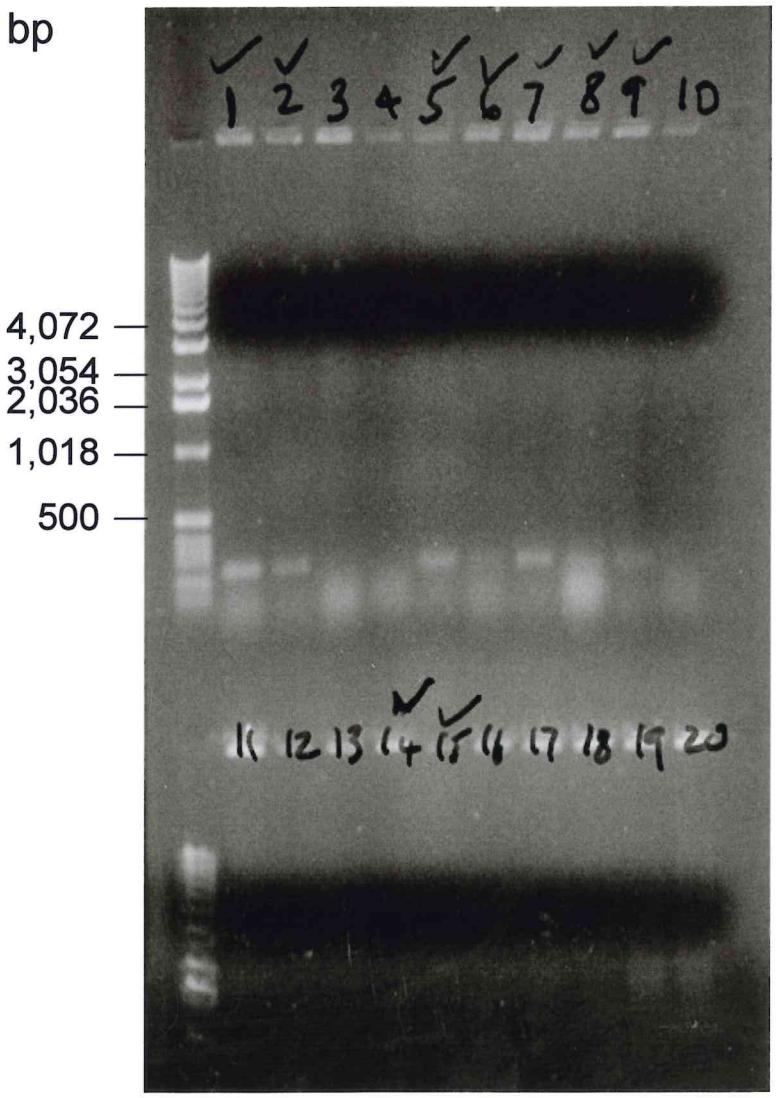

bp
4,072
3,054
2,036
1,018
500
1 2 3 4 5 6 7 8 9 10
11 12 13 14 15 16 17 18 19 20

Fig. 20. CsCl-EtBr purified plasmids

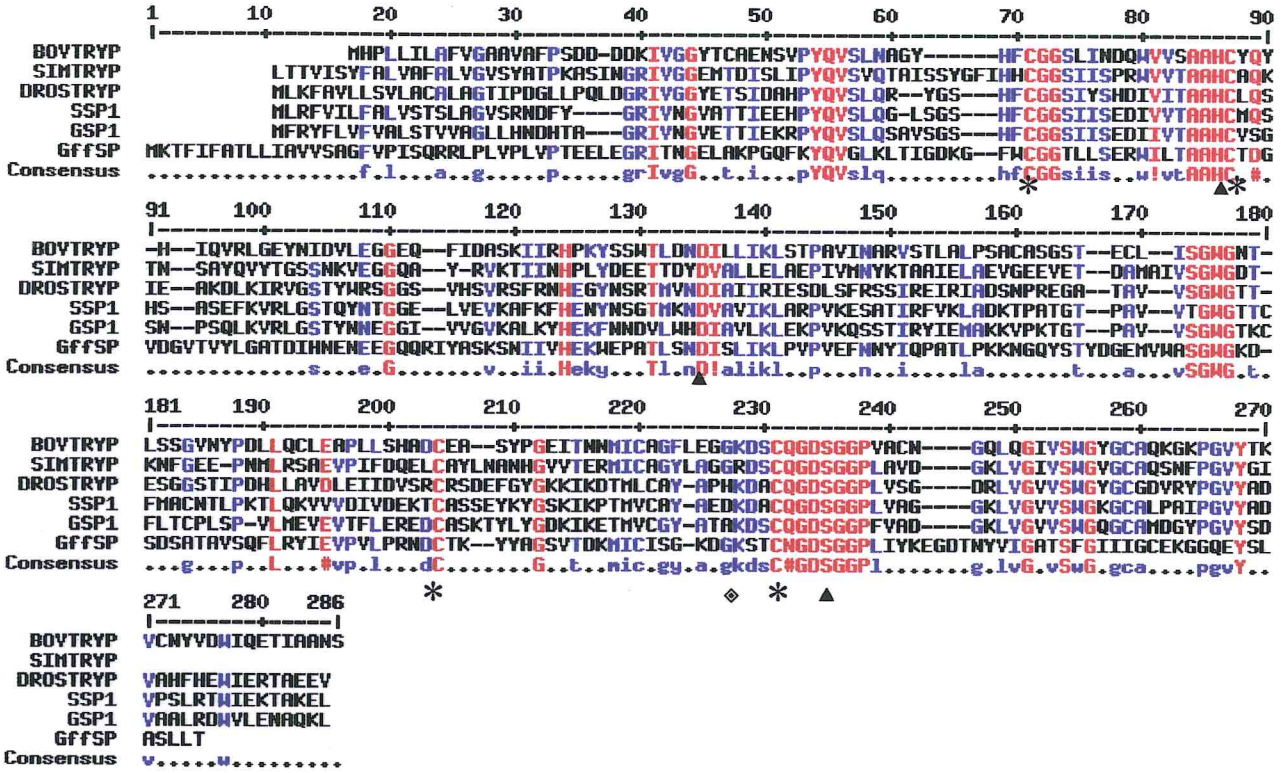
Clones 1,2, 8, and 9 (from Fig.19) were selected and their plasmid DNA purified by equilibrium centrifugation using cesium chloride (CsCl) - ethidium bromide (EtBr) gradient (adapted from Birnboim and Dolly, 1979). Isolated plasmids and the PCR products were separated by electrophoresis using 1% agarose gel containing 0.3 µg/ml ethidium bromide and visualized under UV.

Lanes 1 – 1Kb ladder

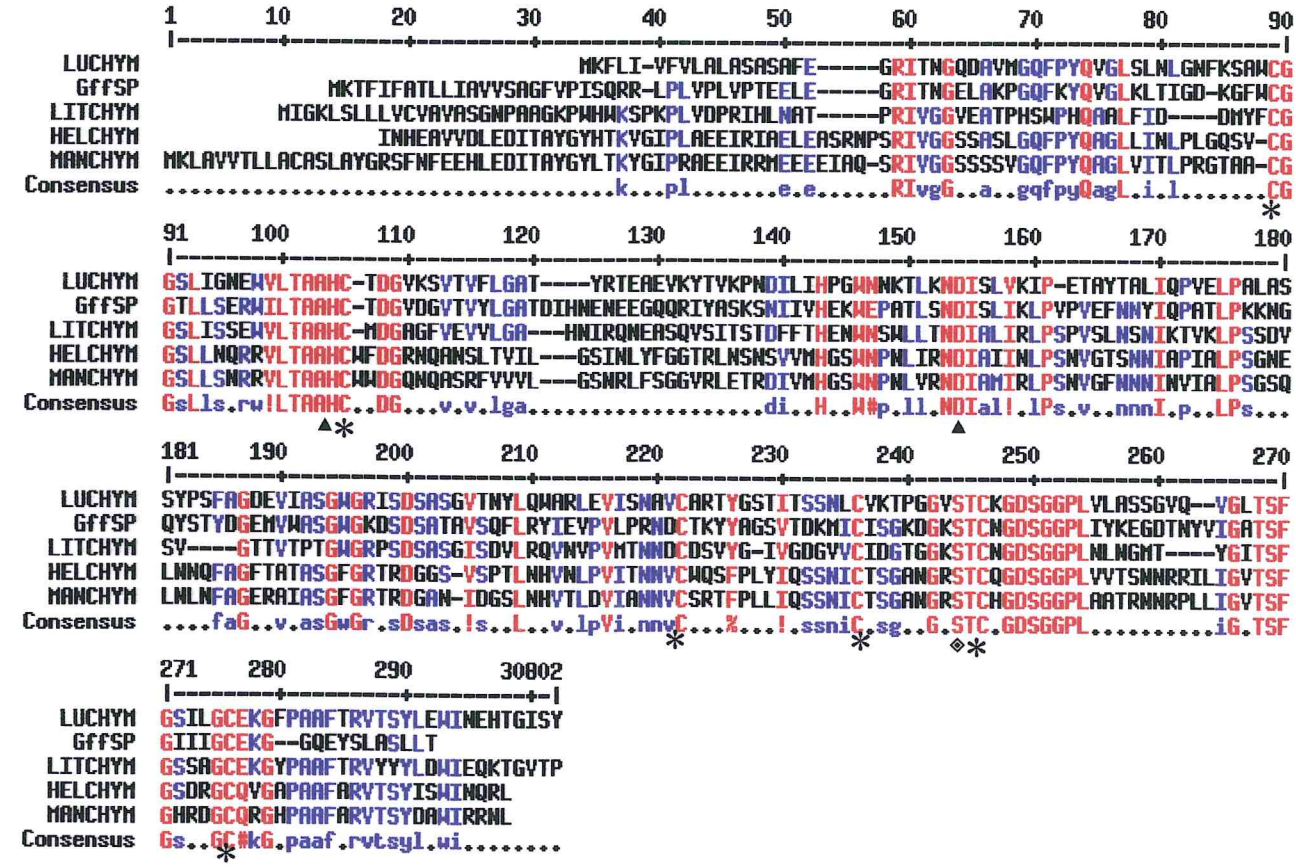
Lanes 2 - 6 show Purified plasmids from clones 1, 2, 8 and 9, respectively Lanes

Lanes 7 -10 show PCR amplified inserts from clones 1, 2, 8 and 9, respectively.

GfSP vs trypsins



GfSP vs chymotrypsins



Multiple alignment of the translated sequence with its best match candidates was carried out using the MultAlign program (Corpet, 1988) (Fig. 23). The results demonstrated that essential scaffold of the catalytic mechanism in trypsins is conserved. The catalytic residues, His-57, Asp-102 and Ser- 195 (using the bovine chymotrypsinogen A numbering system, Brown and Hartley, 1966) and the regions around these residues were highly conserved (indicated by '▲' in Fig. 23). Similarly, the six cysteines, which form three intra-molecular disulphide bonds in invertebrate serine proteases, were also conserved (indicated by '*' Fig. 23). Surprisingly, the Asp residue, which confers trypsin substrate specificity was absent. The presence of a serine residue at this position is indicative of chymotrypsin activity rather than trypsin (indicated by '◈' Fig. 23) (Kraut, 1977).

4.2.4 Sequence analysis of GfSP

The full-length of GfSP cDNA (GenBank submission AY145892) comprised of 952 bp (Fig. 24). The sequence contained thirty-nine nucleotides upstream to the start codon representing 5' UTR, followed by an ORF of 269 codons. There are 73 nucleotides after the stop codon representing the 3' UTR, followed by a poly A tail. The putative polyadenylation signal was present within the 3'UTR. Using PrositeScan algorithm, the protein sequence revealed a potential tyrosine kinase phosphorylation site at amino acids 236-242 (randomized probability 4.074e-04), several protein kinase C phosphorylation sites (randomized probability 1.423e-02), casein kinase II phosphorylation sites (randomized probability 1.482e-02) and N-myristoylation sites (randomized probability 1.397e-02).

Fig. 24. Sequence analysis of GfSP

The nucleotide and deduced amino acid sequences of GfSP cDNA, isolated from G. fuscipes midgut cDNA library is shown (Genbank submission AY145892). Serine and histidine active sites and their surrounding conserved residues are indicated in red.

952 residue sequence "Full-length GfSP (*Glossina* fuscipes serine protease)"
(Coding sequence in upperlane)

```
                                                  M  K  T  F  I  F  A  T  L  L
    1  gccattacggccggggagtcaatttgataccgtttcacaATGAAGACTTTTATTTTTGCAACTTTACTG
   11     I  A  V  V  S  A  G  F  V  P  I  S  Q  R  R  L  P  L  V  P  L  V  P
   70  ATTGCTGTTGTATCGGCTGGCTTCGTACCCATCTCCCAAAGACGTCTCCCATTGGTGCCTCTTGTGCCT
   34   T  E  E  L  E  G  R  I  T  N  G  E  L  A  K  P  G  Q  F  K  Y  Q  V
  139  ACAGAGGAATTGGAGGGCCGCATTACCAATGGAGAATTAGCAAAGCCTGGTCAATTTAAATACCAAGTT
   58     G  L  K  L  T  I  G  D  K  G  F  W  C  G  G  T  L  L  S  E  R  W  I
  208  GGTTTAAAATTAACAATCGGTGATAAAGGCTTCTGGTGCGGTGGCACATTGTTATCTGAACGATGGATT
   81     L  T  A  A  H  C  T  D  G  V  D  G  V  T  V  Y  L  G  A  T  D  I  H
  277  TTAACTGCTGCCCATTGTACTGATGGCGTTGACGGTGTTACCGTTTACTTAGGAGCTACCGATATACAC
  104   N  E  N  E  E  G  Q  Q  R  I  Y  A  S  K  S  N  I  I  V  H  E  K  W
  346  AACGAGAATGAAGAAGGCCAACAAAGAATCTATGCTTCAAAGTCAAACATCATCGTTCATGAAAAATGG
  127   E  P  A  T  L  S  N  D  I  S  L  I  K  L  P  V  P  V  E  F  N  N  Y
  415  GAACCAGCTACGTTAAGCAATGACATATCTTTAATCAAATTACCCGTACCTGTTGAGTTTAACAATTAC
  150   I  Q  P  A  T  L  P  K  K  N  G  Q  Y  S  T  Y  D  G  E  M  V  W  A
  484  ATTCAGCCAGCTACTCTGCCCAAGAAAAATGGCCAATATTCCACCTATGACGGGGAAATGGTTTGGGCT
  174   S  G  W  G  K  D  S  D  S  A  T  A  V  S  Q  F  L  R  Y  I  E  V  P
  553  TCTGGATGGGGTAAAGATAGTGATTCAGCCACTGCAGTTTCACAATTTCTTCGCTATATCGAAGTACCT
  198   V  L  P  R  N  D  C  T  K  Y  Y  A  G  S  V  T  D  K  M  I  C  I  S
  622  GTATTACCTCGCAACGATTGTACAAAATATTACGCTGGCTCGGTCACTGATAAAATGATTTGCATAAGC
  221   G  K  D  G  K  S  T  C  N  G  D  S  G  G  P  L  I  Y  K  E  G  D  T
  691  GGAAAAGATGGTAAATCAACTTGCAATGGAGATTCCGGTGGCCCATTAATTTACAAGGAGGGCGATACT
  244   N  Y  V  I  G  A  T  S  F  G  I  I  I  G  C  E  K  G  G  Q  E  Y  S
  760  AATTACGTTATTGGTGCTACATCCTTTGGTATTATTATTGGTTGCGAGAAAGGTGGCCAGGAGTATTCA
  266   L  A  S  L  L  T
  829  CTCGCGTCACTTCTTACCTAGattggattgaagagaagtctggtgtagttaatgcataatatgcaataaa
  898  aaaatatattctttcttgccacctaaaaaaaaaaaaaaaaaaaaaaaaaaaaaa
```

Serine proteases, trypsin family, histidine active site.
Site : 80 to 85 LTAAHC. Identity.
Randomized probability: 2.601e-07.

Serine proteases, trypsin family, serine active site.
Site : 223 to 234 STCNGDSGGPLI. Identity.
Randomized probability: 7.319e-08.

aataaa = Possible polyadenylation signal

Several structural requirements canonical to serine proteases were also identified. A serine active site (STCNGDSGGPLI) belonging to the serine proteases trypsin family was identified between amino acids 223 to 234 with a randomized probability of 7.319e-08. Similarly, a histidine active site belonging to the serine proteases, trypsin family was identified at amino acids 80 to 85 (LTAAHC) with a randomized probability of 2.601e-07.

4.2.5 Analysis of GfSP recombinant protein

Regulated expression of the cDNA was achieved in *E. coli* JM109. A protein band of M_r ~ 26,807 ± 2748 Da was detected by the antibodies in immunoblotting experiment (Fig. 25, lane 2). In contrast to GpI, this protein neither showed agglutination activity nor could it induce transformation of parasites. However, it exhibited remarkable lysis activity against bloodstream trypanosomes and rabbit RBCs.

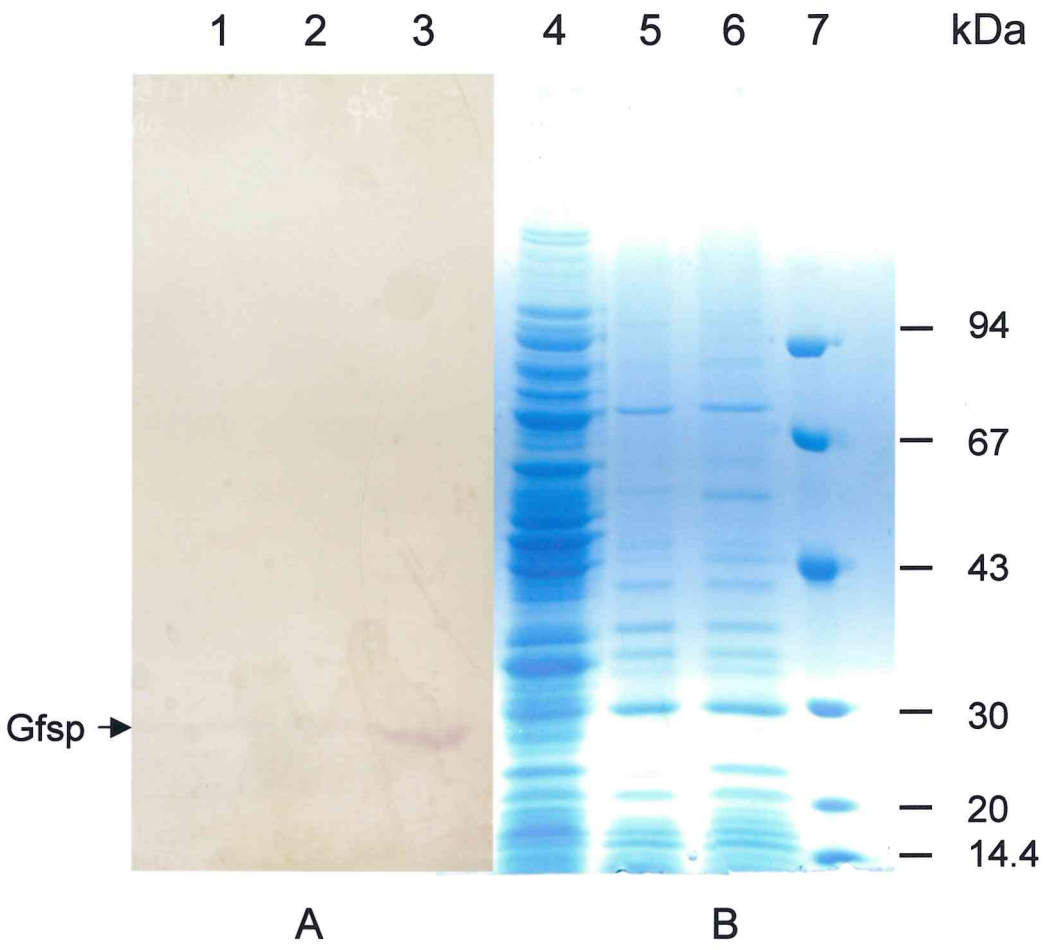
Trypsin activity of the recombinant GfSP molecule was assessed using a trypsin specific-chromogenic substrate, carbo-benzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY). Recombinant GfSP lysate exhibited trypsin activity of 4.390 units x 10^{-4} compared to the bacterial control lysate of 0.303 units x 10^{-4}.

Fig. 25. SDS-PAGE and immunoblot of expressed GfSP.

A culture of *E.coli* transformed with pTriplEx2-GfSP was grown at 37° C. IPTG was added (0.1 mM final concentration) in early exponential growth phase to induce expression of recombinant GfSP. The culture was incubated (7 h, 37° C) and the resultant lysate centrifuged (10,000 g, 15 min, 4° C). The proteins separated by gradient SDS-PAGE (4-20%) and then transferred onto nitrocellulose paper. The blots were then reacted with antiserum.

A. Immunoblot:
1- unexpressed *E.coli* –GfSP lysate (~ 30 µg)
2- *E.coli* JM109 lysate (control) (~ 35 µg)
3- recombinant *E.coli* –GfSP lysate (~30 µg).

B. Coomasie-stained gel:
4- recombinant *E.coli* –GfSP lysate (~30 µg)
5- *E.coli* JM109 (control) (~ 35 µg)
6- unexpressed *E.coli* –GfSP lysate (30µg)
7- low molecular weight standards (Pharmacia) (5 µl)


1
2
3
4
5
6
7
kDa
94
67
43
30
20
14.4
Gfsp
A
B

4.3 Discussion

4.3.1 Isolation of *Glossina* proteolytic lectin (Gpl) cDNA by immuno-screening the *G.f.f* midgut cDNA expression library

We have characterized the cDNA for a novel proteolytic lectin (Gpl1), which is synthesized and secreted in the midgut of the tsetse fly, *G. f. fuscipes* in response to a bloodmeal. The 5' terminus of the deduced amino acid sequence of Gpl was found to be rich in hydrophobic residues (e.g. G, A, V, L, F, P) suggesting that it is secretory in nature (von Hijne, 1983). Expression of the gene in *E. coli* yielded a proteolytic protein capable of agglutinating bloodstream-form trypanosomes and RBCs. In addition, the recombinant Gpl was able to stimulate transformation of bloodstream-form trypanosomes into procyclic forms *in vitro*.

A comparison of the amino acid sequence of the Gpl with the protein database using BLASTP showed that it exhibits similarity to various serine proteases, most notably to the sequence of *G. morsitans* serine protease 1 (Gsp1) (Yan *et al.*, 2001) that was reported while this work was in progress. Multiple alignment showed that Gpl possess the catalytic triad, His, Asp and Ser, and the highly conserved regions surrounding the His and Ser residues that are typical of serine proteases (Fig. 12) (Kraut, 1977). They also have the highly conserved cysteine residues at the position that allows the formation of the three cysteine bonds typical of invertebrate serine proteases and differentiating them from the vertebrate enzymes, which have four such bonds (Lehane *et al.*, 1998). At the substrate specificity site, Gpl contains the aspartate-189 (using the bovine chymotrypsinogen A numbering system, Brown and Hartley, 1966; Fig. 12) at position 212 (numbering as for Gpl; Fig. 13), which is

diagnostic of tryptic specificity (Stroud *et al.*, 1974). The aspartic acid, which lies at the bottom of the substrate-binding pocket, is responsible for trypsin's preference for lysine and arginine (Hedstrom *et al.*, 1992), and occurs in all known trypsins with the exception of a blood meal-induced *A. aegypti* trypsin (Barillas-Mury *et al.*, 1991). Alterations to this critical aspartate residue have generated proteases with poor catalytic rates towards arginine/lysine substrates (Evnin *et al.*, 1990; Czapinska and Otlewski, 1999). On the other hand, the glycine residue in the substrate-binding pocket is replaced by a serine at position 251 (numbering as for Gpl; Fig. 13), which is characteristic of chymotrypsin (Kraut, 1977). Chymotrypsin and trypsin have very similar three-dimensional structures but different substrate specificities. Chymotrypsin prefers bulky aromatic side chains whereas trypsin preferentially interacts with positively charged side chains (e.g. Lys or Arg of a substrate) at the bottom of the specificity pocket (Kraut, 1977; Warshel *et al.*, 1989). In this study, the substrate specificity of Gpl1 was determined empirically using a trypsin-specific chromogenic substrate, carbo-benzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY). The observed trypsin activity suggests that the expressed Gpl1 is closer to trypsin-like proteases than chymotrypsin-like proteases.

In order to assess the role of Gpl in tsetse-trypanosome interactions, the expressed protein was assayed for both agglutination and transformation activities. Gpl agglutinated both RBCs as well as bloodstream-form trypanosomes, suggesting that the protein had lectin activity. In both cases, agglutination was specifically inhibited by D-glucosamine. Several proteins bearing carbohydrate-binding and serine protease motifs, with diverse biological roles in development and immunity have been reported (Ohashi *et al.*, 1999; Vyse *et al.*, 1994). In *Anopheles gambiae*, a trypsin-like serine

protease (Sp22D) encompassing chitin binding, low density lipoprotein receptor and scavenger receptor cysteine-rich domains has been identified (Danielli *et al.*, 2000). In addition, lectins that have hydrolytic enzymatic activities have been reported in the mung bean species (Hankins and Shannon, 1978). Recently, a midgut defensin in association with serine protease was reported in *Stomoxys calcitran* (Hamilton *et al.*, 2002). In *Glossina* species, a lectin with trypsin-like activity has been isolated from the midgut of fed flies (Osir *et al.*, 1995). The non-covalently-linked α and β subunits of the holoprotein were shown to be associated with the trypsin and glycosyl residues, respectively. The presence of glycosyl residues on the β subunit was used as the basis for proposing that this subunit was the lectin component. The fact that the sequence of GpI did not reveal any potential glycosylation sites would suggest that it is probably closer to the α subunit of the complex. Further, the observation that GpI had lectin activity is not surprising since unglycosylated lectins such as calnexin have already been reported (Lis and Sharon, 1998). Further work will be needed to discover nature of the β subunit.

An interesting observation was the ability of GpI to stimulate transformation of bloodstream-form trypanosomes into procyclic forms *in vitro*. Initially, we concluded that this was a property of the lectin-trypsin complex (Chapter 3). The present findings suggest that GpI can by itself stimulate the transformation process. The role of the second subunit in this process deserves further detailed studies.

4.3.2 Isolation of *Glossina fuscipes* serine protease (GfSP) cDNA using trypsin DIG-labeled probe

We have identified a *Glossina fuscipes* serine protease (GfSP) cDNA belonging to the trypsin family, which is expressed in the midgut of the tsetse fly, *G. f. fuscipes* in response to a bloodmeal. The translated sequence of GfSP contains several phosphorylation sites. The string of hydrophobic residues at the N- terminal probably represents a signal sequence as identified in other serine proteases. Expression of the gene in *E. coli* yielded a proteolytic protein of M_r ~ 26,807. The recombinant GfSP was able to lyse bloodstream-form trypanosomes and RBCs.

A comparison of the amino acid sequence of the GfSP with the protein database using BLASTP showed that it exhibits similarity to various serine proteases from insects. Further comparison with all known insect trypsins and chymotrypsin genes confirmed that GfSP does not encode any previously identified digestive serine protease. Multiple alignment showed that GfSP possess the catalytic triad, His, Asp and Ser, and the highly conserved regions surrounding the His and Ser residues that are typical of serine proteases (Fig. 23). Several other structurally conserved regions diagnostic of serine proteases showed considerable conservation in GfSP. For example, the characteristic VSWG motif, which is found in most serine proteases is present (Geer, 1990). Similarly, the amino-terminal motif, GRI(T)NG, which indicates the start of the active enzyme in various mature serine proteases, is also conserved (Kalhok *et al.*, 1993; Han *et al.*, 1997). In addition, GfSP also have the highly conserved cysteine residues at the position that allows the formation of cysteine bonds typical of invertebrate serine proteases.

In *Glossina* species, at least six proteolytic enzymes have been shown to be involved in the digestion of the bloodmeal (Cheeseman and Gooding, 1985). These include trypsins, trypsin-like enzymes, chymotrypsin, chymotrypsin-like enzymes, carboxypeptidases and aminopeptidases (Cheeseman and Gooding, 1985). The most prominent of these are the serine proteases, trypsins and chymotrypsins (Applebaum, 1985). More recently, two gut-specific cDNA products from *Glossina morsitans morsitans* (Gsp1 and Gsp2) have been molecularly characterized and found to contain serine protease motifs (Yan *et al.* 2001). A comparison of the amino acid sequence of the GfSP in this study showed that it had limited similarity with the reported sequences of Gsp1 and Gsp2.

In contrast to the *Glossina* proteolytic lectin, the amino acid sequence of GfSP contains serine-189 at the substrate specificity site (using the bovine chymotrypsinogen A numbering system, Brown and Hartley, 1966; Fig. 23), which is typical of chymotrypsin. Chymotrypsin, which hydrolyses peptide bonds following an aromatic side chains, possess a deep cylindrical cavity with serine-189 replacing the negatively charged aspartate. However, GfSP displayed trypsin activity, albeit lower, with the trypsin-specific chromogenic substrate, carbo-benzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY). Several dual-specificity proteases, exhibiting chymotrypsin and trypsin-like activities have been reported (Sokolova *et al.*, 2001; Pemberton *et al.*, 1997). Moreover, recent studies on localized mutagenesis on trypsin have shown that even substitution of all amino acids in the S1 site of trypsin with their counterparts in chymotrypsin failed to transfer chymotryptic specificity to the variant enzyme (Perona and Craik, 1997). Transfer of specificity requires the additional exchange of amino acids

in at least three distal segments of the enzyme, none of which directly contacts substrate. In the well-studied bovine enzymes, seven separate surface loops have been implicated in distal site substrate or inhibitor contacts with different enzymes of the serine protease family (Perona and Craik, 1997). The exchange of surface loops 1 (residues 185-188, preceding Ser-195) and 2 (residues 217-225) is sufficient to confer high acylation rates towards hydrophobic-aromatic substrates, whereas further mutation in surface loop 3 (residues 169-174), improves binding by 50-fold (Hedstrom *et al.*, 1992).

In order to assess the potential involvement of GfSP in transmission of trypanosome infection in tsetse, the recombinant protein was assayed for parasite agglutination and transformation activities. In both cases, GfSP did not display agglutination activity nor could it induce transformation of parasites. However, the expressed protein exhibited remarkable lysis activity against bloodstream trypanosomes and rabbit RBCs. These results were interesting, since midgut proteases have been proposed to play an important role in parasite survival in addition to bloodmeal digestion. For example, in *A. gambiae*, a serine protease (ISP13) expressed primarily in the midgut was discovered to be transcriptionally upregulated in response to bacteria and *Plasmodium* infections (Dimopoulos *et al.*, 1996, 1997). Serine proteases have also been implicated in activation of the pro-phenoloxidase cascade and subsequent melanization of malaria parasites in refractory strain of *A. gambiae* (Paskewitz *et al.*, 1989; Gorman and Paskewitz, 2001). Recent studies in *Anopheles gambiae* revealed that the *Plasmodium*-refractory traits expressed higher levels of a serine protease gene (AgSp24D) than susceptible mosquitoes (Han *et al.*, 1997). Earlier, Shahabudin *et al.*

(1995) observed that unique specificity of *in vitro* inhibition of mosquito midgut trypsin activity correlated with *in vivo* inhibition of malaria parasite infectivity. Previously, in *Aedes aegypti*, midgut trypsin was shown to activate the *Plasmodium*-produced chitinase, which the parasite uses to traverse the peritrophic matrix barrier (Shahabudin *et al.*, 1993; 1996). Likewise, in the sandflies *P. papatasi*, a specific component of trypsin-like activity has been shown to prevent the survival of *Leishmania donovani* while modulation of this component by *Leishmania major* enables it to thrive (Borovsky and Schlein, 1987). The discovery of a serine protease (GfSP) in the midguts of *Glossina fuscipes fuscipes*, capable of lysing bloodstream trypanosomes, is therefore intriguing. The role of GfSP *in vivo* merits further study in order to completely understand the process of establishment of midgut infection in tsetse.

CONCLUSION

Molecular characterization of the lectin-trypsin from the midgut of the tsetse fly, *Glossina fuscipes fuscipes*, have demonstrated that it plays an important role in determining susceptibility to trypanosome infection. Indeed, the ability of native lectin-trypsin complex and the recombinant *Glossina* proteolytic lectin to stimulate transformation of bloodstream-form trypanosomes into procyclic forms *in vitro* clearly suggests that it may be the physiological trigger in the tsetse midgut.

Initial evidence for the presence of a lectin with trypsin-like activity in *Glossina* was obtained from the midguts of fed *Glossina longipennis*. The non-covalently-linked α and β subunits of the holoprotein were shown to be associated with the trypsin and glycosyl residues, respectively. The observation in this study that the *Glossina* proteolytic lectin had both lectin and proteolytic activities was therefore not surprising. The fact that the sequence of Gpl did not reveal any potential glycosylation sites would suggest that it is probably closer to the α subunit of the complex. Further studies are required to find the role of the ß subunit.

In this study, we have also identified a serine protease from the midguts of *Glossina fuscipes fuscipes*. This protein has a sequence similar to chymotrypsin-like proteases. Substrate specificity analysis however revealed that it had trypsin-like activity. Several dual-specificity proteases have been reported before, suggesting that the isolated gene might display both trypsin and chymotrypsin. Chymotrypsin activity need to be determined empirically using a chymotrypsin-specific substrate to prove this hypothesis. The ability of GfSP to lyse trypanosomes suggests that it may be a trypanolytic molecule involved in the elimination of trypanosome infections in the tsetse

midgut. Further characterization of gene expression and function will be necessary to determine the precise role of this gene.

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APPENDIX

1. BUFFERS

Buffered insect saline (BIS)

10 mM Tris-HCl, pH 7.9

130 mM NaCl

5 mM KCl

1 mM $CaCl_2$

Running buffer for PAGE

25 mM Tris-HCl, pH 8.3

192 mM glycine

1% SDS (for dissociating gel)

T.E buffer

10 mM Tris-HCl

1 mM EDTA, pH adjusted to 8.0

Separating gel buffer for PAGE

1.5 M Tris base. Adjust pH to 8.8 with HCl

Stacking gel buffer for PAGE

0.5 M Tris base. Adjust pH to 6.8 with HCl

Sample loading buffer for PAGE

0.13 M Tris-HCl, pH 6.8

20% (v/v) glycerol

0.002% bromophenol blue

4% SDS, 1% β mercaptoethanol (for dissociating gels)

2. SOLUTIONS AND MEDIA

LB Medium

10g Bacto-tryptone

5g Bacto-yeast extract

10g NaCl

dissolved in 1L distilled water, pH adjusted to 7.0, then autoclaved

LB Agar

LB medium in 1% agar

NZY + Broth

10g of NZ amine (casein hydrolysate)

5g yeast extract

5g NaCl

The above components were dissolved in 1L distilled water and autoclaved. Prior to use, the following solutions were added per 100 ml of 100ml of NZY+ broth:

1.25 ml of 1 M $MgCl_2$

1.25 ml of 1 M $MgSO_4$

1ml of 2 M filter-sterilized glucose solution or 2 ml of 20% (w/v) glucose

The final solution was filter-sterilized

Solution 1

50 mM glucose

10 mM EDTA

25 mM Tris-HCl, pH 8.0

5 mg/ ml lysozyme added immediately before use

Solution 2

0.2 N NaOH

1% SDS

Solution 3

60 ml of 5 M Potassium acetate

11.5 ml glacial acetic acid

28.5 ml distilled water