UNIVERSITY OF MALAWI

THE MIDGUT LECTIN-TRYPSIN COMPLEX OF *GLOSSINA MORSITANS MORSITANS* WESTWOOD (DIPTERA: GLOSSINIDAE): SITES OF SYNTHESIS AND ITS ROLE IN THE DIFFERENTIATION OF *TRYPANOSOMA BRUCEI BRUCEI* (KINETOPLASTIDA: TRYPANOSOMATIDAE).

BY

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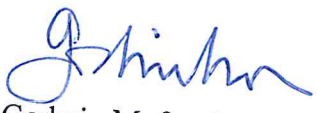
AUGUST, 1998

"Great are the works of the Lord studied by all who have pleasure in them."

Psalms 111 v. 2 (RSV)

DECLARATION

I, Godwin Mufwa Mannex Zimba, hereby declare that this thesis is my original work and the work described here has not been presented to any other University.



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DEDICATION

This thesis is dedicated to my late wife, Joyce Mantong'a Nyalanga Zimba and to our children; Kondwani, Temwanani and Nora who had to spend all this time without their parents around.

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LIST OF ABBREVIATIONS AND ACRONYMS USED

ADP	adenosine diphosphate
ARQU	Animal Rearing and Quarantine Unit
ARRPIS	African Regional Postgraduate Programme in Insect Sciences
BIS	buffered insect saline
cAMP	cyclic adenosine monophosphate
Da	daltons
DFP	diisopropyl fluorophosphate
DMF	dimethuyl formamide
DMFO	difluoromethyl ornithine
DNA	deoxyribonucleic acid
EATRO	East African Trypanosomiasis Research Organisation
FPLC	fast protein liquid chromatography
g	relative centrifugal force
GlcN	D(+) glucosamine
GlcNAc	N-acetyl glucosamine
ICIPE	International Centre of Insect Physiology and Ecology
Ig	immonoglobulin
LIT	lethal insect technique
M	molar
mM	millimolar
M_r	relative (apparent) molecular weight
NAD(H)	nicotinamide adenine dinucleotide
PBS	phosphate buffered saline
PEG	polyethylene glycol
pH	power of hydrogen (measure of acidity/alkalinity)

PMSF	phenyl methyl sulphonyl fluoride
RBC	red blood cells
RLOs	rickettsia-like organisms
SDM	semidefined medium
SDS	sodium dodecyl sulphate
SIT	sterile insect technique
STI	soybean trypsin inhibitor
TCA cycle	tricarboxylic acid cycle
TPCK	tosyl-phenyl alanine chlomethyl ketone
VAT	varaiable antigen type
VSG	variable surface glycoprotein

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ABSTRACT

An agglutinin with trypsin activity was purified from the midgut extracts of *Glossina morsitans morsitans* Westwood using a combination of ion exchange and affinity column chromatography procedures. The molecule had a native apparent molecular weight (M_r) of ~65,700 and consisted of two non-covalently linked subunits; an α subunit (~28,800 Da) and a ß subunit (~35,700 Da). The ß subunit was shown to have a glycosyl residue. This agglutinin had a bloodstream trypanosome agglutination titre of 64 and a trypsin specific activity of 69.3 μmoles/min./mg protein. The molecule compared favourably with the lectin-trypsin, complex previously purified from the midgut of *G. longipennis*.

The *G. m. morsitans* midgut lectin-trypsin complex was immunologically detected in midgut extracts of other *Glossina* species but not in the gut extracts of other haematophagous insects. The lectin-trypsin complex was also shown to be widely distributed in tsetse tissues as it was immunologically detected in the fat body, haemolymph, midgut tissues and the peritrophic membrane. Biological activity (ability to agglutinate trypanosomes) was detected only in the peritrophic membrane homogenate. In the other tissues, the molecule was detected at higher molecular weights. These results suggest that the molecule was present in these tissues either as a precursor or bound to some other molecule which affected the biological activity.

The subunits of the *G. m. morsitans* midgut lectin-trypsin complex were shown to be synthesised by the fat body and the midgut tissues as ~42,000 Da and ~62,000 Da molecules, respectively. The trypsin moiety was shown to be synthesised only by the midgut tissues. It was suggested that the lectin-trypsin complex is activated after the two subunits link up in the midgut lumen. The synthesis of proteins by the fat body was also

shown to be stimulated by the bloodmeal, which corresponded to the release of the agglutinin into the midgut.

The purified agglutinin was also able to induce *in vitro* transformation of trypanosomes from bloodstream to procyclic forms more than the crude midgut homogenate. Increasing the concentration of the lectin-trypsin complex resulted into higher transformation rates accompanied by an increase in trypanosome mortality. Of all the tissues shown to be associated with the midgut lectin-trypsin complex, only the peritrophic membrane induced the *in vitro* parasite transformation, although the mortality rates of the parasites were high. It is suggested that the peritrophic membrane due to its close association with the lectin-trypsin complex is involved in trypanosome tropisms and trafficking within the tsetse midgut.

In vivo studies also showed that elevation of the midgut concentrations of the lectin-trypsin complex significantly lowered the establishment rates of midgut *T. b. brucei* infections in *G. m. morsitans*. On the other hand lowering the midgut concentrations of the lectin-trypsin complex did not significantly affect the establishment of the trypanosome midgut infection in the tsetse. It was proposed that the tsetse midgut lectin-trypsin complex has a dual role in tsetse. In addition to acting as an immune molecule at higher concentrations, clearing the trypanosomes from the midgut, at lower concentrations the same molecule is involved in the induction of differentiation of trypanosomes and hence facilitate the establishment of midgut infections.

The lectin moiety of the molecule was shown to appear bound to the bloodstream trypanosomes as a distinct ~35,000 Da band and inclusion of the inhibitor D(+) glucosamine completely abrogated its binding. A lower distinct band of ~19,400 Da was not affected by any of the inhibitors for the complex subunits. The ~35,000 Da band was proposed to represent

the glycosyl residue of the molecule and is involved in the binding to trypanosomes.

It has been proposed that further work on the identification of genes encoding for the subunits of the molecule would aid in using the molecule in a novel trypanosomosis control. Through genetic manipulation, creation of tsetse strains with over-expressed genes for the subunits would have higher concentrations of the midgut lectin-trypsin complex, and therefore have lower rates of midgut infection establishment in tsetse.

CHAPTER ONE

GENERAL INTRODUCTION

African trypanosomiases, commonly known as sleeping sickness in humans and nagana in domestic animals, are caused by flagellated protozoa of the genus *Trypanosoma* (Kinetoplastida:Trypanosomatidae). Although trypanosomes are regarded as one of the most primitive organisms in the animal kingdom, they are also one of the most specialised. They are often found in the blood and tissues of their hosts (Molyneux and Ashford, 1983).

African trypanosomiasis distribution is restricted to the sub-Sahara Africa, between latitudes 15° N and 30° S, an area of approximately 11.7 million square kilometres, about 30% of the African continent and an area almost equivalent to the size of the United States of America (Figure 1.1)(Logan-Henfrey *et al.*, 1992; Radostits *et al.*, 1994). This distribution coincides with that of the tsetse flies, genus *Glossina* (Diptera; Glossinidae), the known vector for both human, domestic animals and wild game trypanosomiasis.

These diseases have both direct and indirect constraints on the development of the African continent. Although human trypanosomiasis is currently restricted to defined foci (Dransfield *et al.*, 1991), Goodwin (1985) reported that an estimated 45 million people are exposed to the risk of infection. According to the Tropical Disease Research newsletter (1990), about 25,000 new patients are reported annually. However, due to diagnostic difficulties, poor reporting and inaccessibility of affected areas, this figure could be a gross underestimate.

Trypanosomiasis of domestic animals is, however, a more serious problem than the human disease (Jordan, 1993). This disease is more virulent and causes mortality and morbidity to a number of species of domestic animals, particularly cattle. Vast territories are inaccessible to livestock (Jordan, 1986) while approximately 3 million cattle are estimated to be lost each year due to the disease (Gyening, 1990). It has been estimated that the total area infested with tsetse flies, and precluded from productive

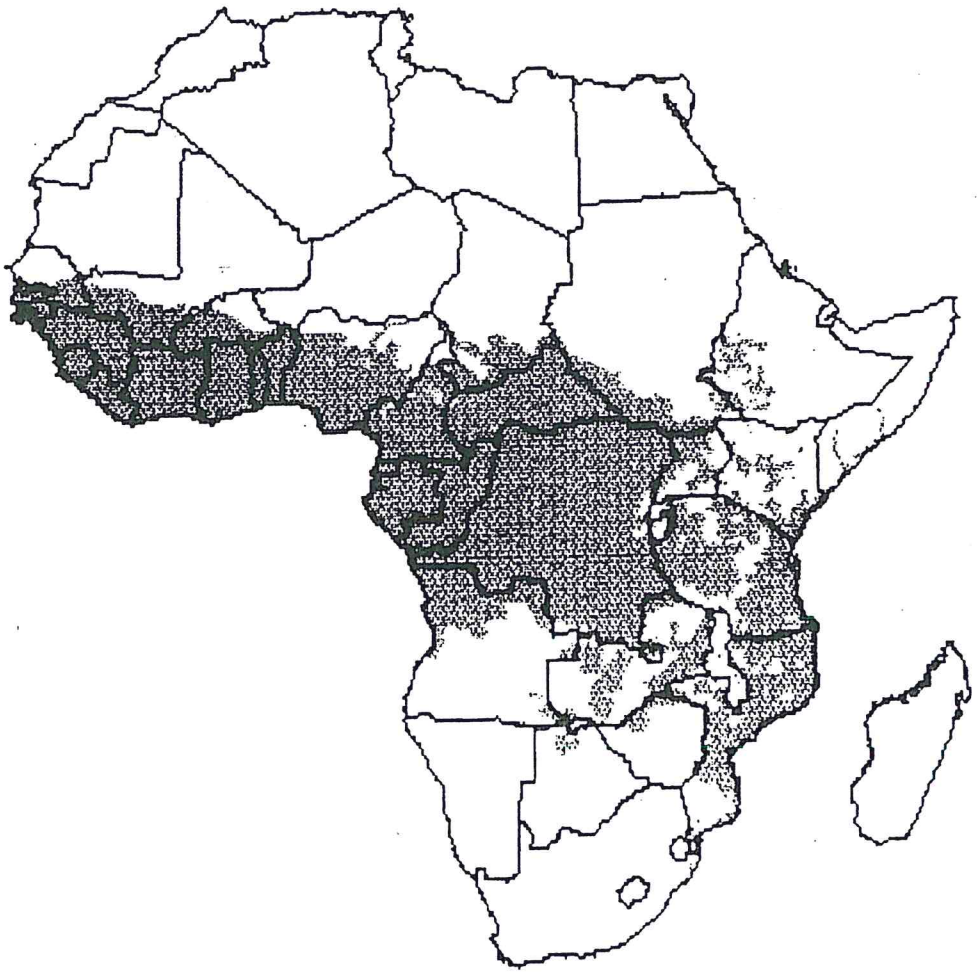

Figure 1.1 Map of the African continent showing the African trypanosomiasis endemic areas (shaded)(Source: Logan-Henfrey *et al.*, 1992).

cattle husbandry has the potential of supporting about 125 million heads of cattle (Odhiambo, 1986).

The problems due to trypanosomiasis are compounded by civic disorder prevalent in Africa, reliance on the central governments to provide solutions and the latters' addictive dependence on foreign aid (Dransfield *et al.*, 1991). It is unlikely that the situation will improve in the near future considering the ever worsening socio-economic situation throughout Africa.

The control of African trypanosomoasis, to date, has depended on two main activities namely: parasite control, which targets the parasite mainly in the vertebrate host, and vector management, which targets the tsetse vector mostly in its habitat.

Parasite control, for both human and animal trypanosomiasis, involves chemotherapy, use of trypanotolerant cattle as well as efforts to develop anti-trypanosome vaccines. Most of parasite control, to date, has relied heavily on chemotherapy for both human and animal trypanosomiasis (Dransfield *et al.*, 1991; Jordan, 1986). Chemotherapy involves use of several anti-microbial drugs either as prophylactics for animals at risk or for the treatment of infected animals. For treatment of early stages of human trypanosomiasis suramin and pentamidine are used while melarsoprol is used in later stages. A newer drug, difluoromethylornithine (DFMO) has also been used in treating the West African sleeping sickness (Jordan, 1986; Seed and Hall, 1992). In domestic ruminants, three compounds are used for prophylaxis and treatment of nagana. These are Diminazene aceturate, Homidium and Isometamidium chloride while suramin and quanapyramine are used for treatment of trypanosomiasis in horses and camels (Logan-Henfrey *et al.*, 1992; Jordan, 1986). However, almost all the anti-trypanosomal drugs currently in use have been shown to have toxic side effects which may result in pathological manifestations such as nephritis and tissue necroses (Stephen, 1986). In addition, most of the anti-trypanosomal drugs do not act on all stages of the parasites and require repetitive administrations (Gutteridge, 1985). Furthermore, the parasites tend to develop resistance to most of the drugs (Finnele, 1983; Peregrine, 1994). Chemotherapy has also been shown to have no

permanent effect on the cycle of the disease as incidences of the disease returning to pre-treatment levels when treatment is relaxed have been reported (Jordan, 1986). It must also be pointed out that since the 1950's no potent new drugs have become available. This is largely due to lack of enthusiasm in the pharmacological industries as profits from drug sales have been low since trypanosomiasis is, generally a diseases of poor countries (Holmes, 1997; Marquardt, 1996).

Vaccination against trypanosomes has remained intractable and elusive. Development of vaccine against trypanosomes has been hampered by the constantly changing antigenic composition on the parasites' surface coat, (Turner, 1985; Borst, 1986). This serves, for the trypanosomes, as a simple but effective strategy of survival in the immune host. Since most vaccines target cell surfaces, this property of the parasite, known as antigenic variation, renders immunological attack on the trypanosomes impossible (Barry, 1989). This means that an effective vaccine will have to contain all the variable antigen types (VATs) whose number is very large, about 1000 antigenically different variable surface glycoproteins expressed sequentially at a time (Eshita *et al.*, 1992; Murray *et al.*, 1983a). The prospects of vaccine development in the near future are, therefore, rather poor (Radostits *et al.*, 1994).

Trypanotolerance, as a means of parasite control, is applicable only in animal trypanosomiasis. This refers to reducing the effects of animal trypanosomiasis by exploiting the natural tolerance to trypanosome infection displayed by some cattle breeds, such as N'dama and Dahomey (Molyneux and Ashford, 1983). Trypanotolerance has been shown to be inherent in many game animals and has been proposed to be a more appropriate control method where effective tsetse control by other methods is not feasible (Murray *et al.*, 1983b).

Vector management in trypanosomiasis control basically aims at reducing the population of tsetse, sufficiently to break the disease transmission cycle. The methods employed include habitat modification, use of insecticides, trapping, genetic control and biological control.

Habitat modification, primarily, involves clearance of vegetation which

consequently eliminates the fly habitat. This vector management method may also include destruction of the wild game and other feeding hosts for the tsetse, thus removing the parasite reservoir from the habitat (Gathuma, 1986). These were the earliest methods that were used to control tsetse fly populations up to the 1940s, before the advent of insecticides and trypanocidal drugs. However, habitat modification results in the destruction of valuable plant and animal resources. Bush clearing, in turn, also leads to soil erosion (Dransfield *et al.*, 1991).

The use of insecticides replaced bush clearing and game destruction in tsetse control and management programmes after the 1940s. Initially residual organochlorines, such as DDT were used for ground spraying. Lower doses of insecticides , such as Endosulfan are currently in use as aerial sprays and this has been augmented by synthetic pyrethroids such as Alpha- and Deltamethrin. Other pyrethroids such as Flumethrin is also used as a spray-on, dip-in and pour-on formulation for livestock, thus making the animals lethal mobile targets for the tsetse flies (Logan-Henfrey *et al.*, 1992; Dransfield *et al.*, 1991; Jordan, 1986). Although the use of insecticides has generally showed considerable success in reducing the tsetse population levels, extensive use of insecticides is harmful to the environment as many non-target organisms are destroyed in the process. This is compounded by failure of most biological systems to completely degrade insecticides (Laird, 1977; Molyneux and Ashford, 1983). Moreover, insecticides are also very expensive and require regular applications.

Unlike insecticides which kill insects immediately following application, traps and screens kill them over an extended period of time consequently reducing the tsetse fly populations (Logan-Henfrey *et al.*, 1992). Different types of traps have been developed and employed though their impact on tsetse fly populations tend to vary from habitat to habitat as well as with species and geographical distribution of the subspecies of *Glossina* (Radostits *et al.*, 1994; Holmes, 1997). Currently the most effective trap is the bioconical trap (Jordan, 1986). Initially, sticky materials were incorporated onto the traps to retain the flies. More recently, odour-baited traps incorporating ingredients of ox breath and urine (mixtures of

acetone, CO_2, 1-octen-3-ol, 4 methyl-phenol and 3-n-propyl-phenol) have shown more promise, being able to reduce tsetse fly populations by over 70% in a community over a 7 month period (Radostits *et a*l., 1994; Molyneux and Ashford, 1983; Jordan, 1986). Odour-baited Deltamethrin traps, used to control tsetse have been shown to be more effective (Logan-Henfrey *et al*,. 1992; Dransfield *et al*,. 1991). The use of traps to manage tsetse populations, although relatively less expensive and easy to manage, is hampered by frequent reinvasion. Secondly the traps can be destroyed by animals or stolen by individuals (Gathuma, 1986).

Due to the low reproductive capacity and infrequent mating of female tsetse flies, genetic control of tsetse seems be more attractive and ideal (Radostits *et al*., 1994; Molyneux and Ashford, 1983). One of the genetic control methods currently in use is the sterile insect technique (SIT) where laboratory reared male tsetse are sterilised by gamma irradiation and then released into the field. The presence of high numbers of sterile males would then lead to eventual reductions in fly populations (Laird, 1977). This method was successfully used to eradicate *G. palpalis gambiensis* from an area in Burkina Faso and *G. m. morsitans* in a ranch in Tanzania (Moloo *et al*., 1988). The complete success of this control method is hampered by subsequent re-invasion by non-sterile males of the target species to supposedly controlled areas. This strategy, however, proved very effective in eradicating screw worm fly from the southern USA (Krafsur *et al*., 1987). SIT is an expensive method requiring high technology which would be unsuitable for developing countries. However, SIT could be more effective if it were incorporated into integrated trypanosomiasis control programmes, where it is used together with insecticides as aerial sprays and bioconical traps (Moloo *et al*., 1988; Laird, 1977).

Biological control of tsetse relies on the use of pathogens, parasites and predators to reduce insect populations. This offers a specific and environmentally-friendly approach to pest control (Jordan, 1985). Although biological control is attractive for tsetse control due to the low reproduction potential (Laird, 1977), only a few, unsuccessful attempts have been made to release the agents into tsetse habitats (Jordan, 1986). The most potential biological control agents of *Glossina* sp.

have been parasitoids such as *Nesolynx* (Hymenoptera; Eulophidae), *Chrestomutilla* (Hymenoptera; Mutillidae) and *Exhyalanthrax* (Diptera; Bombyliidae) (Weiser, 1991; Laird, 1977). However, the low density of wild tsetse populations compared to most other insect pests and their well protected pupae make biological control extremely difficult to carry out (Logan-Henfrey *et al.*, 1992). The only susceptible stage for biological control is when the larva is dropped to the soil prior to burrowing (Weiser, 1991).

From the above précis, it can be seen that most of the trypanosomiasis control methods, currently in use, have proved to be unsustainable. This, therefore, necessitates further search for alternative environmentally benign strategies which can offer long term sustainable tsetse control methods. One such approach has been to look for insect factors responsible for the natural refractoriness to trypanosome infection inherent in both laboratory-reared and natural tsetse populations.

In recent years, there has been considerable interest in the development of new approaches to the control of vector-borne diseases through the development and use of transgenic vectors. By aiming at suppressing vector populations as well as altering the ability of the vectors to transmit diseases, such strategies would have profound and long lasting effects on disease epidemiology (Crampton *et al.*, 1994; Jacobs-Lorena and Lemos, 1995; Hide, 1994). Transgenic technology directly introduces specific deoxyribonucleic acid (DNA) sequences of interest into the germline of the insects which leads to an eventual change in either the vector's reproductive potential or the vectorial capacity.

To effectively produce refractory strains for the pathogen as well as vector strains with low vector competence or low reproductive potential, it is important to understand the complex relationships that exist between the insect vector and the pathogen (Crampton *et al.*, 1994; Billingsley, 1994). The numerous factors involved in determining the outcome of the vector-parasite interactions play a major role in the eventual refractoriness or susceptibility of the vectors to infection by the parasites. Studies on these factors, therefore, can provide important biochemical information which can be used in selectively determining the genes which can be

potential targets for manipulation (Gooding, 1995; Crampton *et al.*, 1994).

In the tsetse-trypanosome system, several factors have been shown to be involved in determining the susceptibility of the tsetse flies to trypanosome infection. Amongst these are lectins (Maudlin, 1991) and trypsins (Imbuga *et al.*, 1992a). The presence of a tsetse midgut glycoprotein with both trypsin and glycosyl activities has shown that both the lectins and trypsins described in previous tsetse-trypanosome studies are in the same molecule (Osir *et al.*, 1995). The fact that this molecule is only present in *Glossina* and not in other haematophagous insects might be of importance in trying to elucidate the vectorial capacity of tsetse flies for trypanosomes. This, therefore, necessitates the study synthesis sites of the molecule and its components as well as the role that the molecule plays in trypanosome development in the tsetse fly.

Studies on the role of the lectin-trypsin complex in trypanosome tropism would be important in explaining the vectorial capacity of tsetse for trypanosome infections. On the other hand, studies on the possible synthesis site(s) of the molecule would help to identify possible target points for blocking parasite transmission, through genetic manipulation. Such information can be used in designing novel trypanosomiasis control strategies which break the parasite's life cycle in the insect by targeting either the vector or the parasite.

The overall objective was to study the synthesis sites and the involvement of tsetse midgut lectin-trypsin complex in determining tsetse fly susceptibility to trypanosome infection. Specific objectives were:

- To determine the sites of synthesis of the components of the lectin- trypsin complex in the tsetse fly as well as investigate the possible site of the complex formation.
- To determine the role of the lectin-trypsin complex in the transformation of bloodstream parasites into procyclics.
- To determine the mode of action (mechanism) of the lectin-trypsin complex in the transformation process.

CHAPTER TWO

2. LITERATURE REVIEW

2.1. The tsetse flies

Tsetse flies belong to the genus *Glossina*, Family Glossinidae and Order Diptera. Being vectors of African salivarian trypanosomes pathogenic to man and domestic animals, tsetse are one of the most economically important insects (Molyneux and Ashford, 1983). The 23 known living species of the genus *Glossina* are restricted to sub-Saharan Africa. On the basis of genitalia features of both sexes and preferential ecological requirements, the *Glossina* species have been divided into three broad taxonomic groups; *fusca, palpalis and morsitans* (Laird, 1977; Jordan, 1983). The *fusca* group, largely confined to the forest areas, are more important as vectors of wildlife infection than of livestock trypanosomiasis (Logan-Henfrey *et al.*, 1992). In this group are; *G. fusca fusca, G. f. congolensis, G. tabaniformis, G. longipennis, G. brevipalpis, G. nigrofusca nigrofusca, G. n. hopkinsi, G. fuscipleuris, G. medicorum, G. severini, G. schwetzi, G. haningtoni, G. vanhoofi* and *G. nashi*. (Jordan, 1986). None of these tsetse species are known to transmit human sleeping sickness (Seed and Hall, 1992).

Tsetse of the *palpalis* group are primarily found in the riverine habitats but can also occupy an array of other ecological habitats. To this group belong; *G. palpalis palpalis, G. p. gambiensis, G. tachnoides, G. pallicera pallicera, G. pallicera newstedi, G. fuscipes fuscipes, G. f. martinii, G. f. quanzensis* and *G. calligenea* (Jordan, 1986). These species feed on a large variety of animals but are not efficient vectors of livestock trypanosomiasis. *G. palpalis* and *G. tachnoides* are the principal vectors of the Gambian sleeping sickness (Seed and Hall, 1992; Jordan, 1986; Logan-Henfrey *et al.*, 1992).

The *morsitans* group of tsetse inhabits the savanna woodlands. Within this group are; *G. morsitans morsitans, G. m. centralis, G. longipalpis, G. pallidipes, G. austeni* and *G. swynertoni* (Jordan, 1986). These flies feed readily on ruminants and are efficient vectors of livestock trypanosomiasis. *G. morsitans* is the most important vector of animal trypanosomiasis and the Rhodesian sleeping sickness (Logan-Henfrey *et al.*, 1992).

Both the male and female tsetse flies have been shown to be vectors of trypanosomes since both sexes feed on blood. Being larviparous insects, the females require more regular bloodmeals, at approximately 2-3 day intervals, for larval development. The blood is obtained from various vertebrate hosts and the bloodmeal sizes range from 20-60 μl depending on the physiological state and the size of the fly (Molyneux and Ashford, 1983).

2.2 The trypanosomes

All the parasitic trypanosomes belong to the order-Kinetoplastida and family-Trypanosomatidae within which are nine genera: *Crithidia, Leptomonas, Herpetomonas, Blastocrithidia, Trypanosoma, Phytomonas, Leishmania, Edotrypanum and Rhynchodomonas.* (Molyneux and Ashford, 1983). In terms of pathogenicity to man and his domesticated animals, the genus *Trypanosoma* is the most important. Depending on their development in the vector and the vertebrate host, the genus *Trypanosoma* is divided into two sections, stercoraria and salivarian. In the stercoraria, the parasite completes its developmental cycle in the gut such that the infective stage appears in the faeces of the vector and transmission is by contamination. On the other hand, the infective forms of the salivarian trypanosomes appear in the salivary medium of tsetse and transmission is by inoculation. The salivarian trypanosomes cause African trypanosomiasis. The genus *Trypanosoma* is further divided into four subgenera: *Duttonella* which

include *T. vivax* and *T. uniforme*; *Nannomonas* in which are *T. congolonse*, *T. simiae* and *T. vanhoofi; Trypanozoon* to which *T. brucei brucei, T. b. gambiense, T. b. rhodesiense, T. equiperdum* and *T evansi* belong. The subgenus *Pycnomonas* has *T.suis*. (Stephen 1986; Molyneux and Ashford, 1983).

The principal trypanosome pathogens of livestock in the sub-Saharan Africa are *T. congolense, T. vivax, T. brucei* and *T. simiae* (Logan-Henfrey *et al.*, 1992). Chronic sleeping sickness in humans is caused by *T. b. gambiense* and is restricted to west Africa and north-central Africa while the more acute disease is caused by *T. b. rhodensiense* and its distribution is restricted to east and central Africa. The major vectors for the *rhodensiense* and *gambiense* sleeping sicknesses belong to the *morsitans* and *palpalis* groups, respectively (Seed and Hall, 1992; Jordan, 1993; Vickerman, 1985).

2.3 The trypanosome life cycle

The life cycle of African salivarian trypanosomes involves the vertebrate hosts and the tsetse vectors (Figure 2.1). During cyclical developments between the vector and the vertebrate host, trypanosomes undergo several developmental stages. The transitions between the host and the vector expose the parasite to completely different physical and biochemical environments (Vickerman, 1985). To successfully cope with this vicissitude of parasitic life, the parasites correspondingly undergo a series of adaptive morphological and biochemical modifications at various stages of the life cycle (Logan-Henfrey *et al.*, 1992).

The life cycle stages often refer to morphological characteristics, identified by shapes of the parasites as well as the organelles, the position of the kinetoplast in relation to the nucleus and the relative length of the flagellar apparatus. Trypomastigotes are characterised by postnuclear kinetoplasts, epimastigotes by pre-nuclear kinetoplasts while amastigotes

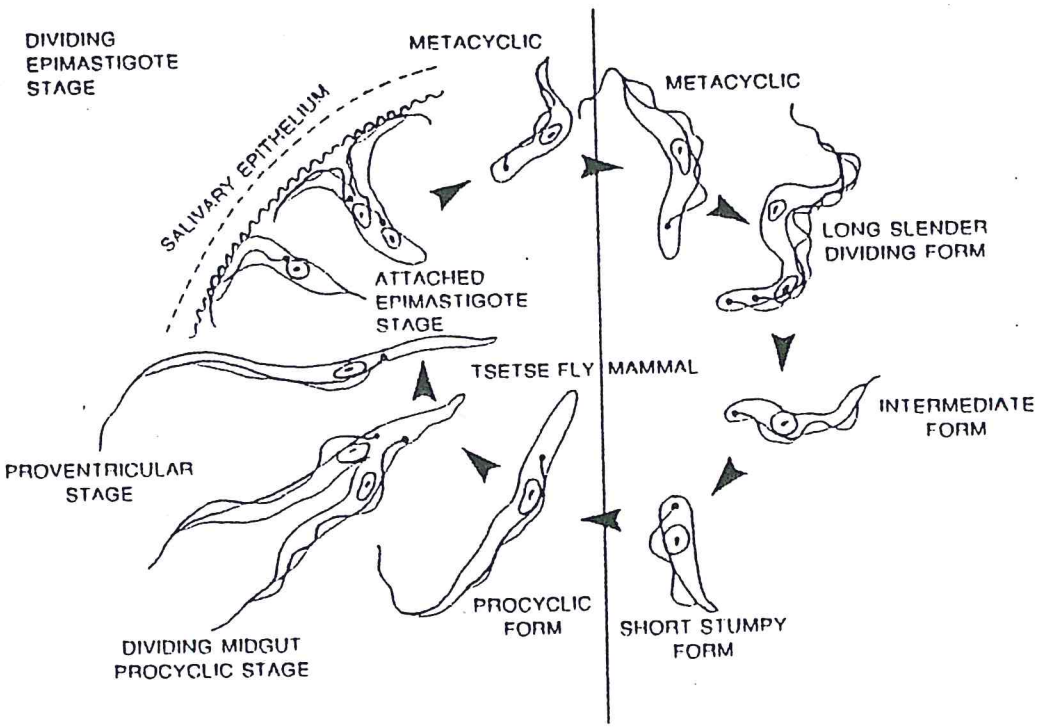

Figure 2.1 Diagram of *Trypanosoma brucei* group life cycle (Source: Seed and Hall, 1992)

lack an emergent flagellum (Vickerman, 1985; Stephen, 1986). Particular life cycle stages can also be designated by location in the host. During the life cycle, in both the vector and the vertebrate host, the trypanosomes alternate between proliferative phase in which they undergo binary fission and the non-proliferative phase where no cell divisions occur (Molyneux and Ashford, 1983).

The infective metacyclic trypomastigote are injected into the vertebrate host when the fly bites. This is followed by a rapid increase in the trypanosome numbers, especially the long slender trypomastigote forms which is a rapidly dividing form and can be found in almost all animal tissues and fluids (Seed and Hall, 1992). A morphological switch from the long slender to intermediate forms and then non-dividing short stumpy forms also occurs. While the long slender bloodstream forms have a semi-terminal kinetoplast, the short stumpy bloodstream forms have a terminal, posteriorly located kinetoplast (Vickerman, 1985).

The slender bloodstream promastigotes forms have mitochondria which are morphologically underdeveloped, inactive and lack cristae. Since these parasite forms lack functional tricarboxylic acid cycles and cytochrome-dependent electron transport chain, energy is generated entirely by glycolysis (Vickerman, 1985; Seed and Hall, 1992). The active glycolytic enzymes are located in microbody-like organelles called glycosomes where glucose is catabolised aerobically to form pyruvate as the end product. Since bloodstream trypanosomes lack lactate dehydrogenase and pyruvate carboxylase, the end product of glycolysis, pyruvate is released into the host's blood and tissue fluids or channeled through phosphoenol pyruvate carboxyl kinase (Vickerman, 1985; Seed and Hall, 1992). Reoxidation of reduced pyridine nucleotides (nucleotide adenine dinucleotide, NADH) from glycolysis is carried out through a glycosomal dihydroxyacetone:glycerol-3-phosphate shuttle linked to the mitochondrial

glycerol-3-phosphate oxidase which has molecular oxygen as a terminal electron acceptor This is also referred to as the trypanosome alternative oxidase (Gutteridge and Coombs, 1977; Logan-Henfrey *et al.*, 1992). The unique compartmentalisation of glycolytic enzymes, adenosine diphosphate (ADP) and glycerol-3-phosphate within the glycosomes facilitates the possible reversal of glycerol kinase activity which results in the net energy generation under anaerobic conditions (Vickerman, 1985; Seed and Hall, 1992; Logan-Henfrey *et al.*, 1992).

The switch from long slender forms to short stumpy forms is accompanied by swelling of the mitochondria, and development of tubular cristae with concomitant appearance of α-ketoglutarate and proline oxidase. These changes herald the switch to an amino acid-based metabolism which occurs in the tsetse midgut-adapted trypanosomes. This explains why the short stumpy forms are the infective forms in the tsetse since they are pre-adapted for the vector environment (Molyneux and Ashford, 1983; Vickerman, 1985; Seed and Hall, 1992).

For all bloodstream trypanosomes, the hosts immune responses are evaded through the numerous variable antigen types (VATs). The surface coat consists of a repertoire of VATs which are characterised by a variant-surface glycoproteins (VSGs) which form a monomolecular layer of the trypanosomes' surface (Barry, 1989). Each VSG is encoded by a separate structural gene and each gene is expressed separately and sequentially in response to the host's immunological attack (Eshita *et al.*, 1992). The continuous replacement of the VSG in response to the host's immunity is known as antigenic variation (Barry and Turner, 1991).

In the vectors' midgut, only the short stumpy forms multiply and transform into the long slender midgut trypomastigotes, also called the procyclics while the long slender forms die or change into short stumpy forms (Vickerman, 1985). The transformation from bloodstream

trypanosomes to procyclics occurs in the posterior midgut, in the endoperitrophic space. Morphologically, transformation is accompanied by increase in body length, including the elongation of the post-kinetoplastic portion of the body, such that the kinetoplast is located between the posterior end and the nucleus. These changes are accompanied by the expansion of the inner mitochondrial membrane into a branched network with discoid cristae as well as loss of the surface coat variable antigens. These changes occur over a period of 48 to 72 hours in the tsetse gut and are accompanied by active division of the trypomastigotes (Molyneux and Ashford, 1983; Logan-Henfrey *et al.*, 1992).

Procyclic development is associated with a switch in energy sources, from glucose to proline. The expansion of the mitochondrion, therefore, correlates with an increase in the activity of succinoxidase and ability to oxidise α-ketoglutarate and proline, which is the principal energy source for tsetse flight muscle (Seed and Hall, 1992). While the activation of the mitochondrion in procyclics is associated with a switch to cytochrome-mediated terminal oxidation, a reduction mainly in hexokinase activity and other glycolytic enzymes occurs. This suggests that during this period, the direct oxidation of glucose is suppressed in the midgut form trypanosomes while proline catabolising enzymes, such as NAD-linked malic enzyme, are activated (Gutteridge and Coombs, 1977; Logan-Henfrey *et al.*, 1992).

The procyclics then invade the ectoperitrophic space, where they form an actively dividing population, in the process losing the VSG coat and acquiring the procyclin coat which is characteristic of the tsetse midgut forms of the parasite (Molyneux and Ashford, 1983; Vickerman, 1985). The parasites then migrate to the salivary glands or mouthparts, depending on the subgenus involved, where they differentiate into epimastigote which through divisions transform into the free swimming metacyclic trypomastigotes. Morphologically, the metacyclic resemble the highly motile short stumpy

bloodstream forms with the nucleus terminally located as well as the development of the VSG (Seed and Hall, 1992).

Establishment of trypanosome infection in tsetse can be considered as a two-step process. Firstly, the trypanosome movement from the ectoperitrophic side, and subsequent migration to either the mouthparts (subgenus *Nannomonas*) or salivary glands (subgenus *Trypanozoon*); and secondly transformation from procyclics through dividing epimastigotes to vertebrate infective metacyclic forms (Vickerman, 1985; Maudlin and Welburn, 1989; Maudlin, 1991; Molyneux and Ashford, 1983: Honigberg, 1986). Metacyclic forms do not divide but are a pre-adapted form for survival and development in the vertebrate blood system where they are injected by the infected tsetse fly during feeding (Molyneux and Ashford, 1983).

2.3.1 Factors that determine the transformation of trypanosomes from bloodstream forms to procyclic forms

Several factors have been shown to be involved in the induction of the transformation of bloodstream trypomastigotes to procyclic trypomastigotes *in vitro*. These factors include; change in temperature, presence of TCA cycle intermediates, types of substrates available, presence of cyclic nucleotides, type of host blood, trypsin or trypsin-like enzymes and crude midgut homogenates.

Initial *in vitro* transformation studies on *Trypanosoma brucei* LUMP 1026 strain by Bienen *et al.* (1980) showed that, in semidefined medium (SDM), parasites showed complete morphological transformation from bloodstream to procyclic trypomastigotes by 72 h post-inoculation when incubated at 25 °C. Increasing the temperature to 30°C and 37°C only increased the rate of cell growth and peak density without having any significant effects on morphological transformation (Bienen *et al.*, 1980).

Induction of transformation by reduction of temperature (25° C) was also shown to be accompanied by loss of the surface coat by 96 h, repression of further VSG synthesis (Czichos *et al.*, 1986) as well as a general decrease in total respiration rate (Bienen *et al.*, 1981). The decrease in incubation temperature which induces the transformation of trypanosomes can be correlated to the natural temperature drop which accompanies ingestion of the parasites with the bloodmeal by tsetse.

Czichos *et al.* (1986) reported that when a variant clone MITat 1.4 (117) of monomorphic *T. brucei* stock 427 was incubated in SDM-79 with 3 mM citrate plus 3 mM *cis*-aconitate at 27° C, the cells transformed to procyclic morphology and divided with the generation time of about 30 h. However, when the acids were omitted from the medium at the same temperature, the cells did not divide and maintained the bloodstream trypomastigote morphology (Czichos *et al.*, 1986). This demonstrated the requirements of the TCA intermediates in the medium at 27° C to serve as signals for triggering transformation (Bass and Wang, 1991). According to Brun and Schonenberger (1981), the combination of *cis*-aconitate and citrate was shown to be more effective in inducing trypanosome transformation while citrate alone had no effect. Other related substrates such as *trans*-aconitate, isocitrate, itaconate or α-ketoglutarate added alone or a mixture of succinate, fumarate, malate, oxaloacetate (3 mM each) neither repressed VSG synthesis nor induced morphological trypanosome. (Czichos *et al.*, 1986). However, Wang and Bass (1991) reported that, at 26° C, elimination of TCA cycle intermediates such as L-proline, L-malate, α-ketoglutarate, fumarate and succinate from the medium increased the rate of cell death with time.

When the effect of different substrates in the incubation medium on trypanosome was investigated, Bienen *et al.* (1981) reported that of the substrates studied (proline, glucose, sucrose, fructose and glycerin), only

proline was shown to support normal growth and transformation rates. With the other substrates, transformation took longer. Using pleomorphic *T. brucei* strain TREU667, Wang and Bass (1991) also showed that depletion of polyamines levels could not trigger parasite transformation.

Rolin *et al.* (1993) showed that the second messenger, cyclic adenosine monophosphate (cAMP) when included in the culture media at 25° C and concentrations of 1 mM resulted in improved synchronous transformation of pleomorphic *T. brucei* clone AnTat 1.1. Although the synchronous differentiation was also shown to be associated with transient activation of adenyl cyclase, an enzyme that catalyse the conversion of adenosine triphosphate (ATP) to cAMP, this was not the case when monomorphic variants were used (Rolin *et al.*, 1993). Wang and Bass (1991) also reported that addition of cAMP to the medium at 25° C had no effect on the transformation of monomorphic *T. brucei* EATRO110.

The type of host blood has been shown to have a role in determining the eventual infection prevalent in tsetse (Mihok *et al.*, 1993). Recent *in vitro* transformation studies using *T. brucei* EATRO 1969 have shown that while the inclusion of red blood cells (RBCs) from different hosts supported transformation of parasites, addition of plasma components led to lower parasite transformation rates (Nguu *et al.*, 1996). The factor(s) responsible for transformation have also been proposed to be associated with the cell membranes of the RBCs.

2.4 Factors that determine the development of trypanosomes in the tsetse fly.

Several factors can influence the successful development of trypanosomes (*Trypanozoon* and *Nannomonas* species) in the tsetse. These factors may be responsible for the low trypanosome infection rates evident in both laboratory-reared and wild tsetse (<10 %). These factors can be

divided into three categories: ecological, parasites and the hosts-related factors and those factors endogenously associated with the fly. Ecological factors include the climate (both micro and macro), availability of infective hosts and availability of hosts for subsequent feeds. The factors related to the parasites and hosts include parasite numbers in the bloodmeal, type of the parasite and its infectivity to the fly, immune state of the host, behaviour of the host and its attractiveness to the fly. Factors associated with the fly are: age at first feed, sex, symbiont infection (viral, bacterial), behaviour of the fly (habitat preference and selection), biochemical and physiological status of the fly, genetic differences between flies as well as the stage of infection in the fly (Ashford and Molyneux ,1983; Jordan, 1986; Seed and Hall, 1992).

2.4.1 Tsetse midgut factors involved in the tsetse-trypanosome interactions

Trypanosomes taken up in the infective bloodmeal by tsetse are subjected to severe biochemical, physiological and physical environmental changes which are very harsh for the survival of the parasite in the midgut. In order to successfully survive in the tsetse, trypanosomes have over years of co-evolution with their vectors developed corresponding physiological and biochemical adaptations (Gutteridge and Coombs, 1977).

Several factors have been shown to be responsible for trypanosome killing in the tsetse midgut. These factors, presumably, contribute to the less than 10% trypanosome infection rates observed in natural and laboratory reared tsetse populations (Maudlin, 1991; Baker, 1974).

The refractoriness and susceptibility of tsetse, to trypanosome infections, have been shown to be different in the three taxonomic groups, with the *palpalis* group being most susceptible (Moloo and Kutuza, 1988). The differences in susceptibility have been suggested to partly reflect

differences in the tsetse gut environment (Maudlin, 1991; Molyneux and Stiles, 1991) and genetic differences between trypanosome stocks in numbers of lectin binding sites on the parasites (Maudlin and Welburn, 1988).

In tsetse, the trypanosomes (*T. brucei* and *T. congolense*) life cycles can be divided into two phases. These are establishment and maturation. During establishment, the parasites transform from bloodstream forms to procyclic (insect midgut) forms. This is the 'immature' infection. The success of this infection determines the eventual vectorial capacity of the flies. This process is thought to be dependent on the quality and quantity of the anti-trypanosomal factors present in the fly's midgut (Molyneux and Stiles, 1991).

Insect factors involved in the establishment of the midgut infection include midgut trypanolysins (Stiles *et al.,* 1990), midgut lectins and presence of gut endosymbionts, especially rickettsia-like organisms (Stiles *et al.*, 1990; Maudlin and Welburn, 1988; Ingram and Molyneux, 1991; Maudlin, 1991; Molyneux and Stiles, 1991), midgut trypsins (van den Abbelle and Decleir, 1991; Imbuga *et al.*, 1992a), and the peritrophic membrane (Lehane and Msangi, 1991; Miller and Lehane, 1993a). The second phase, maturation, involves the differentiation of procyclics to metacyclics, the mammalian infective forms (Maudlin and Welburn, 1989). A haemolymph galactosamine-binding lectin is believed to be necessary for the triggering of trypanosome differentiation from procyclic to vertebrate infective metacyclic forms (Welburn and Maudlin, 1990).

2.4.1.1 The peritrophic membrane

Peritrophic membranes are a special secretory product formed by the midgut cells, surrounding food and food residues in the gut of many arthropods. Peritrophic membranes are reinforced with chitin containing

microfibrils and proteins in the form of glycoproteins (Richards and Richards, 1977). Although the physiological role of the peritrophic membrane still remains controversial, there is evidence that the peritrophic membrane may be involved in providing a mechanical protection for midgut cells from abrasion by food particles. The peritrophic membranes also serve as a physical barrier for microorganisms (bacteria and parasites such as trypanosomes, malaria ookinetes and microfilaria) as well as assisting in the digestion process (Terra and Ferreira, 1994; Peters *et al.*, 1983)

Arthropod peritrophic membranes have been classified as either type I or type II. Type I peritrophic membranes are produced from a diffuse secretory area over the length of the midgut and are discontinuously secreted, commencing in response to feeding. In contrast, type II peritrophic membranes are formed from a localised area at the foregut-midgut junction, usually within the proventriculus. These are continuously produced and form permanent barriers between ingested food and midgut epithelium (Miller and Lehane, 1993a). In both Dipteran adults and larvae, a type II peritrophic membrane exists. Specialised cardia cells at the anterior end of the proventricular region secrete a single or a multilayered tube which extends the whole of the midgut and the hindgut (Richards and Richards, 1977; Miller and Lehane, 1993a). In *G. m. morsitans,* the peritrophic membrane has been shown to be a trilaminate tube (Miller and Lehane, 1993b).

The peritrophic membrane in the tsetse compartimentalises the gut into an endoperitrophic and ectoperitrophic spaces; representing the area immediately around the bloodmeal and the area between the gut epithelial cells and the membrane, respectively. For the *brucei* group of trypanosomes, establishment of midgut infections depends on successful differentiation of bloodstream forms to procyclic forms and the subsequent migration to the ectoperitrophic space where active mitosis occurs (Vickerman, 1985). Two

routes have been proposed to be possibly used by the trypanosomes during the ectoperitrophic space invasion. These are, by either circumventing the peritrophic membrane through the posterior end of the midgut (Vickerman, 1985) or penetration through the newly synthesised peritrophic membrane site where the membrane is still 'soft' (Lehane and Msangi, 1991).

Tsetse fly susceptibility to infection by the *brucei* group of trypanosomes has been shown to be age dependent, with teneral flies being more easily infected than the non-teneral flies (Maudlin, 1991). The explanation of this age-dependent variation in the ability of tsetse to support trypanosome infections have, to a greater extent, centred on the development of the peritrophic membrane. It has been proposed that the faster peritrophic membrane growth rates in teneral flies, after a bloodmeal, offers larger surface areas of semi-liquid membrane posterior to the proventriculus. Trypanosome penetration of the peritrophic membrane has been proposed to be possible in this area (Maudlin, 1991; Lehane and Msangi, 1991). This has been thought to correspond to the higher trypanosome infection rates characteristic of teneral and starved flies. Differential peritrophic membrane development rates between some *Glossina* sp., as well as teneral and non-teneral flies have been postulated as responsible in determining susceptibility differences to *brucei* group infection (Miller and Lehane, 1993a; Lehane and Msangi, 1991).

It has however been shown that *T. b. rhodesiense* can penetrate the peritrophic membrane of *G. m. morsitans* in the middle of its length, which is not the 'soft' part of the membrane (Evans and Ellis, 1983). This means that a fully formed peritrophic membrane might not necessarily be a barrier to trypanosome penetration. Moreover, no physical differences have been shown to exist between newly formed and mature peritrophic membranes in *G. m. morsitans* (Lehane and Msangi, 1991).

2.4.1.2 Presence of rickettsia-like organisms

Rickettsia-like organisms (RLOs) have been reported in tissues of several species of both wild and laboratory-reared tsetse (*G. m. morsitans, G. m. centralis, G. pallidipes, G. f. fuscipes and G. gambiensis*). The RLOs have been reported in ovaries and midgut epithelial cells (Pinnock and Hess, 1974; Moloo and Shaw, 1989). Although the RLOs reside intracellularily, in the cytoplasm of the host cells, they have been shown to have little or no pathological damage to the host cells (Shaw and Moloo, 1991). The actual role of the RLOs in the host cells remain unknown although they have been shown to be maternally inherited through the egg cytoplasm (Pinnock and Hess, 1974; Maudlin and Ellis, 1985).

Tsetse infection by trypanosome was previously thought to depend mainly on the availability of infected hosts. However, the observation that trypanosome infection rates in both laboratory-reared and wild tsetse are very low (less than 10%) suggests that tsetse flies have an intrinsic refractoriness to trypanosome infection (Maudlin, 1985; Maudlin and Welburn, 1988). Using simple breeding experiments, Maudlin (1982) and Maudlin and Dukes (1985) showed that the susceptibility of *G. m. morsitans* to infection by *Nannomonas* and *Trypanozoon* species was maternally inherited and it was possible to select laboratory-reared lines for susceptibility and refractoriness to trypanosome infection over generation. Maudlin (1982) proposed that the prime candidates in the maternal acquisition of trypanosome transmission factor(s) were the RLOs, since susceptible lines of *G.m morsitans* showed higher numbers of RLOs than the refractory flies. The RLOs have also been shown to be present in cells in the mycetome region of the midgut (Moloo and Shaw, 1989).

Rickettsia-like organisms have been shown to produce endochitinases both *in vitro* and *in vivo* (Welburn *et al*, 1993) , the chitinolytic activity leads to build up of glucosamine (GlcN) in the midgut

which consequently inhibits lectin activity (Welburn and Maudlin, 1991). Since chitinase activity has been postulated to be high during the larval/pupal stage, the build up in GlcN will inhibit midgut lectins at the teneral feed, making teneral flies more susceptible to trypanosome infection than the non-tenerals (Maudlin and Welburn, 1988; Welburn and Maudlin, 1991; Welburn *et al.*, 1993). *In vitro* tests have shown that tsetse midgut extracts from refractory flies have higher agglutination titres and were more trypanocidal (Maudlin and Welburn, 1988). It has been postulated that susceptible flies, therefore, do not secrete enough lectins or the lectins are inactivated.

Moloo and Shaw (1989), however, showed that teneral *G. m. centralis* had fewer numbers of RLOs in the midgut cells compared to 30- and 60-day old flies. This implies that there might be no causal association between presence of RLOs within the midgut cells of *G.m. centralis* and susceptibility to *T. congolense* infection. Studies on the infection rates of *T. brucei* and *T. congolense* in eight tsetse species and subspecies (*G .m. centralis, G. austeni, G. f. fuscipes, G. p. gambiensis, G. brevipalpis, G. p. palpalis, G. tachnoides and G. pallidipes*) showed that the number of RLOs increase with the fly age (Shaw and Moloo, 1991). Since teneral flies are the more susceptible and were, in this case, shown to have lower numbers of RLOs, it is unlikely that the relationship between RLOs and trypanosome infection in the tsetse is quantitative in character albeit not an all-or-none phenomenon as described by Welburn and Maudlin (1990). According to Shaw and Moloo (1991), the relationship between RLOs and trypanosome infection in the tsetse midgut might not be as straight forward as previously thought. Other midgut factors might be involved.

2.4.1.3 Midgut lectins

Lectins are polyvalent oligomeric, non-immunoglobulin, structurally diverse proteins or glycoproteins that bind carbohydrates, agglutinate cells or precipitate polysaccharides, glycoproteins or other glycoconjugates (Barondes, 1981; Sharon and Lis, 1989). Although initially discovered in plants, these cell-agglutinating proteins have also been shown to occur in other organisms, on cell surfaces or intracellular particles (Sharon and Lis, 1972). Lectins function by binding determinant carbohydrate moieties of membrane glycoproteins and glycolipids, precipitating soluble glycoconjugates leading to eventual agglutination of the respective cells (Renwratz, 1986). Although lectins bind specifically, reversibly and non-covalently, they are neither enzymes nor antibodies (Jackobson and Doyle, 1996; Sharon and Lis, 1989). Since almost all cells have carbohydrate moieties on their surfaces in form of glycoproteins, glycolipids and polysaccharides, the lectin-carbohydrate interactions, together with other non-carbohydrate ligands, provide ideal cell recognition systems characterised by reversibility and specificity (Sharon and Lis, 1989). In most insects, lectins have been shown to act as immune defence molecules (Kaaya, 1989) by recognising and clearing invading pathogens and parasites (Kanost *et al.*, 1990). Insects agglutinins have been widely reported in haemolymph and tissue extracts from different species in the orders; Orthoptera, Coleoptera, Dictyoptera and Diptera (Ingram and Molyneux, 1991; Pathak, 1993).

Haemolymph and gut lectins have been reported in several Glossina species. These agglutinins have been reported in *Glossina morsitans morsitans* (Ingram and Molyneux, 1991; Abubakar *et al.*, 1995), *G. austeni* (Molyneux and Stiles, 1991), *G. fuscipes fuscipes* (Ingram and Molyneux, 1990), *G. longipennis* (Osir *et al.*, 1995), *G. pallidipes* and *G. brevipalpis* (Grubhoffer *et al.*, 1994) and *G. palpalis palpalis* (Stiles *et al.*, 1990).

Earlier studies (Ibrahim *et al.*, 1984) showed that *Glossina* haemolymph and midguts have anti-trypanosomal properties which could be inhibited by specific sugars suggesting they had lectin-like activities. Maudlin and Dukes (1985) also reported that susceptibility of some *Glossina* sp. to pathogenic trypanosomes was maternally inherited and was correlated to the levels of lectins in the tsetse midgut. The pattern of susceptibility inheritance was shown to be associated with the presence in the midgut of endosymbionts, rickettsia-like organisms (RLOs) (Welburn and Maudlin, 1988; 1991). This D(+)glucosamine build up is as a result of chitin breakdown from the peritrophic membrane by an endochitinase released by RLOs during this stage. Susceptibility of teneral flies was postulated to be due to D(+)glucosamine build up in the midgut during the larval and pupal stages. The GlcN from chitin breakdown then binds to the lectins secreted into the gut after bloodmeal stimulation, in the process decrease the free lectins in the gut. This then would lead to establishment of a midgut infection (Maudlin, 1991; Maudlin and Welburn, 1988).

Genetic differences have also been shown to exist between trypanosome stocks with respect to numbers of lectin binding sites on the trypanosomes. The relative numbers of binding sites have also been suggested to play a role in infection susceptibility. Trypanosomes with more lectin binding sites have been proposed to require higher lectin concentration to clear them from the tsetse gut and would therefore establish more easily than would trypanosomes with fewer lectin binding sites (Maudlin and Welburn, 1988; Molyneux and Stiles, 1991; Maudlin and Welburn, 1988; Gooding, 1992).

Insect lectins are reported to be synthesized *de novo* mainly in the fat body, haemocytes and sex organs; and constitute most of the haemolymph and midgut agglutinins (Ingram and Molyneux, 1991; Yeaton , 1981).

In tsetse flies, lectins released into the midgut exert bifunctional roles. In addition to killing the trypanosomes, they also act as a signal to the surviving trypanosomes to differentiate into epimastigotes and then to the vertebrate infective metacyclic forms (Ingram and Molyneux, 1991; Molyneux and Stiles, 1991; Maudlin and Welburn, 1994; Welburn and Maudlin, 1987). The release of lectins into the midgut has been shown to be stimulated by bloodmeal resulting into periodic activity, fluctuating with normal digestive cycles with peak activity occurring between 48 and 72 h after blood ingestion (Stiles *et al.*, 1990; Maudlin, 1991). This, therefore, means that successful transformation might depend, partly, on the feeding behaviour and quantity of midgut lectins present after a bloodmeal.

The midgut lectin concentrations have been shown to vary in different *Glossina* species and strains. Welburn *et al.* (1994) showed that *G. pallidipes* and *G. palpalis palpalis* have relatively higher midgut lectin titres. Midgut lectin titres have also been shown to be related to the numbers of RLOs present (Maudlin and Welburn, 1988; 1991; Molyneux and Stiles, 1991), the RLOs are thought to release chitinases which leads to chitin breakdown and eventual release of glucosamine, a known inhibitor of tsetse fly midgut lectins.

The importance of RLOs in tsetse trypanosome infection has, however, been disputed by Moloo and Shaw (1989) who reported no association between RLOs infection in the gut cells and the susceptibility of *G. morsitans centralis* to infection by *T. congolense*. Lehane and Msangi (1991) also reported that a rapid drop in infection rates in *G. palpalis* with *T. gambienses* was more closely correlated with development of the peritrophic membrane rather than lectin levels. This then led to the suggestion that the role of lectin in trypanosome differentiation may not be as straight forward as previously thought (Moloo *et al.*, 1994).

2.4.1.4 Midgut trypsins

Haematophagous insects, feeding mainly on vertebrate blood, ingest diets which may consist of up to 70% protein (Applebaum, 1985). Since the chemical composition of the ingested diet determines the types of digestive enzymes released, the most important of the digestive enzymes in the alimentary canals of haematophagous insects are the proteases. These enzymes are responsible for complete digestion of proteins. Midgut proteases include the more predominant proteinases (endopeptidases) and exoproteinases. On the basis of catalytic mechanisms, structure of the active centre and amino acid specificity, proteinases fall into three broad sub-classes: serine proteases (with histidine and serine at the active centre), thiol-proteinases (with cysteine at the active centre) and carboxyl proteinases (with acidic residues at the active centre) (Terra and Ferreira, 1994; Applebaum, 1985). The most predominant proteinases found in the midguts of haematophagous insects are the serine proteinases of which trypsins and chymotrypsins are the most common and generally most active (Applebaum, 1985; Gooding, 1975).

Midgut proteinases (mostly trypsin and trypsin-like enzymes) of some haematophagous insects have been shown to be synthesised by several tissues. Columnar epithelial cells of the midguts have been shown to synthesise proteinases in *Anopheles* sp.. (Horler and Briegel, 1995), *Stomoxys calcitrans* (Moffat *et al.*, 1995), *Culex nigripalpus* (Borovsky, 1986a) and *Aedes aegypti* (Felix *et al.*, 1991). In *Stomoxys calcitrans* and *Culex nigripalpus* the fat body and thoracic tissues have also been shown by Borovsky (1986a; b) as possible synthesis sites of trypsin-like enzymes.

In the adult tsetse flies (*Glossina morsitans morsitans* Westwood), at least six proteolytic enzymes have been shown to be involved in the digestion of the bloodmeal (Cheeseman and Gooding, 1985). These include trypsin (apparent molecular weight (M_r ~20,000), carboxypeptidase A (M_r

~34,200), carboxypeptidase B (M_r ~22,500), aminopeptidases (M_r >100,000), trypsin-like enzyme (proteinase VI; M_r ~22,800), chymotrypsin (M_r ~35,500) and chymotrypsin-like enzyme (proteinase VII; M_r ~38,200) (Cheeseman and Gooding, 1985; Applebaum, 1985; Terra and Ferreira, 1994). The activities of most of these enzymes have been shown to be cyclical in response to bloodmeals and this coincides with the activities of trypanoagglutinins and trypanolysins, showing peak activity at about 48-96 hours post-bloodmeal (Molyneux and Stiles, 1991; Stiles *et al.*, 1990; 1991). It is probable that some or all of these factors might be responsible for the observed high mortality of bloodstream trypanosomes in the midgut after ingestion by the tsetse fly. It has been reported that only about one third of the population of *T. b. rhodesiense* could survive in the midgut of *G. m. morsitans* (Turner *et al.*, 1988). Proteolytic enzymes secreted by *Aedes aegypti* have been shown to damage ookinetes of *Plasmodium* sp. (Gass and Yeates, 1979) as well as affect the survival of *Leishmania donovani* in *Phlebotomus papatasi* (Borovsky and Schlein, 1987).

Yabu and Takayanagi (1988), using bovine pancreatic trypsin, showed that *in vitro* tryptic removal of the surface coat of bloodstream monomorphic *T. b. gambiense* stimulated their transformation to procyclic forms. The importance of trypsin and trypsin-like enzymes in the stimulation of trypanosome transformation from bloodstream to procyclic forms was further reported by Imbuga *et al.* (1992 a) using crude *G. morsitans morsitans* midgut homogenates. It was noted that a close correlation existed between trypsin activity and the ability of midgut homogenates to induce the transformation of bloodstream forms to procyclics both *in vitro* and *in vivo*. It was also shown that optimal trypsin or trypsin-like enzyme concentrations were crucial for effective trypanosome transformation, as higher concentrations resulted in complete lysis of the parasites (Imbuga *et al.*, 1992 b). It has, therefore, been proposed that during transformation of

bloodstream trypanosomes to procyclics, the trypsin or trypsin-like enzyme presumably functions by cleaving off the glycoprotein coat on bloodstream trypanosomes (Yabu and Takayanagi, 1988; Imbuga *et al.*, 1992 a; van den Abbelle and Decleir, 1991). This, then sets in motion the transformation process in association with other factors within the tsetse midgut. One such factor could possibly be a lectin (trypanoagglutinin), since like the midgut trypsins, the lectins also show periodic activity cycles in response to bloodmeals (Molyneux and Stiles, 1991; Stiles *et al.*, 1990).

Inhibition studies on tsetse midgut trypsins by Osir *et al.* (1993) showed that a monosaccharide (D(+)glucosamine), which is a known inhibitor of the midgut lectin (Ibrahim *et al.*, 1984), has an inhibitory effect on the midgut trypsin activity. D(+)glucosamine inhibition of trypsin activity has been reported to be as much as three times in *G. m. centralis* and *G. m. morsitans* crude midgut preparations (Mihok *et al.*, 1994). In addition, since both lectin and trypsin have been shown to kill trypanosomes as well as facilitate establishment in the midgut (Maudlin, 1991; Imbuga *et al.*, 1992a), it has been proposed that the trypsin-like enzyme and the lectins of tsetse midgut are either the same or closely related molecule(s) (Osir *et al.*, 1993).

2.5 The tsetse midgut lectin-trypsin complex

Abubakar *et al.* (1995) in recent studies reported the existence of a close relationship between bloodmeal-induced *G. m. morsitans* midgut trypsins (trypsin-like enzyme) and a midgut trypanoagglutinin. In addition to the midgut trypsin activity and bloodstream trypanosome agglutination titres being inhibited by a protease inhibitor (soybean trypsin inhibitor) and a specific lectin inhibitor (GlcN), both the agglutination and trypsin activity coeluted at 50% NaCl (0.0-0.5 M NaCl gradient) during partial purification on an anion exchange column (Abubakar *et al.*, 1995). Using a two step ion exchange column chromatography procedure, a bloodmeal-induced

agglutinin with proteolytic activity has been purified from *G. longipennis* midgut extracts (Osir *et al.*, 1995). This molecule, aptly called the lectin-trypsin complex, has been shown to be a glycoprotein with a native apparent molecular weight (M_r) ~61,000. The molecule has also been shown to consist of two noncovalently-linked subunits; an α subunit (M_r ~27,000) having tryptic activity and a ß subunit (M_r ~33,000) with glycosyl residues (Osir *et al.*, 1995). Using antibodies raised against the molecule, it was also shown that the molecule was present in *Glossina* species and not other haematophagous insects.

CHAPTER THREE

3. GENERAL MATERIALS AND METHODS

3.1 Insects and animals

3.1.1 Tsetse flies and other haematophagous Insects

Tsetse flies (*Glossina morsitans morsitans* Westwood, *G. morsitans centralis* Machado, *G. fuscipes fuscipes*, Newstead,) were supplied by the Animal Rearing and Quarantine Unit (ARQU) of the International Centre of Insect Physiology and Ecology (ICIPE). *Glossina longipennis* Corti were captured by trapping in the field at Nguruman, south eastern Kenya. The tsetse flies were maintained on a 12 h:12 h light:dark photoperiod at 25° C and a relative humidity of 75-80% (Wanyonje, 1994).

Other haematophagous insects, mosquito (*Aedes egypti*) were also supplied by ARQU of the ICIPE. Sand flies (*Phlebotomus duboscqi*) were provided by Dr. Muhinda Mugunga of the ICIPE while stable flies (*Stomoxys calcitrans*) were trapped near the large animal stable of ARQU at the ICIPE.

3.1.2 Rabbits and rats

New Zealand white rabbits (*Oryctolagus cuniculus*) were used for raising polyclonal antibodies and male Wistar rats (2 -3 weeks old) were used for feeding tsetse flies and maintenance of trypanosme parasites. All animals were supplied by ARQU of the ICIPE.

3.1.3 Trypanosome Parasites

The parasites, pleomorphic *Trypanosoma brucei brucei* Plimmer and Bradford, used in these studies were derived from the EATRO 1969 stock (Otieno *et al.*, 1983). These parasites had initially been kept as stabilates in liquid nitrogen before inoculation into the Wistar rats.

Culture procyclic *T. b. brucei* were provided by Dr. J. Kiaira of Biochemistry Department, University of Nairobi.

3.1.4 Preparation of the crude midgut homogenates

Prior to dissections, male teneral flies (24 h after emergence) were fed on rat blood, starved for 72 h. After immobilising the flies by brief chilling, the midguts were dissected out into phosphate buffered saline (PBS; 50 mM NaH_2PO_4 (Sigma, St. Louis, MO) 150 mM NaCl (Serva, Westbury, NY, USA) (pH 8.0). The midguts were washed several times in PBS to remove haemolymph and lipids and then suspended in ice cold PBS. The midguts were then homogenised using a Virtis homogeniser (Gardiner, USA) and centrifuged (10 000 x *g*, 4° C, 30 min.) in a minifuge (Eppendolf, Germany). The supernatant was filtered through a 0.22 μm pore filter (Nalge, Rochester, NY, USA), extensively dialysed against 20 mM Tris-HCl (Sigma, St. Louis, MO), pH 8.0 and stored at -20° C. (Abubakar *et al.*, 1995).

The midguts homogenates of the other haematophagous insects were prepared in the same way.

3.1.5 Preparation of other tissue homogenates

Whole gut homogenates were prepared as follows: About 200 male tsetse flies were, 24 h after emergence, fed on unparasitised blood and then starved for 72 h. After immobilisation by brief chilling, whole guts were pulled out and placed into ice cold 20 mM Tris-HCl, pH 8.0. The dissected guts were washed three times in 20 mM Tris-HCl (pH 8.0) to remove contaminating haemolymph and lipids and thereafter homogenised using a Virtis homogeniser (Gardiner, USA), centrifuged for 30 min. (10 000 x *g*, 4° C). The supernatant was filtered through a 0.22 μm millipore filter, extensively dialysed against 20 mM Tris-HCl, pH 8.0 and stored at -20° C (Abubakar *et al.*, 1995).

To dissect out the fat body and the posterior midgut tissues; about 50 male flies were fed on unparasitised rat blood, starved for 72 h and then immobilised by brief chilling. A lateral incision was made on the abdomen and the sternites were removed. Fat body and the posterior midgut were carefully dissected out into ice cold 20 mM Tris-HCl, pH 8.0 to which a few crystals of phenylthiourea had been added to prevent melanisation. The peritrophic membrane was also carefully pulled out and put in a separate Eppendorf tube with the same buffer. To remove contaminating haemolymph and lipids, the tissues were washed in three changes of ice-cold 20 mM Tris-HCl, pH 8.0.

The dissected tissues were separately homogenised using a Virtis homogeniser (Gardiner, USA), centrifuged for 30 min. (10 000 x *g*, 4° C) in Eppendorf minifuge. The supernatants were then filtered through 0.22 μm pore filters and stored at -20° C until when needed for use.

3.1.6 Preparation of the parasites

After infecting male Wistar rats with *T. b. brucei*, parasitised blood was collected at the peak of parasitaemia by cardiac puncture. Heparin was used as the anticoagulant. The parasites were isolated using a modification of the method of Lanham and Godfrey (1970). The blood was mixed with an equal volume of phosphate saline glucose (PSG; PBS with 1% glucose)(w/v) (Sigma, St. Louis, MO) and centrifuged in a refrigerated centrifuge (Sigma, St.Louis, MO)(3 000 x *g*, 4° C, 10 min.). The creamy layer of parasites between the supernatant and the red blood cells, was loaded on to a 20 ml column (Bio-Rad, Econno-Pac, Richmond, CA) pre-packed with an anion exchanger, diethyl aminoethyl cellulose (DE52; Whatman Ltd., Kent England) and thoroughly washed with PSG. The parasites were eluted from the column as the unbound material in PSG. A haemocytometer equipped with improved Neubauer ruling was used to count the parasites.

3.2 Preparation of dialysis membranes

Dialysis membranes (Spectra/ Por membrane, MWCO 12-14 000; Spectrum Medical Industries, Houston, Texas) used for the dialysis and concentration of samples were prepared according to the method described by Copeland (1994). Briefly, the membranes were boiled in 100 mM $NaHCO_3$ ((Serva, Westbury, NY, USA) and 10 mM EDTA (Sigma, St. Louis, MO) pH 7.0 for 5 min. The membranes were thereafter soaked for 10 min. in distilled water and then thoroughly rinsed with more distilled water prior to use. Alternatively, the prepared membranes were stored in 20% (v\v) ethanol (Analar, UK) at 4° C.

3.3 Trypsin assay

A chromogenic substrate, carbo-benzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY, Boerhringer, Mannheim, FRG), was used to assay for trypsin activity (Imbuga *et al.*, 1992b). The reaction mixture (950 µl of 100 mM Tris-HCl, pH 8.0, 5 µl of either the tsetse crude midgut or the pooled fractions eluted from the columns) was equilibrated at 30° C for 10 min. The reactions were initiated by addition of Chromozym-TRY (40 µmol.) and the total assay volume maintained at 1.0 ml. Change in absorbance at 410 nm was monitored for 5 min. using a GBC 916 spectrophotometer linked to a computer system (GBC, Victoria, Australia). The change in molar extinction coefficient at 410 nm (ε_{410} =8800) was used to determine the amount of substrate hydrolysed; 1 unit trypsin activity was considered as the amount of enzyme required to hydrolyse 1 µmol. Chromozym-TRY / min. at 30° C. (Erlanger *et al.*, 1961).
The enzyme activity was determined using the formula :

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

where v = volume of Chromozym-TRY used in assay

V = total volume of the assay.

3.4 Agglutination Assay

Agglutination activity was assayed at 27° C by serial two-fold dilutions of 25 µl of either midgut extracts or fractions eluted from the columns with 25 µl of phosphate buffered saline (PBS; 50 mM KH_2PO_4, 150 mM NaCl, pH 8.0) in plastic, flat bottomed microtitre plate wells (Nunc., Denmark). After sample dilution, 25 µl of bloodstream forms of *T. b. brucei* (~5 x 10^6 parasites/ml) were added to each dilution. The total assay volume was maintained at 75 µl. The mixture was then incubated at 27° C for 2 h. Agglutination was scored using an inverted microscope (Leitz Dialux, Germany). Reciprocals of the highest dilutions causing noticeable parasite agglutination were designated as agglutination titres. In the controls, PBS was used instead of the samples. All assay were carried out in triplicate (Stebbins and Hapner, 1985; Osir *et al.*, 1995).

3.5 Protein estimation

Protein estimation was carried out using the Bicinchonic acid (BCA) protein assay kit (Pierce, Rockfold, I11., USA). Bovine serum albumin was used as a standard.

3.6 Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was performed as described by Laemmli (1970) with some modifications. For the resolving gel, gradients (4-15% polyacrylamide) were cast using a gradient maker (BRL, Gaithersburg, MA, USA) while the stacking gels were 3.13% polyacrylamide (APPENDIX A). Samples were dissolved in equal volumes of sample 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 minutes) centrifuged (5 000 x *g*, 5 min., 4° C) before loading the supernatant on to the gel.

The running buffer consisted of 25 mM Tris-HCl, pH 8.3; 192 mM glycine and 0.1% SDS. Electrophoresis was carried out at room temperature at a constant current of 20 mA per gel until the dye front ran into the anode running buffer tank.

Non-denaturing PAGE (native-PAGE) was carried out on gradient gels under the same conditions as the SDS-PAGE but 0.1% SDS was omitted from the gel. Before application on to the gel, samples were dissolved in an equal volume of non-denaturing sample buffer (0.13 M Tris-HCl,; 20% glycerol and 0.002% bromophenol blue). Running buffer used consisted of 25 mM Tris-HCl, pH 8.3 and 192 mM glycine. Electrophoresis was carried out at 4° C with a constant voltage of 70 V until the dye front ran into the anode buffer tank. After electrophoresis, the gels were stained for proteins with 0.6% Coomassie Brilliant Blue (APPENDIX A) for 2 h. The gels were then treated with several changes of destainig solution 1 (APPENDIX A) followed by destain solution 2 (APPENDIX A). For both SDS-PAGE and native-PAGE, appropriate molecular weight marker standards were ran alongside the samples. Molecular weights of the markers and the protein in the samples were estimated from plots of $\log_{10}$ molecular weight of markers versus their relative migration on the gels (R_m).

3.7 Staining for carbohydrates

The periodate acid-Schiff (PAS) stain method of Kapitany and Zebrowski (1973) was used to stain for carbohydrates in the gels. The purified agglutinin was separated on PAGE as already described (section 3.6). The gels were fixed in 12.5% trichloroacetic acid (w/v) for 2 h at 27° C and thereafter thoroughly rinsed with several changes of distilled water. Oxidation of the proteins on the gel was done by incubating in 1% periodic acid (w/v) for 2 h in the dark with gentle shaking. Periodic acid was removed from the gels by washing with 15% acetic acid (v/v) for 2 h with 4 changes. Schiffs reagent (Appendix B) was used to stain the gels in the dark

with shaking (4° C, overnight). The gels were then destained with 4 changes of 7% acetic acid (v/v) with gentle shaking in the dark (24 h, 27° C).

3.8 Immunological studies

3.8.1 Preparation of polyclonal antibodies

Antibodies against the purified agglutinin were raised in a female New Zealand White rabbit according to the method described by Osir *et al.* (1989). The purified agglutinin (~1.0 mg protein) was emulsified in an equal volume of Freund's complete adjuvant and intradermally injected at several sites on the hind thigh of the rabbit. A booster injection (~0.5 mg protein in incomplete Freund's adjuvant) was injected intradermally at several sites on the other hind thigh of the rabbit four weeks later. The animal was bled after 2 weeks through the main ear artery. Fresh blood was kept at room temperature for clot formation for 2 h then kept at 4° C overnight. The serum was separated form the clot and centrifuged (1 000 x *g*, 30 min., 4° C) the kept at -20° C.

3.8.2 Preparation of crude Immunoglobulin mixtures.

Crude immunoglobulins were prepared according to the method described by Mayer and Walker (1987). The serum was precipitated in ammonium sulphate (50% saturation), centrifuged in a Sigma 3 K 10 refrigerated centrifuge (9 000 x *g*, 4° C, 15 min.), washed several times in 1.75 mM ammonium sulphate until the precipitate was white and then dialyzed against water for about 4 h. The immunoglobulin mixture (Ig mixture; ~2.15 mg proteins/ml) was aliquoted into small volumes and kept at -70° C.

3.8.3 Double radial immunodiffusion

Double radial immunodiffusion was carried out according to the original method of Ouchterlony (1958) with modifications (Mayer and

Walker, 1987). A 1% agarose gel with 2% PEG in phosphate buffered saline (PBS; 0.1 M sodium phosphate (pH 7.2) containing 0.15 M NaCl) was poured on glass plates (8.3 x 9.5 cm). Using a template, a well was punched in the centre of the gel on the glass plates and others punched circumferentially around the central well. To test the presence of antibody against the agglutinin, Ig mixture was poured into the central well while the agglutinin, flow through and the crude midgut homogenate were poured in peripheral wells. The immunodiffusion glass plates were then placed in a moist chamber for 24-48 h at 27° C to allow for protein diffusion. The glass plates were then washed in distilled water followed by PBS to remove excess proteins, dried in an oven (70° C), stained with Coomassie Brilliant Blue and destained for examination of immunoprecipitin lines.

3.8.4 Immunoblotting

A modified Western blot procedure of Towbin *et al.* (1979) as described by Copeland (1994) was used. Protein samples were separated on PAGE as described in section 3.6. Using a Novablot 2117 Multiphore II electrophoresis transfer unit (Pharmacia, Uppsala, Sweden), the separated proteins were transferred to a 45 μm pore nitrocellulose sheet (Schleicher and Schuel, Dassel, Germany). Whatman 3 mm filter paper was used for the blotting process after thoroughly soaking them in Towbin's transfer buffer (48 mM Tris-HCl, pH 8.3, 29 mM glycine, 20% methanol (v/v); 0.0375% SDS (optional for transfer of protein from denaturing gels)). A constant current of 0.8 mA per cm^2 of nitrocellulose membrane was applied to the set-up for 2 h at 27° C .

The nitrocellulose membrane was transiently stained with Rouge Ponceau (0.5% (w/v) in 1% (v/v) glacial acetic acid for 10 min. and destained with distilled water. The molecular weights positions on the nitrocellulose membrane were carefully marked. The blots were rinsed in phosphate buffered saline (PBS: 8 mM $Na_2HPO_4.2H_2O$, 1.5 mM KH_2PO_4, 3

mM KCl, 137 mM NaCl, pH 7.2) with 0.3% (v/v) Tween-20, and blocked in gelatin (3% v/v in PBS with Tween) for 4 h. at 27° C. The blots were then reacted with the anti-agglutinin Ig solution (1:300) in PBS-Tween 20 for 4 h with constant shaking at 4° C. The primary antibody was removed and the blots were briefly rinsed in distilled water followed by 4 washes of 15 min. each with PBS-Tween 20. The blots were then incubated for 2 h at 4° C in the secondary antibody, goat anti-rabbit horseradish peroxidase conjugate (Bio-Rad, Richmond, CA) (1:1000) in PBS-Tween 20 and thereafter washed four times in PBS-Tween 20, 10 min. per wash. The Tween 20 was removed from the blots by rinsing in PBS, pH 7.2 for 10 min. The nitrocellulose membrane was further rinsed in 10 mM Tris-HCl, pH 6.8 for about 5 min. The substrate, 4-Chrolo-1-napthol (1 part diluted in 4 parts 10 mM Tris-HCl, pH 6.8 from a stock solution of 0.3 % (w/v) 4-Chrolo-1-napthol in absolute methanol) containing 0.33 µl hydrogen peroxide/ml was added and the blots allowed to stain. Colour development was stopped by immersing the membrane into distilled water.

CHAPTER FOUR

SYNTHESIS SITE(S) OF A MIDGUT LECTIN-TRYPSIN COMPLEX IN TSETSE FLY (*GLOSSINA MORSITANS MORSITANS* WESTWOOD).

4.1 Introduction

Trypanosome transformation from bloodstream forms to midgut forms is one of the most crucial steps preceding establishment of trypanosome infection. This process, therefore, to a great extent plays an important role in determining maturation of the infection in the tsetse fly (Vickerman, 1985). Yabu and Takayanagi (1988), using commercial bovine pancreas trypsin, showed that the enzyme was able to induce the transformation process of monomorphic *T. b. gambiense* from bloodstream to procyclic forms *in vitro*. Studies by Imbuga *et al.* (1992a) also showed that crude midgut homogenates of *G. m. morsitans* induced the transformation of pleomorphic *T. b. brucei* from bloodstream forms to procyclic forms. The ability to induce transformation correlated with midgut trypsin activity and was shown to be inhibited by trypsin inhibitors (Imbuga *et al.*, 1992a). It has been shown that tsetse (*G. m. morsitans*) midgut trypsin activity, like the midgut lectin, is stimulated by a bloodmeal (van den Abbelle and Decleir, 1991; Stiles *et al.*, 1990) and the two also have similar activity profiles peaking at 48 - 72 h post-bloodmeal. The trypsin activity is inhibited by D(+) glucosamine, the known inhibitor of the tsetse midgut lectin (Osir *et al.*, 1993). It has, therefore, been proposed that the two molecules might be closely related or even be the same molecule. A chimeric molecule with both agglutination and trypsin activity has been purified from *G. longipennis* by Osir *et al.* (1995); with trypsin subunit having M_r ~27,000 and the agglutinin subunit with ~33,000 Da.

In order to understand and infer the possible physiological function(s) of the tsetse midgut lectin-trypsin complex, it is important to investigate the synthesis site(s) of the native molecule or its components and also assess the distribution in the fly tissues. The most likely synthesis site(s) of the midgut trypsins or trypsin-like enzymes in haematophagous insects are the midgut epithelial cells, where the columnar cells are the major cell type (Terra and Ferreira, 1994: Billingsley, 1990). However in the mosquito, *Culex nigripalpus* and the stable fly, *Stomoxys calcitrans*, the fat body has also been shown to be involved in the synthesis of trypsin-like enzymes (Borovsky, 1986a; b). On the other hand, *de novo* biosynthesis of haemolymph agglutinins (lectins) has been reported in several insect tissues. The fat body has been reported as the synthesis site of haemolymph lectins in the grasshopper, *Melanoplus differentialis* (Stiles *et al.*, 1988), in the flesh fly, *Sarcophaga peregrina* (Komano *et al.*, 1983) and the beet army worm, *Spodoptera exigua* (Boucias and Pendland, 1993). In some insects, such as the cockroach, *Leucophaea maderae* (Amirante and Mazzalai, 1978) and in the moth, *Hyalophora cecropia* (Yeaton, 1981), haemocytes have been reported as possible synthesis sites of the haemolymph lectin. Also, the sex organs (ovary and testicular tissues) were reported as probable synthesis sites of the haemolymph lectin in the grasshopper, *M. differentialis* (Stiles *et al.*, 1988). However, studies on the synthesis site(s) of midgut lectins and trypsins as well as their distribution in the tsetse flies (*Glossina* species) have not yet been reported.

This chapter presents work on the purification of a *G. m. morsitans* midgut agglutinin with trypsin activity using a two-step column chromatography procedure (ion exchange and affinity column chromatography). Localisation of the lectin-trypsin complex in the tsetse tissues as well as the sites of synthesis are also reported.

4.2 Material and Methods

4.2.1 Purification of the agglutinin

A two step column chromatography procedure was used to purify tsetse fly midgut agglutinin from *G. m. morsitans.* Ion exchange chromatography was used as the first step and affinity column chromatography was used as the second purification step. All chromatographic procedures were carried out at 4° C.

4.2.1.1 Ion exchange chromatography

Diethylaminoethyl Sepharose CL-6B (DEAE-Sepharose CL-6B; Pharmacia, Uppsala, Sweden) was used as an anion exchanger to purify the midgut agglutinin from the crude midgut preparations. The resin was soaked in 20 mM Tris-HCl, pH 8.0 and packed in a Pharmacia XK column (1.6 x 20 cm). The column was equilibrated with about 200 ml of 20 mM Tris-HCl buffer, pH 8.0. and connected to a peristaltic pump (P-1, Pharmacia, Uppsala, Sweden) and the flow rate adjusted to 10 ml/h.

The supernatant of midgut homogenate from about 300 male tsetse flies was first filtered through a 0.22 μm pore filter (Nalge, Rochester, NY, USA), dialysed against 20 mM Tris-HCl, pH 8.0 and then 85-100 mg protein loaded on to the column. Unbound proteins were eluted in 20 mM Tris-HCl, pH 8.0. Fractions (~1.5 ml) were collected using a fraction collector (Model 2128, Bio-Rad, Richmond, CA, USA) and their respective absorbances read at 280 nm using a GBC UV/VIS 916 spectrophotometer attached to a computer system (GBC, Victoria, Australia). Fraction collection was continued until the absorbences at 280 nm reached near zero. The bound proteins were eluted from the column using a 0.0 - 0.5 M NaCl gradient in 20 mM Tris-HCl, pH 8.0 and the absorbencies of the collected fractions at 280 nm, likewise, were recorded. The NaCl gradient used in the elution of the bound proteins from the column was created using a G-1 gradient maker (Pharmacia, Uppsala, Sweden).

Fractions were assayed for trypsin activity as described in section 3.4 and for their ability to agglutinate bloodstream forms *T. b. brucei* as described in section 3.5 Fractions with respective activities were pooled and dialysed against 3 changes of 20 mM Tris-HCl, pH 8.0 (4° C) and concentrated using polyethylene glycol (PEG-2000; Serva, Westbury, NY, USA). The purity of the samples was assessed by polyacrylamide gel electrophoresis (PAGE).

4.2.1.2 Affinity chromatography

Biospecific affinity chromatography was used in further purification of the tsetse midgut agglutinin. An inhibitor of the agglutinin, D(+)glucosamine (Sigma, St. Louis, MO, USA), was used as the ligand to be immobilised on to insoluble polymer, epoxy-activated Sepharose 6B (Sigma, St. Louis, MO, USA), by the bifunctional oxiranes.

4.2.1.2.1 Coupling of D(+)glucosamine to epoxy-activated Sepharose 6B and preparation of the column.

D(+)glucosamine was covalently coupled to epoxy-activated Sepharose 6B 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 min. and thoroughly washed with 100 ml distilled water on a G1 sintered glass filter (Corning, Straffordshire, UK) for 1 h. The wet gel (about 3.0 ml) was mixed with D(+)glucosamine (35 mg/ml) pre-dissolved in coupling solution (0.1 M carbonate\bicarbonate buffer (pH 8): dimethylformamide (50:50), and incubated in a water bath with a shaker (37° C, 16 h). Excess ligands were washed away with the coupling solution, followed by distilled water, carbonate\bicarbonate buffer (0.1 M, pH 8.0) and then acetate buffer (0.1 M, pH 4.0). Remaining reactive groups on the gel were blocked by soaking the gel in 1 M ethanolamine overnight at 45° C in a water bath with a shaker.

The coupled product was finally washed with the 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 gel was then packed on to a 10 ml Bio-Rad Poly Prep column (Bio-Rad, Richmond, CA, USA) attached to peristaltic pump and the column equilibrated with insect buffered saline (BIS; 10 mM Tris-HCl, pH 7.9, 130 mM NaCl, 5 mM KCl (Sigma, St. Louis, MO) 1 mM $CaCl_2$ (Sigma, St. Louis, MO)(McKenzie and Preston, 1992b; Komano *et al.*, 1983) and the flow rate adjusted to 10 ml/h.

4.2.1.2.2 Purification of agglutinin by Affinity chromatography

Samples from ion exchange chromatography (section 4.2.1.1), which agglutinated bloodstream trypanosomes (~3.5 - 5.0 mg protein), were dialysed against BIS and concentrated using PEG before loading on to the affinity chromatography column. The unbound fractions were eluted in BIS, pH 7.9. Using a Bio-Rad model 2128 fraction collector, 1.5 ml fractions were collected and their absorbencies at 280 nm recorded using a GBC UV/VIS 916 spectrophotometer linked to a computer system (GBC, Victoria, Australia). Fraction collection was continued until the absorbance at 280 nm was almost zero. Bound proteins were eluted from the column in BIS, pH 7.9 containing 0.2 M D(+)glucosamine. To increase the amount of the eluted product, the column was left standing for 2 h with D(+)glucosamine in BIS, pH 7.9 (McKenzie and Preston, 1992b; Bradley *et al.*, 1989). Fractions of 1.5 ml were collected and their absorbance at 280 nm recorded. For both the unbound and bound proteins, fractions were assayed for trypsin activity and for their ability to agglutinate bloodstream trypanosomes, as already described above. Fractions with respective activities were pooled, concentrated using PEG and dialysed against BIS, pH 7.9 with three changes (4° C). Polyacrylamide gel electrophoresis (PAGE) was used to assess the purity of the proteins.

4.2.2 Localisation of lectin-trypsin complex in tsetse tissues.

Different tissues were dissected from a total of 50 teneral male tsetse (*Glossina morsitans morsitans*), 72 h post-bloodmeal. The flies were immobilised by brief chilling at 4° C. After making a lateral incision on the abdomen and removal of the sternites, fat body, sexual organs (accessory reproductive gland, testes, epididymis and ejaculatory duct) and the posterior midguts were carefully dissected into ice cold 20 mM Tris-HCl, pH 8.0 (with a few crystals of phenylthiourea added to prevent melanisation). The peritrophic membrane was carefully pulled out and placed in a different Eppendorf tube with the same buffer. The cuticle from tergites was also dissected out after carefully removing the contaminating fat body tissues.

Haemocytes were collected from the haemolymph according to McKenzie and Preston (1992a). Chilled flies were bled for haemolymph through an incision on the coxa of the proleg. By gently squeezing the abdomen, the haemolymph was collected in capillary tubes containing ice cold bleeding buffer (130 mM NaCl, 5 mM KCl, 1.9 mM $NaH_2PO_4.H_2O$ (Aldrich, Dorset, UK) 1.7 mM K_2HPO_4, 10 mM EDTA, pH 7.5). To the haemolymph collected, were added 30 µl of bleeding buffer containing 50 mM glutathione, 1.0 mM diisopropylphosphofluodate (DFP)(Sigma, St. Louis, MO) 2 mM aprotinin and 5 mM phenylmethyl sulphonyl fluoride (PMSF)(Sigma, St. Louis, MO) and a few crystals of phenylthiourea (Pharmacia, Uppsala, Sweden). The samples were centrifuged (10 000 x *g*, 4° C, 10 min.) in an Eppendorf minifuge (5415 C, Germany) and the supernatant was kept as haemolymph while the pellet was collected as the haemocytes. The haemocytes were washed 3 times in ice-cold 20 mM Tris-HCl, pH 8.0.

The dissected tissues were homogenised for 2 min. using a motorised pestle and centrifuged (10 000 x *g*, 4° C, 30 min.) in the Eppendorf minifuge. The supernatants were then filtered through a 0.22 µm

syringe filter. About 50 μg protein of the homogenates were then electrophoresed on native PAGE (4-15% polyacrylamide) as previously described . The immunoblotting procedure and detection of the immunogens on the nitrocellulose membranes was carried out as described in General Materials and Methods.

4.2.3 Investigation of synthesis sites of the lectin-trypsin complex

A total of 50 teneral male tsetse flies (*G. m. morsitans*) used in these studies. The fat body and the posterior midgut were dissected as described in General Materials and Methods. Buffered insect saline (BIS) with 0.1% sucrose (w/v)(Sigma, St. Louis, MO) was used in this case. The peritrophic membranes were also carefully pulled out of the midgut.

4.2.3.1 Metabolic labeling and immunoprecipitation

In order to follow the secretion of newly synthesised proteins, the tissues were metabolically labeled with a radioactive substrate. The tissues dissected from the flies (fat body and midgut) were placed in separate Eppendorf tubes and rinsed 3 times in BIS and then incubated (27° C, 30 min.) in buffered insect saline with 0.1% (w/v). The tissues were then transferred to tubes with fresh buffer (100 μl) containing 45 μCi ^{35}S-methionine (2 mCi, 74 MBq; Amersham, UK). The tubes were incubated for 16 h at 27° C. Protease inhibitors, aprotinin (1 mg/ml) and PMSF (100 μg/ml; Sigma, St. Louis, MO) were added. The samples were then centrifuged (10 000 x *g*, 4° C, 30 min.) in an Eppendorf minifuge and the supernatant transferred to fresh tubes. The proteins in the sample were then solubilised in 0.5 ml NET-gel buffer (150 mM NaCl, 0.1% NP-40, 1 mM EDTA, 0.25% gelatin, 0.02% sodium azide).

Immunoprecipitation procedure used was modified from the original method of Ivarie and Jones (1979) as described by Sambrook *et al.* (1989). The anti-agglutinin immunoglobulins mixture (70 μl) was added to the

solution and the tubes were incubated at 4° C for 3 h on a Sarstedt end-to-end mixer (H. Jurgens GmbH, Bremen, Germany). Protein A-Sepharose 6MB (70 µl; Sigma, St. Louis, MO) was added and the mixture incubated at 4° C on the mixer for another 2 h. The antigen-antibody-protein A complex was thereafter washed twice with NET-gel buffer, with shaking on the mixer, followed by one wash with BIS and then distilled water. Each wash lasted about 15 minutes. SDS-PAGE sample loading buffer (30 µl) was added to the pellet and the mixture boiled for 5 minutes to denature the proteins from protein A-Sepharose 6MB. After centrifugation in a minifuge (10 000 x *g*, 27° C, 5 min.), the proteins in the supernatant were separated on SDS-PAGE as described in General materials and Methods.

The gel was stained with Coomassie brilliant blue and destained as previously described (section 3.6). To enhance the radioctivity, the gel was then soaked in AMPLIFY (Amersham, UK) for 45 minutes, dried on a gel slab drier (GSD-4, Pharmacia) and exposed to a Fuji AIF new RX x-ray film at -70° C for 7 days for autoradiography. The film was thereafter soaked in a Kodak GBX developer (Kodak Co., USA) for 5 minutes, washed in distilled water for 3 minutes, soaked in the Kodak GBX fixer (Kodak Co., USA) for 5 minutes and washed again in distilled water to remove the excess fixer. The X-ray film was air dried and signals from the incorporated radiolabel examined.

4.2.3.2 Identification of the synthesis site(s) of the trypsin moiety of the lectin-trypsin complex.

The synthesis site(s) of the trypsin moiety of the lectin-trypsin complex was identified using a modified procedure of Borovsky and Schlein (1988). This was based on the selective binding to serine proteases by the inhibitor diisopropylfluorophosphate (DFP) (Terra and Ferreira, 1994).

From about 25 male *G. m. morsitans* the fat body and the posterior midgut were carefully dissected out in BIS with 0.1% sucrose as described

in section 4.2.3.1. Care was taken to completely remove the peritrophic membrane from the midguts. The dissected tissues were then washed three times in BIS with 0.1% sucrose and then incubated in 100 µl fresh buffer for 16 h at 25° C. After incubation, the sample was centrifuged (10 000 x *g*, 4° C, 30 min.) and the supernatant collected as the materials secreted by the tissues into the buffer. A specific inhibitor of chymotrypsin, tosyl-phenylalanine chloromethyl ketone (TPCK; Sigma, St. Louis, MO) was added to the samples (100 µg/ml reaction solution from a stock solution of 3 mg/ml ethanol) (Sambrook *et al.*, 1987) and the mixture incubated at 4° C for 4 h. To this mixture, 5 µCi of 1,3-^{3}H-DFP (NEN-Dupont, Boston, MA) with specific activity of 250 µCi (9.25 MBq) was added and incubated at 4° C for 16 h. The proteins were then separated on SDS-PAGE (~35 mg protein) as described in General Materials and Methods. After SDS-PAGE, the gel was stained with Coomassie Blue, destained, dried and exposed to X-ray film for autoradiography as described in section 4.2.3.1.

4.2.3.3 Time course studies on the appearance of the lectin-trypsin complex in the fat body and haemolymph.

About 100 male *G. m. morsitans* were fed on uninfected rat blood 24 hr post-emergence. At 12 h intervals post-feeding, 10 flies were sacrificed and bled for haemolymph as described in section 4.2.2. Fat body tissues were also carefully dissected out in 20 mM Tris-HCl, pH 8.0 and homogenised in the same buffer using a motorised pestle. Teneral flies were also bled for haemolymph and the fat body was also dissected from them. Total protein determination was carried out using the BCA protein assay kit for both the fat body homogenate samples and the haemolymph. Both the fat body and the haemolymph samples (~50 µg protein) were separated on native PAGE and immunoblotted as described in General Material s and Methods.

4.2.3.4 Stimulation of protein secretion by the fat body post-bloodmeal

From a total of about 50 teneral male *G. m. morsitans*, fat body tissue was dissected out immediately after a bloodmeal into a total of 500 µl of BIS with 0.1% sucrose . The tissues were incubated at 27° C. At 12 h intervals, aliquots of 50 µl were taken from the incubation medium and frozen at -20° C. The total proteins of the aliquots were then determined using the BCA protein assay kit.

4.3 Results

4.3.1 Purification of the lectin-trypsin complex

The elution profile from ion exchange chromatography is shown in Figure 4.1. Four prominent peaks were obtained. The fractions in each of the four peaks were assayed for trypsin activity and their ability to agglutinate bloodstream *T. b. brucei* (Table 4.1). Fractions with respective activities were pooled and concentrated. Maximum agglutination titres (128) were recorded in peak III (fractions 45-65), which eluted at about 0.14 M NaCl (about 28% of the 0.5 M NaCl). No observable agglutination was recorded from peak II (fractions 36-40) which were eluted at about 0.047 M NaCl and peak IV (fractions 74-90) which was eluted in about 0.28 M NaCl. However, an agglutination titre of 8 was recorded in peak I (fractions 8-26), the unbound

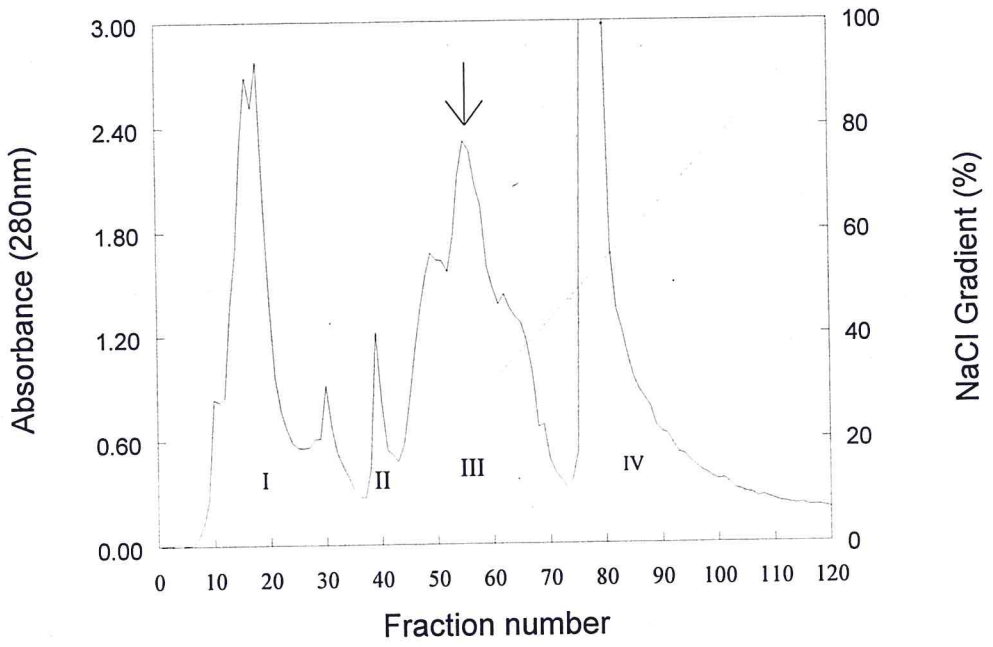

Figure 4.1 Ion exchange chromatography profile of the crude gut homogenate on DEAE-Sepharose CL-6B. In peak I, unbound proteins were eluted while in peaks II - IV the bound materials were eluted in a NaCl gradient (.....). The arrow indicates the peak with the highest agglutination titre and trypsin activity.

Table 4.1. Agglutination and trypsin activity of samples fractionated from tsetse midgut homogenate by ion exchange column chromatography using DEAE-Sepharose 6B as an anion exchanger. (- indicates that no agglutination was observed) .

Peak	**Agglutination Titre**	**Trypsin activity (μmoles/min./ml)**
I	8	2.15×10^{-2}
II	-	5.67×10^{-2}
III	128	10.4×10^{-2}
IV	-	1.63×10^{-2}
crude gut homogenate	256	13.2×10^{-2}

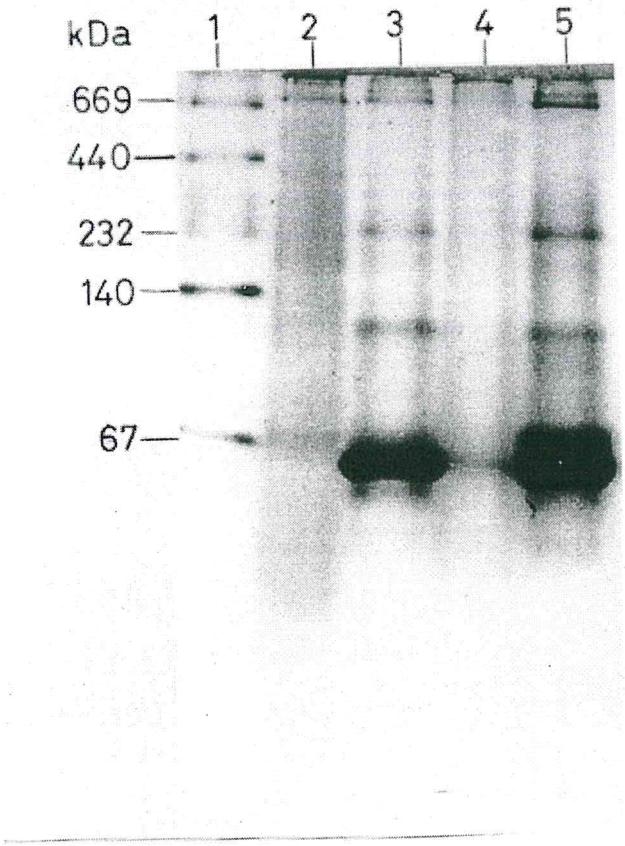

Figure 4.2 Polyacrylamide gel electrophoresis of pooled fractions fractionated from ion exchange column chromatography.
Lane 1 - high range molecular weight markers (Pharmacia, Uppsala, Sweden).
Lane 2 - samples from peak IV.
Lane 3 - sample from peak III.
Lane 4 - sample from peak II.
Lane 5 - sample from peak I.

Comparatively, the *G. m. morsitans* crude midgut homogenate agglutinated bloodstream trypanosomes up to a titre of 256.

Table 4.1 also shows that the highest trypsin activity, was in peak III (10.4×10^{-2} µmoles/min./ml). While trypsin activity of 2.15×10^{-2} µmoles/min./ml was recorded in peak I and 5.67×10^{-2} µmoles/min./ml from peak II, the lowest trypsin activity was detected from peak IV (1.63×10^{-2} µmoles/min./ml). Trypsin activity of 13.2×10^{-2} µmoles/min./ml was obtained from the crude midgut homogenate.

Native-PAGE of the four peaks fractionated from the crude midgut homogenate of *G. m. morsitans* by ion-exchange column chromatography is shown in Figure 4.2. The protein bands representing the agglutinin are evident
in lanes 3 and 5, representing samples from peaks I and III as indicated in the profile (Figure 4.1). The presence of more than one protein band on PAGE were indicative of impurities in the sample despite the agglutination titre of 128 recorded in Table 4.1 for peak III. This necessitated further purification of the agglutinin.

The peak from ion exchange chromatography with the highest agglutination titre (peak III from Figure 4.1) was further purified using affinity column chromatography. To enhance the maximal release of the agglutinin from the column, after addition of 0.2 M GlcN in BIS, the gel was resuspended in the column and incubated for 2 h at 4° C, prior to elution. The affinity chromatography elution profile shows two peaks (Figure 4.3). Peak I represents the fractions with unbound proteins and peak II represents fractions with the bound material. After assaying for trypsin and agglutination activities, the fractions with respective activities were pooled and concentrated. Table 4.2 shows that while the pooled bound fractions (peak II) gave an agglutination titre of 64 and trypsin activity of 1.78×10^{-2}

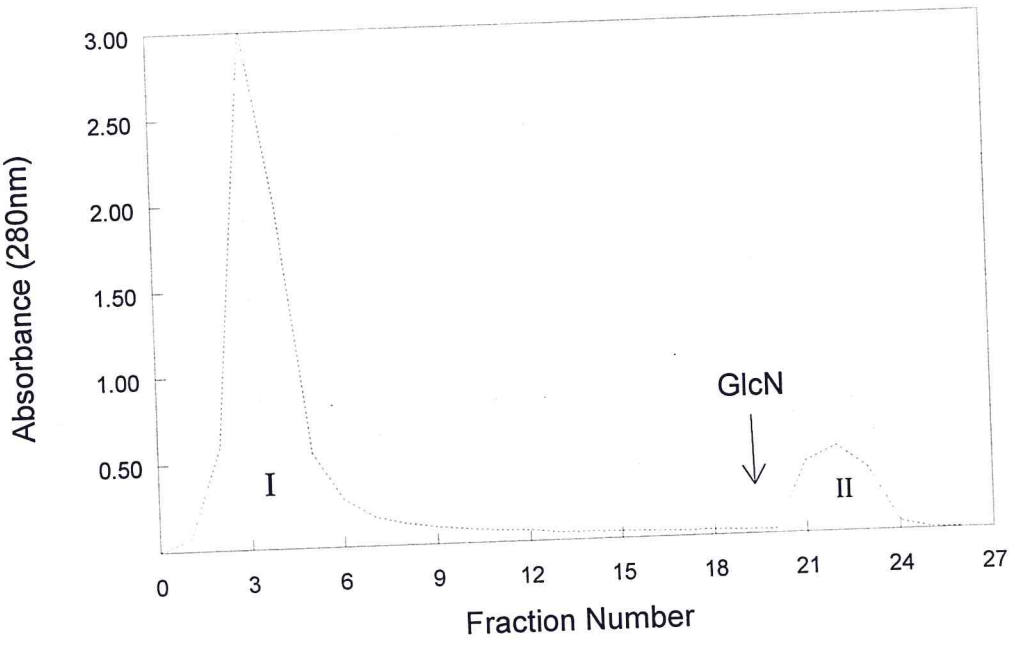

Figure 4.3 Affinity chromatography of pooled fractions that gave high agglutination titres from ion exchange chromatography. The arrow indicates the point at which 0.2 M glucosamine in BIS was added to elute the bound proteins. Peak I represents the unbound fractions while peak II represents the bound materials.

Table 4.2 Agglutination titres and trypsin activity of fractions from affinity column chromatography with D(+) glucosamine coupled to epoxy-activated Sepharose 6MB.

Peak	**Agglutination titre**	**Trypsin activity (μmoles/min./ml)**
1	4	1.84×10^{-3}
2	64	1.78×10^{-2}

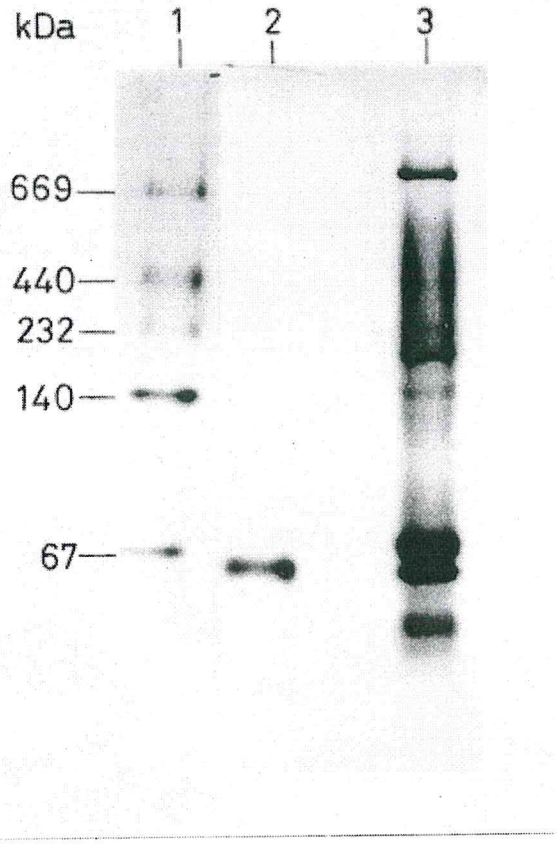

Figure 4.4 Non-denaturing PAGE of pooled fractions from affinity chromatography.
Lane 1 - high range molecular weight markers (Pharmacia, Uppsala, Sweden)
Lane 2 - the purified agglutinin (peak 2; ~35 μg protein)
Lane 3 - unbound fractions (peak 1; ~45 μg protein).

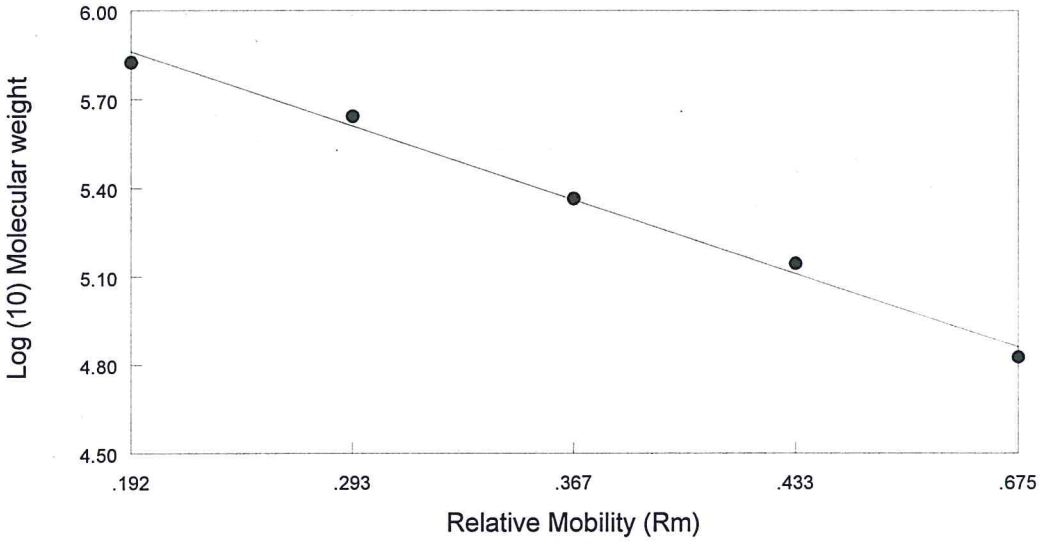

Figure 4.5 Standard curve for molecular weight determination on native PAGE. Pharmacia high range molecular weight markers (thyroglobulin = 669,000, ferritin = 440,000, catalase = 232,000, lactate dehydrogenase = 140,000 and bovine serum albumin = 67,000). The relative mobility (R_m) of the protein bands were plotted against $\log_{10}$ molecular weights.

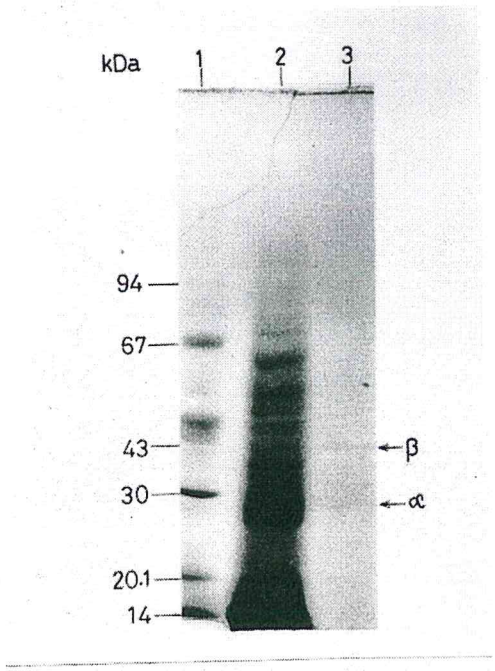

Figure 4.6 Dissociating gel electrophoresis of samples from affinity chromatography of agglutinating fractions.
Lane 1 - low range molecular weight markers (Pharmacia, Uppsala, Sweden)
Lane 2 - crude gut extract (~30 μg protein)
Lane 3 - agglutinin (~30 μg protein).
(α and β are designated bands resulting from dissociation of the agglutinin).

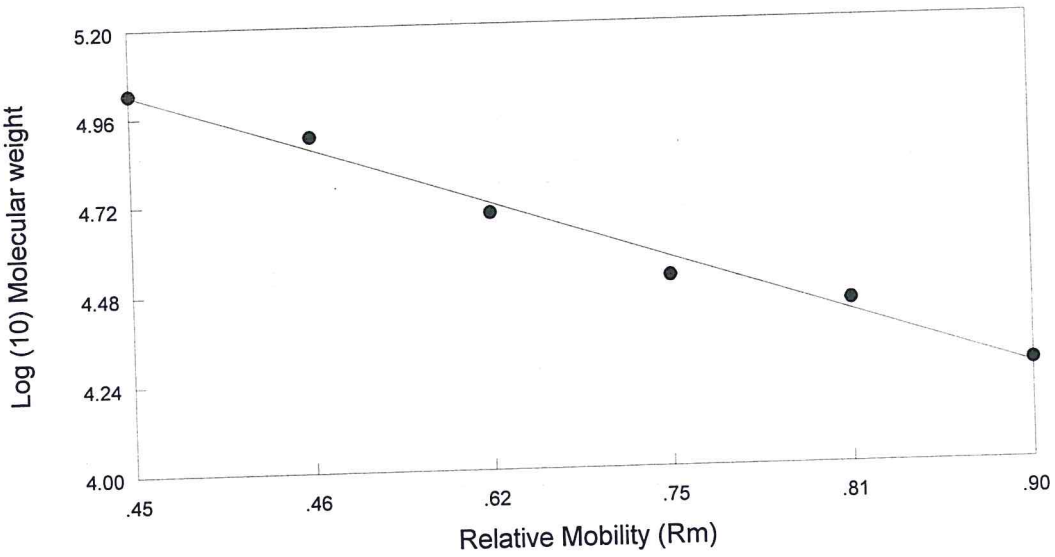

Figure 4.7 Standard curve for molecular weight determination on SDS-PAGE. Pharmacia low range molecular weight markers were used (phosphorylase b = 94,000, bovine serum albumin = 67,000, ovalbumin = 43,000, carbonic anhydrase = 30, 000, trypsin inhibitor = 20,100 and lysozyme = 14,000). The relative mobility of the protein bands of the markers (R_m) were plotted against $\log_{10}$ molecular weights.

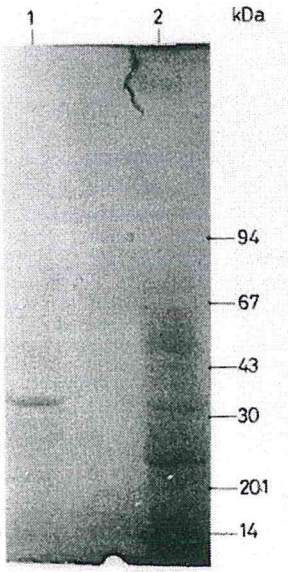

Figure 4.8 Periodate-Schiff (PAS) stain of the agglutinin purified by affinity chromatography.

Lane 1 - purified agglutinin
Lane 2 - low molecular weight markers (Pharmacia, Uppsala, Sweden).

μmoles/min./ml, the pooled unbound fractions (peak I) gave an agglutination titre of 4 and trypsin activity of 1.84×10^{-3} μmoles/min./ml.

On native PAGE (Figure 4.4), samples from peak I (lane 2) showed more than one protein band. Peak II samples (lane 3) showed only one protein band. Using Pharmacia high molecular weight markers standard curve for native-PAGE (Figure 4.5), the apparent molecular weight (M_r) was estimated at ~65,700 ± 1,232 (n=3). On a denaturing gel (SDS-PAGE) (Figure 4.6) of peak 2 from affinity chromatography, the lane of the agglutinin gave two prominent protein bands. Using the Pharmacia low molecular weight standard curve (Figure 4.7), the apparent molecular weight (M_r) were estimated as an α band of ~28,800 ± 277 (n=3) and and a ß band of ~35,700 ± 425 (n=3).

To determine which of the two subunits of the molecule had the glycosyl residue, the purified agglutinin (peak II from affinity chromatography) was electrophoresed on SDS-PAGE (4-15%) and the gel stained for carbohydrates using the PAS staining method. The results (Figure 4.8) show that only one band stained positive for the carbohydrate stain with M_r ~34,800 ± 3,813 (n=3)(lane 2).

4.3.2 Detection of polyclonal antibodies

The double radial immunodiffusion (Figure 4.9) shows that antibodies were shown to have reacted with the crude midgut homogenate (well 3) and the purified agglutinin (well 1), where precipitin lines were observed. A very weak precipitin line was observed form the sample of the unbound material (well 2).

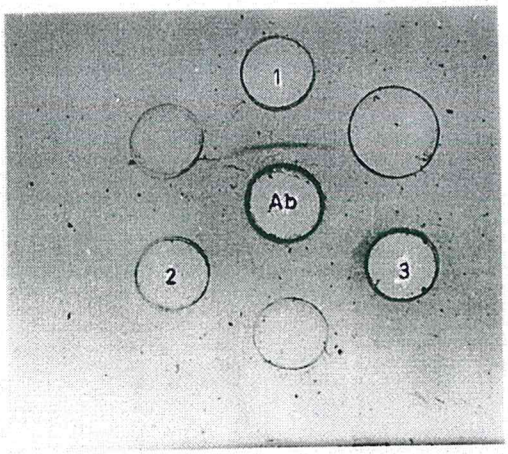

Figure 4.9 Radial immunodiffusion of polyclonal anti-agglutinin antibodies and samples.
Ab- anti-agglutinin polyclonal antibody
1 - purified agglutinin (peak II from affinity chromatography)
2 - peak I from affinity chromatography
3 - crude gut homogenate.

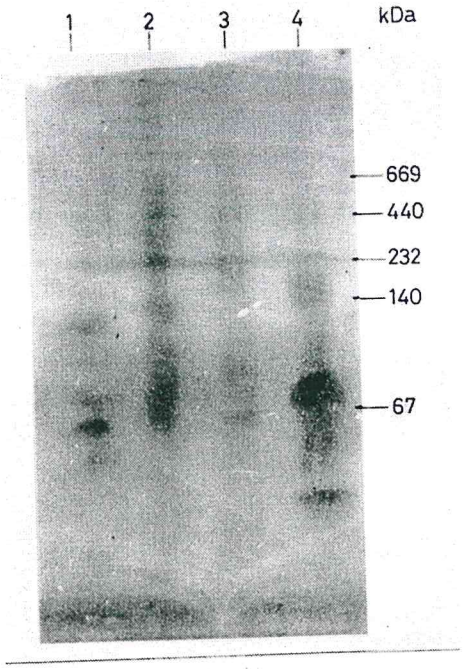

Figure 4.10 Immunoblot of crude midgut homogenate samples from different *Glossina* species.
Lane 1 - *G. f. fuscipes*
Lane 2 - *G. m. centralis*
Lane 3 - *G. m. morsitans*
Lane 4 - *G. longipennis*

4.3.3 Immunological cross-reactivity

Using the immunoblotting procedure, the presence of the agglutinin in selected tissues of the tsetse fly was investigated. The polyclonal antibodies raised against the agglutinin were also used to probe for the presence of the agglutinin in other tsetse species. By the immunobloting procedure, it was shown that there were observable cross-reactions between the anti-agglutinin antibody and all the *Glossina* species studied (Figure 4.10). From lane 1 (*G. f. fuscipes*) two bands were observed at M_r ~92,000 and ~66,000, the same banding pattern was also observed in lane 2 (*G. m. centralis*), although the ~66,000 band appeared as a smear in this case. The anti-agglutinin antibody reaction with *G. m. morsitans* midgut homogenate (lane 3) appeared as a single distinct band at M_r ~65,000. From lane 4 (*G. longipennis*), three bands were observed with M_r ~54,000, ~70,000 and ~92,000 Da.

Midgut samples from selected haematophagous insects were also investigated for cross-reactivity with the anti-agglutinin antibody as shown in Figure 4.11. Cross-reactions were observed only against *G. m. morsitans* midgut (lane 4) and *Aedes aegypti* (lane 1). From *A. aegypti* midgut sample, 2 broad bands M_r >140,000 were observed while against the *G. m. morsitans* midgut one distinct band M_r ~63,000 was prominent. The midgut samples from the sand fly, *Phlebotomus dubosqci*, showed a smear with no evident bands appearing. No bands were detected from midgut homogenate of *S. calcitrans* (lane 3).

4.3.4 Localisation of the lectin-trypsin complex in tsetse tissues.

Using the immunoblotting procedure, the presence of the agglutinin in selected tissues of the tsetse fly was investigated. The polyclonal antibodies raised against the agglutinin were used to probe for the presence

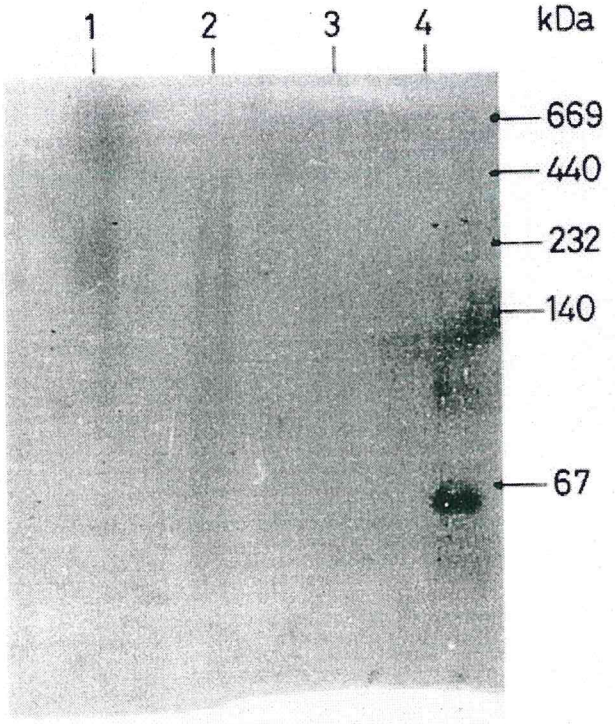

Figure 4.11 Immunostained Western blots of crude midgut homogenates from different haematophagous insect.
Lane 1 -*Aedes aegypti*,
Lane 2 - *Phlebotomus duboscq*i,
Lane 3 - *Stomoxys calcitrans*
Lane 4 - *G. m. morsitans*.

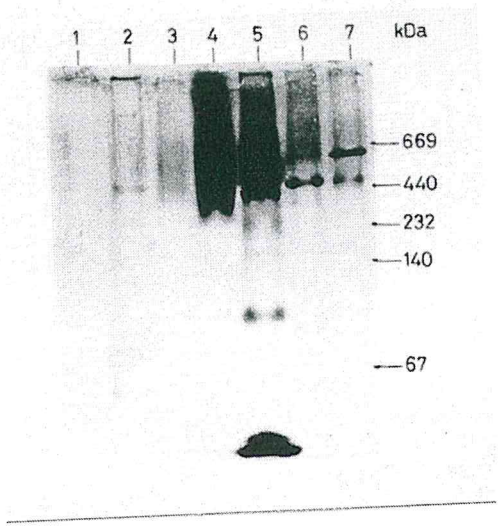

Figure 4.12 Immunoblot analysis of homogenates of different tsetse tissues.
Lane 1 - cuticle extract
Lane 2 - peritrophic membrane homogenate
Lane 3 - sex organs homogenate
Lane 4 - haemocyte homogenate
Lane 5 - haemolymph
Lane 6 - fat body homogenate
Lane 7 - midgut tissues homogenate.

of the agglutinin in tissues previously reported to be associated with insect lectins. These included homogenates of fat body, the posterior midgut, peritrophic membrane (Ingram and Molyneux, 1991), haemocytes (Yeaton, 1981), sexual organs, the cuticle and haemolymph (Stiles *et al.*, 1988). The agglutinin was shown to be associated with the fat body, the posterior midgut, haemolymph and the peritrophic membrane; and not with the sexual organs, haemocytes and the cuticle as shown in Figure 4.12. Homogenate of midgut tissues (lane 7) showed 2 bands; a distinct band of M_r ~600,000 and a lighter band of ~450,000. The same banding pattern was observed in lane 6 (fat body homogenate) although the ~450,000 Da band was more intense and the ~600,000 Da lighter. The ~600,000 Da band can, therefore, be considered characteristic of the midgut tissues and the ~450,000 Da band for the fat body.

From the haemolymph sample (lane 5) four bands were picked by the antibody. Two intense bands, one at ~480,000 Da and another at ~96,000 Da. Two lighter bands at ~430,000 Da and ~65,500 Da were also observed. Two bands appeared in lane 2 (peritrophic membrane homogenate), a lighter ~450,000 Da band and a more intense ~65,000 Da band. It must be noted that the apparent molecular weights of ~65,000 and ~65,500 Da bands observed in the peritrophic membrane homogenate and the haemolymph homogenate respectively, compare favourably with that of the agglutinin purified from the crude gut homogenate of *G. m. morsitans*. It is also possible that the ~450,000 Da band observed in the peritrophic membrane (lane 2) is due to contamination from gut tissues during dissections.

When the homogenates of various tissues dissected out from tsetse were assayed for trypsin activity and their ability to agglutinate both bloodstream and procyclic trypanosomes (Table 4.3), it was observed that there was no detectable trypsin activity in homogenates of sexual organs,

Table 4.3 Agglutination and trypsin activity of different tissues dissected out from *G. m. morsitans.* (- indicates no agglutination was observed; ND = assay was not determined due to high titre in bloodstream forms).

Tissue	Agglutination Titres		Trypsin Activity
	Bloodstream	Procyclic	μmoles/min./ml)
Fat body	-	-	1.83×10^{-3}
Midgut tissue	-	-	2.54×10^{-2}
Peritrophic membrane	8	512	4.6×10^{-4}
Sexual organs	-	-	0
Haemolymph	-	-	0
Cuticle	-	-	0
Haemocytes	-	-	0
Gut homogenate	256	ND	13.2×10^{-2}

cuticle and haemocytes as well as in the haemolymph. The highest activity was found in the homogenate of midgut tissues (2.54×10^{-2} μmoles/ml/min.) followed by the fat body homogenate (1.83×10^{-2} μmoles/ml/min.). It must be noted that in all of these tissues, no positive agglutination of both bloodstream and procyclic trypanosomes was reported. The peritrophic membrane, however, agglutinated bloodstream and procyclic trypanosomes to titres of 8 and 512 respectively and had a very low trypsin activity (4.6×10^{-4} μmoles/ml/min.), which represents only about 0.4% of the total trypsin activity in the tsetse gut. Comparatively, the crude gut homogenate agglutinated bloodstream trypanosomes to a titre of 256 and had trypsin activity of about 13.2×10^{-2} μmoles/ml/min.

4.3.5 *In vitro* synthesis of the lectin-trypsin complex

In vitro synthesis of the agglutinin was evaluated by the incorporation of the radiolabel. After immunoprecipitation and processing the gels for autoradiography, the results are presented in Figure 4.13. One distinct band from the lane 2 (of the fat body) with M_r ~42,000 and 2 lighter bands of ~62,000 and ~67,000 were detected. On the other hand, from the lane of the midgut tissue (lane 1), a distinct band was observed at ~62,000 Da and 2 lighter bands at ~42,000 and ~67,000 Da.

In order to identify which of the tissues above is involved in synthesising the components of the lectin trypsin complex, a specific radiolabeled inhibitor of serine proteases, 1,3-^{3}H-DFP, was used. Since the other midgut serine protease, chymotrypsin, was inhibited by TPCK, the signals detected from the autoradiograph represents the trypsin or trypsin-like enzymes synthesised and secreted by the tissues. Figure 4.14 shows that the whole gut (lane 1) secreted three types of trypsins or trypsin-like enzymes which appeared as bands at ~29,000 Da, ~26,000 Da and ~22,000 Da. The midgut tissues (lane 3) secreted two forms of trypsin or trypsin like enzymes which appeared as bands at ~27,800 Da and ~22,000 Da. No trypsins were detected from the proteins secreted by the fat body tissue (lane 2). This implies that of the two tissues investigated as the possible synthesis sites of the components of the lectin-trypsin complex, the trypsin moiety could only be synthesised by the midgut tissues.

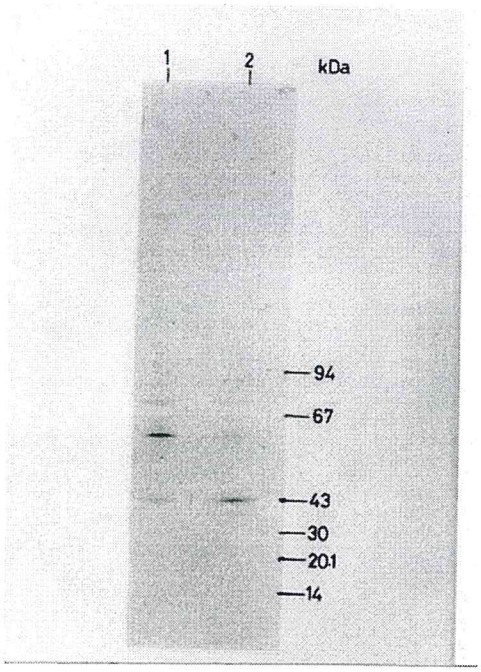

Figure 4.13 Autoradiogram of proteins synthesised by the fat body and midgut tissues.

Lane 1 - sample immunoprecipitated from proteins secreted by the midgut tissues

Lane 2 - sample immunoprecipitated from proteins secreted by the fat body.

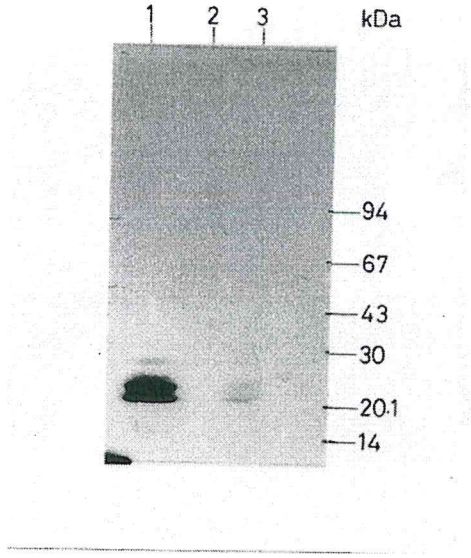

Figure 4.14 Autoradiogram of trypsin or trypsin-like enzymes secreted by tissues.
Lane 1 - sample from proteins secreted by the whole gut,
Lane 2 - sample from proteins secreted by the fat body
Lane 3 - sample from proteins secreted by the posterior midgut tissues.

4.3.6 Time course studies on the appearance of the agglutinin in the fat body and haemolymph.

4.3.6.1 Fat body

When fat body tissues were dissected from tsetse at different times, post-feeding and through immunoblotting probed for the appearance of the agglutinin, it was shown that for both the material secreted into the incubation medium (BIS with 0.1% sucrose) and those retained in the tissues, the agglutinin was present at all the time intervals investigated (Figure 4.15.) A protein band of M_r ~480,000 was evident in all the samples. However, the bands were weak from samples from teneral flies for both the secreted material (lane 8) and the materials retained in the tissues (lane 4).

4.3.6.2 Haemolymph

The anti-agglutinin antibody was also used to probe for the appearance time of the agglutinin in the haemolymph. By immunoblotting, a protein band was detected at M_r ~480,000 in lanes 3, 4, and 5 (Figure 4.16), which represent haemolymph samples bled from tsetse at 48 h, 72 h, and 96 h post feeding. The protein band was evidently absent in teneral flies (lane 1) and in haemolymph samples bled from tsetse flies 48 h post-feeding.

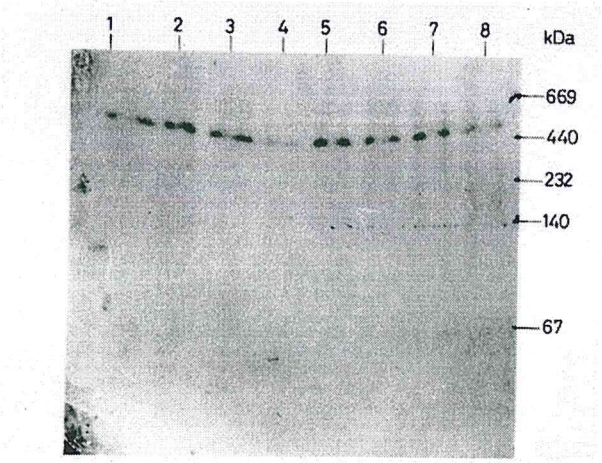

Figure 4.15 Immunoblot of fat body dissected from tsetse at different times, post-feeding.
Lanes 1 to 4 represent materials retained in the fat body
Lanes 5 to 8 represent materials secreted by the fat body
Lanes 4 and 8 - fat body from teneral flies
Lanes 3 and 4 form fat body of 24 h post-fed flies
Lanes 2 and 6 - fat body of 48 h post-fed flies
Lanes 1 and 5 - fat body of 72 h post-fed flies.

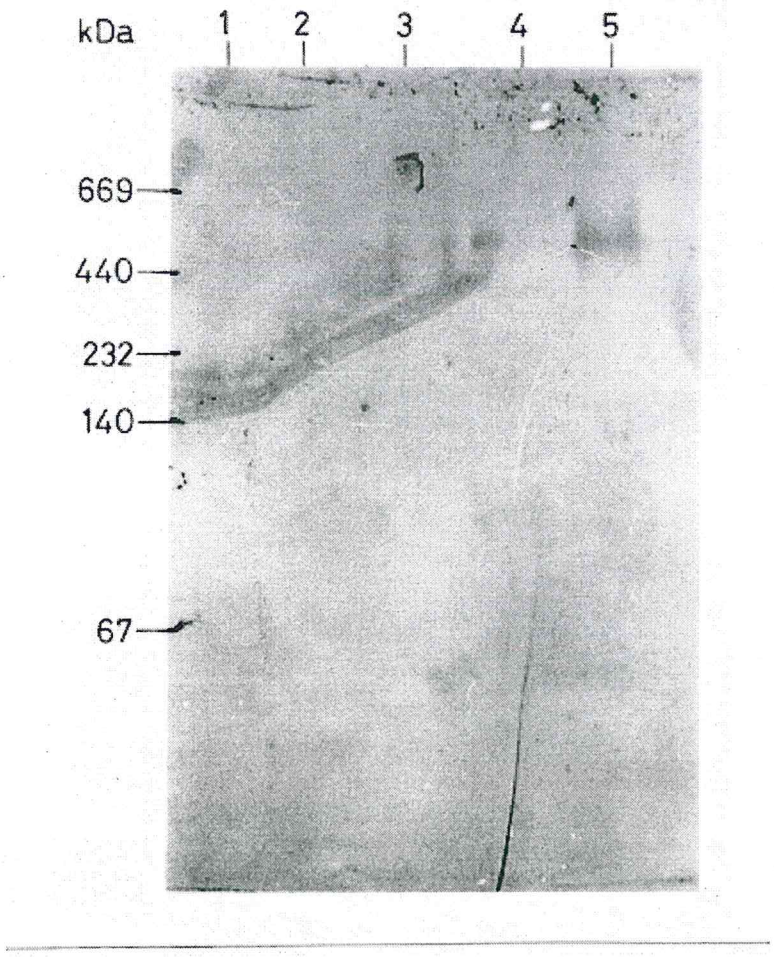

Figure 4.16 Immunoblot analysis of the appearance of the agglutinin in the haemolymph.
Lane 1 - haemolymph bled from teneral flies
Lane 2 - haemolymph from tsetse 24 h after feeding
Lane 3 - haemolymph from tsetse 48 h after feeding
Lane 4 - haemolymph from tsetse 72 h after feeding
Lane 5 - haemolymph from tsetse 96 h post-feeding.

4.3.7 Stimulation of protein secretion by the fat body

The effect of bloodmeal on the secretion of proteins by the fat body was investigated. From fat body tissues incubated at 27° C total protein was determined from aliquots taken at different time intervals post bloodmeal. Figure 4.17 shows that soon after a bloodmeal total proteins secreted by the fat body were high at 0.53 mg protein/ml followed by a drop to 0.22 mg protein/ml at 12 hr post-bloodmeal. This trend is followed by a gradual increase in protein secretion with time up to a maximal peak at 0.80 mg protein/ml, 72 h post bloodmeal. Then there is a gradual decrease in the protein secretion up to 0.42 mg protein/ml at 96 h. It must be pointed out that at 108 h post bloodmeal the fat body tissues had completely decomposed as visualised microscopically.

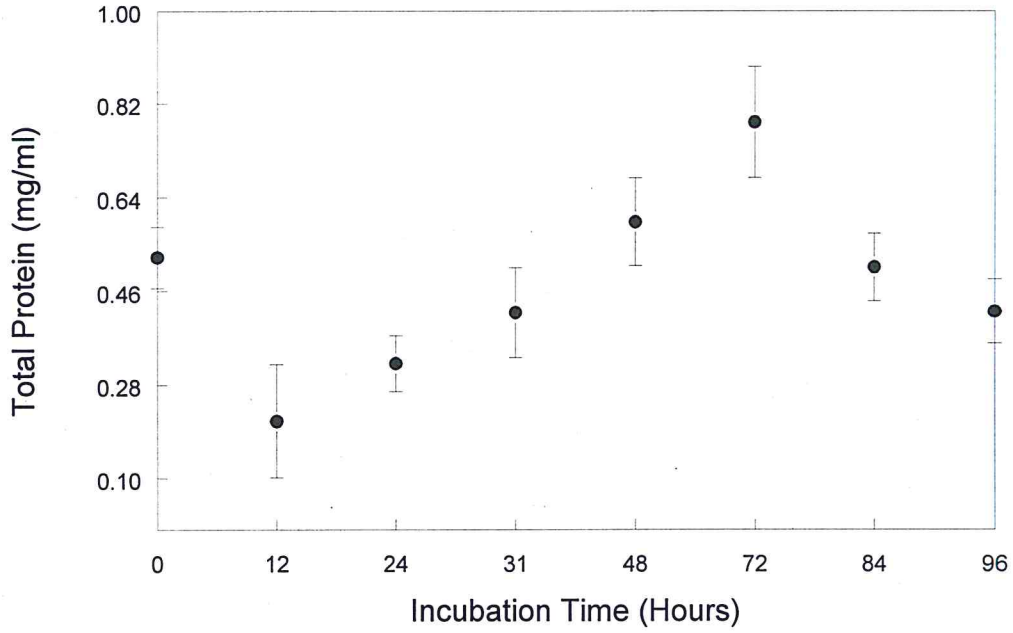

Figure 4.17 Stimulation of fat body protein secretion by bloodmeal. Fat body tissue was incubated at 27° C. Each data point represents a mean ± SD (n=3).

4.4.0 Discussion

4.4.1 Purification and properties the lectin-trypsin complex

Isolation and purification are paramount in protein studies (Scopes, 1996). Several methods are used in the fractionation of proteins. Ion exchange chromatography is the most widely used method in the fractionation of complex mixtures of biological substances (Scopes, 1996). Separation in ion exchange chromatography is achieved on the basis of charges on the surfaces of the solute molecules. The insoluble matrix in ion exchange chromatography consists of ion exchangers which can associate with mobile counter-ions of the solute molecules. Ion exchangers can be positively charged (cations) or negatively charged (anions) (Pharmacia, 1981). The strength of the interaction between the protein in the sample and the adsorbent with the ion exchanger is, therefore, determined by the net charge on the proteins in the sample (Scopes, 1996). Ion exchange (and reverse phase) chromatography, therefore relies on resolution during the elution stage such that each protein species will interact differently allowing a separation during chromatographic elution (Pharmacia, 1981). Electrophoresis also exploits the differential net charges and is mostly used as an analytical tool.

Affinity chromatography exploits the unique biological property of macromolecules to bind ligands specifically and reversibly (Cuatrecasas, 1970). The protein to be purified is passed through a column containing a gel (agarose and polyacrylamide beads) to which a specific competitive inhibitor or ligand has been covalently attached (Pharmacia, 1986b). Protein molecules with no affinity to the ligand pass through the column while those that recognise the ligand are retarded in the column (Scopes, 1996; Pharmacia, 1986b).

Affinity chromatography has been used to isolate and purify lectins from several insect species. Using D(+) galactose as a ligand coupled to

cyanogen bromide activated Sepharose 4B, haemolymph lectins have been reported to have been isolated from the following insects: beet army worm, *Spodoptera exigua* by Boucias and Pendland (1993) the blow fly, *Calliphora vomitoria*, (McKenzie and Preston, 1992); the grasshopper *Melanoplus sanguinipes* (Stebbins and Hapner, 1985) and from silk moth, *Hyalophora cecropia* (Castro *et al.*, 1987). Using methyl D-galactose as a ligand coupled to divinyl sulfone-activated Sepharose 6B, Drif and Brehelin (1994) also purified a haemolymph agglutinin from the locust, *Locusta migratoria*.

Using a two step column chromatography procedure (ion exchange affinity chromatography), an agglutinin with trypsin activity has been isolated from the midgut homogenate of *G. m. morsitans*. The purified agglutinin has been shown to have a native apparent molecular weight of ~65,700 ± 1232. This molecule had an agglutination titre of 64 (for bloodstream trypanosomes) and trypsin activity of 1.78×10^{-2} µmoles/min./ml and specific activity of 69.3 µmoles/min./mg protein. A similar agglutinin (M_r ~61 000) with trypsin activity had previously been purified from *G. longipennis* midgut homogenate by two cycles of ion exchange chromatography (Osir *et al.*, 1995). While the *G. longipennis* agglutinin was shown to be composed of two subunits of M_r ~27,000 and ~33,000 for the α and ß subunits respectively, the *G. m. morsitans* agglutinin purified in this study has been shown to have an α subunit of M_r ~28,800 and a ß subunit of M_r ~35,700. Like the *G. longipennis* agglutinin, the ß subunit of *G. m. morsitans* agglutinin stained for carbohydrates, suggesting that it represents the glycosylated residue of the molecule. The observed differences in the sizes of the molecules might be indicative of the inter-species differences of the midgut agglutinins. This is further confirmed by the findings of Stiles *et al.* (1990), who identified a midgut agglutinin of M_r ~67,000 from *G. palpalis* spp. It is also possible that the midgut agglutinins identified from the three species might be of the same sizes and that the

different molecular weights recorded may be as a result of the different purification procedures used.

Different methods have been used to purify agglutinins from insect tissues. The most commonly used are ion exchange and affinity chromatography. Ingram and Molyneux (1990) purified a M_r ~710,000 haemolymph lectin from *G. f. fuscipes* using a Mono Q HR5/5 anion column attached to a fast protein liquid chromatography (FPLC) system. Also Stiles *et al.* (1990) purified a M_r ~67,000 *G. palpalis* spp midgut agglutinin using an FPLC system with a Mono Q HR 5/5 column. Using a similar type of system, Osir *et al.* (1995) purified a M_r ~61,000 Da agglutinin with trypsin activity from the midgut homogenate of *G. longipennis*. Ion exchange chromatography has also been successfully used to isolate trypsin and trypsin-like enzymes in *Stomoxys calcitrans* (Borovsky, 1986a), *G. m. morsitans* (Cheeseman and Gooding, 1985) and *G. palpalis palpalis* (van den Abbelle and Decleir, 1991). It must also be pointed out that molecular weight of the trypsin moiety of the lectin-trypsin complex (~28,800 Da for *G. m. morsitans* and ~27,000 Da for *G. longipennis*) compare favourably with those of the trypsins isolated from guts of other *Glossina* species. The trypsin and trypsin-like enzyme in the *Glossina* species studied so far have been reported to have molecular weights ranging from ~19,000 Da to ~35,000 Da (Cheeseman and Gooding, 1985; Applebaum, 1985; van den Abbelle and Decleir, 1991). This might imply that the same trypsin or trypsin-like enzymes that are involved in digestion are associated with the lectin in the tsetse gut.

In the initial stages of this study, a Mono Q HR 5/5 anion exchange column attached to a FPLC system with a model GP-250 gradient programmer was used to purify the midgut agglutinin from *G. m. morsitans* (Data not shown). The procedure was however abandoned due to the low amounts of agglutinin that was obtained per purification run. This was

primarily due to the capacity of the column which could not take more than 0.5 ml crude gut homogenate.

At present, no other study has reported the use of affinity chromatography to purify tsetse agglutinins, despite the indication that there are specific inhibitory carbohydrates to tsetse lectins. However, using D(+) galactose as a ligand, haemolymph lectins have been isolated from: the beet armyworm, *Spodoptera exigua*, an aggregate of ~100-170,000 Da with subunits of ~33 200 and ~34,400 Da (Boucias and Pendland, 1993); the blow fly, *Calliphora vomitoria*, a ~130,000 Da tetramer with ~32,000 Da subunits (McKenzie and Preston, 1992b); the grasshopper, *Melanoplus sanguinipes*, a ~600-700,000 Da lectin with aggregates of ~70,000 Da (Stebbins and Hapner, 1985); the silk worm *Hyalophora cecropia*, a ~160,000 Da lectin with subunits of ~41,000 and ~38,000 Da (Castro *et al.*, 1987); the locust, *Locusta migratoria*, a ~650,000 Da lectin with eight subunits of ~ 80 000 Da each.

It is important to note that most of these insect lectins, like those found in the guts of *G. m. morsitans* (this study) and *G. longipennis* (Osir *et al.*, 1995) are oligomers of more than one subunit. It has been proposed that through an unknown mechanism, lectins are responsible for inducing the removal of foreign materials from the insect's body as part of the insects' immune system (Komano *et al.*, 1981). The presence of more than one subunit and probable binding sites suggests the presence of synergistic effects in the lectins' binding and functional mechanisms. This may be as a result of concerted specific reactions (Barondes, 1981).

The presence of lectins or lectin-like molecules in the crop, midgut and haemolymph of *Rhodnius prolixus* which could selectively agglutinate *Trypanosoma cruzi* as reported by Pereira *et al.* (1981) is of importance as regards the vector-parasite interactions. Although the presence of midgut lectins in tsetse was first demonstrated by Ibrahim *et al.* (1984), the possible involvement of this lectin in the establishment of the parasite was reported

by Maudlin and Welburn (1987), where incorporation of GlcN in the bloodmeal resulted in higher rates of midgut infection establishment. From immunological studies carried out in this study, it has been established that molecules related to the *G. m. morsitans* midgut agglutinin are present in all the *Glossina* species investigated, *G. longipennis, G. f. fuscipes* and *G. m. centralis*. These molecules were detected as several bands. The bands of ~70,000 Da detected in *G. longipennis*, ~66 000 Da in *G. f. fuscipes* and *G. m. centralis* and the *G. m. morsitans* homogenates compared favourably with the M_r ~65,700 agglutinin that was purified from the *G. m. morsitans* midgut homogenate. The purified *G. longipennis* midgut agglutinin has been reported to have M_r ~61,000 (Osir *et al.*, 1995), the differences in the M_r with the *G. m. morsitans* agglutinin might mean that the agglutinins in the two species are not as closely related. It is also possible that *G. longipennis* might be having more than one type of agglutinin systems as it has been reported for *G. tachnoides* (Grubhoffer *et al.*, 1994). Comparatively *G. palpallis* spp has been reported to have a midgut agglutinin of ~67,000 Da (Stiles *et al.*, 1990). From the limited data available, it can be stated that the tsetse midgut agglutinins range from ~60,000 to ~70,000 Da and the agglutinins in different *Glossina* species might represent different systems of agglutinins.

The reactions detected from midguts of *A. aegypti* and *P. dubosqci* as well as the different sizes of the molecules detected in the different tsetse species could be indicative of epitope homology between the *G. m. morsitans* midgut agglutinin and other protein molecules in the midguts of insects of the Order, Diptera. Lectins have been shown to have broad specificity for mono-, oligo- and polysaccharides and tend to have stronger affinity for glycoproteins (Yeaton, 1981). This broad specificity which, in lectins, is determined by saccharide sequences proximal to the terminal sugar of the glycopeptide (Sharon and Lis, 1989), might be reflected in the

non-specific or broad specificity exemplified by the polyclonal antibodies raised against the *G. m. morsitans* midgut agglutinin.

4.4.2 Tissue localisation of the lectin-trypsin complex in tsetse

In most arthropods, lectins have been proposed to act as binding molecules which lead to eventual detection of invading micro-organisms by the phagocytes (Renwratz, 1986). Lectins can occur either in the soluble form or they can be bound to cells such as haemocytes. In the cockroach, *Leucophaea maderae*, Amirante and Mazzalai (1978) reported the presence of lectins associated with the plasma membrane of the haemocytes. Similar observations have been made in *Calliphora vomitoria* (McKenzie and Preston, 1992a), *Melanoplus differentialis* and *M. sanguinipes* (Bradley *et al.*, 1989), *Sarcophaga peregrina* (Komano *et al.*, 1983) and *Spodoptera exigua* (Boucias and Pendland, 1983). The haemocyte-bound lectins are most likely associated with the haemolymph which bathe the cells. Other insect tissues shown to be associated with the lectins include the oesophagus and the crop as shown in *R. prolixus* (Pereira *et al.*, 1981); gut in tsetse (Ibrahim *et al.*, 1984; Abubakar *et al.*, 1995; Stiles *et al.*, 1990), *Triatoma infestans* (Hypsa and Grubhoffer, 1995) and in *R. prolixus* (Pereira *et al.*, 1981). Haemolymph lectins have however been more widely reported in several insect species (Kanost *et al.*, 1990).

In this study the tsetse midgut agglutinin has been shown not to be associated with the haemocytes but was detected in the haemolymph. This means that the agglutinin circulated in the haemolymph as a soluble lectin, albeit in an inactive form since it could not agglutinate trypanosomes. Tsetse haemolymph agglutinins have previously been reported in several species. Ingram and Molyneux (1988) reported of *G. tachnoides* and *G. p. gambiensis* haemolymph agglutinins which was able to agglutinate human ABO(H) erythrocytes. Ingram *et al.* (1984) also detected haemolymph agglutinins from *G. m. morsitans* and *G. austeni* that were able to

agglutinate trypanosomatid flagellates. A galactose-specific haemolymph agglutinin has been isolated by Ingram and Molyneux (1990) from *G. f. fuscipes*. This agglutinin has been show to have M_r of ~710,000 Da with subunits of ~70,000 Da.

Although it has been shown that the tsetse midgut agglutinin was also detected in the fat body, midgut tissues and the peritrophic membrane, only the peritrophic membrane homogenate showed detectable agglutination of both bloodstream and procyclic forms of trypanosomes. The tsetse gut agglutinin, therefore, although associated with other tissues, is only biologically active in the gut lumen as evidenced by agglutination activity detected in crude midgut and peritrophic membrane homogenates. In the fat body and midgut, the agglutinin was detected as high molecular weight molecules, ~450,000 Da and ~600,000 Da respectively. Presumably the agglutinin is complexed to other molecule(s) in these tissues which results in the large apparent molecular weights observed. The absence of agglutination activity from the homogenates of these tissues might imply that the agglutinin is present in these tissues in inactive forms.

Two possible explanations have been proposed for the appearance of several reactive bands in the haemolymph. It is possible that the bands that were detected represent different forms of the agglutinin as it is transported after synthesis. Since, as already pointed out, the agglutinins are not associated with the haemocytes, it is very likely that they are transported in the haemolymph bound to the usually abundant haemolymph peptides (Kanost *et al.*, 1990; van Heusden, 1996). The second explanation is based on the possibility that the lectin-trypsin complex or it components may be synthesised as zymogens in the tissues. Further processing, through cleavage of some peptides, would reduce the sizes resulting in the initiation of the biological activity in the gut lumen. This phenomenon is common in the processing of vertebrate proteases (Moffatt and Lehane, 1990). The evidence

for the occurrence of such a process in insects is, however, circumstantial (Applebaum, 1985).

It is interesting to note that the only tissue that showed positive biological activity, the peritrophic membrane, is closely associated with the tsetse gut lumen, where the lectin-trypsin complex is supposedly secreted after induction by a bloodmeal. Most likely the agglutination property of the *G. m. morsitans* peritrophic membrane is as a result of the lectins attached to it. The association between peritrophic membranes and lectins has been demonstrated by Peters *et al.* (1983) who reported of a mannose-specific lectin closely associated with the peritrophic membrane of *Calliphora erythrocephala* Meigen. Lehane and Msangi (1991) also reported that the peritrophic membrane of *G. m. morsitans* agglutinated human erythrocytes of the ABRh- to a titre of 512. The association between lectins and peritrophic membranes is facilitated by the glycoprotein nature of the peritrophic membranes (Miller and Lehane, 1993). Also, the more intense band detected from the peritrophic membrane with ~65,000 Da compares favourably with the size of midgut lectin purified from *G. m. morsitans* crude midgut homogenate. The presence of this band probably explains the agglutination property of the peritrophic membrane homogenate.

4.4.3 Synthesis sites of the lectin-trypsin complex

Several insect tissues have been shown to be involved in synthesising proteins. These include the fat body (Boucias and Pendland, 1993), haemocytes (Yeaton, 1981), thoracic tissues (Borovsky, 1986b), the epidermis, midgut epithelium and sexual organs (Stiles *et al.*, 1988; Pathak, 1993).

In insects, the fat body is the principal tissue of intermediary metabolism, the function of which range from biosynthesis to storage of biomolecules. The fat body can be considered as an insect analogue of the liver in the vertebrates (van Heusden, 1996). Since the fat body is a diffuse

tissue, concentrated mainly in the abdomen, most of the biomolecules synthesizes are released directly into the haemolymph. This explains the presence of a ~450,000 Da molecule related to the lectin-trypsin complex detected in both the fat body and the haemolymph of *G. m. morsitans*, in the tissue localisation study (Figure 4.12). The midgut epithelium has also been shown to be involved in the synthesis of proteins mostly proteases, hydrases and esterases in haematophagous insects, (Ramoser, 1996).

This study has demonstrated that the midgut lectin-trypsin complex of *G. m. morsitans* or either of its components are synthesised in the fat body and the midgut. However, neither of the distinct bands detected from the autoradiograph after immunoprecipitation of the fat body (~42,000 Da) and the midgut tissues (~62,000 Da), compare with either of the subunits of the purified agglutinin. Since the use of non-immune serum in the first immunoprecipitation cycle removed most of the non-specific immunoglobulin binding complexes, there can be two explanations for this observation. Firstly, protein A-Sepharose has been shown to have very high affinity for most immune complexes such that solubilisation of the protein of interest after immunoprecipitation, prior to SDS-PAGE is often incomplete. This has also been reported by Borovsky (1986b) during the immunoprecipitation of trypsin-like enzymes from *S. calcitrans* tissues and by Stiles *et al.* (1988) during the immunoprecipitation of haemolymph lectin from *M. differentialis* tissues. Secondly, it might be possible that the molecule or its subunits are synthesised in a different form (zymogen) which has a higher molecular weight, such that cleavage of part of the molecule leads to its activation. Such mechanisms, characteristic of vertebrates, are rare in insects and have been reported only in trypsin synthesis in *S. calcitrans* (Moffat and Lehane, 1990) and in the synthesis of a trypsin-like enzyme, cocoonase of the silk moth (*Antheraea pernyi*), which facilitates its emergence from the cocoon (Berger *et al.*, 1971; Applebaum, 1985).

Although synthesis sites of midgut lectins have not been documented to date, the fat body has been shown to be involved in the synthesis of haemolymph lectins in several species including the grasshopper, *M. differntialis* (Stiles *et al.*, 1988), the flesh fly *S. peregrina* (Komano *et al.*, 1983), the beet armyworm *S. exigua* (Boucias and Pendland, 1993) and the locust, *Locusta migratoria* (Drif and Brehelin, 1994).

In *S. peregrina*, lectins have been shown to be secreted into the haemolymph by the fat body upon induction by either injury to the body wall, microbial challenge and during larval development (Komano *et al.*, 1983). On the other hand lectins secreted into the haemolymph of *M. differentialis, M. sanguinipes,* and *S. exigua* have been shown not to require any induction as agglutination has been maintained regardless of the presence or absence of stimuli (Stiles *et al.*, 1988; Boucias and Pendland, 1993). In *Bombyx mori*, Amanai *et al.* (1991) reported that the production of a haemolymph lectin by the haemocytes was stimulated by 20-hydroxyecdysone in the 5th instar larvae. The agglutination activity of this lectin was also shown not to be affected by injury on the body wall (Amanai *et al.*, 1991).

In *Glossina* species, the midgut agglutinin titres have been shown to follow a periodic cycle following a bloodmeal (Maudlin, 1991). It must be pointed out that the synthesis of proteolytic enzymes by the midgut epithelium of haematophagous insects is also induced by proteins in the bloodmeal by any of the following stimulational mechanisms: neural, secretagogue, osmotic and mechanical (Romoser, 1996; Applebaum, 1985; Gooding, 1975). Since protein feeding activates the endocrine system, which leads to accumulation of protein synthesis in the trophocytes, the major fat body cells (van Heusden, 1996) it is likely that a bloodmeal would result in increasing the synthesis of the lectin-trypsin complex or its components in both the fat body and in the midgut epithelium. Since the lectin-trypsin complex was detected in the fat body and haemolymph at all times post

bloodmeal, and significant agglutination activity in the midguts of tsetse has been shown to peak only after 48 h post-bloodmeal (Maudlin, 1991), the lectin-trypsin complex could be present in different form in the fat body and presumably serving a completely different function there. A similar mechanism has been reported in *S. peregrina* haemolymph lectin, where the larger α subunit is processed by partial proteolytic cleavage to form the biologically active ß subunit after induction by injury (Komano *et al.*, 1983).

In the tsetse situation, it is likely that following a bloodmeal, there follows either an increase in the synthesis of the lectin-trypsin complex components in both the fat body and the midgut tissues or release of the proteins already synthesised by these tissues. The material synthesised in the fat body are secreted into the haemolymph, as evidenced by the corresponding increase of proteins secreted by the fat body *in vitro* (Figure 4.17). Since the active agglutinin has been shown to be associated only with the gut lumen, the lectin circulating in the haemolymph, after their secretion by fat body, presumably transverses the basal lamina then the epithelial cells, through the microvilli into the gut lumen. It is likely that the activation of the molecule occurs in the lumen either by proteolytic cleavage of part of the molecule or linkage of the glycosyl moiety to proteolytic enzymes synthesised and secreted by the midgut epithelial cells. The possibility of such a transport route is yet to be established. However, should such a route exist, it is also possible that the lectin-trypsin complex forms in the epithelial cells and then later secreted into the gut lumen as an active molecule. It is highly unlikely that the trypsin moiety of the agglutinin would be synthesised and stored in the active form by the midgut epithelial cells due to the proteolytic effect they might have on the cellular proteins. This postulate can be tested by immunocytochemical staining of the midgut epithelial cells using monoclonal antibodies against the subunits of the lectin-trypsin complex.

CHAPTER FIVE

ROLE OF THE TSETSE MIDGUT LECTIN-TRYPSIN COMPLEX IN PARASITE DIFFERENTIATION AND ITS MODE OF ACTION.

5.1 Introduction

Several factors have been shown to be involved in the induction of the transformation of bloodstream trypomastigotes to procyclic trypomastigotes *in vitro*. These factors include; change in temperature, presence of TCA cycle intermediates, types of substrates available, presence of cyclic nucleotides, type of host blood as well as tsetse midgut factors found in the crude midgut homogenates including trypsin or trypsin-like enzymes.

Treatment of culture adapted bloodstream monomorphic *T. b. gambiense* (Wellcome strain) with 5000 IU/ml bovine pancreas trypsin has been shown to stimulate parasite transformation from bloodstream to procyclic trypomastigotes at 27° C (Yabu and Takayanagi, 1988). As much as 95% of bloodstream forms were shown to transform into procyclics in this system. Parasite morphological changes induced by a proteinase has also been observed by Frevert *et al.*(1986) in clone Be Tat 1 of *T. congolense*. Imbuga *et al.* (1992a) also reported that both pancreas trypsin and midgut trypsin from *G. m. morsitans* induced pleomorphic *T. b. brucei* EATRO 1969 transformation *in vitro*. Comparatively, lower concentration of midgut trypsin (~3 x 10^{-2} units) induced parasite transformation while much higher concentrations of bovine trypsin were required. Inhibition of tsetse midgut trypsin activity also led to a concomitant inhibition of parasite transformation (Imbuga *et al*, 1992a).

In addition to the factors listed above trypanoagglutinins, trypanolysins and other unknown factors have also been postulated to be

responsible for the killing as well as establishment of the trypanosome midgut infections in tsetse (Ingram and Molyneux, 1991; Maudlin, 1991). The pathogenecity of trypanosomes to some *Glossina* sp. has been shown to be maternally inherited and correlated to the levels of the lectins secreted into the tsetse midgut (Maudlin and Dukes, 1985). The midgut lectin titres have also been reported to be associated with the numbers of the rickettsia-like organisims, which through their chitinolytic activity on chitin release glucosamine, the inhibitor of tsetse midgut lectins (Maudlin and Welburn, 1988). It has been proposed that in addition to killing the trypanosomes in the midgut, these midgut lectins also act as signals for surviving trypanosome to differentiate into epimastigotes and then to the vertebrate-infective forms (Maudlin and Welburn, 1994).

Since only a small proportion of trypanosomes that have established in the midgut infection eventually develop into mature infections, the most crucial step in the life cycle of the parasite is, therefore, successful transformation of bloodstream to procyclic forms in the tsetse midgut. The purification of a tsetse midgut molecule, from *G. longipennis*, with both lectin and trypsin activities (Osir *et al.*, 1995) might be important in elucidating some of the likely factors that are responsible for the refractoriness and susceptibility to establishment of tsetse midgut infection. It is possible that both the lectin and trypsin activities that have been shown to be associated with trypanosome differentiation are on the same molecule, the lectin-trypsin complex.

Lectins function by binding the specific carbohydrate moieties on the cell surfaces of the parasites, which provides ideal cell recognition systems (Renwratz, 1986). In order to deduce the mechanism of action of a lectin it is therefore necessary to study its binding properties. It is possible to probe for binding proteins in a system by using the biotin-avidin system. The biotin reacts with primary amines on the proteins to form amide bonds. The

biotin can be coupled to proteins and being a small molecule, the biological activity of the protein remains unaltered. The highly specific and strong non-covalent binding of avidin to biotin facilitates easy location of biotin on nitrocellulose membrane with enzyme-conjugated avidin (Van den Abbelle *et al.*, 1996).

This chapter presents work on the possible role of this chimeric molecule, purified from *G. m. morsitans* midgut, as well as the possible mechanism of action.

5..2 Materials and methods

5.2.1 In vitro parasite transformation

The *in vitro* parasite transformation assays used in these studies were modified from the methods of Imbuga *et al.* (1992a) and Nguu *et al.* (1996). No media was used in the incubation mixtures. The preparation of tissue homogenates and parasites were carried out as described in General Materials and Methods.

5.2.1.1 Influence of the purified lectin-trypsin complex on parasite transformation.

Parasitised blood (~5 x 10^6 parasites/ml) was mixed with the purified agglutinin (~0.467 mg protein/ml) and the crude gut homogenate (~1.26 mg protein/ml) and incubated at 27° C. The total volume of the assay was maintained at 600 µl. The control consisted of a mixture of parasitised rat blood and 20 mM Tris-HCl, pH 8.0. At 2 h intervals, the incubation mixture was vortexed and 15 µl aliquots were withdrawn for preparation of thin wet smears.

The thin wet smears were air dried, fixed in absolute methanol (10 min.) and stained for 1 h with Giemsa's stain (Appendix B), rinsed with distilled water and dried. The course of transformation from bloodstream forms to procyclics was followed using a Dialux compound microscope (Leitz Wetzlar, FRG). The criteria used to analyse the change in the morphology of the parasites were those described by Vickerman (1985) and Ghiotto *et al.* (1979). Trypanosomes where the kinetoplast was located between the nucleus and the posterior end and which showed a poorly developed undulating membrane with no or very short flagellum were scored as transforming (Ghiotto *et al.*, 1979; Bienen *et al.*, 1981; Vickerman, 1985).

For each time interval, 300 parasites were counted from different smears and classified, according to their morphology, as being typical bloodstream, midgut forms and transition forms. The percentage of remaining bloodstream forms was then determined for each of group at the respective time intervals. The transformation assay was also repeated at a different concentration of the lectin-trypsin complex (~0.827 mg protein/ml). Observations on the parasite behaviour as well as mortality were also made.

5.2.1.2 Induction of parasite transformation by peritrophic membrane

Peritrophic membrane homogenate (~0.15 mg protein) was mixed with the parasitised blood and the mixture incubated as described in section 5.2.1.1. At 2 h intervals, thin smears were likewise prepared and stained with Giemsa. The progress of parasite transformation was also followed using the same criteria used in the above section (5.2.1.1.). Parasite motility and mortality with time was also recorded.

5.2.1.3 Influence the midgut and fat body homogenates on parasite transformation

Midgut and fat body homogenates were also assayed for their ability to induce trypanosome transformation *in vitro*. Fat body homogenate (~0.18 mg protein/ml) and posterior midgut homogenate (~0.21 mg protein/ml) were incubated with parasitised blood as described in section 5.2.1.1. Their effect on the induction of trypanosome transformation from bloodstream to procyclic forms *in vitro* was similarly investigated as for the lectin-trypsin complex and the peritrophic membrane homogenate.

5.2.1.4 Effects of inhibitors of lectin and trypsin on parasite transformation

Inhibitors of the subunits of the lectin-trypsin complex were included to investigate their effect on the molecule's ability to induce *in vitro* trypanosome transformation. Prior to addition of parasitised blood, the lectin-trypsin complex was incubated with the inhibitor concentrations that could completely inhibit either agglutination or trypsin activity. In one vial, 150 mM D(+) glucosamine (Sigma, St. Louis, MO) was incubated with the molecule for 30 min. at 4° C. In another vial, ~2 μg soybean trypsin inhibitor (STI; Pierce, Illinois, USA) / ml was also incubated with the lectin-trypsin complex at 4° C for 30 min. In a separate experiment, ~120 mM phenyl methyl sulphonyl fluoride (PMSF; Sigma) was included in the assay as a trypsin inhibitor. Transformation of trypanosomes from bloodstream to procyclic forms was monitored as already described (section 5.2.1.1).

5.2.2 Establishment of midgut trypanosome infections

These experiments were carried out to investigate the influence of the midgut lectin-trypsin complex on the establishment of midgut trypanosome infections in tsetse.

5.2.2.1 Effect of the lectin-trypsin complex

Upon peaking of parasitaemia, parasitised blood was collected from infected rats by cardiac puncture. Heparin was used as the anticoagulant. The parasitised blood (5 x 10^6 trypanosomes / ml) was mixed with the agglutinin (0.233 mg protein/ ml) purified from tsetse crude gut preparations. The volume of the mixture was maintained at 2 ml. The agglutinin-parasitised blood mixture was then fed to groups of male *G. m. morsitans* (24 h post-emergence) (n=67), using a silicon membrane.

Subsequent feeds of the tsetse were on unparasitised rats on day 3 after the first feed. Midgut infection rates were assessed by dissecting the fly guts on day 6. Whole guts were dissected in PSG and then examined for presence of parasites under the microscope (x400). For each group of tsetse, mortality rates were also recorded at the end of the experiments. Three replicates were made.

In the control experiment, parasitised rat blood (5×10^6 trypanosomes /ml) was mixed with PSG and the total mixture volume was, likewise, maintained at 2 ml and fed to tsetse (n=62). The initial infective feed and subsequent feeds to groups of tsetse were carried out as described above. Dissection of the guts was also carried out on day 6 and infection and mortality rates assessed as described above.

5.2.2.2 Effect of polyclonal antibodies

In another experiment, 63 male *G. m. morsitans* were initially fed on a mixture of parasitised rat blood and polyclonal antibodies raised against the purified agglutinin (~0.72 mg protein/ml) using a silicon membrane. During the second feed, 3 days later, the flies were fed on unparasitised rat blood. The total volume mixture was maintained at 2 ml. Whole guts were dissected and assessed for infection. Mortality rates were, likewise, recorded.

In another set of experiments, parasitised blood (5×10^6 parasites /ml) was mixed with polyclonal antibodies raised against the agglutinin (~0.72 mg protein/ml). The total volume was maintained at 2 ml. The mixture was fed to a group of male tsetse (n=108) using a silicon membrane. Subsequent feeds on the flies were given every 3 days on unparasitised rat blood. The salivary glands and midguts were dissected from the flies on day 35, following 3 days of starvation, and assessed for infection. A longer duration of 35 days was used to allow for the slow maturing parasites

(Mihok *et al.*, 1992). The same protocol was followed for the control (n=101), except that the initial meal consisted of a mixture of parasitised blood and PSG. For each of the treatments in the experiments above on infection rates, 3 replicates were made.

5.2.3 Mode of action of the lectin-trypsin complex

5.2.3.1 Biotinylation of proteins

The purified tsetse midgut agglutinin was biotinylated according to the manufacturer's instructions (Pierce, Illinois, USA). NHS-biotin was dissolved in water-free dimethylformamide (DMF)(Sigma, St. Louis, MO) at a concentration of ~5 mg/ml. The purified lectin-trypsin complex was then mixed with the dissolved NHS-biotin at a ratio of 7.5:1 (protein : reagent; w:w) and incubated for 2 h at 27° C. Excess reagent was removed from the solution by extensively dialysing against phosphate buffered saline (PBS, pH 7.2). Proteins in the crude midgut homogenates were also biotinylated in the same way. Prior to binding of biotinylated proteins to trypanosomes, the solutions were pre-incubated with; (a) 150 mM GlcN, (b) 2 μg STI and (c) both inhibitors at the same concentrations. These treatments were carried out on the crude midgut homogenates. The purified agglutinin was included for comparison. In the control, the proteins were pre-incubated with PBS.

5.2.3.2 Binding of biotinylated agglutinin to trypanosomes

The biotinylated agglutinin was bound to the trypanosomes according to the method of Van den Abbelle *et al.* (1996). The labeled protein was incubated with live trypanosomes (~5 x 10^6 parasites/ml) for 1 h at 4° C. Unbound proteins were removed by washing four times in PBS, pH 7.2. After labeling, about 10 μl of the mixture was microscopically examined for viability of the parasites.

5.2.3.3 Preparation of crude membrane proteins.

Crude membrane proteins from trypanosomes were prepared using the "freeze-thaw" method described by Barbet and Mcguire (1982) with some modification by Imbuga *et al.* (1992b). The parasites were pelleted by centrifugation (1 000 x *g*, 15 min., 4° C) and the pellet later suspended in 50 µl PBS, pH 7.2. The parasites were lysed by a minimum of four "freeze-thaw" cycles. Further centrifugation (4 000 *g*, 15 min., 4° C) removed large particulate material. Before SDS-PAGE, the supernatant containing the crude membrane proteins were solubilised by boiling in 30 µl SDS-PAGE sample buffer for 10 min.

5.2.3.4 PAGE and localisation of proteins bound to trypanosomes

The samples were co-electrophoresed with Pharmacia molecular weight markers (Pharmacia, Upsalla, Sweden) on SDS-PAGE (4-15% polyacrylamide gradient separating gel and 3.13% polyacrylamide stacking gel) as already described in General Materials and Methods. The separated proteins were transferred onto a nitrocellulose 45 µm paper (Schleicher and Schuel, Dassel, Germany) using a Novablot 2117 Multiphore II electrophoresis transfer unit at 0.8 mA/ cm^2 for 2 h, 27 ° C. The paper was transiently stained with Rouge Pounceau, destained with distilled water and the position of the molecular standards marked as described in General Materials and Methods. The blot was then incubated with avidin-horseradish peroxidase (Pierce, Illinois, USA) diluted 1:200 in PBS, pH 7.2 for 4 h at 4° C. Excess conjugate was removed by three washes in PBS, pH 7.2 followed by one brief wash in 10 mM Tris-HCl, pH 6.8. The substrate, 4-chloro-1-naphthol (1 part diluted in 4 parts 10 mM Tris-HCl, pH 6.8 from a stock solution of 0.3 % (w\v) 4-chloro-1-naphthol in absolute methanol) with 0.33

µl hydrogen peroxide/ml was added and the blot allowed to stain. Colour development was stopped by immersing the blot in distilled water.

5.3 Results

5.3.1 Trypanosome transformation

5.3.1.1 Effect of the lectin-trypsin complex on the transformation of bloodstream trypanosomes.

Figure 5.1 presents results of *in vitro* trypanosome transformation studies in the presence of the lectin-trypsin complex and crude tsetse gut homogenate. When the purified lectin-trypsin complex (~0.467 mg protein/ml) was incubated with the parasitised blood, approximately 50% of the bloodstream trypanosomes had transformed into procyclic forms after 10 h incubation.

Increasing the concentration of the lectin-trypsin complex (0.827 mg protein/ml) in the transformation assay resulted into a higher transformation rate of bloodstream trypanosomes. About 50% bloodstream forms transformed into procyclic forms after 6 h incubation compared to about 35% transformed bloodstream forms at the same incubation time when a lower agglutinin concentration was used in the assay (Figure 5.1). Increasing the agglutinin concentration also resulted in higher parasite mortality such that no live parasites were observed in the assays after 14 h incubation. In the control experiment, more than 95% of the parasites were still bloodstream trypanosomes and viable after 18 h incubation.

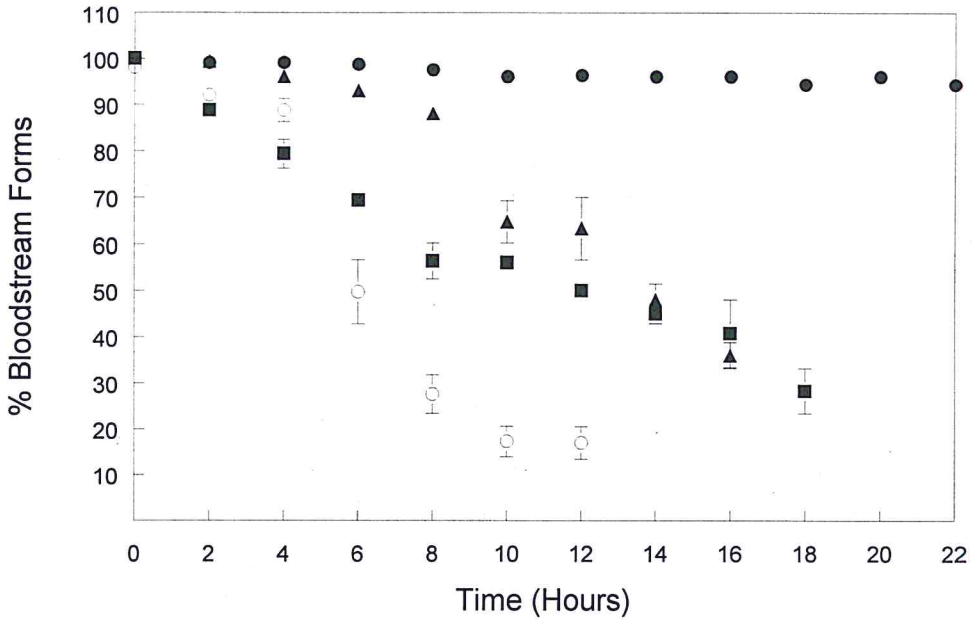

Figure 5.1 Influence of the midgut lectin-trypsin complex on trypanosome transformation. Data points represent mean values ± SD (n=4).
(●) - control
(▲) - crude gut homogenate (~1.26 mg protein/ml)
(■) - lectin-trypsin complex (0.467 mg protein/ml)
(○) - lectin-trypsin complex (0.827 mg/ml)

5.3.1.2 Trypanosome transformation by the peritrophic membrane

Figure 5.2 shows the effect of the peritrophic membrane on trypansome transformation. The peritrophic membrane homogenate was shown to induce the transformation of bloodstream trypanosomes to procyclic forms by about 35% after 12 h incubation. However, the lectin-trypsin complex, by the same incubation time had induced transformation of more than 50% bloodstream trypanosomes to procyclic forms. The induction of transformation by the peritrophic membrane homogenate compared favourably with that of the crude gut homogenate, about 35% parasites transforming in both cases after 12 h incubation. The parasite mortality was higher in assays where the peritrophic membrane was included. All the parasites in the incubation mixture had died after 14 h incubation compared to the assay which included the lectin-trypsin complex, crude gut homogenate and the control where the parasites were still alive after the same incubation time.

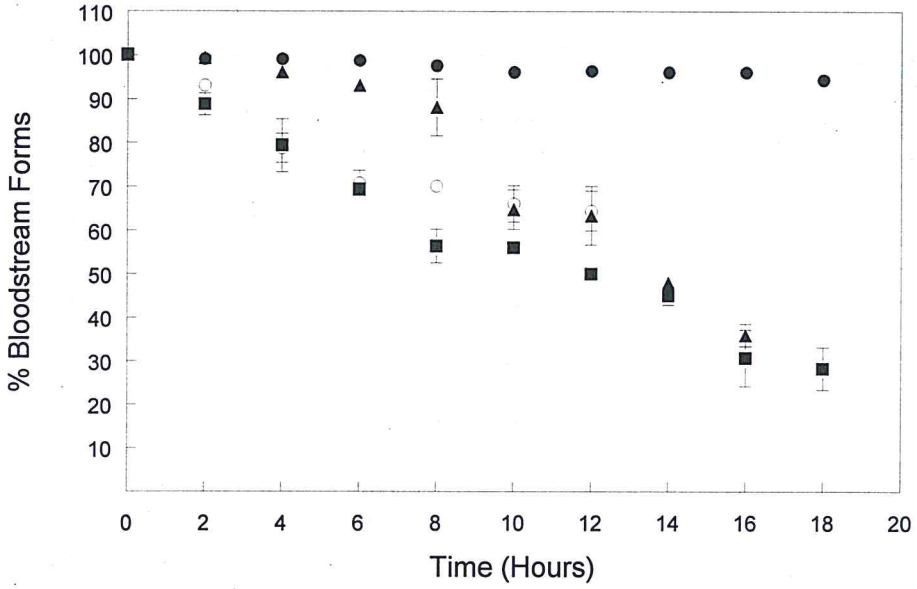

Figure 5.2 Influence of the peritrophic membrane on trypanosome transformation. Data points represent mean values ± SD (n=4).
(●) - control
(▲) - crude gut (~1.26 mg protein/ ml)
(■) - lectin-trypsin complex (~0.467 mg protein/ml)
(○) - peritrophic membrane (~0.15 mg protein/ml)

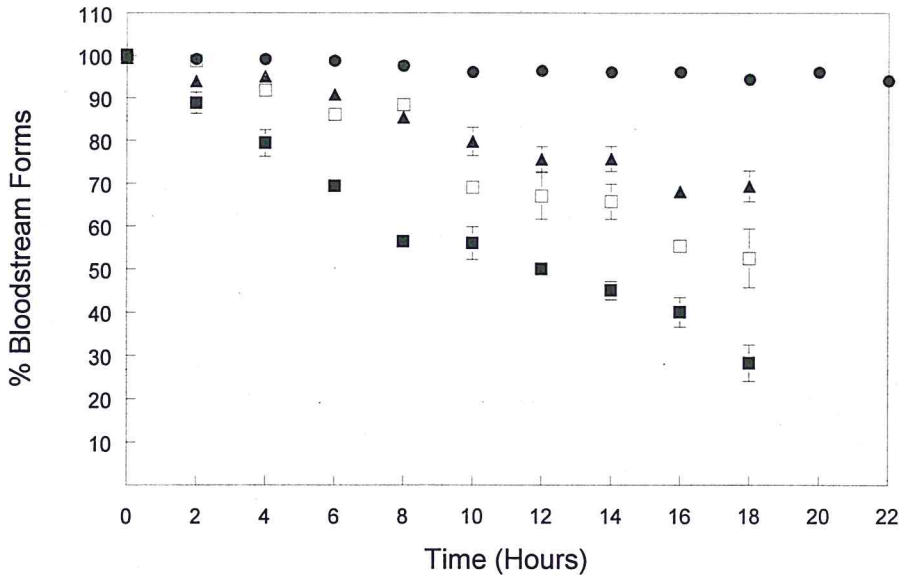

Figure 5.3 Influence of the fat body and midgut tissues homogenates on trypanosome transformation. Data points represent mean values ± SD (n=4).
(●) - control
(■) - lectin-trypsin complex, (~0.467 mg protein/ml)
(❑) - midgut (~0.21 mg protein/ml)
(▲) - fat body (~0.18 mg protein/ml).

5.3.1.3 Influence of fat body and posterior midgut tissue homogenates on transformation.

Fat body and posterior midgut tissues have been shown to be associated with the agglutinin but showed no agglutination activity (section 4.3.4). The effect of these homogenates on parasite transformation, *in vitro,* was investigated. Figure 5.3 shows that the posterior midgut tissue homogenate induced parasite transformation (45%) more than the fat body homogenate (30%) after 18h incubation, at concentrations of 0.21 mg protein/ml and 0.18 mg protein/ml respectively. Comparatively, the lectin-trypsin complex induced transformation of about 70% bloodstream to procyclic trypanosomes at 18 h incubation. The effect of midgut, fat body, homogenates and the lectin-trypsin complex on trypanosome mortality was the same. In all the three cases, the parasites were still viable after 18 h incubation. More than 95% of the parasites were still in the bloodstream form after 18 h incubation in the control.

5.3.1.4 Inhibition of trypanosome transformation

Figure 5.4 shows that inclusion of STI in the transformation assays had higher inhibitory effect on the induction of *in vitro* transformation by the agglutinin than when GlcN was included. While addition of STI to the assay resulted in only about 20% of the bloodstream trypanosomes transforming to procyclics, addition of GlcN resulted in transformation of about 30-35% bloodstream to procyclic forms after 14 h incubation. Absence of the inhibitors resulted in 60% of the bloodstream trypanosomes transforming after the same incubation time. Addition of GlcN in the assay led to higher parasite mortality. All parasites were dead by the 16th h after incubation. In

the control experiment, more than 95% of the parasites were still in the bloodstream form and the parasites were still alive after 20 h incubation.

When PMSF was used as an inhibitor of the trypsin moiety of the agglutinin, there were no live parasites after 2 h incubation (Figure 5.5) while addition of GlcN resulted in the transformation of about 15-20% after 16 h incubation.

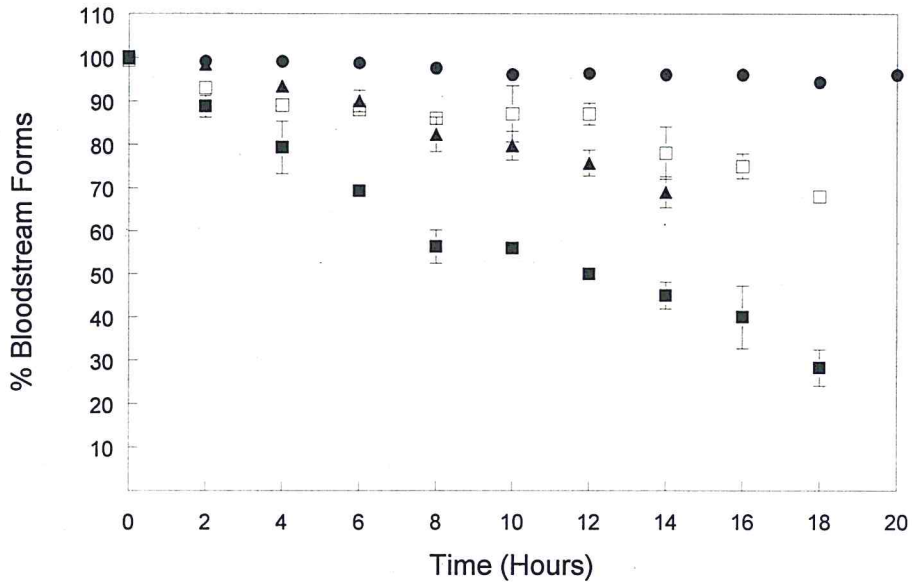

Figure 5.4 Effect of inhibitors on transformation of trypanosomes. Data points represent mean values ± SD (n=4).

(●) - control
(■) - lectin-trypsin complex
(▲) - lectin-trypsin complex + GlcN
(❑) - lectin trypsin complex + STI.

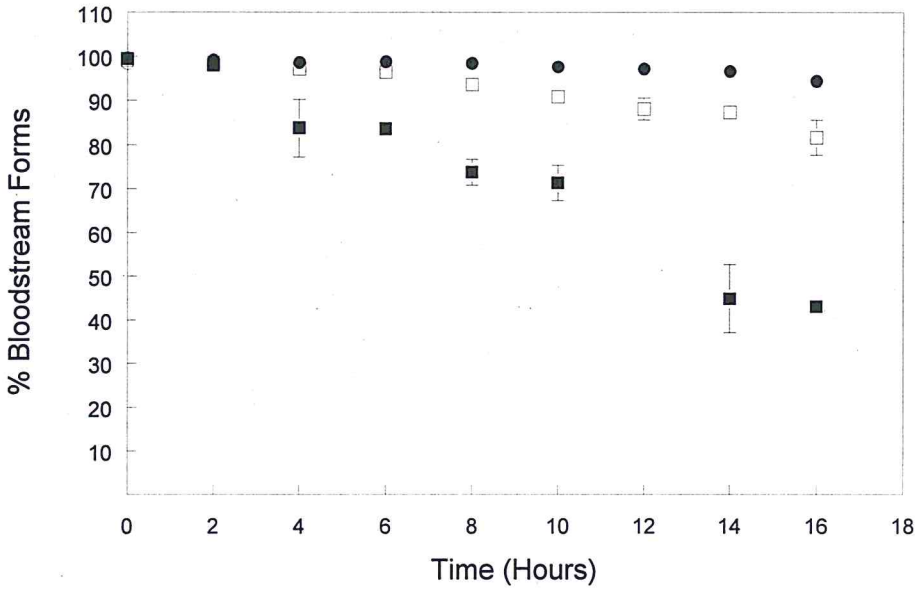

Figure 5.5 Effect of PMSF on trypanosome transformation. Data points represent mean values ± SD (n=4).

(●) - control
(■) - lectin-trypsin complex
(○) - lectin-trypsin complex + PMSO
(□) - lectin trypsin complex + GlcN

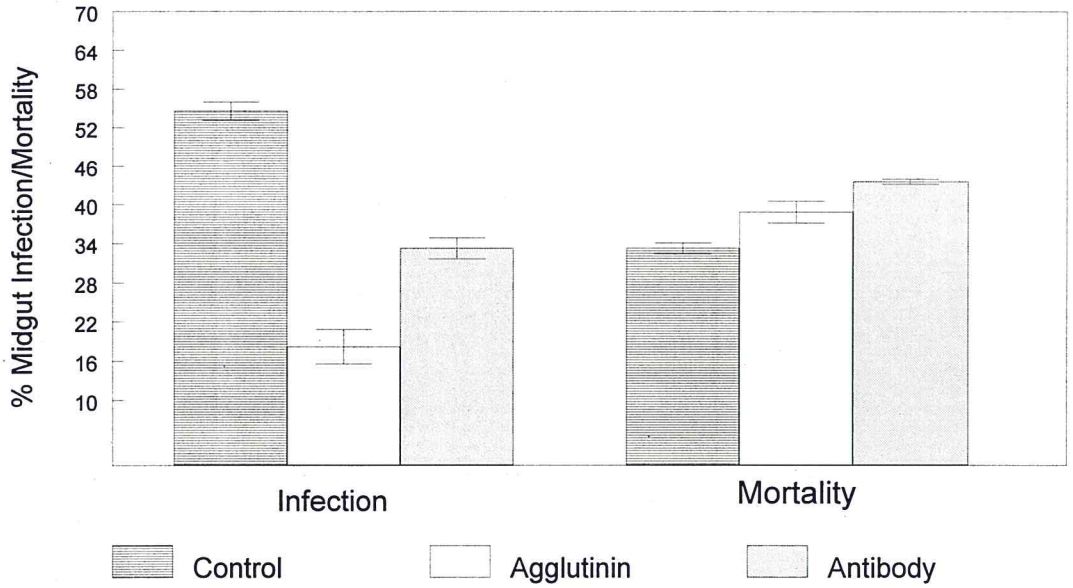

Figure 5.6 Gut infection and fly mortality rates in *G. m. morsitans* fed on meals with either the agglutinin or the anti-agglutinin polyclonal antibodies mixed with rat blood parasitised with *T. b. brucei*. Each plot represents a mean ± SD (n=3).

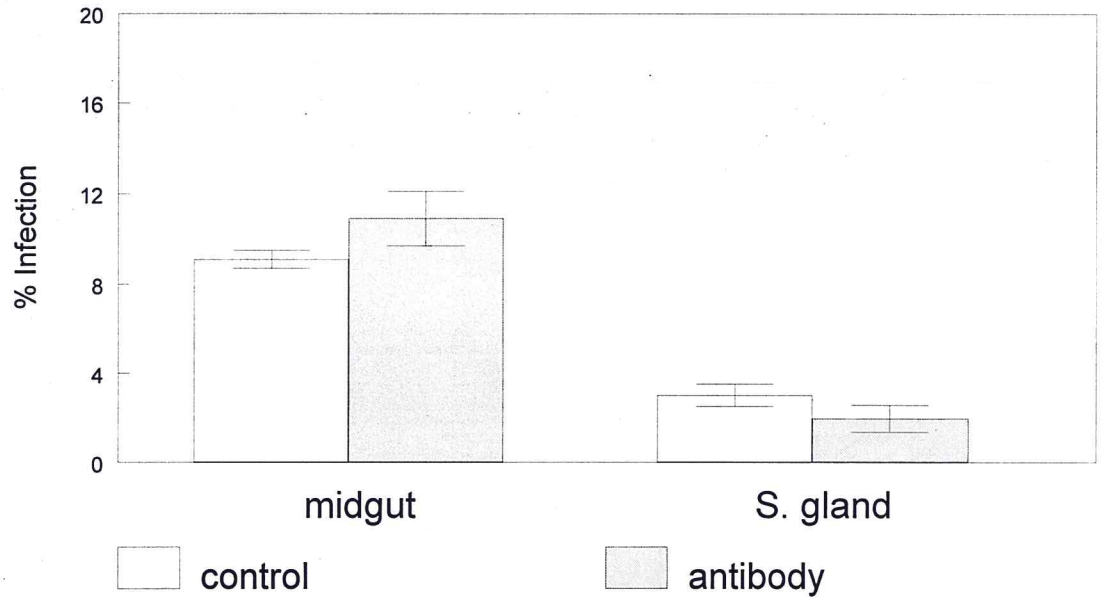

Figure 5.7 Midgut and salivary gland (s. gland) infection rates of *Trypanosoma brucei brucei* in *Glossina morsitans morsitans*, 35 days post-feeding with a mixture of parasitised blood and polyclonal antibodies raised against the midgut lectin-trypsin complex of *G. m. morsitans*. Each plot represents a mean ± SD (n=3).

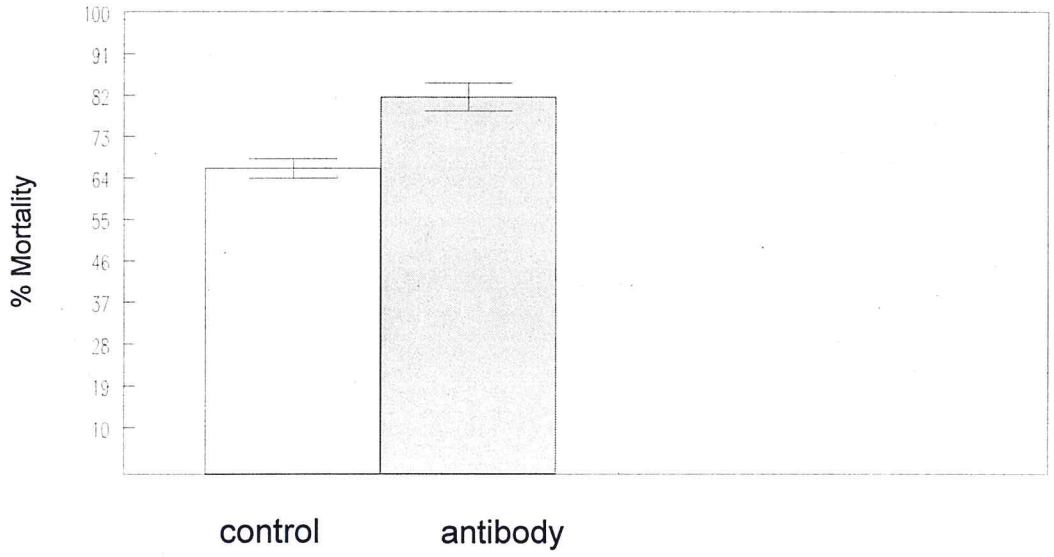

Figure 5.8 Mortality rates of *Glossina morsitans morsitans* 35 days after feeding on rat blood parasitised with *Trypanosoma brucei brucei* mixed with anti-tsetse midgut agglutinin polyclonal antibodies.

5.3.2 Establishment of midgut infections

5.3.2.1 Influence of the lectin-trypsin complex on the establishment of trypanosome gut infection

In order to investigate the effect of the lectin-trypsin complex on the establishment of midgut infections, the purified lectin-trypsin complex was included in the bloodmeal fed to tsetse. This resulted in considerable reduction of midgut infection rates in *G. m. morsitans* from 54.6 % in the control to 18.2% (Figure 5.6), which is about a three-times reduction in fly gut infections. This reduction in the midgut infection when the bloodmeal was inundated with the lectin-trypsin complex is significant ($\chi^2 = 4.812$, $p < 0.05$).

When the polyclonal antibodies were included in the bloodmeal, the midgut infection rates were only reduced to 33.3% representing about a 1.5 times reductions in the infection rates compared to the control (Figure 5.6). This reduction in midgut infection rates due to inclusion of antibodies and hence reduction in the total midgut concentration of the lectin-trypsin complex was not significant ($\chi^2 = 1.327$, $p > 0.05$).

5.3.2.2 Influence of the lectin-trypsin complex polyclonal antibodies on infection

The effects of including the antiagglutinin polyclonal antibody to the initial meal of flies on on infection with trypanosomes is shown in Figure 5.7. Although the midgut infection rates increased from 9.1% for the control treatment to 10.9%, this change was not statistically significant (Fischer exact test; $p>0.05$). Inclusion of the antibody in the bloodmeal decreased the salivary gland infection rate from 3.03% (control) to 1.98%. This decrease in tsetse salivary gland infection rate was also not statistcally significant (Fischer exact test; $p>0.05$). However, presence of the antibodies in the

initial bloodmeal increased the overall mortality rate of the flies after 35 days; 81.5% compared to 66% for the control (Figure 5.8).

5.3.3. Trypanosome-binding studies

These studies were carried out to determine the possible mode of action of the lectin-trypsin complex. Figure 5.9 shows the immunoblot of biotinylated tsetse midgut proteins that bound to bloodstream trypanosomes in the presence and absence of inhibitors. In the absence of the inhibitors, proteins that bound to trypanosomes were detected as seven bands, the prominent ones being of M_r ~35,200 and ~19,400 Da (lane 1). Proteins that bound to biotynylated agglutinin appeared as six bands, with the prominent ~35,200 Da band evident while the lower ~19,200 Da band was not detected (lane 5). An extra ~42,300 Da band was also detected in lane 5. When GlcN was incubated with crude midgut homogenate and then bound to trypanosomes, there was a considerable reduction in the number of bands that were detected (lane 2). Pre-incubation with this inhibitor resulted into the loss of the ~35,200 Da bands, which is prominent in the other treatments; while the lower ~19,200 Da band was present. Even the higher bands of >40,000 Da were absent.

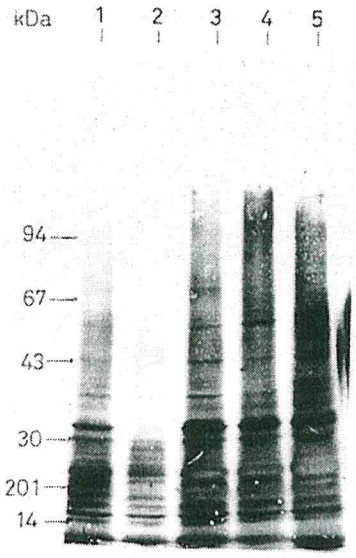

Figure 5.9 Midgut proteins of *G. m. morsitans* that bound to *T. b. brucei*.
Lane 1 - crude midgut homogenate pre-incubated with PBS
Lane 2 - midgut homogenate pre-incubated with 150 mM GlcN
Lane 3 - midgut homogenate pre-incubated with 2μg/ml STI
Lane 4 - midgut homogenate pre-incubated with 150 mM GlcN and 2 μg STI
Lane 5 - purified agglutinin pre-incubated with PBS

Incubation of the crude midgut homogenate with STI resulted into more bands being detected as proteins bound to the trypanosomes. In both lane 3, where only STI was used , and lane 4 where both STI and GlcN were used in the pre-incubation mixture, the prominent ~35,200 and ~19,400 Da bands were detected as proteins bound to trypanosomes. When STI alone was used as the inhibitor, a higher band of ~63,000 Da was also detected, albeit as a light band. However, when both STI and GlcN were used as inhibitors an extra distinct band appeared at ~54,300 Da. Fewer bands were detected when both inhibitors were used (lane 4) than when only STI was used (lane 3); seven bands compared to 9 bands, respectively.

5.4. Discussion

5.4.1 Role of the lectin-trypsin complex in trypanosme differentiation.

Lectins are ubiquitously-occuring specific carbohydrate-binding proteins or glycoproteins whose major property is the saccharide-binding sites (Pathak, 1993). These molecules, together with proteases (especially trypsin, trypsin-like and chymotrypsin) are the most prominent in the midguts of most haematophagous insects (Ham, 1992). Several previous studies have demonstrated that both the midgut lectins and proteases are involved in the interactions between the insect vectors and the parasites they transmit. Lectins have been proposed to be involved in both the establishment of trypanosome infections in tsetse midgut as well as elimination of procyclic trypanosomes (Maudlin, 1991; Maudlin and Welburn, 1994). In *Rhodnius prolixus,* Perreira *et al.* (1981) reported the presence of lectins in the haemolymph, midgut and crop which selectively interacted with specific stages of the parasite, *T. cruzi*. Also, lectins from different species of *Phlebotomus* have been shown to agglutinate different *Leishmania* parasites to different titres reflecting differential vectorial competence leading to infection establishments (Ingram and Molyneux, 1991). In the black flies, *Simulium ornatum*, Ham *et al.* (1988) reported of haemolymph lectins that affected the motility of *Onchocerca lienalis* more than *O. vulgalis*. The involvement of a midgut agglutinin in parasite establishment has also been proposed in the association between the tick, *Rhipicephalus appendiculatus* and the parasite it transmits, *Theileria*. In the tick, high agglutinin titres were reported in the haemolymph, salivary gland and gut preparations, post-feeding (Kamwendo *et al.*, 1993).

In malarial transmissions, lectin-like binding has been proposed to play a role in the initial orientation of the parasites on their way out of the midgut lumen. The lectins have been shown not to be responsible for the

susceptibility of vectors to ookenete invasions in *A. aegypti* and *Anopheles stephensi* (Rudin and Hecker, 1989). However there is considerable evidence that lectins are involved in recognition of sugar residues in hosts/vectors for the sporozoites on the basal lamina of mosquito salivary glands as well as during the invasion of erythrocytes by *Plasmodium falciparum* merozoites (Perrone *et al.*, 1986).

Like lectins, midgut trypsin or trypsin-like enzymes have also been reported to be involved in the interactions between the insect vectors and the parasites they transmit. In the mosquito-malaria parasite interaction, the insect midgut proteases have been shown to have a dual function, protecting the mosquito from establishment of infections as well as in the development of the parasite. *In vitro* studies by Gass and Yeates (1979) showed that digestive proteinases from susceptible *Aedes aegypti* specifically damaged the ookenetes of *Plasmodium gallinaceum*, in the process preventing their establishment. The significance of the mosquito proteases in the development of malarial parasites has been reported in relation with peritrophic membrane penetration by the ookenetes (Shahabuddin *et al.*, 1993). A mosquito midgut protease was shown to activate chitinase, an enzyme that ookenetes of *P. gallinaceum* use to degrade and penetrate the peritrophic membrane of *A. aegypti* and consequently invade the midgut cells. The property of the mosquito proteases in this instance would , most likely, depend on their relative concentrations, where low concentrations would facilitate further development of the parasite while higher enzyme concentrations would lead to parasite destruction. This phenomenon was also proposed for the *G. m. morsitans* - trypanosome interaction by Imbuga *et al.* (1992a).

Borovsky and Schlein (1987) also reported of the ability of *Phlebotomus papatasi* midgut trypsin and chymotrypsin-like enzymes to prevent survival of *Leishmania donovani* in the insect midgut. However,

through the modulation of the activities of these enzymes, *L. major* was shown to be able to survive in *P. papatasi*. *L. major* is naturally transmitted by *P. papatasi.*

Using *in vitro* transformation assay of *T. b. brucei*, Imbuga *et al.* (1992a) showed that *G. m. morsitans* midgut homogenate extracts stimulated the transformation of the parasites from bloodstream to procyclics at 27° C. The transformation-stimulating capacity was reported to be correlated to trypsin activity of the midgut extracts and that there was an optimal concentration for the transformation to proceed (Imbuga *et al.*, 1992a). The involvement of midgut insect lectins and trypsins in both the destruction of parasites as well as in the development of the parasites in the insect midguts is of interest from the functional perspective, considering that Osir *et al.* (1995) isolated a midgut agglutinin with proteolytic activity from *G. longipennis*. Enzymes with cell agglutinating properties, such as glycosidases, have previously been reported (Barondes, 1981). In this study, a similar molecule has been isolated from *G. m. morsitans* (Chapter 4) and was used in the *in vitro* transformation studies.

It is worth noting that the lectin-trypsin complex was able to induce the transformation of bloodstream trypanosomes to procyclics more than the crude midgut preparation up to the 12 h (50% and 30% transformed parasites, respectively)(Figure 5.1). Also doubling the concentration of the lectin-trypsin complex in the assay resulted into higher transformation rates (80% bloodstream transformed compared to about 50% of the parasites transformed at a lower concentration after 12 h) (Figure 5.1). The increase in transformation rates and a higher mortality rates of the parasites that was observed are of importance with regard to the proposed role of the agglutinin (lectin-trypsin complex) in both the induction of transformation of bloodstream trypanosomes to procyclics and death of the procyclic forms of the parasites (Maudlin, 1991).

Interestingly, relative gut concentrations of the agglutinin also affected tsetse midgut infection rates (Figure 5.6) as shown by effects of inundating the agglutinin in the bloodmeal, which significantly lowered midgut infection rates from 54.6% to 18.2%. On the other hand, reduction of the gut agglutinin concentrations by adding the polyclonal antibodies raised against the agglutinin did not significantly change the midgut infection rates (54.6% and 33.3% for control and treatment, respectively). This might be a reflection of the importance of optimal agglutinin concentrations in determining the fate of the parasites in the midgut, through either transformation or death. The inclusion of the antibody did not adversely affect the threshold of midgut agglutinin concentration necessary for parasite transformation, implying that relatively low concentrations of the agglutinin are required for the initiation of the transformation process.

Also, it has been established that changes in the lectin-trypsin complex concentrations in the midgut of tsetse had no significant effect on the maturation of infections in tsetse (Figure 5.7). Mihok *et al.* (1992) also reported that addition of D(+) GlcN to a bloodmeal, which reduced the overall agglutinin levels in the midgut, had no effect on infection maturation rates. The low midgut infection rate observed after 35 days compared to those reported for day 6 (Fig. 5.6) may be attributed to the fact that the trypanosomes must have been subjected to a gamut of trypanocidal factors for a longer time in tsetse gut. These observations compare with those made by van den Abbelle and Decleir (1991) who reported that after 30 days, there were no differences in midgut infections when STI was included in bloodmeals fed to *G. p. palpalis* infected with *T. b. brucei*.

Since tsetse midgut lectin and trypsin activities tend to peak at between 48 and 72 h post-bloodmeal (Stiles *et al.*, 1990; van den Abbelle and Decleir, 1991), it is likely that the transformation of bloodstream to procyclic trypanosomes occurs before this peak in the activities of these

trypanocidal molecules. It is possible that migration of the transformed parasites to the ectoperitrophic space confers the protection from the destructive effects of the lectins and proteases in the endoperitrophic space when the activities are elevated, 48 to 72 h after a bloodmeal. Another protective mechanism from the adverse effect of increased agglutinin and trypsin activities is , presumably, the modulation of trypsin activity through inhibition (Imbuga *et al.*, 1992b). However, this is unlikely to occur at high lectin-trypsin complex concentrations.

The observed effect of including antibodies to the bloodmeal on infection by trypanosomes (Figure 5.6) and fly mortality (Figure 5.8) is intriguing with respect to the search for candidate vaccine against trypanosomiasis. Presumably the presence of the antibodies interfered with some binding properties of the lectin-trypsin complex which then resulted into eventual disruption of the tsetse midgut physiology and biochemistry. The same observations were made in *A. aegypti* by Ramasamy *et al.* (1990), where feeding the insects on animals immunised with midgut tissues of the same mosquito species resulted in significant reduction in susceptibility to infection and mortality. In the stable fly, *Stomoxys calcitrans*, Webster *et al.* (1992) also reported the protective effects of anti-gut antibodies to vaccinated animals. In the tsetse situation, this warrants further investigation.

Some lectins, such as phytohaemagglutinin (PHA), have been reported to be mitogenic and can, therefore, stimulate lymphocyte transformation from small "resting" cells into large blast-like cells which then undergo mitosis in vertebrates (Kabelitz *et al.*, 1993). The induction or inhibition of protozoal parasites (*Trypanosoma* spp. and *Leishmania* spp.) survival, proliferation or differentiation by vertebrate extracellular signaling molecules (such as cytokines) have also been reported (Barcinski and Moreira, 1994). It is suggested here that the lectin-trypsin complex may be

functioning in the same way resulting into change in parasite morphology followed by the proliferation of cell divisions. The agglutinating property may be acting as a signal which through formation of glycoconjugates bridges between the cells (Sharon and Lis, 1989) by the lectin-trypsin complex, may lead to recognition of cells with the same surface and genetic properties in the process inducing their transformation from bloodstream to procyclic forms. Welburn and Maudlin (1997) suggested that the tsetse midgut lectin, as a first line of defense, may be actively involved in triggering parasite death within the gut lumen while established trypanosome populations are self-regulated through cell signaling processes. It is possible that at lower concentrations, the lectin-trypsin complex may be involved in these cell signalling processes, together with other unknown midgut molecules. The probable mitogenic property of the lectin-trypsin complex can be explained by the actively dividing population of procyclics which occur in the ectoperitrophic space compared to the predominantly short stumpy bloodstream trypanosomes found in the endoperitrophic space (Vickerman, 1985; Logan-Henfrey *et al.*, 1992). According to Welburn *et al.* (1997), the various external signals that are released by the vector might lead to either cell death (through apoptosis) or proliferation or differentiation. This has been proposed to be a self selection mode in the trypanosome populations within the vector and the hosts.

Most of the insect lectins have been proposed to function as immune molecules. Through agglutination of invading micro-organisms and also by acting as opsonins, lectins enhance the phagocytosis of foreign particles by the haemocytes (Yeaton, 1981; Pathak, 1993). Lectins in some insects have been reported to be responsible for both immunity of the insect against the invading pathogens and development of the insect from one stage to another. The haemolymph lectin of *Sarcophaga peregrina*, has been shown to be involved in the scavenging of decomposed tissues during the early pupal

stages when the larval tissues break down (Komano *et al.*, 1981). The same lectin has also been shown to be essential for the development of imaginal discs from which adult structure develop during the pupal stage (Natori, 1990). The lectin was proposed to be involved in the non-specific immune surveillance, as the titres were elevated when the body wall was injured or when red blood cells were injected into the larvae (Komano *et al.*, 1981). Amanai *et al.* (1991) also reported *Bombyx mori* haemolymph lectins which are developmentally regulated whose secretion was activated by the hormone that controls metamorphosis, 20-hydroxyecdysone. Haemocyte lectin-secreting activity, in this insect, showed a vital change during larval development with fluctuations occurring during the 4th and 5th instar larvae (Amanai *et al.*, 1991).

In the tsetse situation, the midgut lectin-trypsin complex (agglutinin) can also be considered as performing a dual roles of defense and development. Probably, initially the agglutinin was used by the tsetse to clear trypanosome, amongst other pathogens, from the midgut. And, during the tsetse-trypanosome co-evolution, the parasites adapted to the insect defense mechanism by utilising the low agglutinin concentrations to induce their transformation and then escape into the ectoperitrophic space before the agglutinin concentrations become lethal. Parasites still remaining in the endoperitrophic space would, presumably, be eliminated by midgut factors, one of them being the lectin-trypsin complex.

The peritrophic membrane of insects acts as a semi-permeable barrier across which primary and secondary hydrolytic events are separated (Terra and Ferreira, 1994). The peritrophic membrane can also hinder or even block parasite invasion of midgut epithelial cells. The peritrophic membrane has, however, been shown to be associated with lectins as reported by Peters *et al.* (1983) who reported a mannose specific lectin in the peritrophic membrane of *Calliphora erythrocephala*. The lectin-peritrophic

membrane association in *G. m. morsitans* was previously reported by Lehane and Msangi (1991). In their study, the majority of haemagglutinating lectins in the midgut were found to be associated with the peritrophic membrane. It is , therefore, not surprising that the peritrophic membrane, in this study, was able to induce the transformation of bloodstream trypanosomes to procyclic forms (Figure 5.2). In an earlier study (Chapter 4), the midgut agglutinin of *G. m. morsitans* was immunologicaly located in the peritrophic membrane homogenate. The homogenate was also able to agglutinate both bloodstream and procyclic trypanosomes.

The association between peritrophic membranes and lectins is facilitated by sugar residues due to the presence of glycosylated glycoproteins or glycosaminoglycan-containing proteoglycans or structural polysaccharides such as chitin on the peritrophic membranes (Miller and Lehane, 1993 a; b). These sugar residues facilitate binding of lectins secreted into the midgut lumen to the peritrophic membrane. The close association between the lectins and peritrophic membrane might, therefore explain the low lectin levels in teneral tsetse, whose peritrophic membrane is not completely developed (Lehane and Msangi, 1991).

Since all parasites have carbohydrate moieties on their cell surfaces which can be recognised by the lectins, the lectins possibly form bridges by binding to specific sugars on apposing surfaces or cells (Sharon and Lis, 1989). It is highly likely that this is the situation in the peritrophic membrane-parasite association, where the midgut agglutinin would form bridges between the two. Bridge formation is characteristic of soluble lectin (Sharon and Lis, 1989), of which the tsetse midgut lectin is. It has been reported by Evans and Ellis (1983) that both *T. b. rhodesiense* and *T. congolense* can penetrate a fully formed peritrophic membrane and gut epithelial cells of *Glossina* spp. This penetration of the peritrophic membrane and other vector tissues, most likely, requires a recognition

mechanism in terms of complementary surface receptors between the peritrophic membrane and the parasite (Miller and Lehane, 1993a). Through its polyvalent nature, the midgut lectin provides such a mechanism in association with other factors such as surface glycosyltransferases, which are other carbohydrate binding proteins (Sharon and Lis, 1989). The differences in the peritrophic membranes saccharide complements might be responsible for differences in susceptibility of tsetse to trypanosome infections. Interestingly, Okolo *et al.* (1988), through the use of fluoroscein conjugated lectins, were able to identify α-N-acetylglucosamine on the peritrophic membrane of *G. m. morsitans*.

It was earlier reported in Chapter 4 that fat body and posterior midgut tissue homogenates are involved in the synthesis of the subunits of the lectin-trypsin complex. It was also observed that homogenates of the two tissues could not agglutinate both bloodstream and procyclic trypanosomes. The observation that posterior midgut tissue homogenate could induce the transformation of bloodstream trypanosomes more than the fat body homogenate (45% parasites transformed compared to 30% after 18 h incubation) (Figure 5.3) might mean that the molecule is in a relatively more active form in the posterior midgut tissues than in the fat body. It is also possible that this molecule might be present in the precursor form or it is complexed to other molecules which then affects the biological activity in these tissues.

From transformation inhibition studies shown in Figure 5.4, both inhibitors used in this study (150 mM GlcN and ~2 μg/ml STI), could not completely abrogate the induction of bloodstream trypanosomes transformation to procyclics. In both cases, at least 25% of the bloodstream trypanosomes had transformed at 16 h compared to about 60% parasites transformed in the absence of the inhibitors. Two reasons can be expounded for the presence of residual ability to induce transformation after inhibition.

First, it is possible that other unknown factors are also involved in the induction of the transformation of bloodstream trypanosomes and were, therefore, not affected by these inhibitors. On the other hand this situation might represent a case of incomplete inhibition in the assays, which could result from low concentrations as the selected concentrations were those that completely inhibited trypanosome agglutination. It is also possible that incomplete inhibition could have occurred due to the broad specificity for saccharide residues displayed by most lectins (Sharon and Lis, 1989). Specific binding sites for non-carbohydrate ligands, which are also important in recognition, may also be present on a lectin (Goldstein *et al.*, 1980). These binding sites, should they be present on the lectin-trypsin complex, may not have been inhibited in this case.

5.4.2 Mode of action of the lectin-trysin complex

Bloodstream forms of salivarian trypanosomes have a single species of a glycoprotein coat covering their surface coat, which is exposed to the surface. Comparatively, the procyclic culture forms have more than 25 different proteins on the surface (Seed and Hall, 1992). Of these, the procyclin is the most predominant (Roditi and Pearson, 1990). Turner *et al.* (1988) showed that the transformation of trypanosomes from bloodstream to procyclics is accompanied by removal of the glycoprotein coat. Tryptic removal of the trypanosome surface coat has also been shown to be accompanied by changes in the morphology of the parasite, such as flagellar internalisation, membrane adhesions leading to plasma membrane/plasma membrane fusions (Frevert *et al.*, 1986).

Since *G. m. morsitans* midgut trypsin and trypsin-like enzymes have been reported to induce transformation of bloodstream trypanosomes (Imbuga *et al.*, 1992a), it has been proposed that the trypsins are involved in the cleavage of the glycoprotein coat (Osir *et al.*, 1995). However, tryptic

removal of the glycoproteins requires relatively high concentrations of the trypsins, as much as 40 µg trypsin/ml (Frevert *et al.*, 1986). Such concentrations are unlikely to be found in the tsetse midguts. It is, however, possible that the agglutinating property of the lectin-trypsin complex, in addition to acting as a signal for selection of some parasites, might also be acting as a means of locally concentrating the substrates and enzymes. It is therefore important that the agglutinating property of this molecule be investigated from the physiological and biochemical prospectives.

All parasites have carbohydrates on their cell surfaces (Jacobson and Doyle, 1996). Glycosyl groups have been shown to confer some protection against proteolytic degradation (Willadsen and Billingsley, 1996). Therefore, it is also highly unlikely that the tsetse midgut trypsin moiety of the complex would directly interact with the trypanosome surface coat. The presence of band of ~35,200 Da as one of the proteins bound to trypanosomes (Figure 5.9) and its absence when both the crude gut homogenate and the agglutinin were incubated with GlcN is interesting. This band also compares favourably with the glycosyl subunits of the lectin-trypsin complex (Chapter 4). This suggests that the ß subunit of the complex is directly involved in the binding of the agglutinin to the parasites. In a recent study, van den Abbelle *et al.* (1996) also reported of two bands of *G. palapalis gambiense* and *G. m. morsitans* midgut proteins (~28,000 Da and ~14,000 Da) as binding to procyclic trypanosomes and that only the higher molecular weight band, ~28,000 Da was GlcN specific. It is possible that the lectin component of the molecule binds to the sugar residues present on the cell membranes of the parasites, which renders attack by the trypsin/trypsin-like enzymes possible. In this study, therefore, when GlcN was used as inhibitor (Figure 5.9, lane 2), it is likely to have bound to the lectin moiety of the complex resulting into failure of the molecule to bind to the glycosyl residues on the trypanosomes. N-acetyl glucosamine (GlucNAc) has been

identified on cell surfaces of *T. b. brucei, T. b. rhodesiense* and *T. congolense* (Jacobson and Doyle, 1996). This provides one of the binding sites of the lectin-trypsin complex to the parasites. The involvement of the two subunits of the lectin-trypsin complex in parasite tropism is also exemplified by the effects of GlcN and STI on *in vitro* transformation studies as shown in Figure 5.4. Since GlcN has been reported to inhibit both midgut agglutinin and trypsin activities, the steric effects of the substrate/inhibitor when bound to the two subunits warrants further investigation. From the perspective of this study, it is also important to further establish what is the exact role of the trypsin moiety of the complex in this tsetse-trypanosome interactions, considering that its inhibition did not significantly change the number of proteins that bound to the trypanosomes.

CHAPTER SIX

GENERAL DISCUSSION AND CONCLUSIONS

6.1 General Discussion

Trypanosomiasis, a debilitating disease complex of both man and domesticated animals, has had a profound influence on the history of sub-Saharan Africa. In addition to causing human morbidity and mortality, trypanosomiasis has stagnated agricultural development through the limitation of livestock production evident in about 37 countries (Logan-Henfrey *et al.*, 1992). Although incidence statistics of these diseases appear relatively low, their effects on individuals and communities can be quite devastating (Hide, 1994). To a larger extent, rural development in sub-Saharan Africa depends on the successful eradication of tsetse and trypanosomiasis. However, successful control has remained intractable and elusive despite the many attempts that have been made throughout this century (Holmes, 1997).

In the absence of an effective vaccine, the practical control of trypanosomiasis has depended on the use of trypanocidal drugs, trypanotolerant breeds of livestock, tsetse traps and insecticides. In most African countries, the most well established and widespread method of control has been the use of trypanocidal drugs for both therapeutic and prophylactic purposes. However, the use of the trypanocidal drugs across Africa is mostly unsupervised and often under-dosage occurs which has greatly contributed to drug resistance (Peregrine, 1994). Also, due to the relative poverty of the African farmers, there is little enthusiasm in the pharmaceutical industry to develop new drugs. The three drugs readily available against tsetse transmitted trypanosomiasis (diminazene, isometamidium and homidium) have been in the market for over 30 years (Holmes, 1997).

Alternatively, trypanotolerant breeds of cattle have been kept in areas where the trypanosomiasis challenge is low (Murray *et al.*, 1983b). However, this trypanotolerance has been reported not to be absolute, since these breeds succumb to infections in areas of heavy challenge, which is characteristic of most of the African rangelands (Dransfield *et al.*, 1991).

Trypanosomiasis control is also achieved by controlling the tsetse, where attempts are made to eradicate tsetse or to reduce their numbers sufficiently enough to break the transmission cycle. Insecticides are used as sprays, pour-ons or dip-ins. Despite the successes often achieved by the use of insecticides, the effect on the environment are often very drastic, due to the indiscriminate destruction of the fauna (Laird, 1977). Also, insecticide prices are always on the increase. Complete success is also hampered by frequent re-invasions. Alternatively, traps targets and bait technology have been used as a cost effective methods of controlling tsetse (Jordan, 1986).

In recent years there has been emphasis on an integrated approach to trypanosomiasis control, where as many control methods as possible are simultaneously used with the involvement of local communities. In this case, suppression of tsetse populations rather than eradication of the flies is the desired objective (Dransfield *et al.*, 1991; Holmes, 1997). The persistence of the trypanosomiasis despite the many control strategies has therefore necessitated the need for further search for alternative control strategies. Amongst these are biological control, sterile insect technique (SIT) and the lethal insect technique (LIT) mooted at the ICIPE, which might require further consideration.

All the control methods aim at breaking the trypanosomiasis transmission cycle in one way or another. The observation that only a small percentage of tsetse, both in the wild and in laboratory reared populations, are susceptible to trypanosomes infection is of interest from the perspective of the relationships that exist between tsetse and

trypanosomes. Earlier studies indicated that several midgut factors might be involved in the determining the outcome of the tsetse-trypanosome relationships. These include trypanolysins (Stiles *et al.*, 1990), midgut lectins (Maudlin and Welburn, 1987; 1988), midgut trypsins (Yabu and Takayanagi, 1988; Imbuga *et al.*, 1992), the peritrophic membrane (Lehane and Msangi, 1991) and other unknown factors. From the functional perspective of some of these factors, the isolation of an agglutinin with proteolytic activity from midgut extracts of *G. longipennis* (Osir *et al.*, 1995) is intriguing. As mentioned earlier in this thesis, a similar molecule with M_r ~65,700 having two non-covalently linked subunits; α subunit (~28,800 Da) and ß subunit (~35,700 Da) has been isolated from midgut extracts of *G. m. morsitans*. It is also noteworthy that a comparatively similar molecule could not be immunologically detected in midgut extracts of other haematophagous insects studied (*S. calcitrans, P. duboscqi, A. aegypti*) but was found in the midgut extracts of other tsetse studied (*G. longipennis, G. f. fuscipes, G. m. centralis*). The presence of this molecule only in *Glossina* sp. might help to explain the vectorial capacity of tsetse to trypanosome transmission and hence the possible role of this molecule in determining the success of establishment of midgut infection in tsetse flies.

In this study, it has been reported that the midgut agglutinin of *G. m. morsitans* is associated with several tissues such as the fat body, haemolymph, posterior midgut and the peritrophic membrane. In the fat body, posterior midgut and haemolymph the agglutinin has been reported as occurring in the inactive form and mostly as molecules with higher molecular weights. The proximal association between the peritrophic membrane, which was shown to agglutinate both bloodstream and procyclic trypanosomes, and the gut lumen where the agglutinin is supposedly secreted after stimulation post-bloodmeal (Stiles *et al.*, 1990), emphasises the possible involvement of this molecule in the interactions

between tsetse tissues and the trypanosomes, prior to or during parasite tropism. This interaction is most likely a pre-requisite to the penetration of the peritrophic membrane by the trypanosomes as proposed by Miller and Lehane (1993).

This study has also demonstrated that the two moieties of the lectin-trypsin complex are synthesised in different tissues. The glycosyl moiety, most likely the lectin constituent, is synthesised in the fat body while the trypsin unit is synthesised by the posterior midgut tissues. Since homogenates from both the tissues, fat body and posterior midgut, did not agglutinate trypanosomes, it was assumed that the molecules are either present in these tissues in inactive forms or as precursors. It has also been proposed that the lectin-trypsin complex is activated after the linking up of the two constituent subunits, presumably in the midgut lumen.

The ability of trypanosomes to transform from bloodstream to procyclic forms is an important requirement for successful establishment of midgut infections in the tsetse. Midgut infections are, therefore, correlated to the ability of the midgut factors to either clear the parasites or to induce the transformation process. It has been previously reported that normally the parasite transformation process from bloodstream to procyclics is complete by 24 h post-feeding (Vickerman, 1985). It must be pointed out that this is prior to the onset of peak midgut trypsin and lectin activities which have been reported to be between 48 and 72 h post bloodmeal in tsetse (van den Abbelle and Decleir, 1991; Stiles *et al.*, 1990). In this study, it has been postulated that it is very unlikely that the increase in the two activities could be positively correlated to the parasite transformation process.

It has also been demonstrated in this study that the lectin-trypsin complex can induce *in vitro* transformation of *T. b. brucei* from bloodstream forms to procyclics at 27° C. Also, doubling the concentration of the agglutinin resulted in a higher transformation rates with a

concomitant increase in the rates of trypanosome mortality. The significance of this is that probably low agglutinin concentrations enhance the trypanosome establishment in the midgut while higher concentrations result into destruction of the trypanosomes in the tsetse midgut. This has been confirmed in this thesis by the *in vivo* studies on the effects of varying the agglutinin concentrations on midgut infection rates.

Inclusion of the polyclonal antibody raised against the agglutinin in the bloodmeal did not significantly affect the midgut infection rates while when the agglutinin was included in the bloodmeal, there was a significant reduction in the midgut infection rates. The concentration dependence of a tsetse midgut trypsin on trypanosome survival and differentiation was also reported by Imbuga *et al.* (1992a), who observed that that although trypsin induced the transformation of bloodstream trypanosomes to procyclics, there was a threshold above which parasite lysis ensued. Protozoal parasites have, however, been shown to inhibit trypsin activities in the midguts of the vectors, to some extent. This has been reported for *T. b. brucei* in *G. m. morsitans* (Imbuga *et al.*, 1992b) and *L. major* in *P. papatasi* (Schlein and Borovsky, 1987). Such a trypsin activity modulation mechanism may also apply to the activities of the lectin-trypsin complex during the tsetse-trypanosome interaction in the midgut. However, such a mechanism can only benefit the parasite either when the parasitaemia is very high which is unlikely in most natural situations or when the concentrations of the lectin-trypsin complex have not reached maximum activity, especially before 48-72 h post-bloodmeal.

The chemical, locational and proposed functional properties of the tsetse midgut lectin-trypsin complex can be exploited with the eventual aim of developing other novel and more sustainable methods of trypanosomiasis control. The ultimate goal of such an approach will be to interfere with the either differentiation, replication or transmission of the parasite in the vector (O'Brochta and Atkinson, 1997). A detailed

understanding of the interrelationships between insect vectors and the pathogens they transmit is paramount in this respect as insects that are susceptible or refractory to infection or transmission of micro-organisms occur naturally in vector populations (Beard *et al.*, 1993).

The advent of recombinant DNA technology and transgenic techniques provides a means for controlled genetic manipulations of insect vector genome by direct introduction of DNA that encodes for refractoriness phenotypes into the vector germline (Crampton *et al.*, 1994; Zheng, 1997).

In order to develop transgenic strains of tsetse, identification of genes which encode for the lectin and the trypsin moieties of the lectin-trypsin complex in the fat body and the posterior midgut cell, respectively, is imperative. As earlier stated in this thesis, elevation of concentrations of the lectin-trypsin complex, both *in vitro* and *in vivo* led to the increased mortality rates of the parasites and hence a significant reduction in the midgut infection rates. Reduction in the establishment of midgut infections will, obviously, lead to a significant reduction in the rates of infection maturation in the insects. It is therefore proposed here that over-exepression of the genes that encode for the subunits of the lectin-trypsin complex would significantly reduce the competence of tsetse to transmit trypanosomes.

On the other hand, this study has demonstrated that low concentrations of the lectin-trypsin complex are responsible for the induction of parasite transformation. It is also proposed that severe reduction in the concentrations of the molecule will deny the parasites the signal for transformation as well as interfere with the peritrophic membrane-parasite interaction, which has been emphasized as very important in trypanosome tropism in the tsetse midgut. Under-expression of the lectin and trypsin encoding genes would lower the overall agglutinin concentration in the midgut and therefore block the establishment of

midgut infections. Maudlin (1996) proposed that introduction of N-acetyl glucosamine (GlcNAc) permease genes into tsetse would lead to accumulation of GlcNAc and GlcN, known inhibitors of midgut lectins, hence reducing the overall agglutinin concentration, enough to interrupt induction of the parasite transformation. However, this would be advantageous to the insect only if the agglutinin does not have another physiologically important role in the tsetse. For trypanosomiasis control purposes, replacement of field tsetse populations with genetically engineered strains would result in the establishment of new tsetse population, refractory to trypanosomes.

It must, however, be pointed out that that the use of genetically manipulated tsetse would involve considerable research and development which will have to encompass, amongst other things, other midgut factors which function synergistically or antagonistically with the lectin-trypsin complex in determining either susceptibility of refractoriness to trypanosome infection. This is due to the fact that in many cases, the naturally occurring refractoriness may have complex genetic bases (Beard *et al.*, 1993).

6.2 Conclusions

This thesis has established that the *G. m. morsitans* midgut agglutinin is a ~65 700 Da molecule with two non-covalently linked subunits; α subunit (~28 000 Da) and ß subunit (~35 700 Da). The ß subunit has been shown to have a glycosyl residue and has been proposed to be involved in the binding of the agglutinin to the parasite. Related molecules have been detected only in *Glossina* sp. but not in other haematophagous insects. The agglutinin has been shown to be widely distributed in tsetse tissues as it was detected in fat body, haemolymph, posterior midgut tissues and peritrophic membrane. In these tissues, the agglutinin was found in the active form only in the peritrophic membrane

while in the other tissues it was found in the inactive form. This molecule was found not to be associated with the cuticle and haemocytes.

Radiolabeling experiments with ^{35}S-methionine showed that the subunits of the lectin-trypsin were synthesised in the fat body and the posterior midgut tissues. Using 1,3-3H-DFP, it has been established that the trypsin moiety of the lectin-trypsin complex is synthesised by the posterior midgut tissues.

Both *in vitro* and *in vivo* experiments have shown that the tsetse midgut lectin-trypsin complex is involved in both the immune defense of tsetse against the trypanosomes and the development of the parasites. While lower concentrations of the molecule induced the transformation process and hence enhance establishment of midgut infections, higher concentrations result into higher mortality rates of the parasites and hence reducing the midgut infection rates in the tsetse flies.

6.3 Recommendations

From the work carried out in this thesis , it is recommended that further work on the lectin-trypsin complex should be on:

6.3.1 Dissociation of the molecule in its constituent components and determine the relative biological significance of each of the subunits in relation to the tsetse-trypanosome interactions. The antibodies raised against the subunits can help in determining the actual assembly point of the subunits to form the biologically active complex.

6.3.2 Investigation of the ability of the antibodies against each of the complex components to block parasite development in the tsetse gut.

6.3.3 Determining the amino acid sequences of the subunits of the molecule and hence identify the genes that encode for them in the fat body and the posterior midgut epithelial cells.

6.3.4 Investigating fully the close association between the peritrophic membrane and the lectin-trypsin complex and identification of the actual ligands that are involved in this association. Studying the involvement of the lectin-trypsin complex in trypanosome attachment and penetration of the peritrophic membrane.

6.3.5 Investigation of other midgut factors that may act synergistically or antagonistically with the lectin-trypsin complex in the parasite tropism or death within tsetse midgut.

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APPENDICES

APPENDIX A

1. Stock solutions and buffers for slab gel electrophoresis

(a) 30% Acrylamide stock solution

29.2% w/v acrylamide

0.8 % w/v N,N-methylene-bisacrylamide

Acrylamide solutions are light sensitive and should be stored in dark bottles at 4° C after filtering. Caution should be exercised when handling acrylamide since it is neurotoxic.

(b) Ammonium persulphate (APS)

10% w/v made up fresh and kept at 4° C.

(c) Separating gel buffer

1.5 M Tris base. Adjust to pH 8.8 with HCl.

(d) Stacking gel buffer

0.5 M Tris base. Adjust to pH 6.8 with HCl.

(e) Sample loading buffer

0.13 M Tris-HCl pH 6.8, 20% (v/v) glycerol, 0.002% bromophenol blue,

4% SDS*, 1% β mercaptoethanol*

* optional for dissociating gels

(f) Formulation for 4-15% separating gels

	4%	**15%**
acrylamide (stock)	2.4 ml	9.0 ml
separating buffer	4.6 ml	4.6 ml
distilled water	10.8 ml	4.2 ml
10% w/v SDS*	180 µl	180 µl
TEMED	6 µl	6µl
10 % APS	130 µl	90 µl

* optional for dissociating gels

(g) Formulation for 3.13% stacking gels

acrylamide (stock)	1.02 ml
stacking gel buffer	2.5 ml
distilled water	6.1 ml
10% SDS*	100 µl
TEMED	10 µl
10% APS	50 µl

(h) Running buffer

25 mM Tris-HCl, pH 8.3,
192 mM glycine
1% SDS*
optional for dissociating gel

(i) Coomassie stain

Dissolve 6 g Coomassie Brilliant Blue in 400 ml absolute methanol and 92 ml acetic acid. Stir until the dye dissolves completely then make up to 1 L with distilled water. Filter and store in a dark bottle.

(j) Destaining solutions

	Destain I	**Destain II**
methanol	50%	5%
acetic acid	9.2%	7.2%
distilled water	41.8%	87.5%

2. Periodate acid-Schiff (PAS) stain

Dissolve 1 g basic Fuschin in 100 ml boiling distilled water and stir for 5 min. then cool to 50° C. Filter and add 20 ml 1 N HCl cool to 25° C and then add 1 gm of sodium metabisulphite. Let the solution stand in the dark for 12-24 h. Add 2 g of activated charcoal and shake for 1 min. and filter. Store at 4° C.

APPENDIX B

Giemsa stain, stock solution preparation.

Dissolve 10 g Giemsa stain in 660 ml glycerol and heat the mixture at 56° C for 90-120 min. Add 660 ml absolute methanol and mix thoroughly. Allow the solution to stand at room temperature for 7 days. Filter and store in dark bottle. Working solution should be diluted 1:10 with PBS, pH 7.2.