PURIFICATION AND CHARACTERIZATION OF TRYPANOAGGLUTININ FROM THE MIDGUT OF TSETSE FLY, *GLOSSINA LONGIPENNIS*

BY

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DECLARATION

I, Laila Uweso Abubaker, hereby declare that this thesis is my original work and the work described here has not been presented to any University.

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DEDICATION

This thesis is dedicated to my parents, brothers and sisters for their understanding and support over the years.

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ABBREVIATIONS

BANA	-	Benzoyl-L-arginine-β-naphthylamide
BCA	-	Bicinconinic acid
BSA	-	Bovine serum albumin
Chromozym TRY	-	Carbobenzoxy-val-gly-arg-4-nitroanilide acetate
DFP	-	Diisopropylfluorophosphate
DIP-trypsin	-	Dilsopropyl-trypsin derivative
DMF	-	Dimethylformamide
FCA	-	Freunds complete adjuvant
FPLC	-	Fast protein liquid chromatography gel electrophoresis
HCl	-	Hydrochloric acid
NaCl	-	Sodium chloride
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PEG	-	Polyethylene glycol
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium dodecyl sulphate-polyacrylamide
STI	-	Soybean trypsin inhibitor
TB	-	Tris-buffer
TBS	-	Tris buffered saline
TCA	-	Trichloroacetic acid
Temed	-	N, N, N', N' - Tetramethylenediamine
TPCK	-	Tosylamide-2-phenylethyl chloromethyl ketone
Tris	-	Tris-(hydroxy methyl) aminomethane
V_e	-	Elution volume

UNIT ABBREVIATIONS

Ci	-	Curie
Ci/mmol	-	Curie/ millimole
cm	-	Centimetre
gm	-	Gram
h	-	Hour
Kd	-	Kilodalton
M	-	Molar concentration
mA	-	Milliamperes
min	-	Minute
mg	-	Milligram
ml	-	Millilitre
mM		Millimolar
M_r	-	Relative molecular weight
nm	-	Nanometre
pH	-	Log_{10} hydrogen ion concentration
rpm	-	Revolutions per minute
μCi	-	Microcurie
μg	-	Microgram
μl	-	Microlitre
μmol	-	Micromolar
V	-	Volt
W	-	Watt

SUMMARY

The tsetse fly is an insect of great economic importance to man as a vector of both human and animal trypanosomiasis. Trypanosomes are ingested by the tsetse fly vector with a bloodmeal taken from an infected host. In the midgut, these parasites are exposed to a hostile environment which comprises of lectins/ trypanoagglutinins, proteolytic enzymes, trypanolysins and other unknown factors. Lectins or agglutinins are a group of proteins of non-immune in origin, that bind carbohydrates specifically. Recent investigations have shown that trypsin, the most predominant protease in the midgut, appears to be very closely related to the midgut lectins. It was therefore considered pertinent to resolve the relationship between the lectins/trypanoagglutinins and trypsin or trypsin-like enzymes in the tsetse midgut.

In this study, the trypanoagglutinin was purified from the midgut of *Glossina longipennis* and its properties studied *in vitro.* Purification was achieved by anion exchange chromatography using Fast Protein Liquid Chromatography (FPLC). It co-eluted with trypsin -like activity at approximately 225 mM NaCl gradient. The molecule was capable of agglutinating *Trypanosoma brucei brucei* and rabbit erythrocytes. The release of the molecule was induced by a bloodmeal since unfed midguts showed negligible agglutination activity. Compared to the bloodstream parasites, a much lower concentration of trypanoagglutinin was required to agglutinate procyclics and erythrocytes. Furthermore, agglutination of the parasites as well as of the erythrocytes was specifically inhibited by glucosamine. Similarly, the soybean trypsin inhibitor abrogated the agglutination of bloodstream parasites while agglutination of erythrocytes was partially inhibited. In contrast, agglutination

of procyclic parasites was not affected by this inhibitor. The molecule was found to be thermo-labile since 90% of the agglutination activity was lost by heating at 60-100° C for 10 minutes. Analysis on native polyacrylamide gel electrophoresis (PAGE) revealed one band of M_r ~ 61,000 which also showed trypsin activity. However, electrophoresis under denaturing conditions gave two subunits (M_r ~ 33,000 and 27,000). Only the 27,000 subunit showed trypsin activity. It is thus postulated that both the agglutination and trypsin-like activity exist in the same molecule.

Antibodies raised against the isolated trypanoagglutinin inhibited agglutination as well as the trypsin activities of the molecule. Out of the blood-sucking insect species tested for immunological cross-reactivity, only members of the *Glossina* family gave a positive reaction. The two bands in *G. m. morsitans* co-migrated with those from *G. longipennis*, suggesting that this protein could be unique to tsetse fly.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Tsetse flies as vectors of trypanosomes

The threat posed to man and his domestic livestock by trypanosomiasis is related almost entirely to the distribution of infected tsetse flies. Tsetse flies are haematophagous insects belonging to the genus *Glossina*. Based on their morphology, ecology, karyotype, and behavior, the tsetse flies can be classified into 3 groups. The *fusca* group which are forest dwellers, consist mainly of *Glossina longipennis* and *Glossina brevipalpis*. The second group, the *morsitans* are savanna dwellers and comprises of *Glossina morsitans, Glossina pallidipes, Glossina austeni* and *Glossina swynnertoni*. The third group, the *palpalis* tend to have a riverine distribution and consists of *Glossina fuscipes, Glossina palpalis* and *Glossina tachinoides*.

By the nature of their feeding habits, tsetse flies act as vectors of trypanosomes. The trypanosomes belong to the genus *Trypanosoma*, a group of protozoal parasites found in the blood and tissues of vertebrate hosts. Their life cycle comprises of various developmental changes both within the vertebrate host and the tsetse fly vector (Hoare, 1972). This circulation of trypanosomes is of practical significance when man and his domestic animals intrude into the circle and become hosts of the parasite. The result is sleeping sickness in man and nagana in domestic animals (Bruce, 1895). The African trypanosomes elicit pathogenicity in these animals, which are generally fatal unless treated (Buxton, 1955). The natural infection rates of tsetse flies have long been used by those

concerned with epidemiology and control to assess the risk or "challenge" created by the disease. Until recently, these natural infection rates were thought to be determined largely by the biology of the parasite. Clearly, an infective feed is an essential prerequisite for a fly infection. However, the number of trypanosomes ingested by the fly during the infective feed does not influence the infection rate. Indeed, a single trypanosome has been found to be sufficient in infecting a tsetse fly (Maudlin, 1989).

1.2 Life cycle of the African trypanosomes

An important part of the life cycle of African trypanosomes occurs within the tsetse fly vector. Bloodstream forms of the parasite are covered by a dense surface coat consisting of the variant surface glycoprotein (VSG). The bloodstream forms are ingested when the fly takes an infected bloodmeal from a vertebrate host. In most cases the parasites pass through the oesophagous to the crop. However, in the case of *Trypanosoma vivax*, the life cycle is restricted within the fly mouthparts. After feeding, the parasites migrate to the peritrophic membrane lining the midgut where the blood is digested. In the midgut the parasites encounter a physiologically hostile environment that includes various digestive enzymes (Cheeseman and Gooding, 1985), lectins (Welburn *et al.*, 1989), agglutinins (Ingram and Molyneux, 1988; Stiles *et al.*, 1990), trypanolytic proteins (Stiles *et al.*, 1990) and possibly other yet unknown factors. Most parasites that enter the midgut are killed by one or more of these factors, and those that survive transform into procyclic midgut forms (Maudlin and Welburn, 1987; Stiles *et al.*, 1990). These parasites establish themselves in the ectoperitrophic space as midgut or "immature" infections. The successful establishment of the parasites within the insect vector is

dependent on transformation from bloodstream to procyclic form. This process of transformation involves complex morphological, biochemical and physiological changes that enable the parasites to adapt to a radically different environment within the fly midgut (Vickerman, 1965; Vickerman and Preston, 1976; Englund *et al.*, 1982). These include loss of the surface coat; cessation of synthesis of the VSG; activation of the mitochondrial enzymes and the cytochrome electron transport system; induction of procyclin synthesis; and displacement of the positions of the nucleus and kinetoplast (Vickerman and Preston, 1976; Barry and Vickerman, 1979; Ghiotto *et al.*, 1979; Roditi and Pearson, 1990). Subsequently, the trypanosomes leave this midgut shelter and migrate to the salivary glands (*T. b. brucei*) or mouthparts (*T. congolense*) where they mature into infective metacyclic forms. This migration is through the proventriculus, where the peritrophic membrane is soft and allows the parasites to penetrate. It is these metacyclic forms that are injected into the vertebrate host when the fly feeds. It takes 18-34 days from the time the tsetse fly feeds on an infected vertebrate host for metacyclics to form (Service, 1986).

Thus, after entering the fly, the probability of the trypanosome completing its life cycle within the vector is inversely dependent on the complexity of the changes elaborated. This in turn is related to the species of trypanosome involved. *Trypanosoma vivax* which is confined to the fly mouthparts and has the simplest life cycle would be expected to have a greater chance of success in the fly than *Trypanosoma congolense*, which goes through a midgut stage before eventually maturing in the mouthparts. *Trypanosoma brucei* seems to have the most complex life cycle within the fly and therefore should have the least chance for successful cyclical transmission (Buxton, 1955). These assumptions have been supported by

field data which show that *Trypanosoma vivax* infection rates in tsetse flies are usually greater than *Trypanosoma congolense*, which in turn are greater than *Trypanosoma brucei* group infections (Pires *et al.*, 1950; Jordan, 1974). Although it may be logical to relate infection rates to behavioral differences between trypanosome species, this does not account for the intriguing fact that infection rates in natural fly populations, irrespective of the species of trypanosome or availability of infected hosts are generally very low (<10%) (Harley, 1966, 1967; Harley and Wilson, 1968; Elce, 1971, 1974; Jordan, 1974)

1.3 Vectorial capacity of tsetse flies.

Tsetse fly species differ greatly in their ability to transmit trypanosomes . *Glossina pallidipes* and *Glossina morsitans centralis* appear to have similar vector competence for *Trypanosoma vivax* but differ greatly with regard to susceptibility to *T. congolense* and *T. brucei*; the former having a lower vector competence than the latter (Moloo *et al.*, 1992b). Studies on the vectorial capacities of tsetse flies using different hosts showed that irrespective of the host species used, the *T. congolense* infection rate was highest in *G. m. centralis*, lowest in the *palpalis* and *fusca* group tsetse, with *G. pallidipes* being intermediate (Moloo *et al.*, 1992a). Generally, it has been observed that the *morsitans* group is more susceptible and therefore efficient vectors of trypanosomes while the *palpalis* and *fusca* groups appear to be poor vectors (Harley and Wilson, 1968; Harley, 1971, Roberts and Gray, 1972; Moloo and Kutuza, 1988b; Moloo *et al.*, 1987, 1992a, b). It is possible that the differences in the infection rates reflect differences in the gut environments of the tsetse studied such that the parasites of the subgenera Trypanozoon and Nannomonas can become established more readily and undergo full cyclical

development in some tsetse flies while arrested in the gut of others (Molyneux, 1983; Moloo and Kutuza, 1988b).

1.4 Tsetse fly midgut environment

The midgut is situated between the distal end of the proventriculus and the junction of the two malphigian tubules (Service, 1986). It is lined along its entire length by the peritrophic membrane (Service, 1986). The midgut is very long and convoluted. During feeding, blood is sucked up the proboscis, passes to the crop and later to the midgut. In case of an infected bloodmeal, the parasites encounter a physiologically hostile environment within the tsetse midgut. Here, the trypanosomes either migrate to regions of low anti-trypanosomal molecules (anterior midgut) or are lysed in the posterior midgut while others survive. The distribution of parasites within the midgut is dependent on how effficient the destructive molecules can be avoided. Trypanosomes like *T. vivax* are unable to withstand the midgut environment and are restricted to foregut, the cibarium and the hypopharynx (Jeffries *et al.*, 1987). The establishment of the trypanosomes in the midgut has also been associated with the presence of *Rickettsia*-like organisms (RLOs) in the midgut epithelial cells (Maudlin and Ellis, 1985).

Recently, the relationship between various midgut factors and the susceptibility of tsetse flies to infection of trypanosomes has attracted considerable interest (Gingrich *et al.*, 1985; Maudlin and Welburn, 1987; Welburn and Maudlin 1989, 1991; Imbuga *et al.*, 1992a, b; Osir *et al.*, 1993). However the precise mechanism of action of these molecules are not clearly understood, hence the need for further research to elucidate the mechanisms.

1.4.1 Role of the peritrophic membrane

The epithelial cells of the midgut are devoid of cuticle, but in most insects they are separated from the contents of the gut by a detached cylindrial sheath called the peritrophic membrane. In tsetse flies, the peritrophic membrane is secreted by cells of the proventriculus (Moloo *et al.*, 1971; Lehane, 1976) and consist of chitin base and associated protein (Wigglesworth, 1972). The membrane is absent in newly emerged flies but develops rapidly after eclosion, lining the entire length of the midgut after 80-90 h (Lehane and Msangi, 1991). The peritrophic membrane has several functions. For example, it protects the midgut cells from abrasion by hard fragments in food and acts as an ultrafilter but offers no hinderance to digestive enzymes and digestion products (Wigglesworth, 1972). More recently, it has been proposed that the peritrophic membrane may also act as a physical barrier to the penetration of trypansosomes (Lehane and Msangi, 1991). Normally in the case of an infective bloodmeal, the trypanosomes enter the gut inside the membrane (endoperitrophic space) and then transform and establish themselves as midgut infections in the ectoperitrophic space between the midgut cells and the bloodmeal. It has been postulated that the parasites penetrate this space through the proventriculus where the peritrophic membrane is soft (Yorke *et al.*, 1933; Fairburn, 1958; Freeman, 1970; 1973). Several studies have shown that, as the tsetse flies grow older they become increasingly resistant to infection with trypanosomes (Van Hoof *et al.*, 1937; Wijers, 1958; Harley, 1970; Makuyamviri *et al.*, 1984; Distelmans *et al.*, 1982; Mwangela *et al.*, 1987). Attempts to explain this age-dependent variation in suceptibility has centred on the nature of the peritrophic membrane (Lehane and Msangi, 1991). Since newly emerged flies lack a peritrophic membrane, the inability of older flies to support trypanosome infection

was thought to be due to the development of the peritrophic membrane. However, Ellis and Evans (1977) observed that *T. b. rhodesiense* could penetrate the fully developed peritrophic membrane of *G. m. morsitans*. Furthermore, older flies can be infected after a period of starvation (Gingrich *et al.*, 1982a; Makumyaviri *et al.*, 1984) suggesting that fully formed peritrophic membrane is not a physical barrier to the penetration of trypanosomes but other factors could be involved.

The ligand affinity reactions of the peritrophic membrane differs between susceptible and refractory *G. m. morsitans* due to exposed carbohydrate residues (Okola *et al.*, 1988). More recently, it has been suggested that gut lectins and not the peritrophic membrane determine the age-dependent susceptibility of tsetse for trypanosomes (Maudlin and Welburn, 1987; 1988). These lectins are produced in response to stimulation from the serum content of the first bloodmeal thus explaining the age dependent infectability of tsetse flies, as only trypanosomes ingested with the first bloodmeal will encounter no protective lectins. Peters *et al.*(1983) reported the presence of a mannose specific lectin on the peritrophic membrane of the blowfly, *Calliphora erythrocephala*. It is not known whether these lectins are produced in conjunction with the peritrophic membrane or are produced elsewhere and become attached to the membrane after there secretion into the gut lumen. However, this association of lectins with the peritrophic membrane may partly explain the paucity of lectins in the newly emerged fly, where the peritrophic membrane is incomplete (Lehane and Msangi, 1991).

1.4.2 Midgut Proteases

Digestion of the bloodmeal occurs in the posterior region of the midgut. As proteins are the most important nutritive components of the bloodmeal, digestion is mostly proteolytic. A comprehensive study of the proteolytic activity of the tsetse fly, *Glossina morsitans morsitans*, has already been carried out by Gooding (1976, 1977). Six enzymes have been identified in the midgut of the flies, namely trypsin, a trypsin-like enzyme, a chymotrypsin-like enzyme, carboxypeptidase A and B and an aminopeptidase.

Gingrich *et al.* (1985) observed an increased midgut infection rate in flies fed on trypanosomes suspended in serum free blood. As a possible explanation he postulated a relationship between the activity of midgut proteinases and the development of a procyclic infection. Similarly, the interaction between proteinases and parasite has also been proposed for other haematophagous insect vectors. For example, trypsin-like proteinases in *Aedes aegypti* are responsible for destruction of ingested ookinates of Plasmodium (Gass, 1977; Gass and Yeats, 1979; Yeates and Steiger, 1981). In *Phlebotomus papatasi*, it has been suggested that a specific component of the trypsin-like activity prevents the survival of *Leishmania donovani* and modulation of this component by *Leishmania major* enables it to thrive (Borovsky and Schlein, 1987). The anterior midgut possess proteinase inhibitor which inhibit *G. m. morsitans* posterior midgut trypsin (Houseman, 1980). The inhibitor is subspecies specific, each anterior midgut lysate inhibits only the homologous lysate. The exact nature of the inhibition mechanisms is not known. It is likely that the inhibitor protects the gut cells from proteolytic activity during periods of starvation (Vogel *et al.*, 1968). The possibility that trypsin activity is involved in the elimination and/or transformation of bloodstream forms,

has been shown by preliminary *in vivo* studies of the midgut trypsin of *Glossina palpalis palpalis* on *T. b. brucei* (Abbeele and Declair, 1991). Likewise, a correlation was established between the transformation activity of the midgut homogenates of *G. m. morsitans* at 27° C and trypsin activity on bloodstream forms of *T. b. brucei* into procyclic (midgut) forms *in vitro* (Imbuga *et al.*, 1992a). Furthermore, trypanosomes/trypanosome membranes from *Trypanosoma brucei brucei* inhibit midgut trypsin activity in *G. m. morsitans* (Imbuga *et al.*, 1992b). This suggests that in order to survive in the midgut, the trypanosomes have to modulate the activity of trypsin and trypsin-like enzymes. Tsetse flies infected with parasites and maintained on a diet of blood containing glucosamine have been reported to develop high midgut infection rates (Maudlin and Welburn, 1987). It could be argued that the reason for the elevated infection rates in these flies involve the inhibition of trypsin by glucosamine (Osir *et al.*, 1993). The different species of *Glossina* when fed before an infective feed could clear trypanosomes from their midguts faster than flies infected as tenerals (Welburn *et al.*, 1989). This difference could be attributed to trypsin levels in teneral and non-teneral flies. Trypsin activity levels rises after a bloodmeal and peaks at 72 h and 24 h in tenerals and non-tenerals respectively (Onyango, 1993). Subsequently, the activity drops but still remain higher than the initial levels prior to the first bloodmeal (Nguu, unpublished). Thus the ability of non-teneral flies to clear trypanosomes after an infective feed is due to the trypsin activity. It has been reported that older G. m. morsitans can be infected with T. b. brucei if they are starved for 3-4 days prior to taking the infective bloodmeal (Gingrich *et al.*, 1982a; Makumyaviri *et al.*,1984). This can be attributed to the reduction in trypsin activity during starvation.

1.4.3 Lectins

During the life cycle of gut adapted trypanosomes, a particularly critical time is the initial establishment of a midgut infection in the fly. Within hours of entering the vector, most bloodstream forms are lysed before transforming into gut adapted procyclic forms (Turner *et al.*, 1988). During the next few days, many gut infections are lost as the few surviving procyclics often fail to establish (Welburn *et al.*, 1989). Although multiple factors probably affect parasite establishment and maturation in the fly, tsetse "immune" processes mediated by lectins appear to be crucial.

Lectins belong to a class of proteins of non-immune origin that bind carbohydrates specifically and non- covalently (Liener *et al.*, 1986; Sharon and Lis, 1989b) They are polyvalent and can thus agglutinate animal or plant cells (unmodified or enzyme treated) and/or precipitate polysaccharides, glycoproteins and glycolipids. Their specificity is defined in terms of the monosaccharide or simple oligosacharide that inhibit the lectin-induced agglutination reactions (Goldstein *et al.*, 1980).

Lectins are not confined to plants as originally believed, but are ubiquitous in nature being frequently found on the cell surfaces and intracellular particles. Recently, it has been proposed that these lectins act as recognition molecules (Sharon and Lis, 1987; Harrison and Chesterton, 1980; Brandly and Schnaar, 1986). This resulted from the realization that surface carbohydrates may function in cell recognition (Liener *et al.* 1986; Lis and Sharon, 1986; Sharon and Lis, 1989a;). The presence of lectins in several insects has also been demonstrated. For example, lectins present in the midgut of *Rhodnius prolixus* can stimulate differentiation of *Trypanosoma cruzi*, the etiological agent of chagas disease (Pereira *et al.*, 1981). Lectins have also been identified in the midgut, hindgut and

haemolymph of *Glossina austeni* (Ibrahim *et al*., 1984); and in the gut extracts of *Phlebotomus papatasi* (Wallbanks *et al*., 1986). These lectins exhibit different carbohydrate binding specificities. For example, the lectins in the crop, midgut and haemolymph of *Rhodnius prolixus* are specific for N-acetyl-D-mannose, ∝-N-acetyl D-galactose and ∝ and *β*-D-galactose respectively (Pereira *et al*., 1981). Those in *Phlebotomus papatasi* are inhibited by turanose and trehalose (Wallbanks *et al*., 1986). On the other hand, *Glossina morsitans* midgut lectins are specific for glucosamine (Ibrahim *et al*. 1984; Welburn and Maudlin, 1989). Tsetse flies infected and maintained on a diet of blood containing glucosamine develop higher midgut infection rates (Maudlin and Welburn, 1987). These elevated infection rates might be due to inhibition of midgut lectins (Ibrahim *et al*., 1984; Welburn and Maudlin, 1989). Hence the normal role of midgut lectins is to mediate the lysis of most trypanosomes entering the midgut (Maudlin and Welburn, 1987). The midguts of teneral flies have been shown to have little lectin activity compared to non-teneral flies. This suggests as in the case of trypsin, that the production of lectins is induced by bloodmeal (Welburn *et al*., 1989; Imbuga *et al*., 1992). The rate of midgut killing varies both between and within species of fly and that these differences correlate with lectin output measured by *in vitro* agglutination tests (Welburn *et al*., 1989). Thus, flies with the highest lectin output are the most successful at preventing trypanosome establishment in the midgut (Welburn *et al*., 1989). On the other hand, *G. m. morsitans* midgut lectin has also been implicated in providing the signal for established midgut trypanosomes to proceed with the process of maturation (Maudlin and Welburn, 1988a). Subsequently, it was shown that inhibition of lectin activity using glucosamine temporarily prevented maturation of trypanosomes, which could proceed once the inhibition was removed (Welburn

and Maudlin, 1989). This suggested that midgut procyclic trypanosomes are not preprogrammed to complete their life cycle in the fly, but remain in the midgut stage until they receive the lectin signal to proceed with maturation.

It has also been proposed that a second lectin stimulus, the haemolymph lectin is part of the normal maturation process of trypanosomes in the tsetse fly (Welburn and Maudlin, 1990). Compared to *G. m. morsitans*, *G. p. palpalis* have been shown to have low haemolymph lectin titre (Welburn and Maudlin, 1990). Hence flies with optimum midgut and haemolymph lectin titres, like *G. m. morsitans* may have high transmission indices for *T. congolense* and *T. b. rhodesiense*. On the other hand, flies with low titres of haemolymph lectin, like *G. p. palpalis*, will mature few infections of these trypanosomes, even in the presence of optimum midgut lectin titres (Welburn and Maudlin, 1990). These differences between species of flies in lectin secretion may be responsible for the species specific barriers which apparently exist between trypanosomes and vectors (Welburn and Maudlin, 1990). The nature of the stimulus produced by the haemolymph lectin is still not clear. However, it has been proposed that the lectin may act in two diffferent ways. Firstly, it may act as a trigger by binding to individual procyclic trypanosome membrane and the parasites differentiate (Welburn and Maudlin, 1990). Secondly, it may simply act as mitogen, a common property of lectins (Goldstein and Poretz,1986).

The precise mechanism by which secreted lectins kill trypanosomes in the fly is not known. On the other hand trypsin treatment removes variable surface glycoprotein (VSG) and other glycoproteins of the trypanosome surface. Consequently, trypsin activity in the tsetse midgut could possibly make the trypanosomes more vulnerable to the action of midgut lectins by exposing the lectin

binding sites.

1.4.4 Role of *Rickettsia* like organisms (RLOs)

Susceptibility to trypanosome infection in *Glossina morsitans morsitans* has been shown to be a maternally inherited trait associated with the presence of tsetse symbionts, the *Rickettsia*-like organisms (RLOs), in the midgut cells (Maudlin and Ellis, 1985; Maudlin *et al*., 1986). On the basis of these findings, it was concluded that the critical events leading to the susceptibility of the teneral fly probably takes place in the larval/pupal period. During this time it is postulated that the RLOs secrete endochitinase which degrade chitin resulting in a build up of glucosamine. The glucosamine in turn inhibits midgut lectin activity at the teneral feed (Maudlin and Welburn, 1988b; Baker *et al*., 1990). On the other hand, lectin levels in the midguts of non-teneral flies when switched on by the bloodmeal are too high to be affected by RLOs activity. Hence the low *Trypanosoma congolense* and Trypanozoon infection rates commonly observed in non- teneral flies of whatever pedigree or species (Welburn and Maudlin, 1991). However, Moloo and Shaw (1989) dispute this theory. Having found RLOs in all *Glossina morsitans centralis*, both infected or uninfected, they concluded that susceptibility is not associated with these organisms. The RLOs were found to be numerous in older flies compared to younger flies, yet the former are more resistant to infection than the latter. Furthermore, the presence of RLOs in these flies did not affect the maturation of *Trypanosoma congolense* (Moloo and Shaw, 1989). Maintaining the puparial stage of *G. m. morsitans* at 3° C lower than normal reduced the numbers of RLOs carried by emerging flies. The susceptibility of these flies to midgut infection with *Trypanosoma congolense* was also significantly reduced compared with control flies

held at normal temperature. These results suggested that the relationship between RLOs and susceptibility is not an all-or-nothing effect but is quantitative in character (Welburn and Maudlin, 1991). Moreover, RLOs are thought to affect only the establishment of midgut infections in teneral flies while maturation of these infections is a distinct process dependent on lectin signalling following the establishment of the midgut infection (Welburn and Maudlin, 1989, 1990).

1.4.5 Trypanoagglutinin

Insects are known to exhibit both cellular and non cellular (humoral) "immune" reactions against various pathogenic organisms (Chadwick and Aston, 1978; Rowley and Ratcliffe, 1981; Whitcomb *et al.*, 1974). The humoral factors upon which much attention has been focused are the agglutinins, so termed because of their ability to agglutinate vertebrate erythrocytes *in vitro* (Bernheimer, 1952; Lackie, 1980), bacteria (Pauley *et al.*, 1971; Pistole, 1978) and Trypanosomatid flagellates (Ingram *et al.*, 1983; 1984). It is now recognised that most of these agglutinins are lectins. Agglutinating activity has been found in the haemolymph, midgut and hindgut of *Glossina austeni* against calf, guinea pig and chicken erythrocytes (Ibrahim *et al.*, 1984). Trypanosoma brucei procyclic forms were also agglutinated by the latter two, but not by haemolymph (Ibrahim *et al.*, 1984). More recently, Stiles *et al.* (1990) described a trypanolysin and a trypanoagglutinin from the midguts of *G. p. palpalis* and *G. p. gambiensis*. The trypanolysin caused lysis of the trypanosomes, while trypanoagglutinin resulted into agglutination of the parasites. As with midgut proteases (Cheeseman and Gooding, 1985; Imbuga *et al.*, 1992; Onyango, 1993), these two molecules were active only in the posterior midgut, were heat labile above 50° C and had a periodic

cycle of activity in response to bloodmeal intake. Interestingly, these molecules were not affected by protease inhibitors or trypsin but were inactivated by pronase. The trypanolysin contained two proteins with molecular weight of M_r~ 10,000 and 12,000. The agglutinin of both subspecies had an M_r~ 67,000. The precise mechanisms of action of these molecules are still not clearly understood, hence the need for further research in this area.

1.5 Importance of the study

In Sub-Saharan Africa, sleeping sickness is still a significant disease. In addition, nagana impedes livestock production directly and crop production indirectly. The control of trypanosomiasis currently relies on tsetse fly control, use of trypanocidal drugs and to a lesser extent, the breeding of trypanotolerant livestock. The traditional methods of controlling tsetse flies involved clearing of bushes and spraying the habitat with insecticides. Unfortunately, intensive and repeated spraying of residual insecticides can have disastrous effects on the local fauna (Koeman *et al.*, 1971). Other control strategies include use of pheromones or attractants, impregnated traps, release of sterile male flies (SIT), use of predators, pathogens and insect growth regulators (Itard, 1975; Abbeele and Declair, unpublished; Denlinger, 1975). Although successful initially, all these measures have had setbacks due to reinvasion of the tsetse flies from other areas. On the other hand, attempts to develop a vaccine against the parasites have been unsuccessful to date. This is largely due to the ability of the parasite to undergo antigenic variation, thus evading the host immune system. Further attempts to interfere with the parasite metabolism by using drugs have been in vain, as the trypanosomes developed drug resistance. A new approach has now been

developed in tackling insect-borne diseases. Instead of trying to eradicate the insects, vector biologists hope to produce transgenic strains that are incapable of transmitting disease (Aldhous, 1993). However, for this to be achieved it is imperative to understand the mechanisms involved in the interaction between vectors and the parasites they transmit.

Susceptibility of tsetse flies to trypanosome infection appears to be a maternally inheritable trait associated with the presence of RLOs in the fly midgut (Maudlin and Welburn, 1988a, b). The RLOs exert this effect by producing endo-chitinase, an enzyme that digests chitin to release glucosamine which in turn inhibits the lectins that normally kill the parasites entering the midguts of refractory flies (Maudlin and Welburn, 1988a). According to this model, a close relationship exists between lectin titres and susceptibility to trypanosome infection. Those flies with the highest lectin titres produce the lowest infection rates (Welburn *et al.*, 1989). *In vitro* studies have shown that trypanosomes are agglutinated by tsetse midgut lectin. The exact mechanism by which midgut lectins kill the parasites remains a matter of conjecture. However, recent studies suggest a close relationship between lectins and trypsin or trypsin-like enzymes in the tsetse midgut (Welburn *et al.*, 1989; Imbuga *et al.*, 1992;.Ibrahim *et al.*, 1984; Osir *et al.* 1993). The assumption that the midgut trypsin-like enzymes and lectins are either the same or closely related would explain the mechanism by which trypanosomes are lysed. Hence the need to purify and characterize these molecules. Eventually, this may open up new avenues for the genetic engineering of vector populations with a view to replacing wild-type vectors with parasite refractory strains. For example, introducing the gene for the midgut lectin in the symbionts will lead to the production of transgenic tsetse flies expressing lectins in their midguts, making them refractory to trypanosomes.

1.6 Aims and objectives

The overall aim of this study was to resolve the relationship between lectins, trypsins and agglutinins in the tsetse midgut. The specific objectives were:

(i) To purify a trypanoagglutinin from the midgut of tsetse fly, *Glossina longipennis*.

(ii) To carry out physico-chemical studies on the isolated trypanoagglutinin

(iii) To carry out immunological studies on the isolated trypanoagglutinin

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

The laboratory chemicals used in the experiments were of analytical grade obtained from Aldrich Chemical Company (Dorset, England), BDH Chemical Company (Poole, England), Bio-Rad Laboratories Ltd (Richmond, CA, USA), Pharmacia-LKB (Uppsala, Sweden), Pierce Chemical Company (Rockford, Ill., USA), Serva Feinbiochemica GmbH & Company (Heidelberg/NewYork) and Sigma Chemical Company (St. Louis, USA).

2.2 Experimental Insects and Animals

Tsetse flies, *Glossina longipennis* Corti, were obtained from the Tsetse Vector Laboratory of International Laboratory for Research on Animal Diseases (ILRAD). They were maintained in a climate controlled room at a temperature of 25 ± 0.5° C with relative humidity of 80-85%. Sandflies, *Phlebotomus duboscqi*, were obtained from Insect and Animal Breading Unit (IABU) of the International Centre of Insect Physiology and Ecology (ICIPE). Stable flies, *Stomoxys calcitrans* were obtained from Dagoreti, Nairobi. Male rats (Wistar strain) and New Zealand white rabbits used were obtained from IABU.

2.3 Preparation of the parasites

Male Wistar rats were infected with a *Trypanosoma brucei brucei* stock derived from EATRO 1969. This stock was isolated as previously described (Otieno *et al.*, 1983). The trypanosomes were originally grown in rats from stabilates that had been cryopreserved in liquid nitrogen. Parasitized blood was obtained from the rats by cardiac puncture, and the parasites isolated on a diethylaminoethyl-cellulose (DEAE) column (Lanham and Godfrey, 1970) and then suspended in phosphate buffered saline (PBS: 0.1 M sodium phosphate, pH 8.0 containing 0.15 M NaCl) and 1% glucose was added.

Procyclic culture forms of the parasites used in the experiments were obtained by transferring the bloodstream forms into an SDM-79 medium (Brun and Jenni, 1977; Brun and Schonenberger, 1979) containing 10% foetal calf serum. The trypanosomes were cultivated at 27° C with twice weekly passages. No antibiotic was included in the medium. Before use, the parasites were pelleted from the medium by centrifugation (3000 rpm, 10 min, 4° C) and washed once in PBS in order to remove serum components. The parasites were then counted using a haemocytometer equiped with improved Neubauer ruling to give a final concentration of 5 x 10^6 parasites/ml in PBS pH 7.4.

2.4 Preparation of erythrocytes

Blood from a clean New Zealand white rabbit was collected in heparin. The erythrocytes were washed five times by centrifugation (2,000 rpm, 10 min, 27° C) and resuspended each time in PBS, pH 7.4. Prior to use in agglutination assay, the erythrocytes were adjusted in the same buffer to give a final erythrocyte suspension of 2% (packed cell volume), which was approximately 10^7 cells/ml.

2.5 Dissection of flies and preparation of the midgut homogenate

Male tsetse flies (24 h after emergence) were fed from the ears of restrained lop-eared rabbits for approximately 15 min and then starved for 72 h. The flies were then immobilized by brief chilling after which the midguts were carefully dissected in ice cold 20 mM Tris/HCl buffer, pH 8.0. Fifty midguts were washed once in ice-cold buffer, suspended in 1 ml of the buffer and homogenized using a Virtis homogenizer (Gardiner, USA). The resulting homogenate was left to stand for 2 h at 4° C and then centrifuged (12,000 rpm, 15 min, 4° C) using a Beckman Microfuge (USA). The supernatant solution was transfered into a microfuge tube and stored at -20° C until required.

2.6 Purification

The trypanoagglutinin was purified using a Mono Q HR 5/5 anion-exchange column (Pharmacia, Uppsala, Sweden) attached to an FPLC system equiped with a model GP-250 gradient programmer. Crude midgut homogenate was thawed and filtered through a 0.2 μm pore filter (Millipore, Swinex B) prior to application onto the column. Buffer A (20 mM Tris/ HCl, pH 8.0) and buffer B (20 mM Tris/ HCl, pH 8.0 containing 0.5 M NaCl) were prepared, filtered and degassed using a Buchner filter apparatus with a 0.2 μm membrane. Midgut homogenate (~13.5 mg protein) was then applied to the column which was pre-equilibrated with buffer A. The column was washed with 20 ml of buffer A at the rate of 0.5 ml/min. The absorbances were monitored at 280 nm. Elution of the bound proteins was carried out using a linear gradient of 0-350 mM NaCl and finally 500 mM NaCl. Each of the fractions obtained (1 ml) was tested for both trypsin and agglutination activities. The fractions which showed agglutination activity were pooled, concentrated using

polyethylene glycol (PEG) at 4° C and then dialyzed against buffer A. This sample was reapplied to the Mono Q column, washed with 5 ml of buffer A and eluted with a gradient of 150-300 mM NaCl. Fractions were monitored at 280 nm and those corresponding to the bound peak were pooled and concentrated using PEG at 4° C.

2.6.1 Agglutination assays

Agglutination assays were carried out in Microtitre plates (Nunc, Denmark). Double serial dilutions of the crude midgut homogenates and the FPLC fractions were prepared using PBS containing 0.1 mg/ ml $MgCl_2$ and 0.13 mg/ ml $CaCl_2$. An equal volume of parasite suspension that contained 5.0 x 10^n parasites/ml or 2% erythrocyte suspension was added to each dilution. The plates were incubated at 27° C for 2 h and scored for agglutination activity using an inverted microscope (Leitz Dialux, Germany). All agglutination tests were carried out in duplicates with controls consisting only of parasites or erythrocytes and PBS. Agglutination activity is presented as reciprocals of end point dilution titres (maximum dilution which showed complete agglutination of the parasites or erythrocytes).

2.6.2 Enzyme assay

The FPLC fractions obtained were examined for trypsin activity using a chromogenic substrate, carbobenzoxy-val-gly-arg-4-nitranilide acetate (Chromozym-TRY, Boehringer-Manhein, FRG). The Reaction mixtures contained chromozym-TRY (40 μmol) and FPLC fractions in 100 mM Tris/ HCl, pH 8.0 in 1 ml assay system. The reactions were initiated by the addition of the substrate. Increase in absorbance at 405 nm was monitored using a Beckman model DU 50

spectrophotometer (Palo Alto, CA, USA) fitted with a thermostat control. The change in molar extinction at 405 nm ($\in_{405}$=8,800 : Erlanger *et al.*, 1961) was used to determine the amount of substrate hydrolysed. One trypsin unit is the amount of enzyme required to hydrolyse 1.0 μmol of chromozym-TRY per min at 30° C

2.7 Protein estimation

Protein concentration was measured by the Bicinchoninic acid (BCA) assay system (Pierce, Rockford,USA), according to the instructions supplied by the manufacturer. Bovine serum albumin (BSA fraction V) was used as the protein standard and absorbance monitored at 562 nm.

2.8 Electrophoresis

Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Gradients (4-20%) were cast using a gradient marker (BRL). Samples were mixed in an equal volume of sample buffer (130 mM Tris/ HCl, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1% β mercaptoethanol, pH 6.8) and boiled for 5 minutes in a water bath, prior to application onto the gel. Running buffer (25 mM Tris/ HCl, 192 mM glycine, 0.1% SDS, pH 8.3) was used and electrophoresis was performed at room temperature at a constant current of 25 mA.

Gels were stained with Coomassie Brilliant Blue (Weber and Osborn, 1969) in a solution of acetic acid, methanol and distilled water (9.2:50:40.8) overnight. The gels were then soaked with several changes of destaining solution (acetic acid, methanol, distilled water (9.2:50:40.8) for 12-15 h at room temperature. Gels were

also stained for protein by the silver staining method of Wray *et al.* (1981). Destained gels were preserved in 7% acetic acid.

Electrophoresis under non-denaturing conditions was carried out as described for SDS-PAGE except that buffers did not contain SDS and β-mercaptoethanol. The samples were also not heated.

2.9 Molecular weight estimation

Both native-PAGE and SDS-PAGE were used to determine the molecular weight of the isolated trypanoagglutinin. For native molecular weight estimation, Pharmacia protein standards used were : α-lactal albumin (M_r~ 14,400), Trypsin Inhibitor (M_r~ 20,100), Carbonic anhydrase (M_r~ 30,000), ovalbumin (M_r~ 43,000), albumin (M_r~ 67,000), phosphory- lase b (M_r~ 94,000), lactate dehydrogenase (M_r~ 140,000), catalase (M_r~ 232,000), Ferritin (M_r~ 440,000), thyroglobulin (M_r~ 669,000). For SDS-PAGE molecular weight estimation, Bio-Rad protein standards were used: lysozyme (M_r~ 14,400), Trypsin inhibitor (M_r~ 21,500), bovine carbonic anhydrase (M_r~ 31,000), ovalbumin (M_r~ 45,000), BSA (M_r~ 66,200), phosphorylase b (M_r~ 97,400). The molecular weight of the trypanoagglutinin was estimated from the plots of log. molecular weights versus the relative migration of the standards.

2.10 Trypsin activity on PAGE

2.10.1 Staining for trypsin activity

Trypsin activity on PAGE was determined using Benzoyl-Arg-β-Naphthalamide (BANA) as substrate. Native PAGE was carried out at 4° C using duplicate of the sample on the gel. One half of the gel was stained for trypsin

activity while the other half was stained for protein. Immediately after electrophoresis, half of the gel was incubated in 50 ml of 0.1 M phosphate buffer, pH 8.0 that contained 0.5 mg/ml BANA, 10% N,N-Dimethylformamide (DMF) (Sigma) at 37° C for 30 min. Hydrolysis of the substrate yields free naphthylamide. Thereafter 5 mg/ml of Diorthonisidine tetrazotised (Sigma) was added. The naphthalamide released is coupled with the diazo blue B resulting in distinct bands of orange colour. The reaction was then stopped by adding 0.1% acetic acid.

Trypsin staining on SDS-PAGE was also carried out but the gel was incubated in 2.5% Triton-X100 (Sigma) at 25° C to remove SDS prior to staining for trypsin activity as described above.

2.10.2 Trypsin activity using autoradiography

Crude midgut and isolated trypanoagglutinin samples were incubated with 5 μCi [1,3-^{3}H] diisopropyl-fluorophosphate (DFP) (specific activity 35 Ci/mmol, Amersham, U.K) for 18 h at 4° C. In the case of crude midgut sample (in 50 mM Tris/HCl, 0.1 M $CaCl_2$, pH 8), 8 mM TPCK (tosylamide-2-phenylethyl chloromethyl ketone; chymotrypsin inhibitor) was added and incubated for 4 h prior to incubation with DFP. Both native and SDS-PAGE of [1,3-^{3}H]DIP-trypsin derivatives were performed as described in section 2.8. Gels were stained with Coomassie brilliant blue and destained overnight. To enhance the radioactivity signal, the gels were incubated in ENH^{3}ANCE (Amersham) for 30 min at room temperature and dried in a GSD-4 slab gel drier (Pharmacia) under vacuum overnight at 12 volts constant heating. The dried gel was then placed in an X-ray cassette with an intensifying screen and exposed to an X-ray film (Fuji-RX, 18cm x 24cm) for 1 day at -70^0 C. The film was developed by soaking in developer (Kodak) for 5 min then washing it

in water (2 min) followed by soaking in fixer (Kodak) for 5 min. The film was then washed in water (2 min) and dried in air for visualization.

2.11 Heat stability tests

Thermal stability of the isolated trypanoagglutinin was determined. Samples (25 μl) of the trypanoagglutinin (0.15 mg/ml) were separately incubated in a waterbath at 27, 37, 42, 50, 60 and 70^0 C for 20 minutes. The samples were then cooled to room temperature and agglutination assay carried out as described above.

2.12 Inhibition tests

2.12.1 Effect of sugars on agglutination activity

The effects of glucose, galactose, mannose, galactosamine (Sigma), glucosamine, N-acetyl glucosamine, methyl α-D-glucopyranoside, methyl- β-D-glucopyranoside (Serva), sucrose, maltose, lactose (Laborama, Nairobi) on agglutination activity of trypanoagglutinin was assessed as follows. Stock solutions (500 mM) of the sugars were prepared in PBS, pH 7.4. Double serial dilutions of trypanoagglutinin (0.15 mg/ml) were preincubated with the various sugars for 30 minutes. Parasites or erythrocytes were then added and agglutination activity determined as described above. Control consisted of the same assay mixture in the absence of sugar.

2.12.2 Effect of Soybean Trypsin Inhibitor (STI) on agglutination activity

The effect of protease inhibitor, soybean trypsin inhibitor (STI; Millipore Corp, Freehad, USA), on agglutination activity was determined. Increasing concentrations

of STI (0-1 mg/ml) were preincubated with isolated trypanoagglutinin (~0.15 mg/ml) and trypsin activity at each STI concentration was assayed. Double serial dilutions of each incubation mixture was then prepared, parasites or erythrocytes added and agglutination activity determined as described above.

2.13 Immunological studies

2.13.1 Raising antibodies against the trypanoagglutinin

Antibodies against the trypanoagglutinin were raised in a male New Zealand white rabbit. The rabbit was bled from the marginal ear vein for preimmune serum and then injected subcutaneously at different sites with 150 μg of the purified trypanoagglutinin emulsified with an equal volume of Freunds complete adjuvant (FCA). A booster injection of 150 μg protein was administered intramuscularly on the 8th and 24th day. The rabbit was then bled from the marginal ear vein 7 days after the 2nd booster.

To obtain antiserum, the blood was allowed to clot for 6 h at room temperature and then overnight at 4° C. Serum was then separated from clots by centrifugation (1500 rpm, 20 min) and stored at -20° C.

2.13.2 Double radial immunodiffusions

Antibodies were detected by double radial immunodiffusion as described by Ouchterlony (1968). Molten agarose (1% in PBS) was carefully poured onto a glass plate and left to set. Six wells were punched peripherally around a central well. To detect the presence of antibodies against the trypanoagglutinin (antigen), antiserum was placed in the central well and the antigen in the peripheral wells. To test for cross-reactivity, the midgut samples from *G. longipennis, G. m. morsitans,*

Phlebotomus duboscqi, stomoxys calcitrans and *Aedis aegypti* were poured in the peripheral wells with the antiserum in the central well. Diffusion was allowed to take place in a humid chamber at room temperature for at least 24 h. The plates were then washed extensively with PBS to remove unprecipitated proteins, then dried by blotting with 3 mm filter papers. The gel was then stained with Coomassie Brilliant Blue and destained as described in section 2.9.

2.13.3 Immunoblotting

The immunological reactivity of the antiserum raised against the trypanoagglutinin was tested by immunoblotting. Midgut samples from the various insect species were also tested for cross-reactivity with the antiserum.

Samples were separated by SDS-PAGE and electro-phoretically transferred onto a nitrocellulose paper as described by Towbin *et al.*, (1979) using an LKB 2117-NOVABLOT electrophoretic transfer kit. The transfer was conducted for 2½ h in 48 mM Tris 39 mM glycine, pH 8.5 that contained 20% methanol, at 0.8 mA sq cm^{-1}. The success of the transfer was ascertained by staining with Rouge Ponceau (0.05% in 3% TCA). The nitrocellulose paper was destained by washing in distilled water and then rinsed briefly in Tris buffered saline (TBS) (20 mM Tris/HCl pH 7.5, 500 mM NaCl) containing 5% fat-free milk powder (TBS-milk 5%). Non-specific binding sites on the paper was blocked in the same solution (TBS-milk 5%). The blots were then washed with TBS for 5 min and incubated overnight in antiserum solution (diluted x50 in TBS-milk 1%) at room temperature with shaking. The blots were then washed 4 times for 15 min each in TBS-milk 1% and then washed once with TBS. After washing, the nitrocellulose blots were immersed in horse- radish peroxidase labelled anti-rabbit IgG diluted 1000x in TBS-milk 1%, and incubated for

2 h at room temperature with shaking. Unbound secondary antibody was washed off with TBS-milk 3x for 10 min each with constant shaking. The blot was then rinsed once in TBS to remove milk and then in 10 mM Tris/HCl buffer pH 6.8 (TB). The blot was then incubated in substrate solution (0.3% 4-chloro-1-naphthol in methanol diluted 5x with TB and containing 0.33 μl of H_2O_2 (30%) per ml of final substrate solution) until the bands became visible (5-10 min). The excess substrate was washed off with distilled water and the blot preserved by keeping it between filter papers.

2.13.4 Immunoinhibition

2.13.4.1 Inhibition of agglutination activity by antisera

The effects of the antiserum raised against the trypanoagglutinin on agglutination activity was investigated *in vitro*. Double serial dilutions of the isolated trypanoagglutinin (0.15 mg/ml) were preincubated with antiserum for 30 min. Agglutination assay was then performed as described in section 2.6.1. Controls consisted of serial dilutions of trypanoagglutinin only and serial dilutions of trypanoagglutinin incubated with normal rabbit serum .

2.13.4.2 Inhibition of trypsin activity by antisera

The effects of antisera raised against purified trypanoagglutinin on trypsin activity of the purified molecule was studied *in vitro*. Commercial kit of casein substrate gels (Bio-Rad, Watford) was used to determine trypsin levels by measuring their casein-lytic activity (Stiles *et al.*, 1991). Trypanoagglutinin samples were placed in 0.3 cm wide wells cut in a casein substrate gel in 8.5 x 6.5 cm diffusion plates. The plates were covered with plastic lids and incubated at 27° C

for 16 h. The resultant lytic zones around the wells were enhanced by flooding the gels with 3% acetic acid (which also stopped the reaction) and then measured with Vernier callipers. This was used as the positive control. To determine the effect of antisera, increasing concentration of antisera (0-12 μg/ml protein) was mixed with trypanoagglutinin in the casein gel wells and assayed for trypsin activity as described above. Serial dilutions of commercial trypsin (Bovine pancreatic, Sigma) in PBS pH 7.4 were also assayed to obtain a standard curve. The enzyme activity measured in terms of diffusion zone diameter, increases linearly with logarithm of trypsin concentration, provided the protease levels exceed the equivalent of 1.25 μg/ml (0.1 IU/ml) trypsin.

CHAPTER 3

RESULTS

3.1 Purification of the trypanoagglutinin

The purification of the trypanoagglutinin from the midgut of tsetse fly, *G. longipennis*, was achieved by a two step procedure involving anion exchange chromatography.

The initial step involved the separation of the crude midgut homogenate on a Mono Q anion-exchange FPLC column. The column was washed with 20 mM Tris/HCl pH 8 and the bound proteins eluted by NaCl gradient. Fractions were monitored for agglutination and trypsin activities. The results showed that both the unbound (Peak I, V_e = 3.5 ml) and bound (Peak II, V_e = 30 ml) fractions had trypsin activity (Fig. 1). However, the agglutination activity co-eluted with trypsin activity in the bound fraction. Agglutination activity was not detected in any other fraction. Fractions 29-31 (Peak II) which showed agglutination activity were then pooled, concentrated using PEG and re-chromatographed on the Mono Q column. The profile showed a single protein peak (Fig. 2).

Purity of the isolated trypanoagglutinin was ascertained by non-denaturing PAGE (Fig. 3) on which a single protein band was observed. The isolated trypanoagglutinin had a trypsin specific activity of 82 μmoles/ min/ mg protein.

Fig. 1 **Separation of crude midgut homogenate by Anion Exchange Chromatography**

Approximately 13.5 mg of protein was loaded onto a Mono Q colum equilibrated with 20 mM Tris/ HCl, pH 8.0. The flow rate was 0.5 ml min^{-1} and elution was by linear NaCl gradient, 0 - 350 mM (-•••-). Fractions (1 ml) were assayed for agglutination activity (ª), trypsin ac (---) and protein content, A_{280} (———).

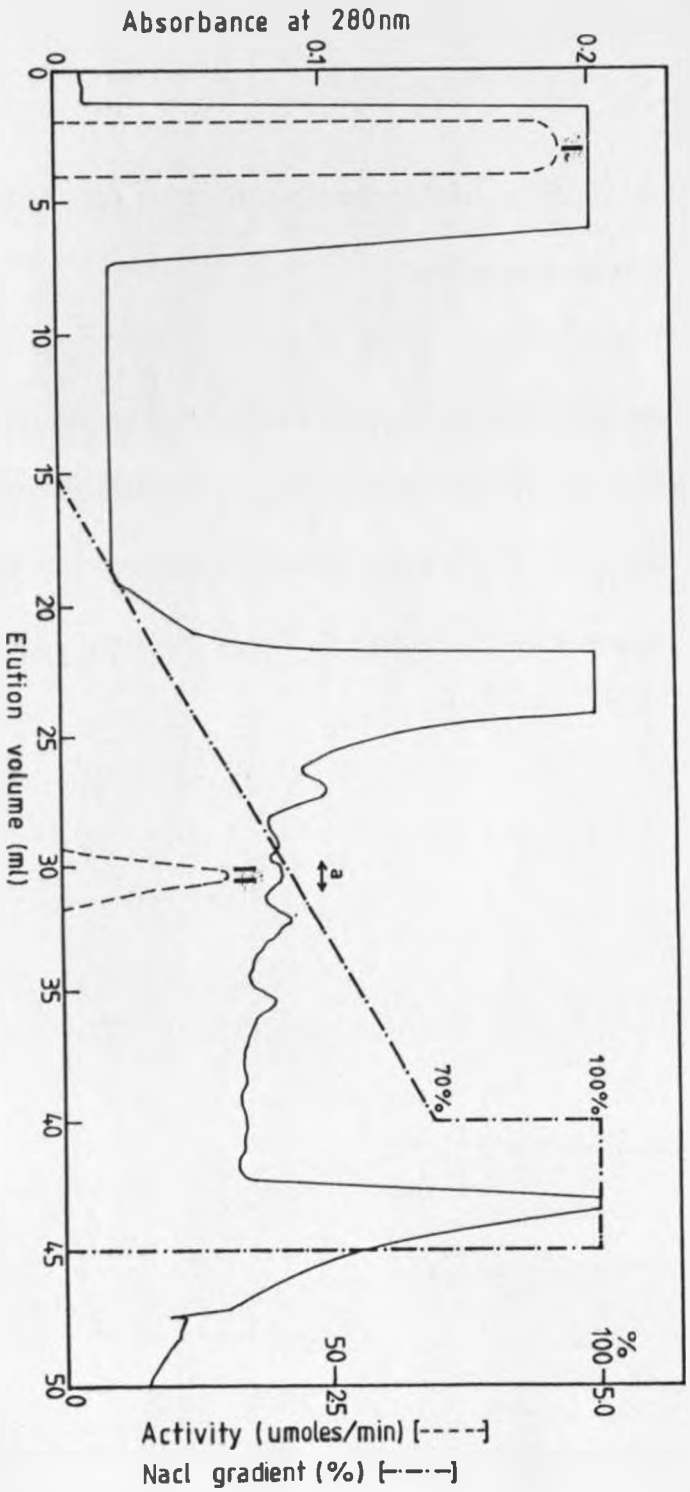
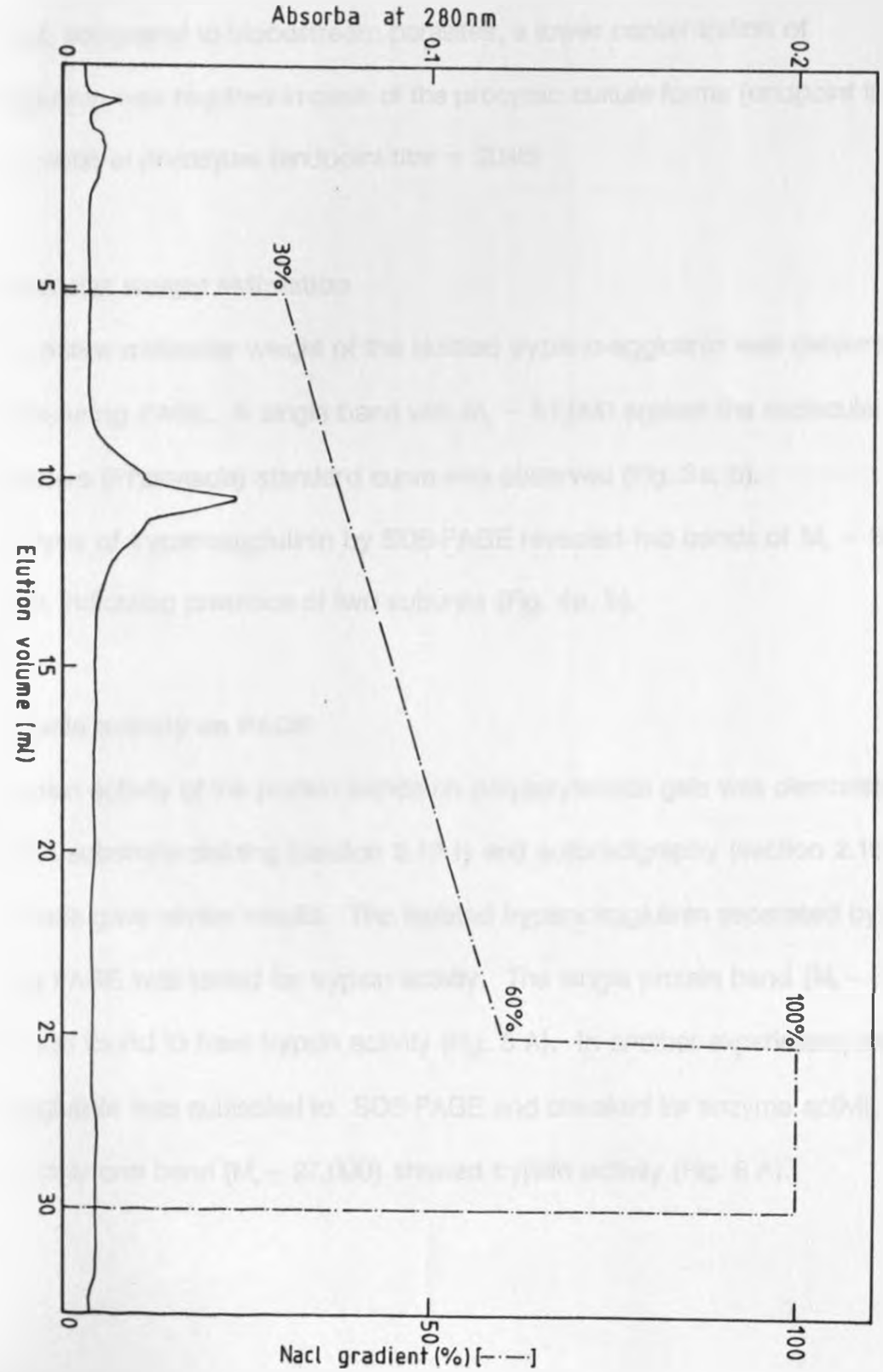

Absorbance at 280nm
0
0.1
0.2
I
II
a
70%
100%
Elution volume (ml)
0
5
10
15
20
25
30
35
40
45
50
0
25
50
%
100
5.0
Activity (umoles/min) [----]
Nacl gradient (%) [-·-·-]

Fig. 2 **Purification of trypanoagglutinin by Anion Exchange Chromatography**

Fractions 29-31 (Peak II) which showed agglutination activity were pooled, concentrated to a volume of 1.0 ml and dialysed against Tris HCl, pH 8. The sample was rechromatographed using a Mono Q column . Elution was by NaCl gradient, 150 mM - 300mM (-•••-) at a flow rate of 0.5 ml min^{-1}. Protein content, A_{280} (__).


Absorba at 280nm
0
0.1
0.2
30%
60%
100%
5
10
15
20
25
30
Elution volume (ml)
0
50
100
Nacl gradient (%) [- · —]

3.2 Properties of the trypanoagglutinin

3.2.1 Agglutination activity

The ability of the trypanoagglutinin to agglutinate erythrocytes, bloodstream and procyclic trypanosomes was assessed. Double serial dilutions of isolated trypano-agglutinin (0.15 mg/ml) were assayed for agglutination activity. The results (Table 1) showed that, compared to bloodstream parasites, a lower concentration of trypanoagglutinin was required in case of the procyclic culture forms (endpoint titre = 4096) and rabbit erythrocytes (endpoint titre = 2048)

3.2.2 Molecular weight estimation

The native molecular weight of the isolated trypano-agglutinin was determined by non-denaturing PAGE. A single band with M_r ~ 61,000 against the molecular weight markers (Pharmacia) standard curve was observed (Fig. 3a, b).

Analysis of trypanoagglutinin by SDS-PAGE revealed two bands of M_r ~ 33,000 and 27,000, indicating presence of two subunits (Fig. 4a, b).

3.2.3 Trypsin activity on PAGE

Trypsin activity of the protein bands on polyacrylamide gels was demonstrated using BANA substrate staining (section 2.10.1) and autoradigraphy (section 2.10.2). Both methods gave similar results. The isolated trypanoagglutinin separated by non-denaturing PAGE was tested for trypsin activity. The single protein band (M_r~ 61,000) obtained was found to have trypsin activity (Fig. 5 A). In another experiment, isolated trypanoagglutinin was subjected to SDS-PAGE and checked for enzyme activity. In this case, only one band (M_r~ 27,000) showed trypsin activity (Fig. 6 A).

Table 1 **Agglutination activity using *T. b. brucei* and rabbit erythrocytes**

Parasites or erythrocytes	Agglutination titre
Control	0
Bloodstream forms	128
Procyclic forms	4096
Rabbit erythrocytes	2048

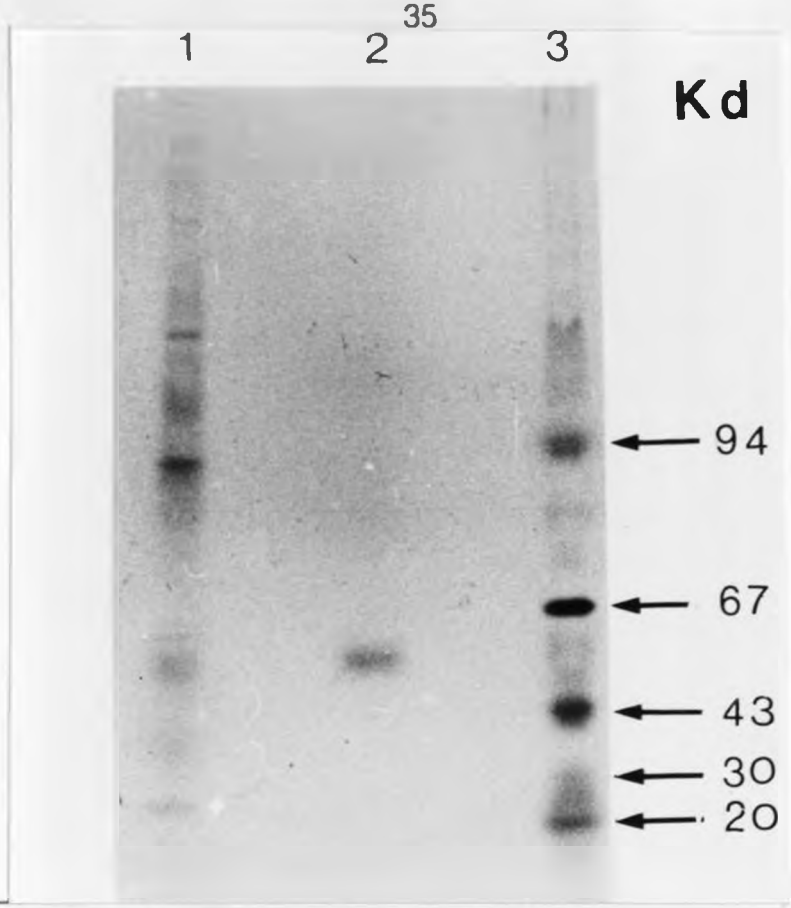
Doubling serial dilutions of isolated trypanoagglutinin (~0.15 mg/ml) were prepared and agglutination activity determined as described in **Materials and Methods.** Agglutination titre is expressed as the reciprocal of end point dilutions (maximum dilution where complete agglutination of parasites or erythrocytes was observed).

Fig. 3a **Molecular weight estimation on non-denaturing PAGE.** Crude mi homogenate and isolated trypanoagglutinin were subjected to native PAGE (4-20%)

1. Crude *G. longipennis* midgut homogenate (~60 μg)
2. Isolated trypanoagglutinin (~9 μg)
3. Low molecular weight standards (10 μl)

   (Pharmacia)

Fig. 3b **Standard curve of log molecular weight against relative mobility o (4-20%) non-denaturing polyacrylamide gel**


1
2
3
Kd
94
67
43
30
20

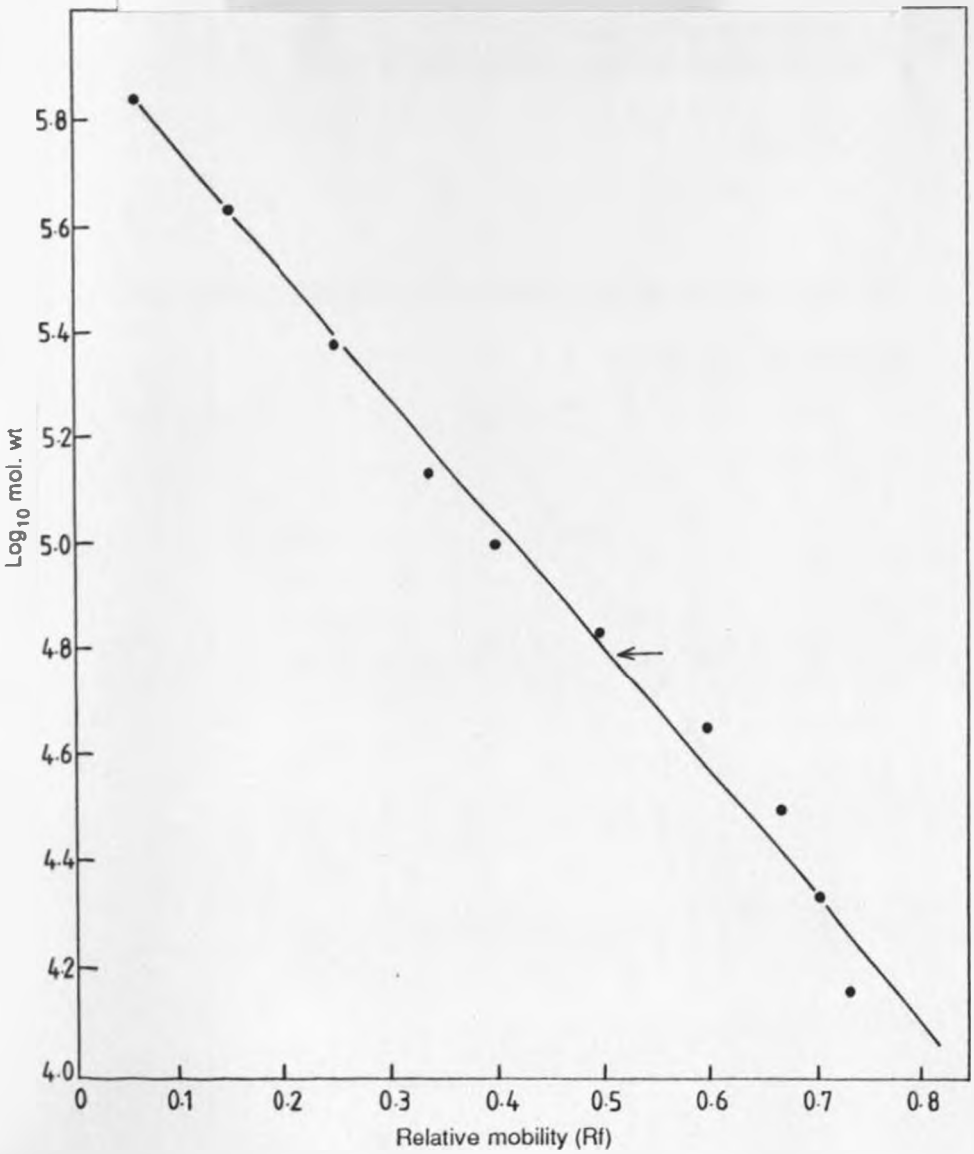

5·8
5·6
5·4
5·2
5·0
4·8
4·6
4·4
4·2
4·0
Log10 mol. wt
0
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
Relative mobility (Rf)

Fig. 4a **SDS-PAGE molecular weight analysis of isolated trypanoagglut**

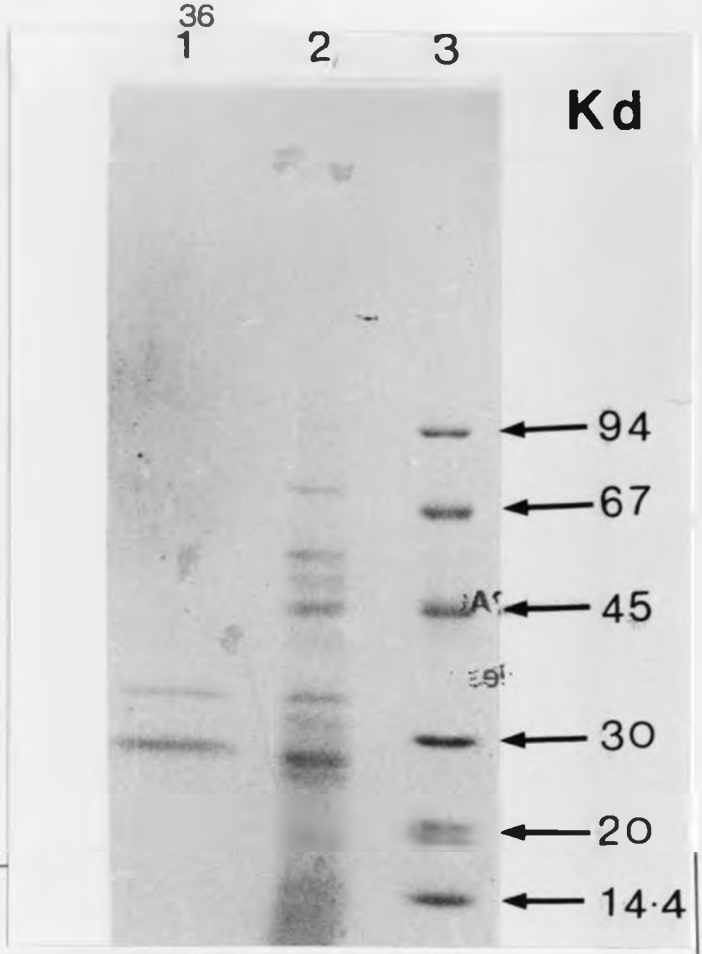
Samples were subjected to electrophoresis on a 4-20% gradient ge
under denaturing conditions

1. Isolated trypanoagglutinin (~4.5 μg)
2. Crude *G. longipennis* midgut homogenate (~30 μg)
3. Low molecular weight standards (2 μl)

   (Bio-Rad)

Fig. 4b **Standard curve of log molecular weight against relative mobility**

SDS-PAGE (4-20%)


1
2
3
Kd
94
67
45
30
20
14·4

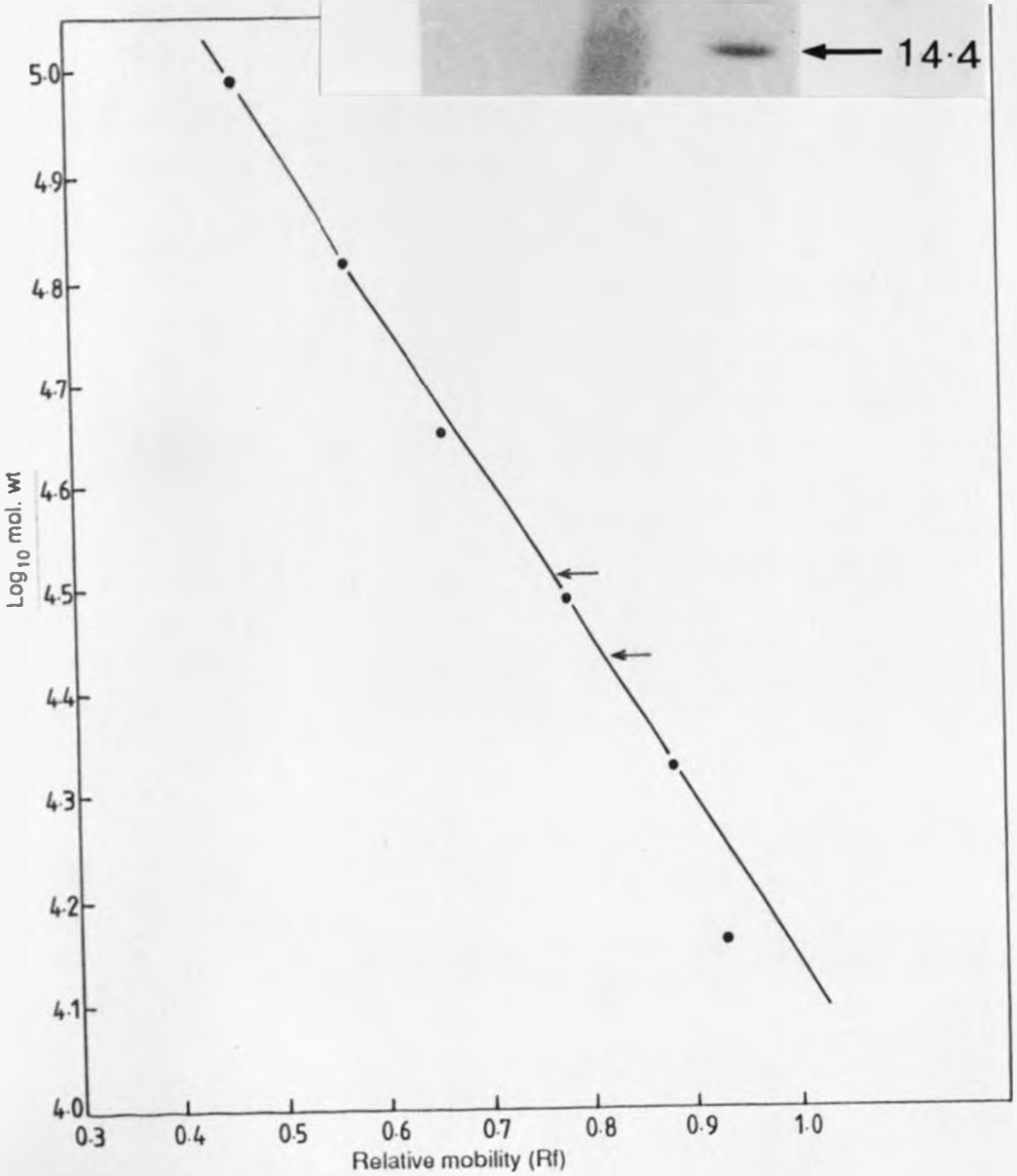

Log10 mol. wt
5·0
4·9
4·8
4·7
4·6
4·5
4·4
4·3
4·2
4·1
4·0
0·3
0·4
0·5
0·6
0·7
0·8
0·9
1·0
Relative mobility (Rf)

Fig. 5 **Trypsin activity on non-denaturing PAGE.** Crude midgut homogenate and trypanoagglutinin were incubated with [1,3-^{3}H]DFP (5 μCi, specific activity 35 Ci/mmol) and separated on native PAGE (4-20%)

A - Autoradiogram of [1, 3-^{3}H]DIP-Trypsin

B - Protein staining

1 and 3: Crude midgut homogenate (~60 μg)

2 and 4: Isolated trypanoagglutinin (~9 μg)

5: Low molecular weight standards (10 μl) (Pharmacia)

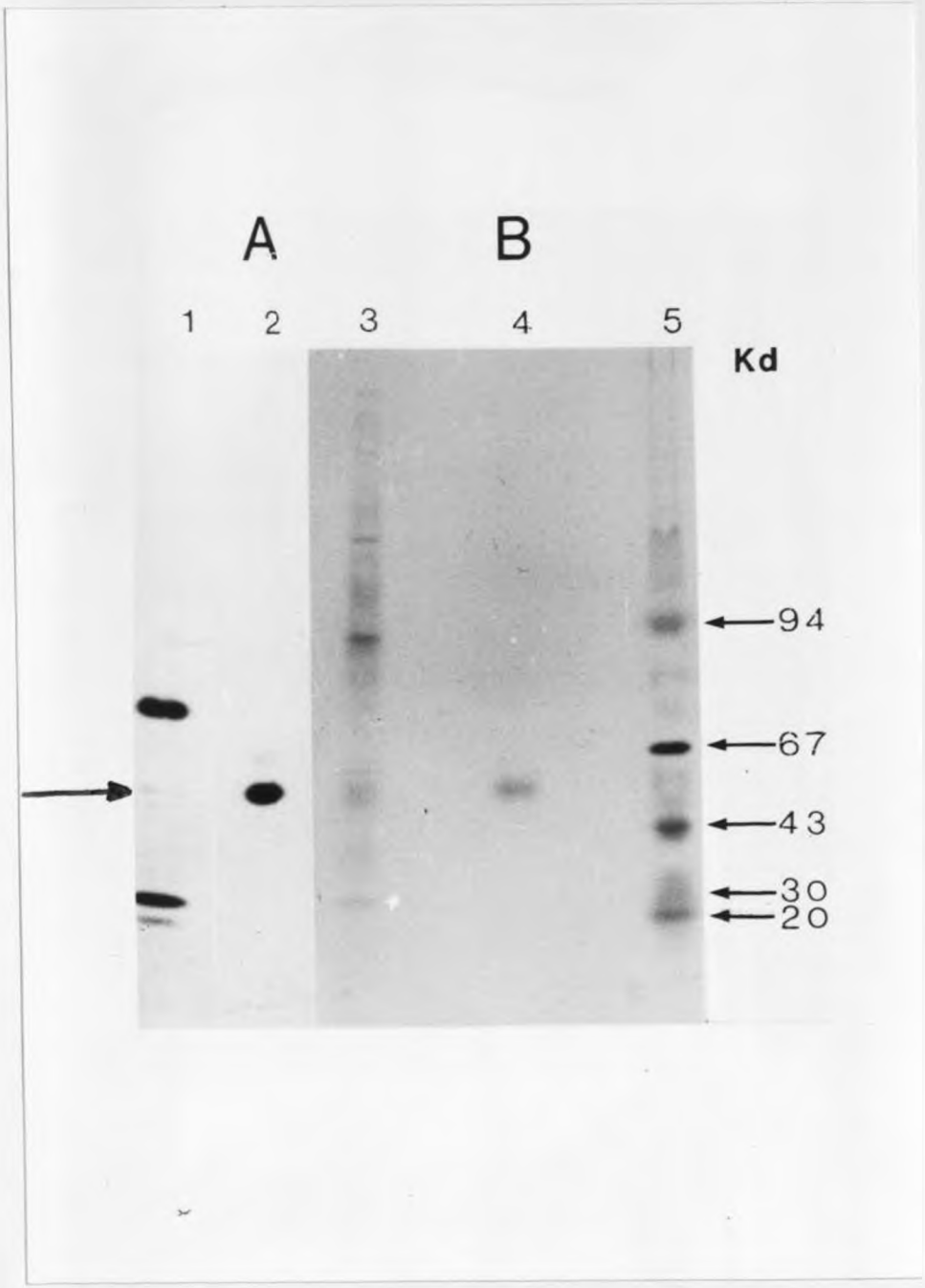

A
B
1
2
3
4
5
Kd
94
67
43
30
20

Fig. 6 **Trypsin activity on SDS-PAGE.** Crude midgut homogenate and isolated trypanoagglutinin were incubated in [1,3-^{3}H]DFP (5 μCi, specific activity 35 Ci/mmol) and separated by electrophoresis (4-20%) under denaturing conditions

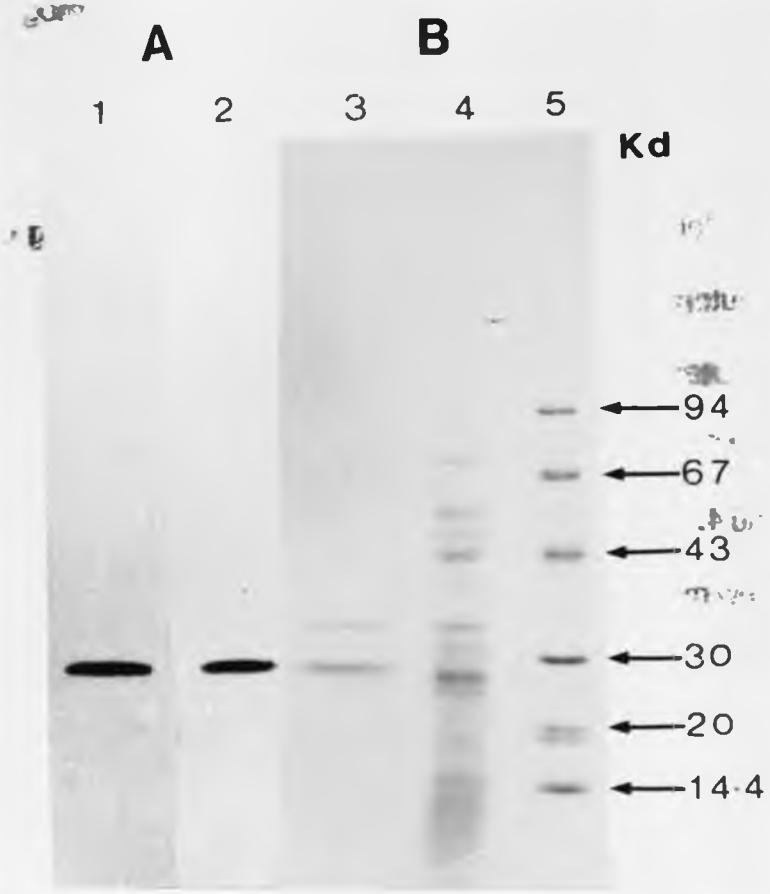
A - Autoradiogram of [1, 3-^{3}H] DIP-Trypsin

B - Protein staining

1 and 3: Isolated trypanoagglutinin (~4.5 μg)

2 and 4: Crude midgut homogenate (~30 μg)

5: Low molecular weight standards (2 μl) (Bio-Rad)


A
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Kd
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67
43
30
20
14·4

3.2.4 Thermal stability

The effect of exposing the trypanoagglutinin to various temperatures was studied. The activity was heat labile, with the agglutination titre being reduced by 90% at temperatures above 50° C (Table 2).

3.2.5 Effect of Inhibitors

3.2.5.1 Effect of sugars on agglutination activity *in vitro*

Agglutination activity was assayed in the presence of the following sugars: glucose, galactose, mannose, glucosamine, galactosamine, N- acetyl glucosamine, methyl α-D-glucopyranoside, methyl β- D-glucopyranoside, sucrose, maltose and lactose (Table 3). Glucosamine strongly inhibited the agglutination activity of the trypanoagglutinin against rabbit erythrocytes and procyclics, and subsequently reduced the agglutination titre by 90.9% and 91.7% for erythrocytes and procyclics, respectively (Table 3). On the other hand, N- acetyl glucosamine reduced the agglutination titre by 27.3% and 25% in the case of erythrocytes and procyclics, respectively (Table 3). All the other sugars had no inhibitory effect on agglutination activity. In another experiment, the effect of decreasing concentrations of glucosamine on agglutination activity was assessed. Double serial dilutions of glucosamine (500 mM) were preincubated with the isolated trypanoagglutinin (4.5 μg/ml) and agglutination assessed. Complete inhibition was observed upto a concentration of 62.5 mM glucosamine below which there was partial inhibition (Table 4).

Table 2 **Determination of thermal stability of trypanoagglutinin**

Temperature (°C)	Agglutination titre	
	Procyclics	Rabbit erythrocytes
27	4096	2048
37	4096	2048
40	2048	1024
50	4	8
60	2	4
70	2	2

Isolated trypanoagglutinin (~0.15 mg/ml) was preincubated for 10 min at the respective temperature and then brought to room temperature. Agglutination activity was determined as described in **Materials and Methods**. Agglutination titre is expressed as the reciprocal of maximum dilution where complete agglutination was observed.

Table 3 Effect of sugars on agglutination activity

Type of sugar	Agglutination titre	
	Procyclics	Rabbit erythrocytes
Control	4096	2048
galactose	4096	2048
mannose	4096	2048
galactosamine	4096	2048
maltose	4096	2048
lactose	4096	2048
methyl α-D-glucopyranoside	4096	2048
methyl β-D-glucopyranoside	4096	2048
glucose	2048	2048
sucrose	1024	1024
N-acetyl D-glucosamine	512	256
D-glucosamine	2	2

Doubling serial dilutions of isolated trypanoagglutinin (~0.15mg/ml) were preincubated (30 min, 27° C) with 500 mM sugar in PBS pH 7.4 and assayed for agglutination activity. Controls consisted of serial dilutions of trypanoagglutinin in the absence of sugar. Agglutination titre is expressed as the reciprocal of end point titre (maximum dilution where complete agglutination of parasites or erythrocytes was observed).

Table 4 Inhibition of agglutination activity by D- glucosamine *in vitro*

	Inhibition titre
Procyclic *T. brucei*	16
Rabbit erythrocytes	8

Double serial dilutions of glucosamine (500 mM) were preincubated with isolated trypanoagglutinin (~4.5 μg/ml) and assayed for agglutination activity. Inhibition titre was expressed as the reciprocal of the lowest dilution of glucosamine where complete inhibition was achieved.

3.2.5.2 Effect of soybean trypsin inhibitor (STI) on agglutination activity

The effect of various concentrations of STI on agglutination activity was assessed *in vitro* using rabbit erythrocytes, bloodstream and procyclic *T. b. brucei*. The results showed that increasing concentrations of STI caused a marked inhibition of agglutination of the bloodstream parasites (Table 5). Compared to control, ~1.0 mg/ml STI resulted in a decrease in agglutination activity of the trypanoagglutinin by ~97%. The same concentration of STI inhibited trypsin activity by 88%. In contrast, agglutination of procyclic parasites was unaffected by STI even at a concentration of ~1.0 mg/ml. On the other hand, the various STI concentrations showed partial inhibition against rabbit erythrocytes (Table 5).

Table 5 **Effect of STI on agglutination activity *in vitro***

Inhibitor	Agglutination titre			Trypsin activity
(mg/ml)	Bloodstream	Procyclics	RBC	(μmol/ml/min)
0.0	128	4096	2048	12.3
0.2	128	4096	p. a.	9.8
0.4	64	4096	p. a.	6.3
0.6	16	4096	p. a.	4.6
0.8	8	4096	p. a.	3.2
1.0	4	4096	p. a.	1.8

Increasing concentrations of STI (0-1 mg/ml) were preincubated with isolated trypanoagglutinin (0.15 mg/ml). Trypsin and agglutination activities were then assayed as described in **Materials and Methods**.

p. a. = partial agglutination was observed in all the wells

3.3 Immunochemistry of the purified trypanoagglutinin

3.3.1 Double radial immunodiffusion analysis

The presence of antibodies against the trypano-agglutinin was detected by double radial immunodiffusion. A high antibody titre was produced after a second booster injection (Fig. 7). A single precipitin band observed when crude midgut from *G. longipennis* was reacted with the antisera confirmed the specificity of the antisera and hence the purity of the isolated trypanoagglutinin (Fig. 8)

Midgut samples from *G. longipennis, G. m. morsitans, Phlebotomus duboscqi, Stomoxys calcitrans* and *Aedis aegypti* were tested for cross-reactivity with antisera against trypanoagglutinin by double radial immunodiffusion. Precipitin bands were observed with midguts from *G. longipennis and G. m. morsitans*. However, no reaction was detected with midguts from other species mentioned above (Fig. 8).

3.3.2 Immunoblots analysis

The immunological reactivity of the isolated trypano-agglutinin separated by SDS-PAGE was tested by immunoblotting. Two discrete bands were observed corresponding to $M_r \sim$ 33,000 and 27,000 (Fig. 9). These results showed that both subunits were immunoreactive towards the antibodies.

Figure 10 shows the results obtained when midgut samples from *G. longipennis, G. m. morsitans, Phlebotomus duboscqi, Aedis aegypti and Stomoxys calcitrans* were reacted with antiserum to trypanoagglutinin. The antibodies detected proteins of mobility close to that of trypanoagglutinin in *G. longipennis* and *G. m. morsitans*. No cross-reactivity was detected in midguts of other species used in this study (Fig. 10).

Fig. 7 : **Double radial immunodiffusion of isolated trypanoagglutinin ag antiserum to trypano-agglutinin.** The central well contained anti (Ab)(20 μl) while the peripheral wells contained isolated trypanoagg (antigen)(5 μg).

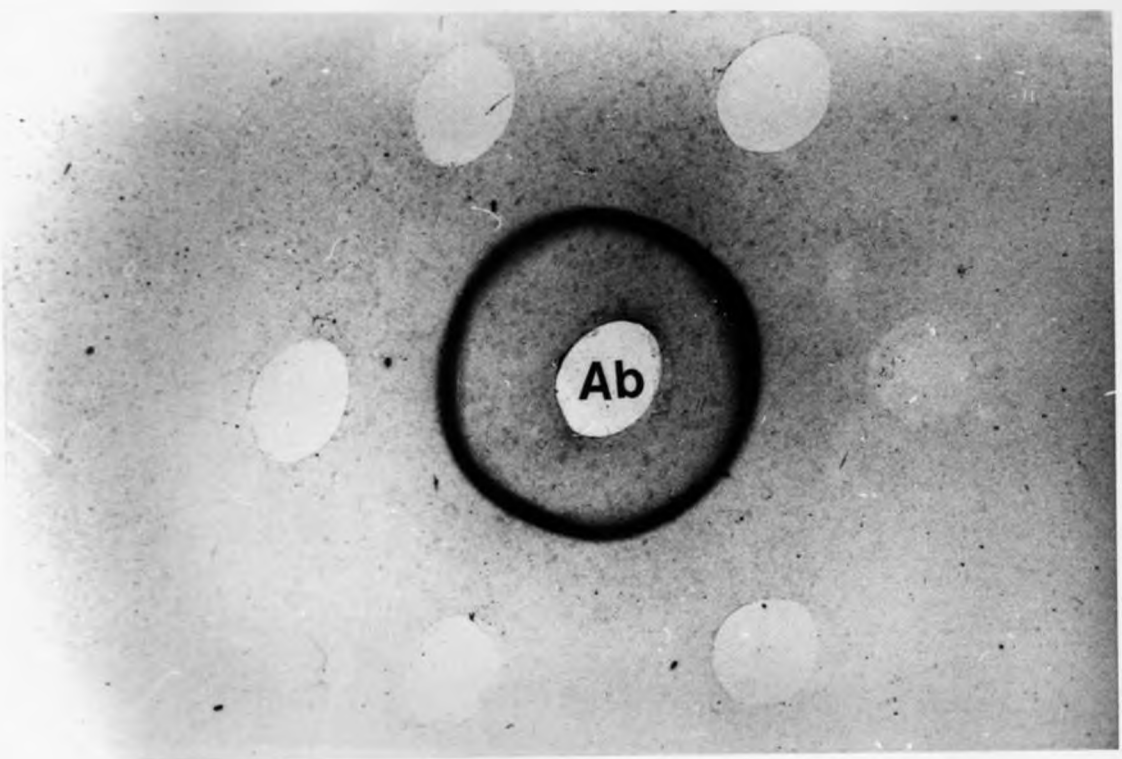
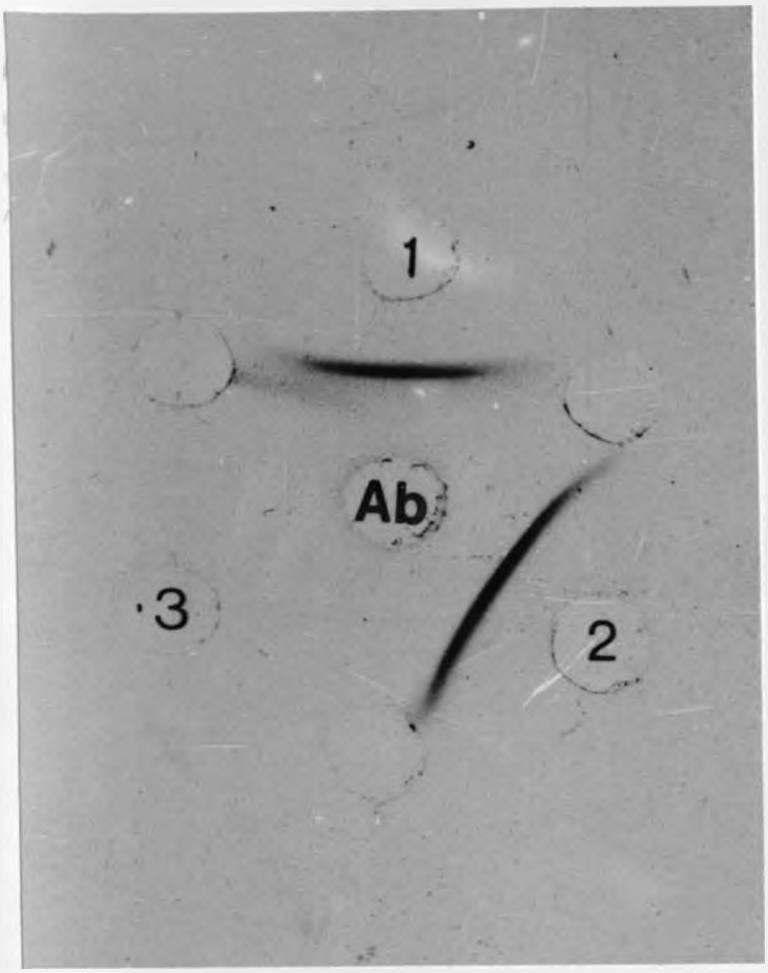

Ab

Fig. 8 : **Double radial immunodiffusion of midgut samples from other insect species against antiserum to trypanoagglutinin.** The central wells contained antiserum (20 μl) while the peripheral wells in A contained:

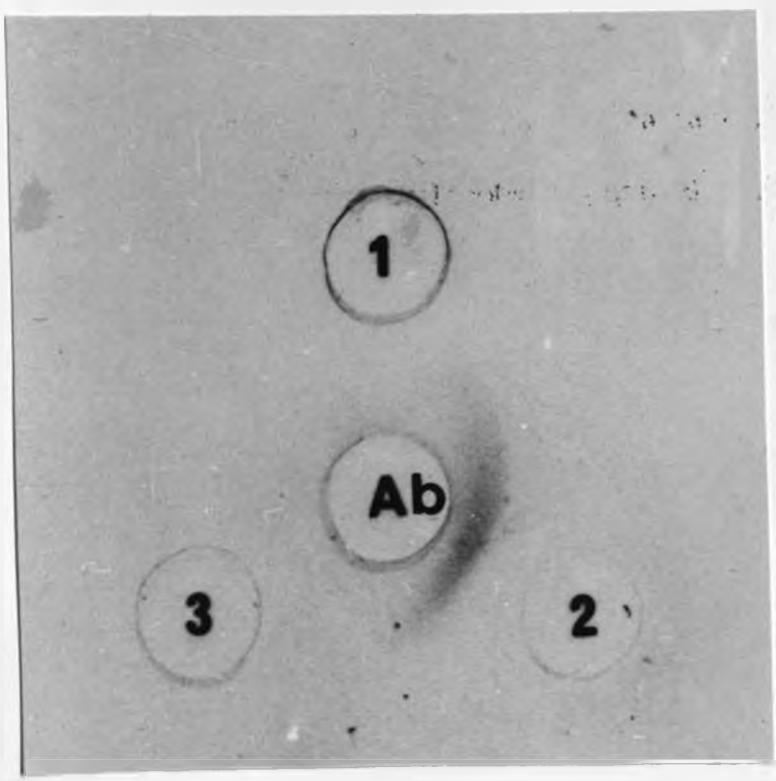
1. *G. longipennis* midgut homogenate
2. Isolated trypanoagglutinin
3. *Phlebotomus dubosqi* midgut homogenate

Peripheral wells in B contained:

1. *Stomoxys calcitrans* midgut homogenate
2. *G. m. morsitans* midgut homogenate
3. *Aedis aegypti* midgut homogenate



A



B

Fig. 9 **Immunoblot analysis of isolated trypanoagglutinin.** Crude *G. longipennis* midgut homogenate (~15 μg) and isolated trypanoagglutinin (~2.5 μg) samples were first separated by SDS-PAGE and then electrophoretically transferred to nitrocellulose paper. The blot was then reacted with the antiserum.

A - Immunoblot

B - Protein staining

1 and 3: Isolated trypanoagglutinin

2 and 4: Crude midgut homogenate

5: Low molecular weight standards (Bio-Rad)

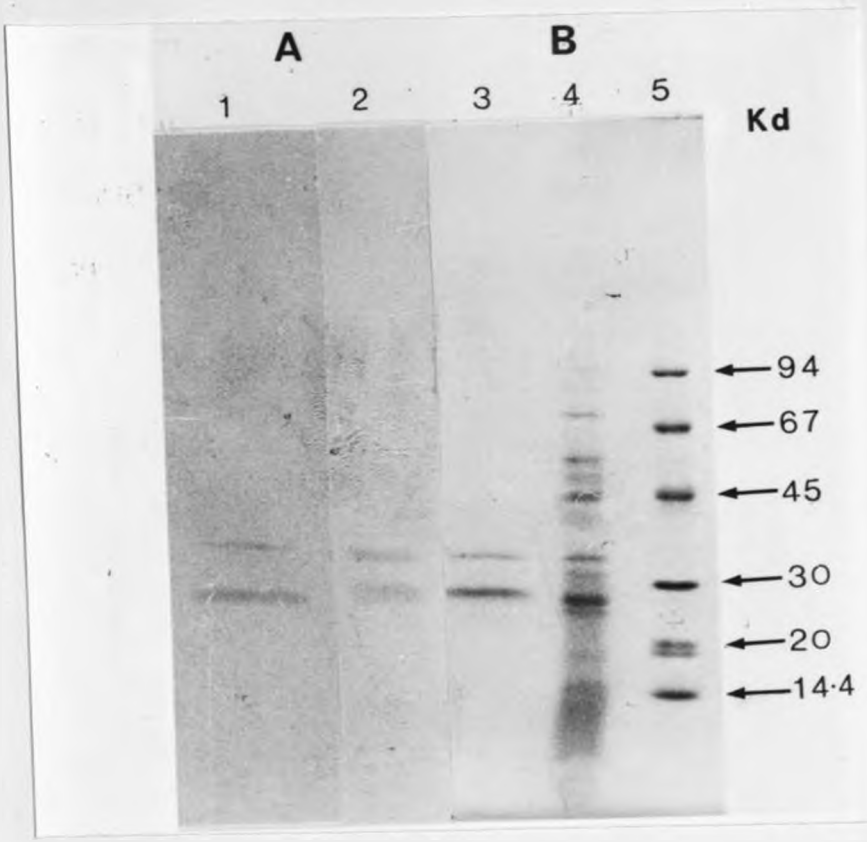

A
B
1
2
3
4
5
Kd
94
67
45
30
20
14·4

Fig. 10 **Immunoblot analysis of midgut homogenates from various insect species.** Aliquots of 40 μl (two fly equivalents) of each midgut homogenate were separated by SDS-PAGE, transferred onto nitrocellulose paper, and reacted with the antiserum.

A - Immunoblot

B - Protein staining

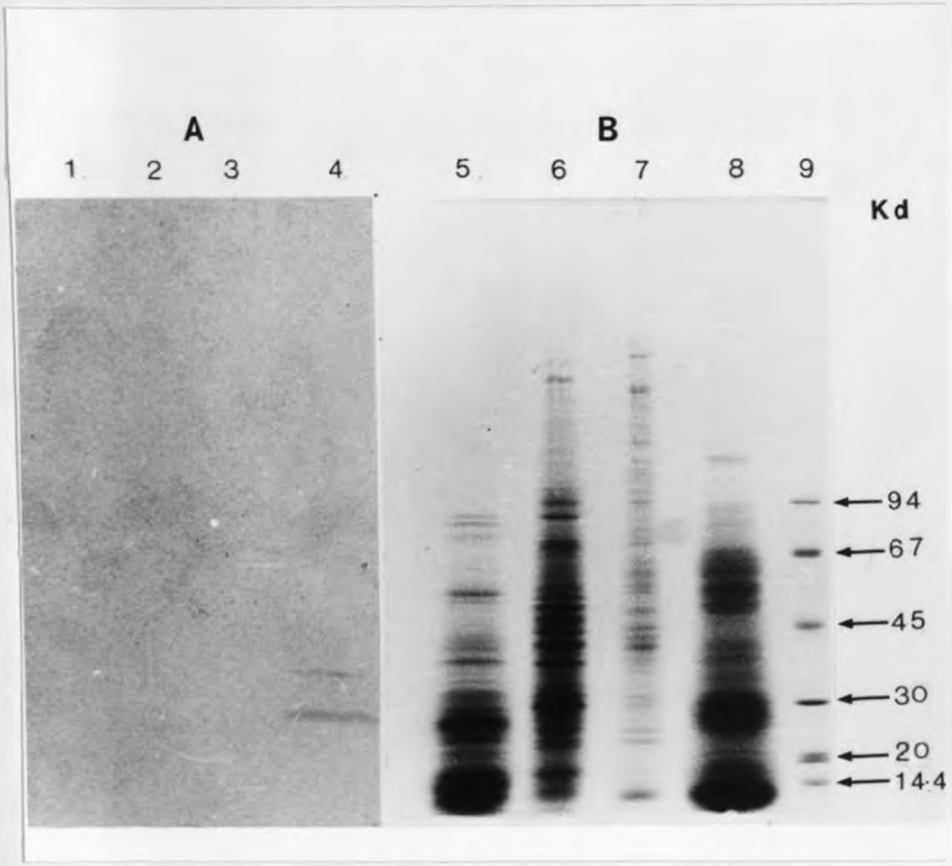
1 and 5: *Stomoxys calcitrans* midgut homogenate

2 and 6: *Aedis aegypti* midgut homogenate

3 and 7: *Phlebotomus dubosqi* midgut homogenate

4 and 8: *G. m. morsitans* midgut homogenate

9: Low molecular weight standards (Bio-Rad)


A
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Kd
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14·4

3.3.3 Immunoinhibition

3.3.3.1 The *in vitro* inhibition of agglutination activity by antisera

The effect of antisera on agglutination activity of the isolated trypanoagglutinin was assessed *in vitro*. The results showed that the agglutination activity against procyclics as well as erthrocytes was strongly inhibited by the antisera raised against the isolated trypanoagglutinin (Table 6). Normal serum had no significant inhibitory effect on agglutination activity (Table 6).

3.3.3.2 Inhibition of trypsin activity by antisera

The ability of antisera raised against trypano-agglutinin to inhibit trypsin activity of the purified molecule was investigated. The results showed that trypsin activity, measured in terms of diffusion zone diameter, decreased progressively with increase in concentrations of antisera (Fig. 12a, b). The activity was reduced by 80% at antisera concentration of $\sim$12 μg/ml protein.

Table 6 **The inhibition of agglutination activity by antisera *in vitro***

Samples	Agglutination titre	
	Procyclics	Rabbit RBC
Trypanoagglutinin	4096	2048
normal serum + trypanoagglutinin	1024	1024
antisera + trypanoagglutinin	0	0

Double serial dilutions of trypanoagglutinin (0.15 mg/ml) were preincubated (30 min, 27° C) with antiserum and agglutination activity assessed. The control incubation consisted of normal serum with trypanoagglutinin, and another consisted of trypanoagglutinin only. Agglutination titre is expressed as the reciprocal of the maximum dilution where complete agglutination of the parasites or erythrocytes was observed.

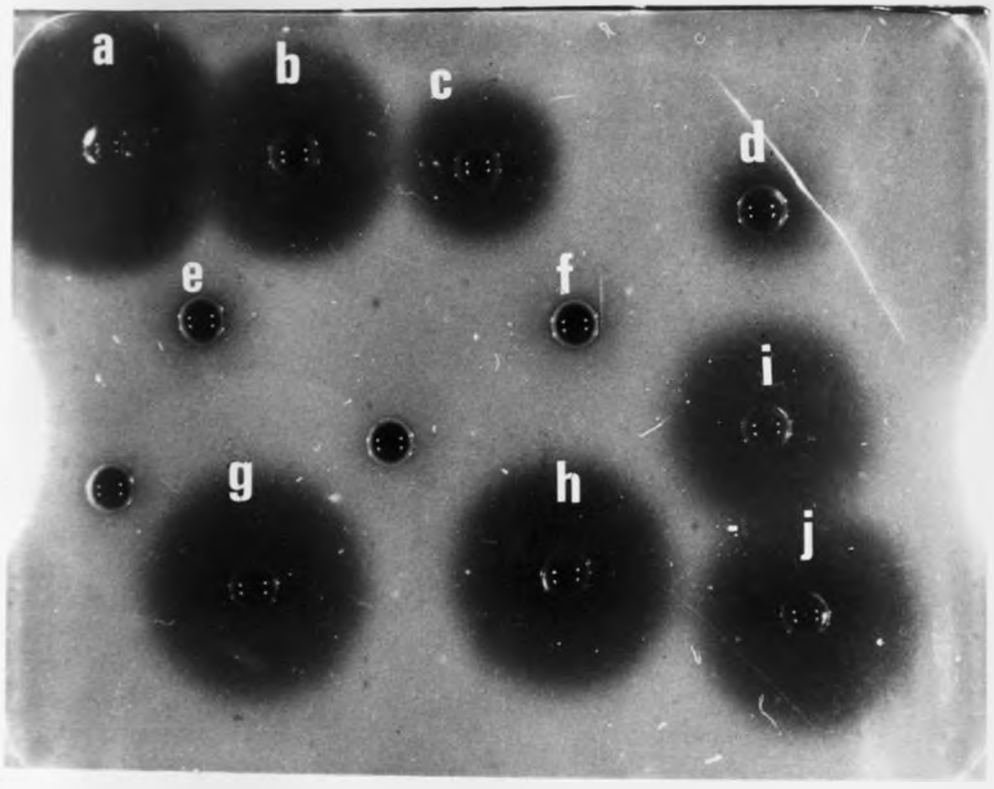
Fig. 11a **Trypsin activity using casein substrate gel.**

Commercial trypsin (Bovine pancreatic, Sigma) and isolated trypanoagglutinin were assayed for trypsin activity using casein substrate gel

a - f Serial dilutions of commercial trypsin for standard curve.

g - j Isolated trypanoagglutinin (~0.15 μg/ml) with normal serum as a control

Fig. 11b **Trypsin standard curve showing diffusion zone diameter vs log$_{10}$ concentration**


a
b
c
d
e
f
g
h
i
j

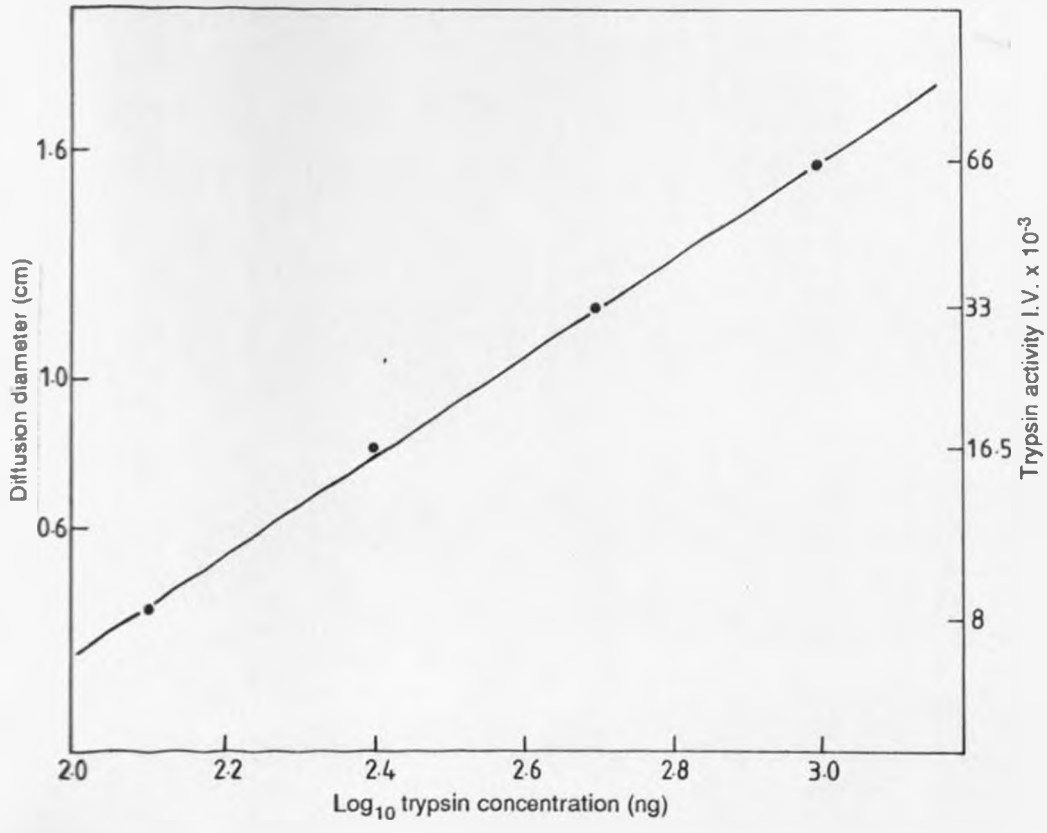
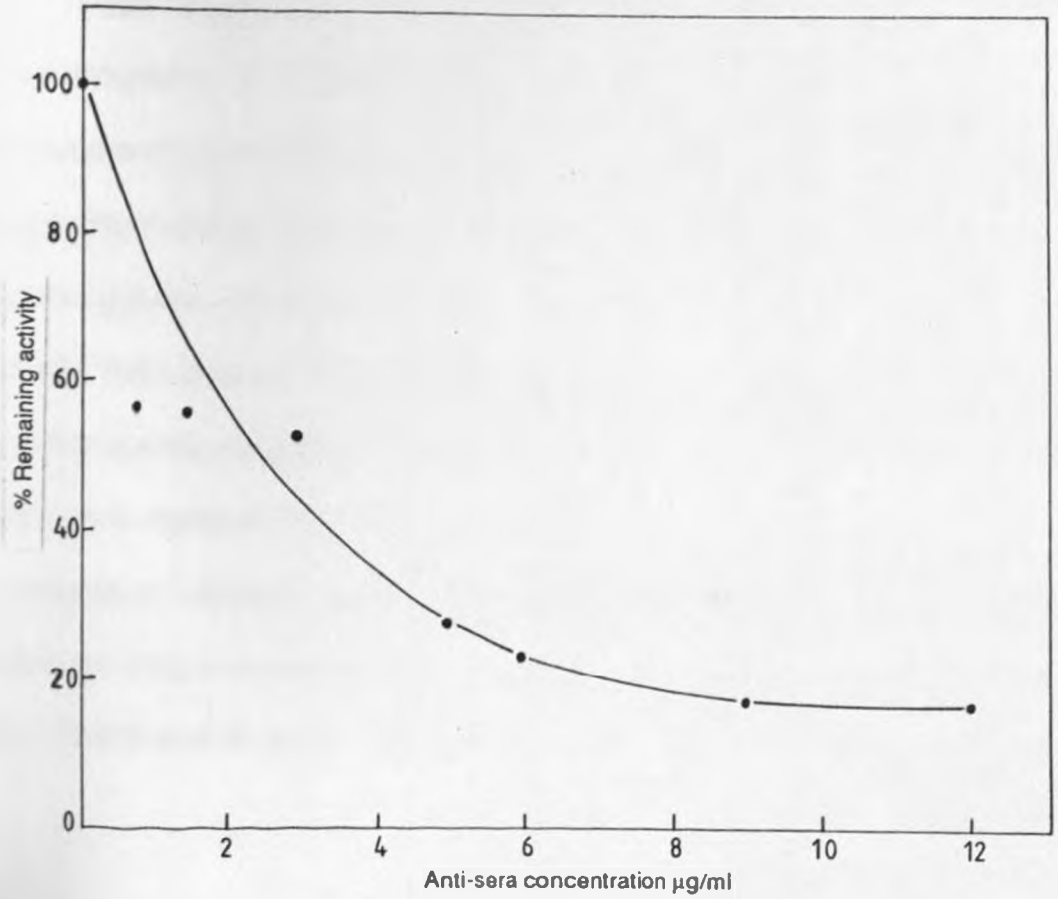
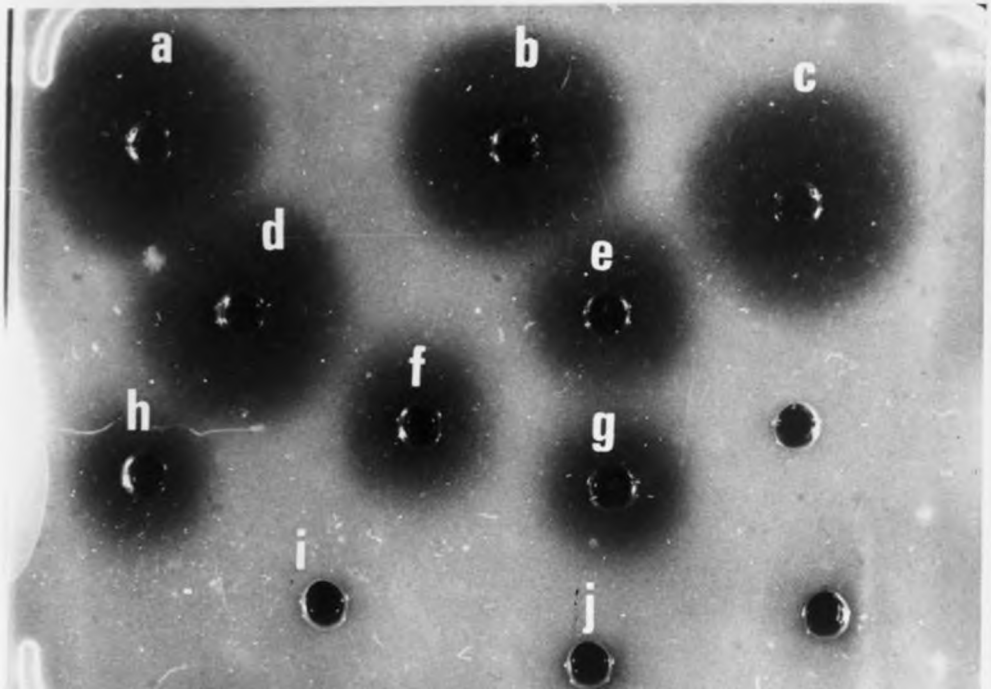

1·6
1·0
0·6
Diffusion diameter (cm)
66
33
16·5
8
Trypsin activity I.V. x 10⁻³
2·0
2·2
2·4
2·6
2·8
3·0
Log10 trypsin concentration (ng)

Fig. 12a **Inhibition of trypsin activity by antisera using casein substrate gel.** Isolated trypanoagglutinin was incubated with increasing concentration o antisera in casein substrate gel. The caseinolytic activity was determine by measuring the diffusion zone diameter

a - j Trypanoagglutinin (~0.15 mg/ml) with increasing concentrations of antisera (~0-12 μg/ml)

Fig. 12b **Trypsin inhibition profile showing % remaining activity vs concentration of antisera**


100
80
60
40
20
0
% Remaining activity
2
4
6
8
10
12
Anti-sera concentration µg/ml


a
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i
j

CHAPTER 4

DISCUSSION

A bloodmeal induced trypanoagglutinin with trypsin activity was isolated from the midguts of *G. longipennis*. The trypanoagglutinin was capable of agglutinating both bloodstream and procyclic *T. b. brucei* as well as rabbit erythrocytes. However, compared to bloodstream trypanosomes, a much lower concentration of trypano-agglutinin was required to agglutinate procyclics and erythrocytes. The agglutinating activity of trypano-agglutinin was strongly inhibited by D-glucosamine and weakly by N-acetyl D glucosamine. None of the other sugars tested had any inhibitory effect suggesting that it is D-glucosamine specific. The trypanoagglutinin was heat labile with the agglutination activity being lost rapidly at temperatures above 50° C.

Trypanoagglutinin was effectively purified by anion-exchange chromatography. The molecule had a net negative charge at pH 8.0. Both unbound and bound fractions (Peaks I and II, respectively) had trypsin activity suggesting that they had different charge properties. However, the trypanoagglutinin co-eluted with the bound trypsin activity (Peak II) at approximately 225 mM NaCl gradient. Further purification of the trypanoagglutinin was achieved by subsequently eluting at a narrow NaCl gradient (150-300 mM). The resultant single peak appeared as a homogeneous protein on a non-denaturing PAGE, with a native M_r of ~61,000. On the other hand, SDS-PAGE of the trypanoagglutinin under reducing and non-reducing conditions showed the presence of two subunits (M_r ~ 33,000 and 27,000). Trypsin activity was further observed on the single band

(M_r~61,000) obtained on non-denaturing PAGE. Interestingly, under denaturing conditions, only the M_r~ 27,000 subunit showed trypsin activity. The protease inhibitor, soybean trypsin inhibitor, could abrogate the agglutination of bloodstream *T. b. brucei*. Similarly, agglutination of rabbit erythrocytes was partially inhibited. In contrast, agglutination of procyclics was not affected by this inhibitor.

Antibodies raised against the isolated trypanoagglutinin reacted with both subunits. Out of the species tested for cross-reactivity using the antibodies, only members of the *Glossina* family gave a positive reaction. The two bands in *G. m. morsitans* co-migrated with the bands in *G. longipennis*. The agglutination activity of the trypanoagglutinin was strongly inhibited by the corresponding antiserum. Similarly, the trypsin activity of the trypanoagglutinin could also be inhibited by the antiserum.

Agglutinins or lectins are a group of carbohydrate-binding proteins with a ubiquitous distribution in nature (Lis and Sharon, 1986; Sharon, 1993). In insects and other invertebrates, agglutinins are believed to be involved in defence mechanisms that contribute to both cell-mediated and humoral immunity (Boman and Hultmark, 1987; Ingram and Molyneux, 1990; Natori, 1990). More recently however, these molecules have attracted considerable interest due to the realization that they may be involved in host-parasite vector interactions, especially in such important vectors as tsetse flies and sandflies. The midgut of *Glossina* contains proteolytic enzymes, lectins/ trypanoagglutinins and trypanolysins. Out of the six proteases present in the posterior midgut, trypsin is the most predominant enzyme (Cheeseman and Gooding, 1985). While trypsins from the different *Glossina* species have been well studied, little attention has been paid to the need to characterize the other midgut factors that have an activity on the trypanosomes.

For example, the midgut lectins of *Rhodnius prolixus* have been shown to be capable of inducing the transformation of *T. cruzi in vitro* (Pereira *et al.*, 1981). Similarly, in *Glossina* species, midgut lectins have been implicated in differentiation (Maudlin and Welburn, 1988). Lectins have also been proposed to be responsible for the destruction of trypanosomes entering the tsetse midgut (Maudlin and Welburn, 1987; Welburn and Maudlin, 1989). Although the importance of lectins in parasite-vector interactions is now widely recognized, there is still insufficient information on the properties of these molecules from different tsetse fly species. Recently, Stiles *et al.* (1990) identified a trypanoagglutinin in the posterior midguts of *G. palpalis* capable of agglutinating procyclic forms of *T. congolense* and *T. b. brucei.* This molecule had a relative molecular weight M_r~ 67,000 on SDS-PAGE. However, in the present study, the trypanoagglutinin isolated from the midguts of *G. longipennis* could agglutinate both bloodstream as well as procyclic forms of *T. b. brucei.* Furthermore, this trypanoagglutinin had a native molecular weight of M_r~ 61,000 on non-denaturing PAGE which resolved into two subunits (M_r~ 33,000 and 27,000) on SDS-PAGE. The presence of two subunits on SDS-PAGE under reducing and non-reducing conditions suggest that they are non-covalently bound. Immunoblot analysis of *G. tachinoides* midgut lectin showed that it also possessed an M_r~ 27,000 protein component under SDS-PAGE (Volf *et al.*, unpublished). Out of the two subunits shown in this study, only the M_r~27,000 had trypsin activity. The molecular weight values of trypsins from other sources like bovine pancreas (M_r ~ 24,000), human pancreas (M_r ~ 21,000 and 20,000), *G. p. palpalis* (M_r ~ 24,000 and 26,000) and *G. m. morsitans* (M_r ~ 24,000) are very close to the trypsin activity reported in this study (Feinstein et al., 1974; Abbeele and Decleir, 1991; Onyango, 1993). Since the unbound trypsin (Peak I) was found to be incapable of

agglutinating trypanosomes or erythrocytes, it can be presumed that the agglutinating activity of the trypanoagglutinin resides in the M, ~ 33,000 subunit which was also found to be glycosylated.

The presence of both trypsin and lectin activities on the trypanoagglutinin is very interesting. Previous investigations have suggested a close relationship between lectins and trypsin or trypsin-like enzymes in the tsetse midgut. The release of trypsin and lectins are induced following a bloodmeal by a component of the serum (Gooding, 1974; Maudlin *et al*., 1984). Furthermore, both lectins and trypsin have been implicated in the differentiation of the trypanosomes (Maudlin and Welburn, 1988a, b; Yabu and Takayanagi 1988; Welburn and Maudlin, 1989; Imbuga *et al*., 1992a) as well as in the destruction of the parasites in the fly midgut (Maudlin and Welburn, 1987; Welburn et al., 1989; Imbuga et al., 1992). More recently, it has been reported that glucosamine inhibits midgut trypsin (Osir *et al*., 1993). This inhibition was found to be reversible, partial and competitive, suggesting that the inhibition is effected most probably in an allosteric manner. In the present study it has been shown that the agglutinating activity of the trypanoagglutinin was specifically inhibited by glucosamine as was the case with *G. m. morsitans* midgut lectins (Ibrahim *et al*., 1984; Welburn and Maudlin, 1989). This new finding that the trypsin and lectin activities are present on the same molecule can therefore explain the mechanism by which the trypanosomes are lysed. In other insects like the mosquito, high levels of protease activity has been reported to cause lysis of *Plasmodium* (Yeates and Steiger, 1981). Similarly, a component of trypsin-like activity has been reported to cause lysis of *Leishmania donovani* and modulation of its activity enables *Leishmania major* to survive in *Phlebotomus papatasi* (Borovsky and Shlein, 1987). The finding in this study that trypanoagglutinin possess both trypsin and agglutinin activities is not unique, since

proteins with protease as well as lectin activities have been reported before (Mirelman, 1986). These proteases with lectin-like activities have been shown to have toxic effects such as inducing the secretion of electrolytes and fluids in colonic loops and cause a reduced incorporation of thymidine and leucine into tissue cultured BHK-21 cells. Other sugar specific enzymes with their multiple binding sites have been reported to agglutinate cells under certain conditions and therefore act as lectin (Goldstein *et al.*, 1980; Barondes, 1981).

Bloodstream trypanosomes and trypanosome membranes (*T. b. brucei*) have been reported to inhibit *G. m. morsitans* midgut trypsin activity (Imbuga *et al.*, 1992b). This suggested that in order to survive in the midgut, the bloodstream trypanosomes have to inhibit trypsin and trypsin-like enzymes. In this study it was shown that the protease inhibitor, soybean trypsin inhibitor (STI), could abrogate the agglutination of bloodstream *T. b. brucei*. This suggested that the agglutination of bloodstream trypanosomes require trypsin activity. Similarly, trypsin activity has been reported to enhance agglutination of erythrocytes. The use of trypsin-treated erythrocytes resulted in significantly higher agglutination titres (Ingram and Molyneux, 1990; Goto *et al.*; 1992). Similarly, Sharon and Lis (1989) reported that cells that could not be agglutinated by low concentration of a lectin often become agglutinable after mild proteolysis. In the present study it has been shown that STI partially inhibits the agglutination of rabbit erythrocytes. It can therefore be suggested that the trypsin associated with agglutinin cleaves off surface molecules thus exposing the sugar residues on bloodstream trypanosomes and erythrocytes, thus making them more vulnerable for agglutination activity.

Tsetse flies fed on an infective feed containing glucosamine have been reported to develop higher infection rates (Maudlin and Welburn, 1987).

Subsequently, Maudlin and Welburn, (1988a, b) proposed that susceptibility to trypanosome infection in tsetse flies is a maternally inherited trait associated with the presence of *rickettsia*-like organisms (RLOs) in the fly midgut. The RLOs produce chitinase which hydrolyses chitin to glucosamine. Presumably, glucosamine inhibits lectin activity and consequently increases susceptibility of tsetse flies to trypanosome infection (Maudlin and Welburn, 1988). Indeed, the present study has shown that the presence of glucosamine in the midgut can inhibit both the trypsin and agglutinin activities of the trypanoagglutinin thus making the tsetse flies vulnerable to infection. Trypanosomes are known to express two abundant stage-specific glycosylphosphatidyl inositol (GPI)-anchored glycoproteins, the procyclin in the procyclic form, and the variant surface glycoprotein (VSG) in the mammalian bloodstream forms (Field *et al.*, 1992). The GPI anchor has been reported to contain glucosamine residues. It is therefore likely that the D-glucosamine-specific trypanoagglutinin reported in this study agglutinates trypanosomes by recognizing these glucosamine residues.

Immunological methods have long been used to check for proteins with similar antigenic determinants. A positive reactivity is always expected when antigen shares epitopes with the protein under investigation. Out of the species tested for cross reactivity using the antibodies raised against the isolated trypanoagglutinin, only members of the *Glossina* family gave a positive reaction. This suggests that the trypanoagglutinin is present only in the *Glossina* family. Sandflies, stable flies, mosquitoes and tsetse flies have been reported to synthesize several trypsin-like enzymes after a bloodmeal (Borovsky and Schlein, 1988; Cheseman and Gooding, 1985). In the present study it was shown that though the trypanoagglutinin had trypsin activity it does not share antigenic determinants with

trypsin-like enzymes from other blood-sucking insect species. Inhibition of enzymes by the corresponding antiserum in an *in vitro* system has been reported previously (Dingle *et al*., 1971; Vundla, 1990). The immunoinhibition is normally the result of steric blockage of the enzyme catalytic site by surrounding antibody molecules (Cinader and Lafferty, 1964; Arnon, 1967; Dingle, 1971). The results of this study have shown that the agglutination activity of the trypanoagglutinin was strongly inhibited by the corresponding antiserum. Similarly, the trypsin activity of the trypanoagglutinin could also be inhibited by the antiserum. Initial attempts to check the immunoinhibition of trypsin activity using chromozym substrate were not successful. This was because a small substrate molecule like chromozym can penetrate between the antibodies surrounding the trypanoagglutinin and reach the active site. On the other hand, a larger substrate like casein can be effectively blocked by the antibodies surrounding the trypanoagglutinin subunits.

CONCLUSION

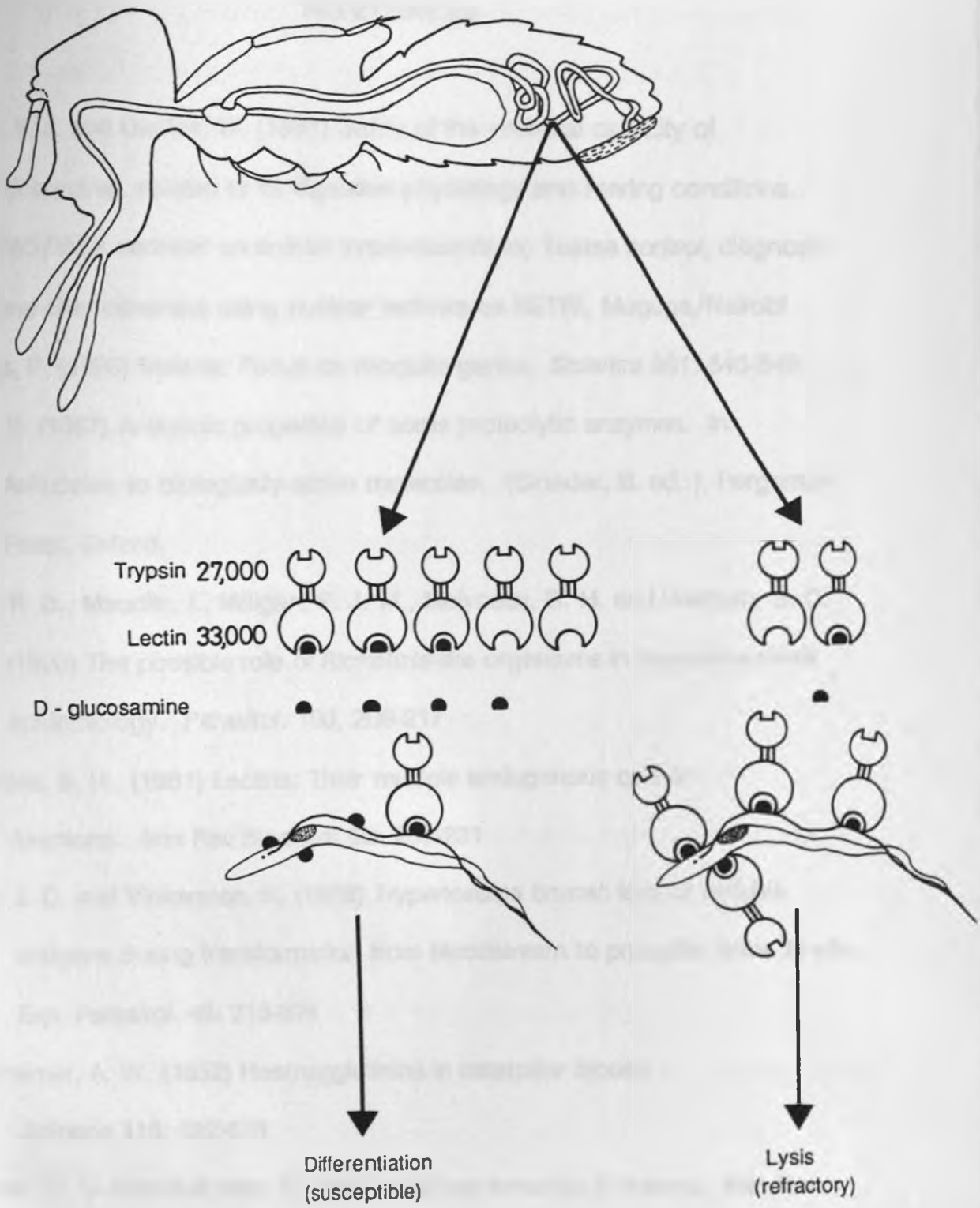
The study of trypanoagglutinin from the midgut of *G. longipennis* has shown that the trypanoagglutinin (M_r ~ 61,000) consist of two non-covalently bound subunits (Mr ~ 33,000 and 27,000). The trypsin activity is present in the M_r ~ 27,000 subunit while the agglutinin activity is present in the M_r ~ 33,000 subunit. This study has also shown that the trypanoagglutinin was specifically inhibited by D-glucosamine. The trypanoagglutinin was capable of agglutinating both bloodstream as well as procyclic forms of trypanosomes. However, trypsin activity was required for the agglutination of bloodstream trypanosomes.

On the basis of these findings, the mechanism by which trypanosomes in the tsetse midguts are lysed can now be explained. In susceptible flies, the action of the enzyme chitinase produced by *Rickettsia*-like organisms (RLOs) leads to the accumulation of glucosamine in the fly midgut, which in turn inhibits the trypanoagglutinin thus lowering the agglutinin activity to optimum levels for the trypanosomes to differentiate. On the other hand, refractory flies with relatively few RLOs produce less glucosamine leading to high trypanoagglutinin activity. Trypanosomes entering such midguts will therefore be agglutinated and eventually lysed (scheme 1). The trypsin activity has a dual function. Firstly, it exposes agglutinin binding sites on the bloodstream trypanosomes. Secondly, it lyses the trypanosomes that have been agglutinated.

Several important aspects of the trypanoagglutinin would form the subject of future investigations. Further studies to elucidate the precise mode of action of the trypanoagglutinin would be necessary to confirm the proposed mechanism of action. The origin of the two subunits as well as their biosynthetic process also need to be established. Studies on the gene(s) coding for the trypanoagglutinin with the view to cloning the gene(s) also stands in need.

Scheme 1 **Role of trypanoagglutinin (agglutinin/trypsin complex) in the establishment of midgut infections in tsetse.**

Tsetse - Trypanosome relationships



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