RELATIONSHIP BETWEEN LECTINS AND TRYPSIN IN THE MIDGUT OF THE TSETSE FLY, GLOSSINA MORSITANS MORSITANS.

BY

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DECLARATION

I, Patrick Onyango, 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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SCHEMES

ABBREVIATIONS

BANA	-	Benzoyl-L-arginine-β-naphthylamide
BApNA	-	Benzoyl-L-arginine-p-nitroanilide
BCA	-	Bicinconinic acid
BSA	-	Bovine serum albumin
$CaCl_2$	-	Calcium chloride
Chromozym TRY	-	Carbobenzoxy-val-gly-arg-4-nitroanilide acetate
Con A	-	Concanavalin A
DMF	-	Dimethylformamide
Σ	-	Molar extinction coefficient
FPLC	-	Fast protein liquid chromatography
HCl	-	Hydrochloric acid
I	-	Inhibitor
NaCl	-	Sodium chloride
PAGE	-	Polyacrylamide gel electrophoresis
PBS	-	Phosphate buffered saline
PEG	-	Polyethylene glycol
SDS	-	Sodium dodecyl sulphate
SDS-PAGE	-	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
STI	-	Soybean trypsin inhibitor
TAME	-	p-Tosyl-L-arginine methyl ester
TLCK	-	N-p-Tosyl-lysine chloromethyl ketone
V	-	Enzyme activity
v/v	-	volume by volume
w/v	-	weight by volume

UNIT ABBREVIATION

gm	-	Gram
h	-	Hour
M	-	Molar
mA	-	Milli Amperes
min	-	Minute
mg	-	Milligram
ml	-	Millilitre
mm	-	Millimeter
mM	-	Millimolar
µM	-	Micromolar
µg	-	Microgram
µl	-	Microlitre
µmol	-	Micromoles
M_r	-	Molecular weight
nm	-	Nanometre
pH	-	$-Log_{10}$ hydrogen ion concentration
rpm	-	Revolutions per minute
SD	-	Standard deviation
sec	-	Second

SUMMARY

Tsetse flies are haematophagous arthropods which feed on a diverse range of vertebrates. The *Glossina morsitans* group of tsetse flies are among the important vectors of the African trypanosomes. The African trypanosomes are the causative agent of the diseases, sleeping sickness in man and nagana in livestock. The trypanosomes are protozoal parasites with a complicated life cycle both within the vertebrate host and the tsetse fly vector. These diseases, are generally fatal unless treated. Once a tsetse fly ingests trypanosomes during feeding, the parasites pass through the oesophagus to the midgut. Here, the parasites are exposed to a hostile environment which consists of the digestive enzymes like trypsin, a trypsin-like enzyme, chymotrypsin, carboxypeptidase A and B, and an aminopeptidase. Out of the six digestive enzymes, trypsin is the most abundant involved in digestion. The tsetse fly midgut trypsin has been implicated in the lysis of the trypanosomes as well as their transformation into procyclic forms. Similarly, "lectins", a group of proteins that bind carbohydrates specifically, have been reported to be secreted by tsetse midgut in response to a bloodmeal. These "lectins" which are specific for glucosamine have been proposed to have two functions. Firstly, they mediate lysis of the parasites that enter the fly midgut.

Secondly, they provide the signal for the surviving parasites to differentiate to procyclic forms. Furthermore, *in vivo* studies have shown that flies infected with parasites and maintained on a diet of blood containing D-glucosamine develop elevated levels of midgut infections. It might be argued that the reason for the increased infections is due to inhibition of the "lectins" by D-glucosamine. Since the "lectins" and trypsin appear to be induced by the same factors, and mediate similar events, it was considered pertinent to explore the relationship between "lectins" and trypsin in midgut of the tsetse fly, *Glossina morsitans morsitans*.

In these studies, it was shown that the sugar D-glucosamine specifically and reversibly inhibited trypsin and trypsin-like enzyme activity. In contrast, galactosamine, mannose, glucose, fructose, galactose, Inositol, glucosamine pentacetate, N-acetyl-glucosamine, methyl-α-D-glucopyranoside, methyl-β-D-glucopyranoside, α-D-mannosamine had no inhibitory effect on tsetse fly trypsin activity even at concentrations as high as 700 mM. The inhibition by glucosamine was determined to be partial, as shown by non linear Dixon plots. The Lineweaver-Burk plots intersected on the y axis, a pattern characteristic of competitive inhibition. The K_i was estimated to be 68 $\pm$ 3 mM. Glucosamine also had similar inhibition effect on bovine pancreas trypsin. However, glucosamine did

not inhibit tsetse fly midgut aminopeptidase activity. Interestingly, glucosamine at a concentration of 100 mM both in vivo and in vitro, inhibited transformation of *Trypanosoma brucei brucei* bloodstream forms to procyclic forms. The inhibition of transformation was fairly constant, and 90% of the parasites were still in the bloodstream forms 8 hours after feeding. On the other hand, transformation proceeded normally in the absence of glucosamine with 80-90% of the parasites in the procyclic form 8 hours after feeding. The results suggest a close relationship exist between tsetse fly midgut lectins which are specifically inhibited by glucosamine and the enzymes, trypsin and trypsin-like.

In addition *Glossina morsitans morsitans* midgut proteases, trypsin and trypsin like were purified and partially characterized. The purification process involved anion exchange, gel filtration, affinity chromatography and electroelution. The two enzymes, trypsin and trypsin-like enzyme had the molecular weight of ~ 24 KD. They differed in charge, trypsin being positively charged at pH 8.0 and trypsin-like enzyme had a net negative charge at the same pH. Soybean trypsin inhibitor (STI) and N-p-Tosyl-lysine chloromethyl ketone (TLCK) had similar inhibitory effect on

the trypsin and trypsin-like enzyme. The K_is' for trypsin and trypsin-like enzyme were determined to be 0.295, 0.24 µg/ml for STI and 0.12, 0.15 mg/ml for TLCK respectively. The tsetse fly midgut trypsin was 2-3 times more active than the trypsin-like enzyme.

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

1.1 Tsetse flies as vectors of trypanosomes

The genus *Glossina*, family Muscidae comprises the tsetse flies of Africa. Tsetse flies are haematophagous arthropods, which feed on a diverse range of vertebrates. Based on their morphology, ecology, karyotype and behavior, tsetse flies can be classified into the following three groups: Fusca group (forest flies), Morsitans group (savanna flies), and Palpalis group (riverine and forest flies). The Morsitans group consists of three species, Glossina pallidipes, Glossina swynnertoni and Glossina morsitans. Of the three, Glossina morsitans is the most important as a vector of African trypanosomes (Service, 1986). The trypanosomes belong to the genus *Trypanosoma*, a group of protozoal parasites found in the blood and tissues of various vertebrate hosts. The parasites are usually transmitted from one host to another by tsetse flies. 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 diseases, which are generally fatal unless treated (Buxton, 1955).

Trypanosomiasis in particular, has had adverse effects on agriculture in the Africa continent. In sub-Sahara Africa, sleeping sickness is still a significant disease. The World Health Organization (WHO,1975) estimates that approximately 20,000 new cases are reported each year. In addition, nagana impedes livestock production directly and crop production indirectly. Besides, tsetse flies occur in the humid and sub-humid zones. These areas total approximately 10 million square kilometers, or about half the non desert area of Africa (WHO, 1975). Furthermore, of the 10 million square kilometers infested with tsetse flies, 7 million are thought to be suitable for mixed agriculture and livestock farming. Thus, in 1963, Africa's animal loss in meat production due to tsetse flies was estimated to be US $ 5 billion.

The control of trypanosomiasis currently relies on trypanocidal drugs, tsetse fly control and, to a lesser extent, the breeding of trypanotolerant livestock. These control strategies are usually used in isolation. Unlike trypanosomes, which elicit drug resistance, there are no insecticide resistance with tsetse flies (Kaminsky and Zweygarth, 1989). Hence, tsetse fly control relies almost exclusively on the use of insecticides. The insecticides used to control tsetse flies include Dieldrin, DDT, HCH,

Endosulfan, Synthetic pyrethroides, Fenthion (Kendrick and Alsop, 1974; Hadaway and Turner, 1975; Spielberger and Na'isa, 1975). Unfortunately, intensive and repeated spraying of residual insecticides can have disastrous effects on the local fauna (Koeman *et al.*, 1971). For example, endosulfan is acutely toxic to fish, Dieldrin and DDT are toxic to wildlife and other non target organisms in the savannah zones (Koeman and Hadden, 1968). Other control strategies include, use of pheromones or attractants impregnated traps, release of sterile male flies (SIT), use of parasites, predators, pathogens, insect growth regulators (Itard, 1975;; Abbeele and Decleir, unpublished; Denlinger, 1975). However, residual application of insecticides would interfere with the release of sterile males, predators and parasitoides, but not the use of pathogens. Hence, the need to integrate the various tsetse fly control strategies in an effective way. Moreover, the use of integrated control measures is more efficient and cheaper than the use of chemical methods (WHO, 1975). On the other hand, control of trypanosomiasis by developing a vaccine, has thwarted the efforts of researchers to date. The major obstacle to finding a trypanosome vaccine, is the ability of the parasite to undergo antigenic variation. In this process, the trypanosomes change the antigenic character of their surface coat and so evade the hosts immune response.

The outer surface coat is made up of the variable surface glycoprotein (VSG). The immune response against each variant is effective in killing the trypanosomes with that particular antigen. However, the same immune response is invariably too late to act against that proportion of the population which has altered its antigenic identity. An effective vaccine would evidently have to contain all variable antigenic types (VATs). Such a vaccine would be difficult to concoct, because of the large numbers of the VATs. The (VSG) is encoded by a multigene family that contains about a thousand genes, each encoding an antigenically distinct VSG. Generally, each trypanosome expresses a single VSG gene which carries a particular VAT corresponding to the VSG on the cell surface. The mechanism of generation of antigenic variation and the switching on and off of the VSG gene expression is not yet entirely understood. It appears that the process involves gene duplication and transposition of the duplicate gene copy to a potential expression site on the genome. Whether the VSGs are released into the circulation during an infection is not clear. The released VSG may serve as "smoke screen" for the parasite to keep activating the immune system against the wrong VAT. Nevertheless, these drawbacks have only resulted into intensive research, in the analysis of the variable surface glycoprotein. It is hence possible that

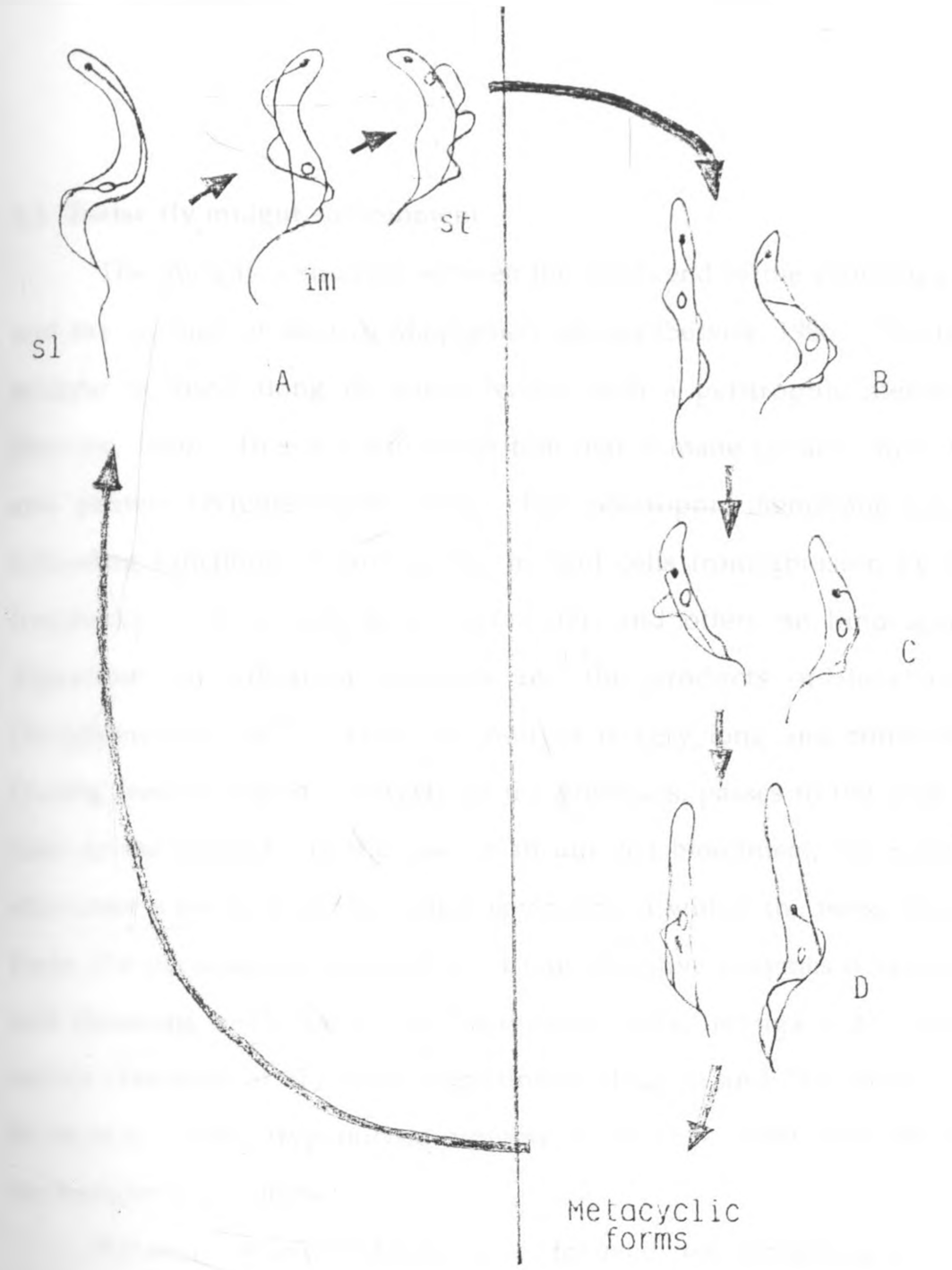
some time in the trypanosome life cycle "weak spots" amenable to immunological control might be exposed. Indeed, it can be concluded that sustainable control of African trypanosomiasis will only be realized by integrating the different control strategies.

1.2 Life cycle of the African trypanosomes

The life cycle of African trypanosomes comprises of various developmental changes, both within the vertebrate host and the tsetse fly vector (Hoare, 1972). The life cycle is illustrated in scheme 1. The cycle of development within the tsetse fly is as follows. Trypanosomes (bloodstream forms) are ingested by the tsetse fly during a bloodmeal from an infected vertebrate host. In most cases the parasites pass through the oesophagus to the crop. However in the case of *Trypanosoma vivax*, the life cycle is restricted within the fly mouth parts. After feeding, the parasites migrate to the peritrophic membrane lining the midgut where the blood is digested. In the midgut the parasites encounter a hostile environment, whereby some of the parasites are lysed. Interestingly, the parasites are not all destroyed (Maudlin and Welburn, 1987). The surviving parasites differentiates to midgut forms (procyclic), which then migrate to the salivary glands. This migration is through the

proventricules, where the peritrophic membrane is soft and allows the parasites to penetrate. It is in the salivary glands where the mature infective metacyclic forms develop. These 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). The transformation from the bloodstream to procyclic forms involves complex morphological and physiological changes (Vickerman, 1965; Vickerman and Preston, 1976; Englund *et al.*, 1982). These changes enable the parasite to adapt to a radically different environment within the fly midgut. The transformation involves the following. Loss of the surface coat; cessation in the synthesis of the variable surface glycoprotein (VSG); activation of mitochondrial enzymes and cytochromes electron transport system; induction of procyclin synthesis; and displacement of the position of the nucleus and the kinetoplast (Vickerman Preston, 1976; Barry and Vickerman, 1979; Ghiotto et al., 1979; Roditi and Pearson, 1990). The establishment of the parasites within the insect vector is dependent on transformation, which is an essential initial first step. Evaluation of the factors involved in triggering the process has been the subject of many

recent investigations (Bienen *et al.*, 1980, 1981; Overath *et al.*, 1983; Simpson et al., 1985; Yabu and Takayanagi, 1988; Turner *et al.*, 1988; Imbuga *et al.*, 1992 a, b).



SCHEME 1: *Trypanosoma brucei brucei.* Developmental changes, during the trypanosome life cycle. Magnification 1400X. (A) Bloodstream population consisting of slender (sl), intermediate (im) and stumpy (st) forms. (B) Transforming cells. (C) Early procyclic forms. (D) Established procyclic forms.

1.3 Tsetse fly midgut environment

The midgut is situated between the distal end of the proventriculus and the junction of the two Malpighian tubules (Service, 1986). The tsetse midgut is lined along its entire length with a peritrophic membrane (Service, 1986). This is a thin tough film that is made up of a chitin base and protein (Wigglesworth, 1972). The peritrophic membrane has the following functions, it protects the midgut cells from abrasion by hard fragments in food, acts as an ultra-filter, and offers no hindrance to digestion; for digestion enzymes and the products of their action (Wigglesworth, 1972). Tsetse fly midgut is very long and convoluted. During feeding, blood is sucked up the proboscis, passes to the crop and later to the midgut. In the case of an infected bloodmeal, the parasites encounter a physiologically hostile environment within the tsetse midgut. Here, the parasites are exposed to various digestive enzymes (Cheeseman and Gooding, 1985: Yabu and Takayanagi, 1981; Imbuga *et al.*, 1992 a), lectins (Welburn et al., 1989), agglutinnins (Ingram and Molyneux, 1988; Stiles *et al.*, 1990), trypanolytic proteins (Stiles *et al.*, 1990) and other yet uncharacterized factors.

Recently many studies have focused on physiological and biochemical factors underlying the tsetse fly-trypanosome interaction (Gingrich et al., 1985; Maudlin and Welburn, 1987; Welburn and Maudlin, 1989, 1991; Imbuga *et al.*, 1992 a , b). Gingrich *et al.*, (1985) observed an

increased midgut infection rate in flies fed on trypanosomes suspended in serum free blood. Thus it was suggested that the presence of serum in an infective feed was essential for induction of proteolytic activity. As a result they 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 the destruction of ingested ookinates of *Plasmodium gallinaceum* (Gass, 1977; Gass and Yeates, 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 possesses proteinase inhibitor which inhibit *Glossina morsitans morsitans* posterior midgut trypsin (Houseman, 1980). The inhibitor is subspecies specific, each anterior midgut lysate only inhibits the the homologous lysate. The exact nature of the inhibition mechanism is not known. It is likely that the inhibitor protects the gut cells from proteolytic activity during periods of starvation (Vogel *et al.*, 1968). Stiles *et al.*, (1990) isolated and partially characterized trypanolysin and trypanoagglutinin

from the midgut of *Glossina palpalis palpalis*. The two molecules were active only in the posterior midgut, were heat labile above 50^0 C, and had a periodic cycle of activity in response to a bloodmeal intake. Interestingly, the molecules were not affected by protease inhibitors or trypsin but were inactivated by pronase. The trypanolysin contained two proteins with molecular weight of ~12 and ~10 KD. The agglutinin of both subspecies had a molecular weight of ~ 67KD. The trypanolysin caused lysis of trypanosomes, while trypanoagglutinin resulted into agglutination of the parasites. On the other hand, the reaction of peritrophic membrane differs between susceptible and refractory Glossina morsitans morsitans due to exposed carbohydrate residues (Okola *et al.*, 1988). Trypanosomes in the midgut either migrate to regions of low anti-trypanosomal substances (anterior midgut) or are lysed in the posterior midgut, while others survive. The distribution of parasites within the midgut is dependent on how efficient the destructive molecules can be avoided. Trypanosomes like *Trypanosoma vivax* are unable to withstand the midgut environment and are restricted to foregut, the cibarium and the hypopharynx (Jeffries *et al.*, 1987). The precise mechanisms of action of these molecules are not clearly understood, hence the need for further research to elucidate the mechanisms.

1.3.1 The role of trypsin in transformation.

Digestion of the bloodmeal takes place 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 on 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.

Yabu and Takayanagi (1988) reported that bovine pancreas trypsin stimulated transformation of *Trypanosoma brucei gambiense* bloodstream forms to procyclic forms in vitro. They used monomorphic stock of *Trypanosoma brucei gambiense* (Wellcome strain) bloodstream forms grown on culture (Yabu and Takayanagi, 1986, 1987). In contrast to the trypsin-treated populations, the transformation of non treated bloodstream forms and forms treated with trypsin inhibited with soybean trypsin inhibitor were prolonged. These results strongly suggested that tryptic removal of the surface coat is a specific inducer for transformation of the bloodstream forms. 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 *Trypanosoma brucei brucei* (Abbeele and Decleir, 1991). Likewise, a correlation was established between the transformation activity of the midgut homogenates of *Glossina morsitans morsitans* at 27^0 C and trypsin activity on bloodstream forms of *Trypanosoma brucei brucei* into procyclic (midgut) forms in vitro (Imbuga *et al.*, 1992 a). Furthermore, trypanosomes/trypanosome membranes from *Trypanosoma brucei brucei* inhibit midgut trypsin activity in *Glossina morsitans morsitans* (Imbuga *et al.*, 1992 b). This suggests that trypanosomes modulate trypsin activity to entail transformation.

1.3.2 Lectins

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, 1989). The existence of lectins has been known for more than 100 years, though the idea that they may act as recognition molecules, has only been recently proposed (Sharon and Lis, 1987; Harrison and Chesterton, 1980; Brandly and Schnaar, 1986). This was brought about by the realization that surface carbohydrates may function in cell recognition (Liener *et al.*, 1986; Lis and Sharon, 1986; Sharon and Lis, 1989 a).

Lectins are not confined to plants, as originally believed, but are ubiquitous in nature being frequently found on the cell surfaces and intracellular particles. The specificity of lectins has proved to be much more exquisite than originally assumed. This is because they distinguish between different monosaccharides and specifically bind to oligosaccarides (Sharon and Lis, 1989 b).

The involvement of insect lectins in transformation of trypanosomes was first proposed by Pereira *et al.*, (1981). Their results suggested that lectins from the midgut of the triatomid bugs could stimulate differentiation of Trypanosoma cruzi, the etiological agent of Chagas disease. The presence of lectins have also been demonstrated in

other insects. For example, lectins have been identified in midgut, hindgut and haemolymph of Glossina austeni (Ibrahim *et al.*, 1984); and in the gut extracts of Phlebotomus papatsi (Wallbanks *et al.*, 1986). These lectins exhibit different carbohydrate binding specificities. The lectins from midgut, hindgut and haemolymph of Rhodnius prolixus are specific for N-acetyl-D-mannose, α-N-acetly-D-galactose and, α and β-D-galactose, respectively (Preira *et al.*, 1981). On the other hand, Glossina morsitans morsitans midgut lectin is specific for glucosamine (Ibrahim *et al.*, 1984; Welburn and Maudlin, 1989). In vitro studies have shown that trypanosomes are immobilized and agglutinated by tsetse haemolymph and midgut lectin (Croft *et al.*, 1982; Ibrahim *et al.*, 1984; Abubakar, unpublished).

The removal of serum from infective feeds significantly increase trypanosome infection rates in tsetse flies (Gingrich *et al.*, 1985; Maudlin *et al.*, 1984). These results suggest that serum is necessary in inducing trypanosome killing. In addition, midguts from teneral flies have been shown to have little lectin activity compared with fed flies, suggesting that some component of serum is responsible for initiating lectin production (Welburn *et al.*, 1989).

Trypanosomes entering the midgut of the tsetse fly are exposed to secreted lectin which can result into trypanosome death (Maudlin and

Welburn, 1987). Furthermore, 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). Based on this, it was concluded that flies with the highest lectin output, are most successful at preventing trypanosome establishment in the midgut and will accordingly produce lower infection rates (Welburn *et al.*, 1989). On the other hand, *Glossina morsitans morsitans* midgut lectin has been implicated in providing the signal for established midgut trypanosomes to proceed with the process of maturation (Maudlin and Welburn, 1988 a). 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 preprogramed 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). Unlike *Glossina morsitans morsitans*, *Glossina palpalis palpalis* has been shown to have high haemolymph lectin titre (Welburn and Maudlin, 1990).

Welburn, 1987). Furthermore, 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). Based on this, it was concluded that flies with the highest lectin output, are most successful at preventing trypanosome establishment in the midgut and will accordingly produce lower infection rates (Welburn *et al.*, 1989). On the other hand, *Glossina morsitans morsitans* midgut lectin has been implicated in providing the signal for established midgut trypanosomes to proceed with the process of maturation (Maudlin and Welburn, 1988 a). 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 preprogramed to complete their life cycle in the fly, but remain in the midgut stage until they receive the lectin signal to proceed with maturation.

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Hence flies with high midgut and haemolymph lectin titres, like the latter, may have high transmission indices for *T. congolence* and T*rypanosoma brucei rhodesiense.* On the other hand, flies with low titres of haemolymph lectin, like the former, will mature few infections even in the presence of high midgut lectin titres (Welburn and Maudlin, 1990). These differences between flies in lectin secretion may be responsible for the 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 different ways. Firstly, it may act as a trigger by binding to individual procyclic trypanosome membrane (Welburn and Maudlin, 1990). Secondly, it may simply act as a mitogen, a common property of lectins (Goldstein and Poretz, 1986).

Susceptibility in *Glossina morsitans morsitans* has been shown to be maternally inherited trait associated with the presence of Rickettsia-like organisms (RLO) 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 RLO

chitinase activity results in a build up of glucosamine. Glucosamine in turn inhibits midgut lectin activity at the teneral feed (Maudlin and Welburn, 1988 b; Baker *et al.*, 1990). On the other hand, lectin levels in the midgut of non-teneral flies when switched on by the bloodmeal, are too high to be affected by RLO 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).

The precise mechanism by which secreted lectins kills trypanosomes or effect maturation in the fly midgut is not known. On the other hand, adaptation of the parasite to the tsetse fly, has been attributed to two factors relating to procyclin, a glycoprotein which consists of glutamate and proline repeats (Roditi and Pearson, 1990). The first is that procyclin protects the parasite from proteases in the tsetse midgut. Secondly, procyclin may play a role in determining tropism in the tsetse fly vector.

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.

1.4 Importance of study

The prevalence of trypanosomiasis in Africa is staggering. Over 10,000 km2 of land is affected. Unfortunately, the available treatment is often quite poor. Trypanosomes have developed exquisite systems to evade host defenses. Although the life cycles as well as basic biological questions have been elucidated, very little is still known about the mechanisms of vector-parasite interactions.

Factors such as lectins, trypsin, trypanolysin and trypanoagglutinin (Maudlin and Welburn, 1987; Yabu and Takayanagi, 1988; Imbuga *et al.*, 1992 a, b; Stiles *et al.*, 1990) and may be others make the midgut environment hostile for ingested trypanosome bloodstream forms. The exact mechanisms of these factors is not clearly understood. Trypanosomes have to conquer this battle and transform to the adapted procyclic form. The release of tsetse fly midgut trypsin has been shown to be induced by a bloodmeal. The released trypsin has been implicated in the lysis of the trypanosomes as well as their transformation into procyclic forms. Similarly, "lectins", a group of proteins that bind carbohydrates specifically, have been reported to be secreted by tsetse midgut in response to a bloodmeal. These "lectins" which are specific for glucosamine have been proposed to have two functions. Firstly, they mediate lysis of the

parasites that enter the fly midgut. Secondly, they provide the signal for the surviving parasites to differentiate into procyclic forms. Furthermore, in vivo studies have shown that flies infected with parasites and maintained on a diet of blood containing glucosamine develop elevated levels of midgut infections. It might be argued that the reason for increased infections is due to the inhibition of the "lectins" by glucosamine. Since the "lectins" and trypsin appear to be closely related it was considered pertinent to explore the relationship between "lectins" and trypsin in the midgut of the tsetse fly, *Glossina morsitans morsitans*. Elucidation of the mechanisms of "lectin" and trypsin action, will give more insight in understanding the process of differentiation (transformation) of bloodstream forms to procyclic forms. This basic knowledge could possibly open new avenues in understanding the epidemiology of trypanosomiasis.

1.5 Aims of the study

1.5.1 Overall aim :

Elucidation of the relationship between lectins and trypsin in midguts of the tsetse fly, *Glossina morsitans morsitans*.

1.5.2 Specific aims:

(i) Establishment of trypsin assay in crude midgut homogenates.

(ii) Elucidation of the mechanism of trypsin inhibition by glucosamine/ analogues or selected hexoses; and the chemical group(s) involved in this process.

(iii) Determination of the role of glucosamine/ analogues or selected hexoses on transformation of bloodstream *T. b. brucei* to procyclic forms in relation to trypsin.

(iv) Purification and partial characterization of the trypsin.

(v) Kinetic studies on glucosamine/ analogues and selected hexoses in relation to trypsin.

CHAPTER 2

MATERIALS AND METHODS

2.1 Reagents

The reagents used in this work were of analytical grade obtained from Aldrich Chemical Company (Dorset, England), BDH Chemical Company (Poole, England), Bio-Rad Laboratories Ltd (Hemel Hempstead Hertfortshire, UK), Boehringer-Mannheim FRG, Gedeon Richter Ltd (Budapest, Hungary) Pharmacia LKB (Bromma, Sweden), Pierce (Rockford, IL. USA), Serva Feinbiochemica GmbH & Company (FRG), Sigma Chemical Company (St. Louis, USA).

2.2 Experimental insects and animals

Tsetse flies, *Glossina morsitans morsitans* Westwood, and animals were supplied by the Insect and Animal Breeding Unit (IABU) of the International Centre of Insect Physiology and Ecology (ICIPE). Male rats (Wister strain) and New Zealand white rabbits were used. The tsetse flies were reared on a 12 h Light: 12 h Dark photoperiod at 75-80% relative humidity and 25^0 C.

2.3 Dissection of flies and preparation of midgut homogenate

The flies were immobilized by brief chilling after which the midguts were carefully dissected in ice cold 0.1 M Tris/ HCl buffer, pH 8.0. Fifty midguts were homogenized in 1 ml the buffer for 45 seconds using a Virtis homogenizer (Gardiner, USA). The resulting homogenate was centrifuged (12,000 g, 15 min 4^0 C) using a Beckman Microfuge (USA). The supernatant solution was transferred into a Microfuge tube, and was designated as the crude midgut homogenate. This sample was either used immediately or stored at -20^0 C until required. Unless stated otherwise all the enzyme handling were carried out at 4^0 C.

2.3.1 Enzyme assay

Benzoyl-L-arginine-p-nitroanilide (BApNA; Σ_{410}=8800; Serva; Erlanger *et al.*, 1961), Carbobenzoxyl-val-gly-4-nitranilide acetate (Chromozym-TRY; Σ_{410}=8800; Boehringer-Mannheim; Erlanger *et al.*, 1961), and p-Tosyl-L-arginine methyl ester (TAME; Σ_{247}= 540; Serva; Gooding and Rolseth, 1976) were used as trypsin substrates. Leucine-para-nitroanilide (LpNA; Σ_{410}=8800; Sigma; Erlanger *et al.*, 1961) was used as aminopeptidase substrate. Stock solutions were made as follows; 0.02 M BApNA, LpNA in 60% DMF (Sigma) and 0.1 M Tris/HCl pH 8.0, 1 mg/ml Chromozym-TRY in freshly prepared 0.1 M Tris/HCl pH 8.0 and 0.02 M of TAME (Serva) was suspended in Butanol and then dissolved in 0.1 M Tris/HCl pH 8.0 containing 0.01 M $CaCl_2$. Different concentrations (0-10

µg/ml) of bovine pancreas trypsin (Serva) or crude midgut homogenate (0-100 µg protein) were used. For each fixed enzyme concentration the concentration of the substrates was varied as follows: (0.4-2 mM) BApNA; (0.156-4.68 mM) Chromozym-TRY and (0.5-2 mM) TAME. For aminopeptidase the concentrations of the substrate (LpNA) used was between 0.4 and 2 mM. The enzyme activity was determined from the change in OD/min with molar extinction for each substrate as stated above , using a Beckman DU-50 Spectrophotometer fitted with a temperature controller. Enzyme assays were carried out at 30^0 C.

After determining the appropriate working assay systems, the following procedure was generally adopted. Trypsin activity was assayed using a chromogenic substrate, Chromozym-TRY. The reaction mixtures contained Chromozym-TRY(80 µmol) and ~100 µg crude midgut homogenate in 0.1 M Tris/ HCl, pH 8.0 in 1 ml assay system. The reactions were initiated by the addition of the substrate. Increase in absorbance at 410 nm was monitored using a Beckman model DU 50 spectrophotometer fitted with a thermostat control. The change in molar extinction at 410 nm (Σ_{410} =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 mol of Chromozym-TRY per min at 30^0 C.

2.4 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) was used as the protein standard.

2.5 Effect of D-glucosamine/analogues and some hexoses on trypsin activity *in vitro*

The effect of varying concentrations (0-700 mM) of D-glucosamine, N-acetyl Glucosamine, Methyl-α-D-glucopyranoside, Methyl-β-D-glucopyranoside, Methyl-α-D-Mannopyranoside (Serva), Glucosamine pentacetate, N,N'-Diacetyl chitobiose, Inositol, α-D-Mannosamine hydrochloride (Aldrich), Galactosamine, Glucose, Mannose, Galactose (Sigma) on trypsin activity was assessed as follows. The various concentrations were preincubated with 100 µg/ml crude midgut homogenate or 5 µg/ml purified trypsin. The reactions were initiated by addition of substrate (Chromozym-TRY or BApNA). Activities were determined as described above.

2.5.1 The effect of sugars on trypsin actiavity *in vivo*

Increasing concentrations (0.0-500 mM) of glucosamine, analogues and other selected hexoses, were mixed with defibrinated rat blood obtained through heart puncture. Tsetse flies were fed on this mixture for 30 min through an artificial silicon membrane maintained at 30^0 C by a temperature regulator as described by Imbuga *et al.*, 1992 a. Thereafter, two flies were sacrificed at hourly intervals for a period of 4 h. The midguts were then dissected and tapped gently in 40 µl of 0.1 M Tris/ HCl buffer, pH 8.0. Trypsin assay was carried out using 2 µl of this crude midgut extract as described above.

2.6 Kinetic studies

Kinetic studies were carried out to determine the mechanism of inhibition, K_M, V_{max} and K_i. To determine the K_M and V_{max}, at different fixed inhibitor concentration (0-500 mM), the substrate concentration was varied between 0 and 0.32 mM while the enzyme concentration was fixed at 100 µg of protein in crude midgut homogenate. After the trypsin assays the K_M and V_{max} were determined from Lineweaver-Burk plots, double reciprocal plots of enzyme velocity against substrate concentration. The double reciprocal plots were carried out for each of the inhibitor

concentrations. The K_M , V_{max} could be inferred from the x and y intercepts respectively. Mechanism of inhibition was determined by varying the sugar concentration (0-300 mM) in different experiments at fixed substrate (Chromozym-TRY) concentrations (0-0.16 mM). The enzyme concentration was kept at 100 µg. The mode of inhibition could be inferred from the nature of the Lineweaver-Burk plots. Dixon plot was also used to cross check the nature of inhibition. The K_i was determined from the Slopes of 1/V (from Lineweaver-Burk plots) versus [Inhibitor] plot.

2.7 Transformation assays

The effect of glucosamine on transformation was assessed both in vitro and in vivo. This was carried out for teneral and non teneral flies (starved for 3 and 4 days). Fifty flies were fed as before on rat blood, containing 100 I.U/ml heparin (Gedeon Richter Ltd) to prevent clotting. The flies were immobilized by brief chilling, after which the midguts were carefully dissected. The midguts were then teased and tapped gently and mixed with parasitized blood (10^6 trypanosomes/ ml) that contained glucosamine at a final concentration of 100 mM. The incubation was

carried out at room temperature. At hourly intervals the mixture was vortexed and 20 µl were withdrawn and used to make wet smears. At the same time 2 µl was also withdrawn for trypsin assay. In the in vivo experiments, flies were fed on parasitized blood (10^6 trypanosomes/ml) that contained 100 mM glucosamine. Thereafter, two flies were sacrificed at hourly intervals. In both cases, the control experiments did not have glucosamine. The effect of glucosamine on trypanosomes was also assessed. In the these experiments, parasitized blood containing 100 mM glucosamine was kept at room temperature, and 3 µl aliquotes were taken at hourly intervals for observation of parasite mortality. The wet smears were left to dry for 14 h. The smears were fixed with methanol for 10-15 min and then stained with Giemsa's stain for between 30 min and 2 h, depending on the freshness of the stain. The slides were then rinsed once with distilled water and air dried. Examination of the slides was carried out using a Dialux compound microscope (Leitz Wetzlar, FRG). Three groups of 100 parasites were counted and classified on the basis of morphological characteristics as follows. Typical bloodstream forms

(kinetoplast positioned at the tip), transition forms (kinetoplast positioned between tip and the nucleus) or midgut forms (kinetoplast positioned with the nucleus or beyond) (Lloyd and Johnson, 1924; Ghiotto *et al.*, 1979). Refer also to scheme 1, for morphological characterization of the parasite forms.

2.8. Binding of *Glossina morsitans morsitans* midgut trypsin to sugar immobilized columns

Three samples of crude midgut homogenates (200 µl) were each dialysed for 24 h at 4^0 C in Con A buffer (0.01 M Tris/ HCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.15 NaCl, 0.04% NaN_3, pH 8.0); lentil lectin buffer (50 mM Tris/ HCl, pH 8.4); N, N'Diacetylchitobiose bead column buffer (20 mM Tris/ HCl, pH 8.0). After dialysis, the homogenate was applied to 7.5 ml Con-A sepharose (Pharmcia), 5 ml of lentil specific (Pharmacia), and 1 ml N, N' Diacetylchitobiose agarose (Aldrich) columns. Elution was carried out at a flow rate of 12 ml/h, trypsin activity as well as absorbance at 280 nm (protein content) were monitored. The elution was stopped when absorbance approached zero. Elution of bound material was subsequently carried out using 500 mM α-methylmannopyranoside for Con-A column, 500 mM Mannose for lentil column, 2 mM BApNA containing 60% (v/v) DMF in N, N'Diacetylchitobiose bead column buffer.

2.9 Trypsin Isolation and Partial Characterisation

Posterior midguts were dissected from either unfed flies or 48 h after a bloodmeal. One hundred midguts were homogenized in 1 ml 20 mM Tris/ HCl buffer, pH 8.0, 4^0 C. The homogenate was then centrifuged at 12,000 rpm for 15 min at 4^0 C. The supernatant solution was used in the isolation procedure. After filtration through a 0.2 µm Millipore filter (Nalge, Rochester NY, USA), the homogenate (500 µl) was applied on a Mono Q HR 5/5 (Pharmacia) and washed with 20 mM Tris/ HCl buffer, pH 8.0 at 20^0 C. Elution was carried out using 20 mM Tris/ HCl that contained 0.5 M NaCl in a gradient of 50% to 100% salt. Fractions of 1 ml were collected and the absorbances monitored continuously at 280 nm. The fractions with trypsin activity were pooled and concentrated by acetone precipitation. In this case, 5 volumes of ice cold acetone were added to the pooled fractions and precipitated at -20^0 C for 10 min. The solution was then centrifuged (15,000 rpm, 25 min, 4^0 C) in Corex glass centrifuge tubes (Du Pont, USA) for 25 min at 4^0 C using the Sorvall RC-5C Automatic Superspeed Refrigerated Centrifuge (Du Pont). The resulting pellet was washed three times with acetone then lyophilized at -40^0 C in a Virtis Freeze Drier model 10-030 (Gardiner, USA). The protein was freeze dried in ice cold 20 mM Tris/ HCl, pH 8.0, and designated Mono Q fraction. For further purification, gel permeation chromatography was used.

Approximately 0.1 mg of the Mono Q fraction was applied on to the Superose 12 column attached to the FPLC. The column was washed with 20 mM Tris/ HCl buffer, pH 8.0, at a flow rate of 0.5 ml/min. Fractions with trypsin activity were pooled and concentrated using acetone. The concentrated lyophilized sample was freeze dried in the washing buffer, and designated Superose fraction. This partially purified sample was further subjected to affinity chromatography or electroelution. Soybean trypsin inhibitor coupled to agarose (Pharmacia) was used as the affinity column. It has been previously shown that the inhibitor used as a ligand is very efficient in complexing all tsetse trypsin activity (Gooding and Rolseth, 1976). Bound trypsin was eluted at 4^0 C with 0.1 M Glycine/ HCl buffer, pH 2.5 that contained 10 mM TAME synthetic substrate. The stability of trypsin under this acidic conditions was determined. The trypsin active fractions were pooled and concentrated on a PM-10 membrane (Amicon, USA). Alternatively, the enzymes were further purified by electroelution using the BIORAD system as described by the manufactures. Purifed trypsin and trypsin-like enzymes were studied for optimal pH activity and stability under denaturing conditions. For the determination of optimal pH activity, the pH was varied between 4.0 to 9.0 while all the other parameters were kept as described under section 2.3.1. Purified enzyme stability was accessed by boiling the samples(~1µg, 1-5 min) in the presence of β-mecaptoethanol prior to enzyme assay.

2.9.1 Electrophoresis

Polyacrylamide gel electrophoresis (PAGE) were conducted on gradient gels (4-20%). A discontinuous buffer system (electrode buffer: 0.025 M Tris/ Glycine, pH 9.0; gel buffer: 0.375 M Tris/ HCl, pH 8.9) were

used. The samples 20% (v/v) glycerol, 0.002% (w/v) bromophenol blue) before application on to the gel.

SDS-PAGE was carried out on a gradient gel (4-20%) according to the method of Laemmli (1970). Molecular weight standards were obtained from Bio-Rad or Pharmacia.

2.9.2 Agglutination assays

The agglutination assays were carried out on Microwell plates (Nunc, Denmark). Bloodstream forms of *Trypanosoma brucei brucei* were isolated using DEAE-52 Cellulose. The column was first equlibrated with 50 mM Phosphate saline glucose buffer, pH 8.0. The parasites which were in the eluent were then centrifuged (4^0 C, 2000 g, 10 min). The pellet was then resuspended in a minimum volume of 50 mM Phosphate saline glucose buffer, pH 8.0. Cultured procyclic forms of *T. b. brucei* (kindly donated by N. Darji, LRU, ICIPE) were also used in the assays. The wells contained 10^6 trypanosomes/ ml and two-fold serially diluted midgut homogenates (100 - 0 µg) or purified midgut trypsins (1 - 0 µg). In the control experiments, 50 mM Phosphate saline glucose buffer, pH 8.0 was used together with the parasites. The incubation was carried out at 27^0 C for 2 h. Thereafter agglutination was observed using inverted microscope (Leitz Labovert, FRG).

2.9.3 Effects of STI and TLCK on trypsin and trypsin like enzymes

Separation of trypsin and trypsin-like enzymes was carried out as follows. The midgut homogenate was filtered using Nalge syringe filters (0.22 m) and applied onto a Mono Q anion exchange column. The enzymes were separated as flow through and bound, respectively (Section 2.9). The two enzymes were studied for effects by STI (Millipore Corp., Freehad, USA) and TLCK (Sigma, St. Louis, Mo., USA). The K_i and K_M values were determined by fixing enzyme concentration at 10 µg and varying substrate (Chromozym-TRY) concentration (0.016-0.16 mM). The concentrations of the inhibitors were varied as follows: STI (0.0-0.4 µg/ml) and TLCK (0.0-1.6 M).

2.9.4 Staining for trypsin activity on PAGE

Native-PAGE were carried out at 4^0 C, using duplicates of the samples 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 at 30^0 C for 20- 50 min, in 50 ml of 0.1 M Posphate buffer, pH 8.0 that contained 0.5 mg/ml BANA, 10% DMF (Sigma). Thereafter, 5 mg/ml of Diorthoanisidine tetrazotised (Sigma) was added. After colour development the reaction was stopped by

adding 0.1% acetic acid (Aldrich). On the other hand, SDS-PAGE was carried out and the gel incubated at 25^0 C in 2.5% Triton-X100 (Sigma) to remove SDS prior to staining for trypsin activity as described above.

CHAPTER 3

RESULTS

3.1 Enzyme assay in crude midgut homogenate

The three trypsin substrates, BApNA, Chromozym-TRY, and TAME were used to establish the trypsin assay in tsetse midgut homogenate. The following assay systems were developed for both the bovine pancreas trypsin (Serva) and the crude midgut homogenate of the tsetse fly, *Glossina morsitans morsitans* :

(i) 0.0945 M Tris/ HCl buffer pH 8.0, 2 mM BApNA, 5 g bovine pancreas trypsin or 100 µg crude midgut homogenate of *G. m. morsitans*.

(ii) 0.0945 M Tris/ HCl buffer pH 8.0, 1.248 mM Chromozym-TRY, 5 µg bovine pancreas trypsin or 10 µg crude midgut homogenate of *G. m. morsitans*.

(iii) 0.0945 M Tris/ HCl buffer pH 8.0, 2mM TAME, 5 µg bovine pancreas trypsin or crude midgut of *G. m. morsitans*.

The assay systems were developed on the basis that the substrate concentrations did not result in feedback inhibition. Moreover, the enzyme activity was linear upto 5 min under the assay conditions with a SD = $\pm$ 0.02, n = 6 (Table 1). While all the assay systems were used method (ii) above was generally adopted.

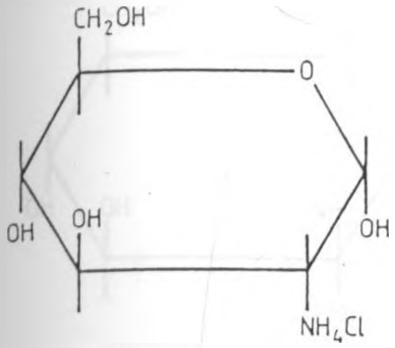
Table 1. Trypsin assay in the presence of three different substrates.

Time (min)	BApNA	Chromozym-TRY	TAME
1	0.0870	0.6750	0.0880
2	0.0850	0.6740	0.0890
3	0.0890	0.6750	0.0870
4	0.0860	0.6730	0.0880
5	0.0850	0.6760	0.0860

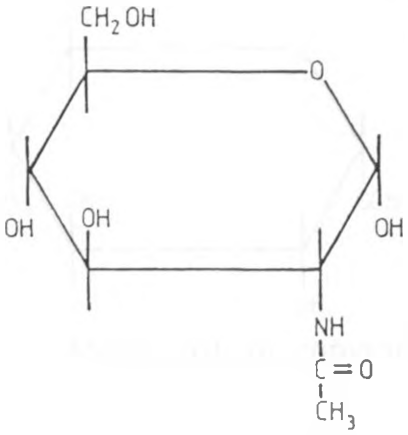
The Table shows trypsin activity (change in OD/ min) as a function of time (min) for the various substrates, BApNA, Chromozym-TRY and TAME. SD= ± 0.02, n=6.

3.2 Effect of the sugars on tsetse midgut trypsin activity, *in vitro*

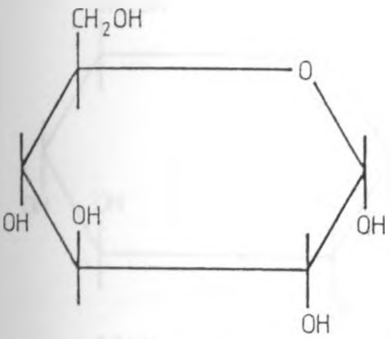
Trypsin activity was assayed in the presence of the following sugars: D-glucosamine, N-acetyl glucosamine, methyl-α–D-glucopyranoside, methyl-β-D-glucopyranoside, methy-α-D-mannopyranoside, glucosamine pentacetate, N,N'-diacetyl chitobiose, inositol, α-D-mannosamine hydrochloride, galactose, glucose, mannose and galactose. The structures of the sugars are illustrated in scheme 2. The concentrations used were as in **Materials and Methods**. D-Glucosamine, inhibited tsetse trypsin activity both *in vivo* and *in vitro* (Fig. 1 a). Increasing D-glucosamine concentrations, resulted in progressive decrease in trypsin activity. In a parallel experiment, D-glucosamine had similar inhibitory effect on bovine pancreas trypsin (Fig. 2). In both cases the type of inhibition was analyzed by Dixon plots (reciprocals of enzyme velocity plotted as a function of D-glucosamine concentration). The Dixon plots for tsetse midgut trypsin activity (Fig. 1 b), and bovine pancreas trypsin (Fig. 2, inset), gave non linear curves characteristic of partial inhibition. The other sugars (listed above) had no effect on tsetse midgut trypsin activity, even at concentration as high as 700 mM (Fig. 1 a).



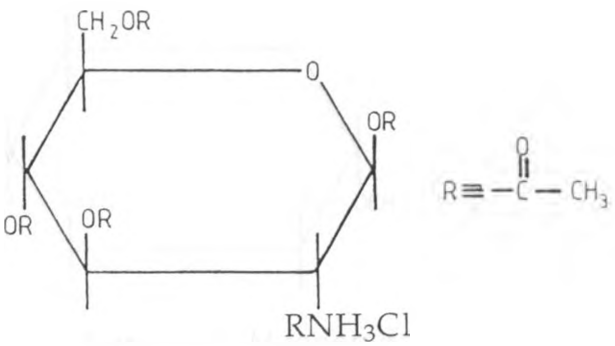
α-D-Glucosamine
Hydrochloride



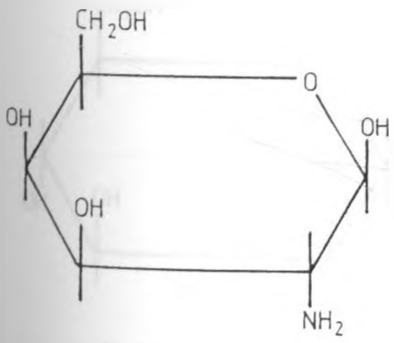
α-D-N-Acetyl Glucosamine



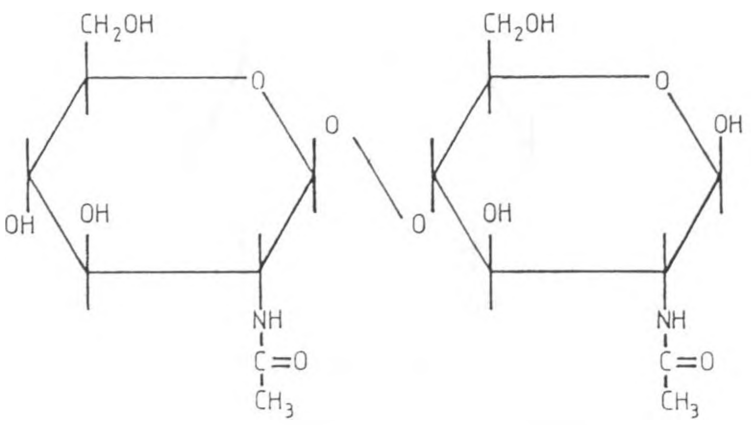
α-D-Glucose



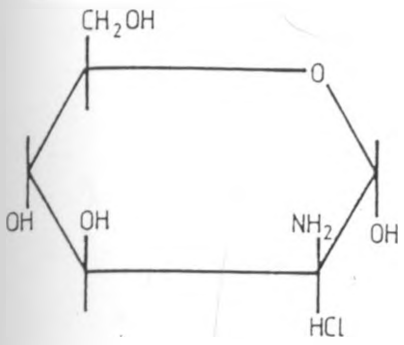
β-D-Glucosamine pentacetate
Hydrochloride



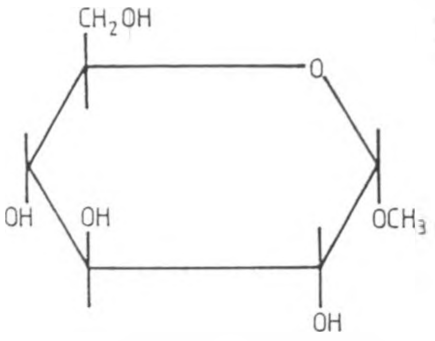
β-D-Galactosamine



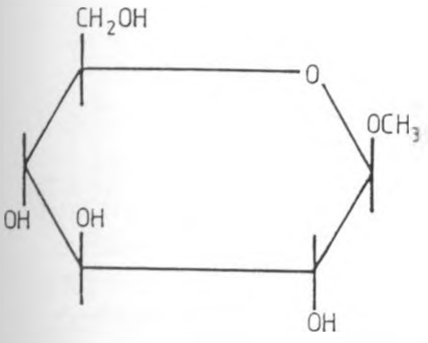
N,N-Diacetylchitobiose



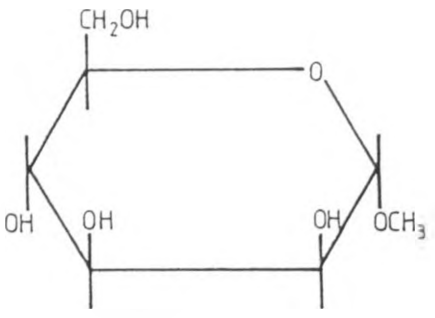
α-D-Mannosamine
Hydrochloride



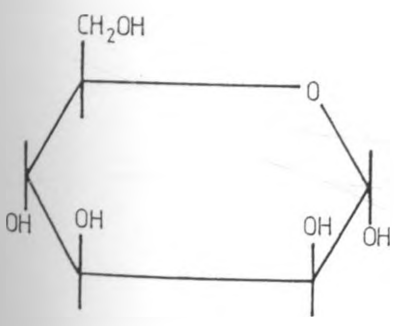
Methyl-α-D-glucopyranoside



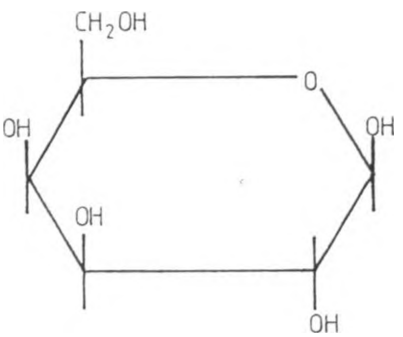
Methyl-β-D-glucopyranoside



Methyl-α-D-Mannopyranoside



α-D-Mannose



β-D-Galactose

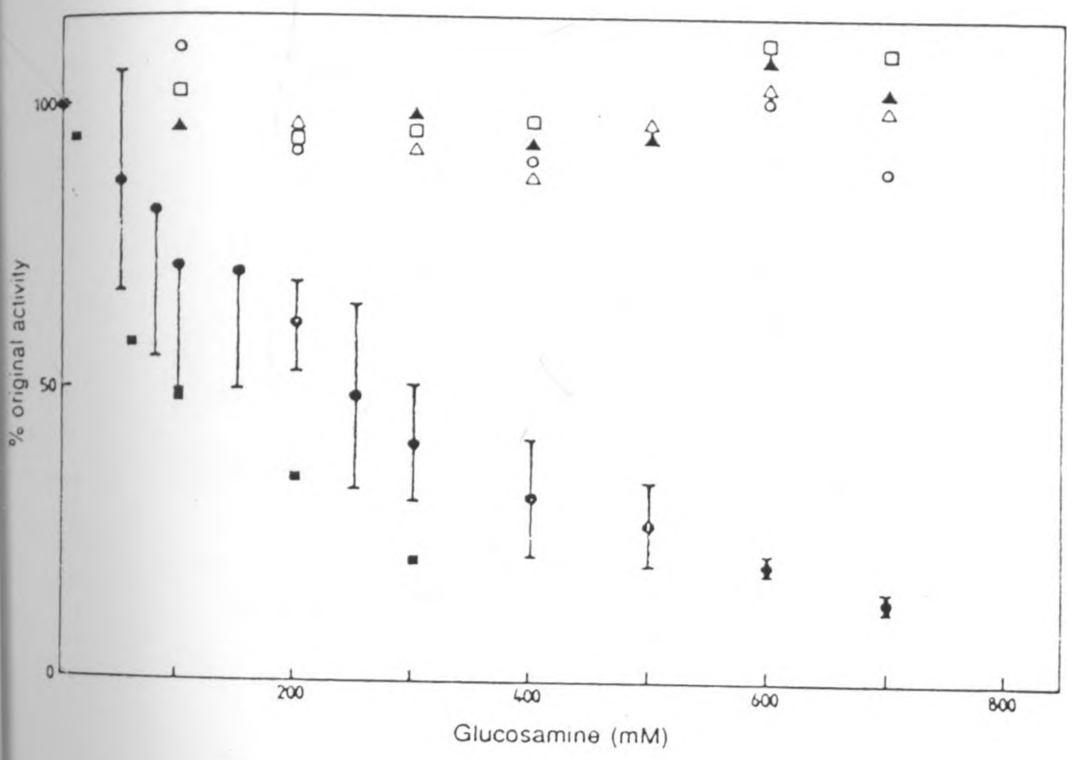

100
50
0
% original activity
200
400
600
800
Glucosamine (mM)

Fig. 1 b Dixon plot of the data in figure 1 a. The plot represents the reciprocal of initial velocity (V) versus increasing concentration of D-glucosamine.

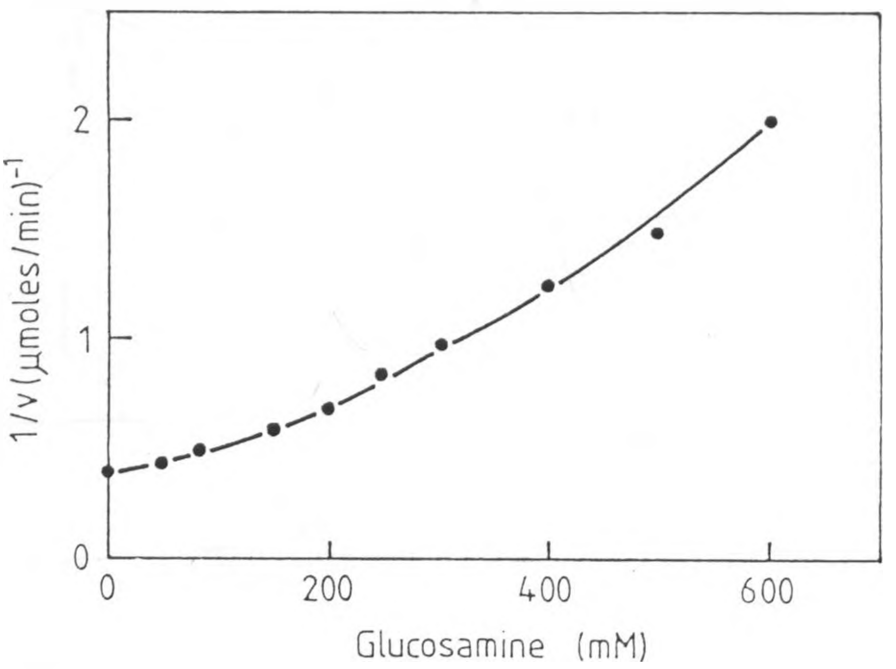

1/v (μmoles/min)$^{-1}$
2
1
0
0
200
400
600
Glucosamine (mM)

Fig. 2 Inhibition of bovine pancreas trypsin activity by D-glucosamine *in vitro*. Bovine pancreas trypsin activity was assayed as described in Figure 1 a. Inset: Dixon plot of the reciprocal of initial velocity (V) versus increasing concentrations of D-glucosamine.

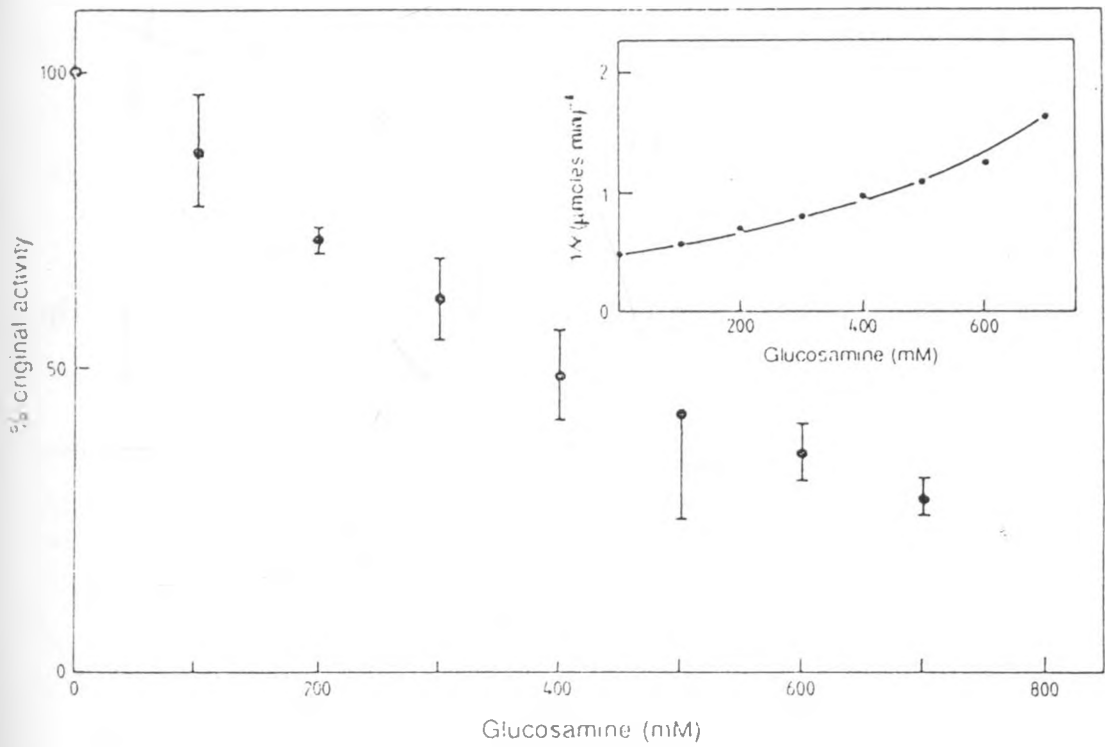
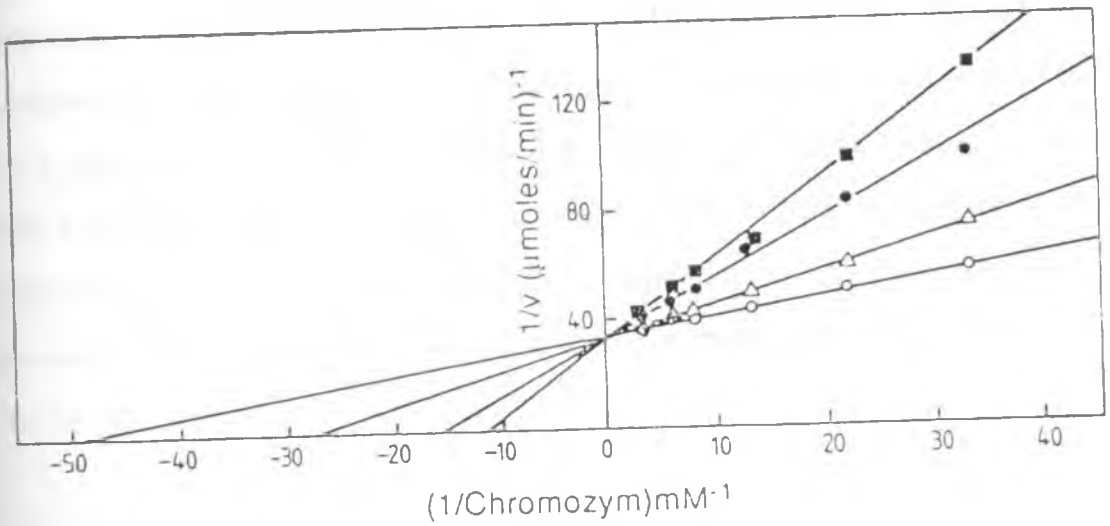

100
50
0
% original activity
0
200
400
600
800
Glucosamine (mM)
2
1
0
200
400
600
Glucosamine (mM)

Fig. 3 Lineweaver-Burk analysis of trypsin inhibition by D-glucosamine. Double reciprocal plot of the initial velocity (V) versus increasing concentrations of substrate (Chromozym-TRY) determined at various fixed D-glucosamine concentrations (○)= 0,(△)=100, (●)=200, (▬)=300 mM.


1/v (µmoles/min)⁻¹
120
80
40
-50
-40
-30
-20
-10
0
10
20
30
40
(1/Chromozym)mM⁻¹

3.2.1 Glucosamine and glucose effects on tsetse trypsin activity, *in vivo*

Teneral flies (24 h after emergence) were fed for 30 min on rat blood containing varied concentrations of glucosamine and glucose (0-500 mM). Trypsin assays were carried out at hourly intervals. The results showed that increasing concentrations of D-glucosamine resulted in a progressive decrease in trypsin activity (Fig. 1 a). The effect of the various D-glucosamine concentrations on the mortality of the flies was also observed for a period of 72 h (Table 2). Flies that were fed on blood containing 500 mM had 100% mortality within 6 h, whereas 300 and 200 mM caused the same rate of mortality after 24 and 48 h, respectively. On the other hand, glucose had no effect on the flies even at concentrations as high as 500 mM (Table 3).

Table 2. Effect of Glucosamine on tsetse flies.

Glucosamine (mM)	0	7.5	60	100	200	300	500
Time (h)							
0	100	100	100	100	100	100	100
12	100	100	100	98	90	50	0
24	100	100	98	80	50	0	0
48	100	100	96	50	0	0	0
72	100	100	94	40	0	0	0

Teneral flies were fed on blood containing various concentrations of D-glucosamine. The table shows the number of flies alive at a given time. Data are the average of 4 determinations.

Table 3. Effect of feeding tsetse flies on various concentrations of glucose

Glucose(mM)	0	60	100	200	300	500
Time (h)						
0	100	100	100	100	100	100
12	100	100	100	100	100	100
24	100	100	100	100	100	90
48	100	100	100	94	94	50
72	100	100	94	92	90	40

Teneral flies fed on blood containing various concentrations of glucose. The table shows the number of flies alive at a given time. Data are the average of 4 determinations.

3.2.2 Reversible effect of D-glucosamine inhibition on tsetse trypsin activity

The inhibitory effect of D-glucosamine could be removed by increasing the concentration of the substrate. In this study, 300 mM (Table 4) or 500 mM (Table 5) D-glucosamine were used to inhibit the trypsin activity. Tripling the substrate concentration in the presence of 300 mM glucosamine resulted in the full recovery of enzyme activity. However, increasing the substrate concentration four fold resulted in substrate inhibition. Similarly, four times the initial substrate concentration in the presence of 500 mM glucosamine resulted into recovery of trypsin activity, however, the recovery was 75.5 %.

Table 4. Reversible inhibition of midgut trypsin activity by D-glucosamine.

D-Glucosamine (mM)	0	300	300	300	300
Chromozym-TRY (mM)	0.078	0.078	0.156	0.234	0.312
Trypsin activity	100	53.2	86.2	100	67.0

Glucosamine (300 mM) was preincubated with undiluted tsetse midgut homogenates (~100 µg/ml) in 0.1M Tris-HCl (pH 8.0) and assayed for enzyme activity in the presence of increasing concentrations of the substrate (Chromozym-TRY). Data are the average of 5 determinations

Table 5. Reversible inhibition of midgut trypsin activity by D-glucosamine.

D-Glucosamine (mM)	0	500	500	500	500
Chromozym-TRY (mM)	0.078	0.078	0.156	0.234	0.312
Trypsin activity	100	41.8	60.2	67.3	75.5

Glucosamine (500 mM) was preincubated with undiluted tsetse midgut homogenates (~100 µg/ml) in 0.1M Tris-HCl (pH 8.0) and assayed for enzyme activity in the presence of increasing concentrations of the substrate (Chromozym-TRY). Data are the average of 5 determinations.

3.2.3 Effect of D-Glucosamine on aminopeptidase activity

The effect of glucosamine (0-700 mM) on aminopeptidase activity was assessed in vitro The results showed that glucosamine even at concentrations as high as 700 mM had no effect on activity of this enzyme (Table 6).

Table 6. Effect of D-glucosamine on aminopeptidase

D-Glucosamine (mM)	0	100	200	300	400	500	600	700
% original activity	100	109	102	98	103	100	101	104

Table 6. Aminopeptidase (100 µg/ml) activity was assayed in vitro in the presence of glucosamine (0-700 mM). The assays were carried out using 2 mM LApNA as the substrate. Data are the average of 3 determinations.

3.3 Kinetic studies on the effect of D-glucosamine on tsetse trypsin activity

The mode of inhibition by D-glucosamine on tsetse trypsin activity was competitive as shown by Lineweaver-Burk plot (Fig. 3) (Double reciprocal plots, depicting initial velocity (V) versus substrate concentration). The concentrations of the substrate were varied between 0.032 and 0.32 mM. The double reciprocal plots gave the same V_{max}, while the apparent K_M values increased with increasing glucosamine concentrations. The V_{max} and K_M values were determined to be 250 µmoles/min/mg and 42 ± 3 µM, respectively (Fig 3). The K_i value was determined from a replot of $Slope_{1/V}$ versus glucosamine concentration (Fig. 4). The values of $Slope_{1/V}$ and D-glucosamine concentrations were derived from figure 3. The intercept of the x-axis of Fgure 4 gave the K_i value as 68 ± 3 mM.

The competitive inhibition equation is:

$$V = \frac{V_{max}[S]}{K_M(1+[I]/K_i)+[S]}$$

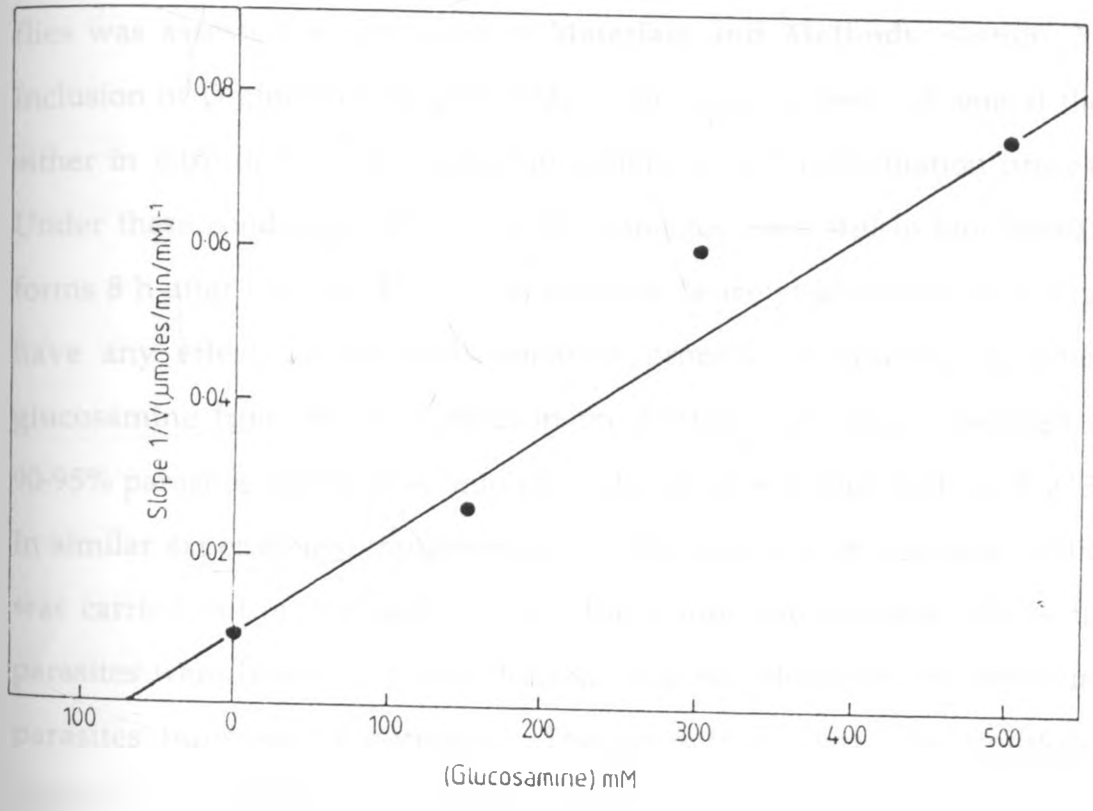
Therefore, $1/V = \frac{1}{[S]}\left[\frac{K_M 1+[I]/K_i)}{V_{max}}\right] + \frac{1}{V_{max}}$

Note that y intercept = $1/V_{max}$, Hence unchanged V_{max} in double reciprocal plots of 1/V versus 1/[S]

Subsequently, slopes of double reciprocal plots gives

$Slope_{1/v} = [I]\frac{Km}{K_i V_{max}} + \frac{Km}{V_{max}}$ Note that when $Slope_{1/v}=0$ $[I]=-K_i$, hence K_i= -x axis intercept.

Fig. 4 Plot of $Slope_{1/v}$ versus D-glucosamine concentration. Data were derived from Figure 3.


0·08
0·06
0·04
0·02
Slope 1/V(μmoles/min/mM)-1
100
0
100
200
300
400
500
(Glucosamine) mM

3.3.1 Effect of D-glucosamine on transformation of Trypanosoma brucei brucei

The effect of D-glucosamine on transformation of Trypanosoma brucei brucei bloodstream to procyclic forms in teneral and non-teneral flies was assessed as described in **Materials and Methods,** section 2.7. Inclusion of D-glucosamine (100 mM) in the infective feed of teneral flies either in vitro or in vivo resulted in inhibition of transformation process. Under these conditions, 87-90% of the parasites were still in bloodstream forms 8 h after feeding (Fig. 5). In contrast, N-acetyl-glucosamine did not have any effect on the transformation process. Likewise, excluding glucosamine from the bloodmeal in the control experiment, resulted in 90-95% parasites transformed into procyclic forms 8 h after feeding (Fig. 5). In similar experiments, transformation of the parasites in non-teneral flies was carried out *in vitro* and *in vivo.* The results showed that 90% of the parasites were lysed 1-2 h after feeding (Fig. 6). However, the surviving parasites transformed normally. Trypsin activity was also monitored during the transformation experiments. The results showed that trypsin activity was reduced by 50% in the presence of 100 mM glucosamine and by 80-75% in the presence of glucosamine (100 mM) and parasites (Fig. 7). A comparison of midgut trypsin levels induced by a bloodmeal in teneral and non-teneral flies was monitored for 8 h (Fig. 8). Two midguts were carefully dissected and the enzyme released by gentle teasing. The results showed that the midgut trypsin levels were 2-3 times higher in the non-teneral compared to the teneral flies (Fig 8). In addition, the effect of

glucosamine (100 mM) on transformation in non-teneral flies (starved for 3 and 4 days) was carried out. As shown in Figure 9, the transformation was partially inhibited. In this case 65 % of the parasites had transformed into the procyclic forms by 8 h. Interestingly, 16% of the parasites had transformed in the non-teneral flies fed on 100 mM D-glucosamine after 1-2 h. This observation was in contrast to teneral flies fed on glucosamine free blood.

3.4 Binding of tsetse trypsin onto immobilized sugar columns.

The possibility of binding of crude midgut trypsin onto the following sugar immobilized columns; Con A, lentil and N, N'diacetylchitobiose was carried out. As shown in figure 10, none of the columns could bind the midgut trypsin. Elution of bound proteins were carried out from fraction 10 using the respective specific sugars and buffers as stated in section 2.8.

Fig. 5 Effect of D-glucosamine, N-acetyl-glucosamine on transformation of *T. b. brucei* bloodstream to procyclic forms in vivo and in vitro in teneral flies. The transformation experiments were carried out as previously described. The % bloodstream forms were determined at hourly intervals in the presence of D-glucosamine *in vivo* (■), *in vitro* (△); N-acetyl-glucosamine *in vivo* (○); in the absence of glucosamine *in vivo* (●), *in vitro* (▲).

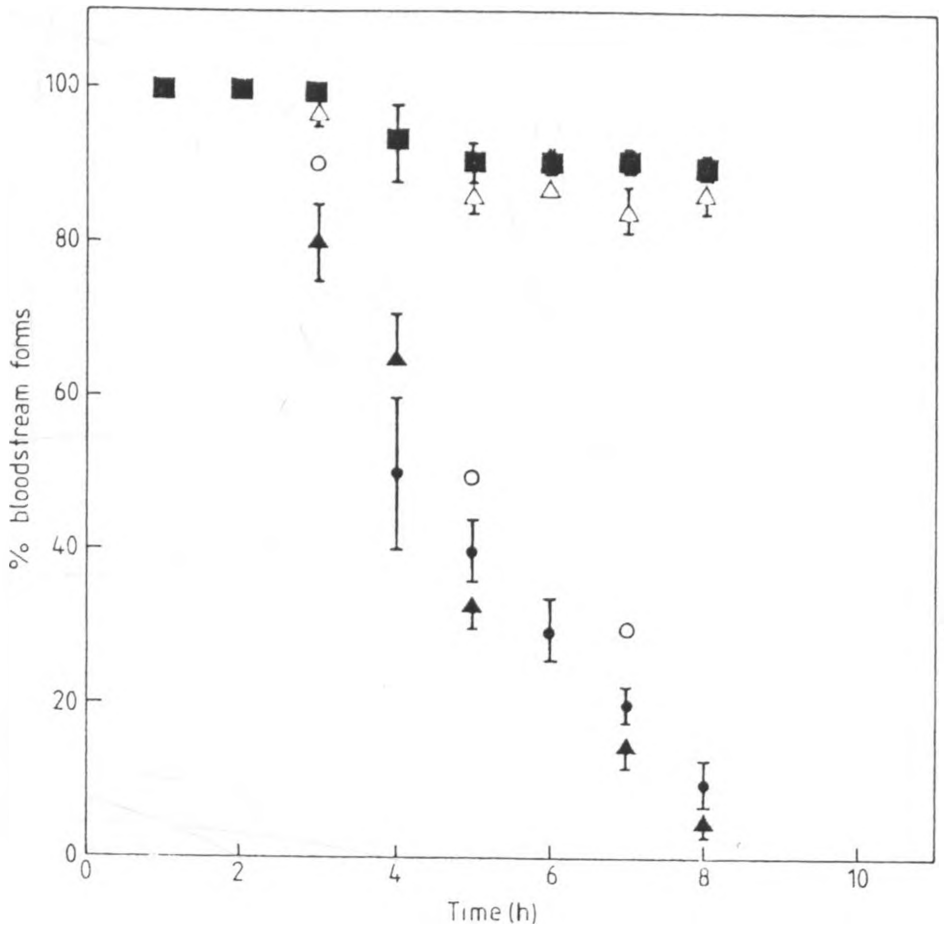

100
80
60
40
20
0
% bloodstream forms
0
2
4
6
8
10
Time (h)

Fig. 6 **Transformation of *T. b. brucei* bloodstream to procyclic forms in teneral and non-teneral flies (starved for 3 days), in vivo and in vitro.** Teneral *in vivo* (□), *in vitro* (■), non-teneral flies *in vivo* (○) *in vitro* (●), teneral parasite numbers (△) and non-teneral parasite numbers (▲).

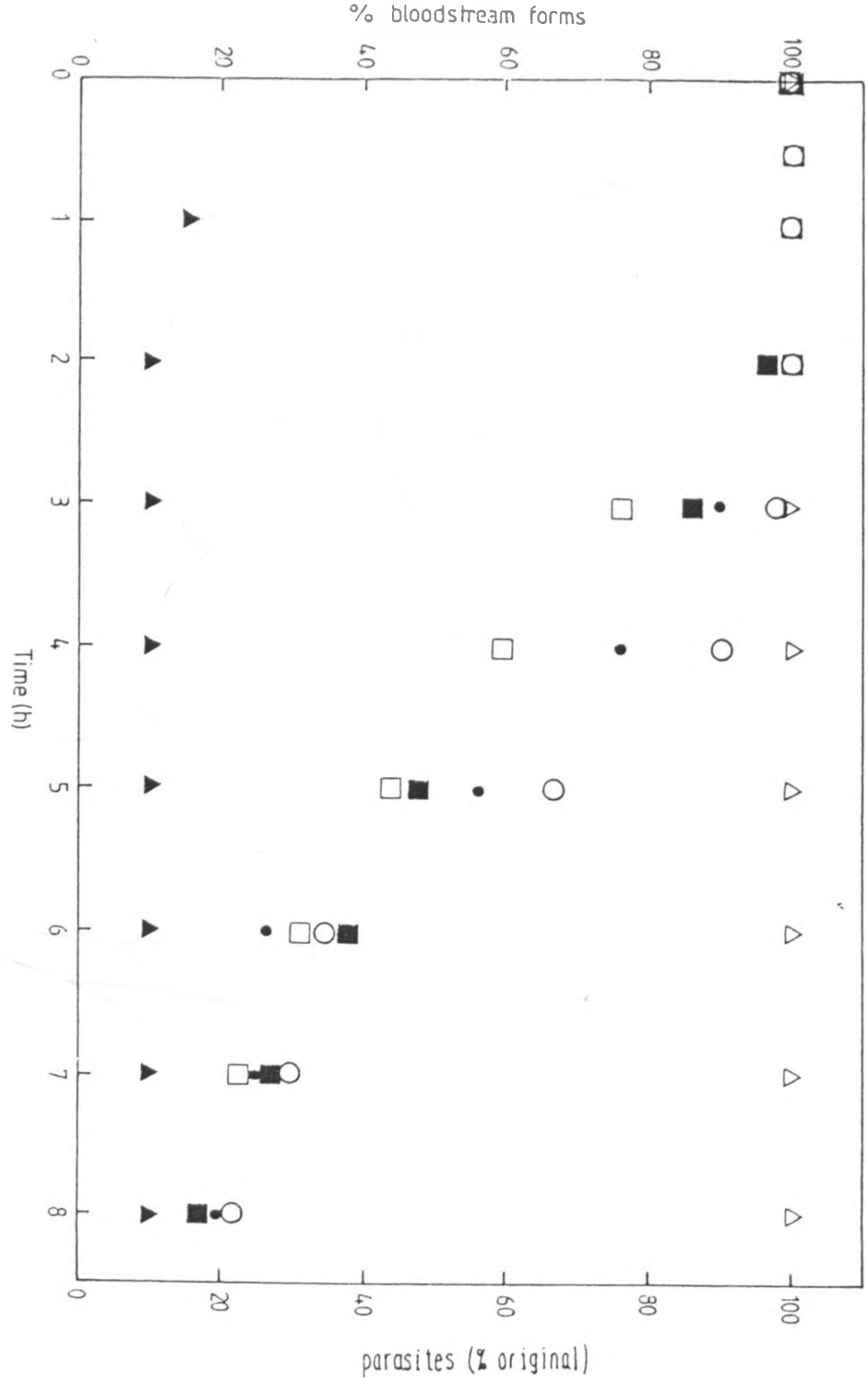

% bloodstream forms
0
20
40
60
80
100
Time (h)
0
1
2
3
4
5
6
7
8
parasites (% original)

Fig. 7 Trypsin activity monitored at hourly intervals during the course of transformation, *in vivo* and *in vitro*. Activity was assayed in the presence of D-glucosamine in vivo (■), in vitro (△); in the absence of glucosamine *in vivo* (●), *in vitro* (▲); in the presence of N-acetyl-glucosamine *in vitro* (○); in the absence of D-glucosamine and parasites *in vivo* (□).

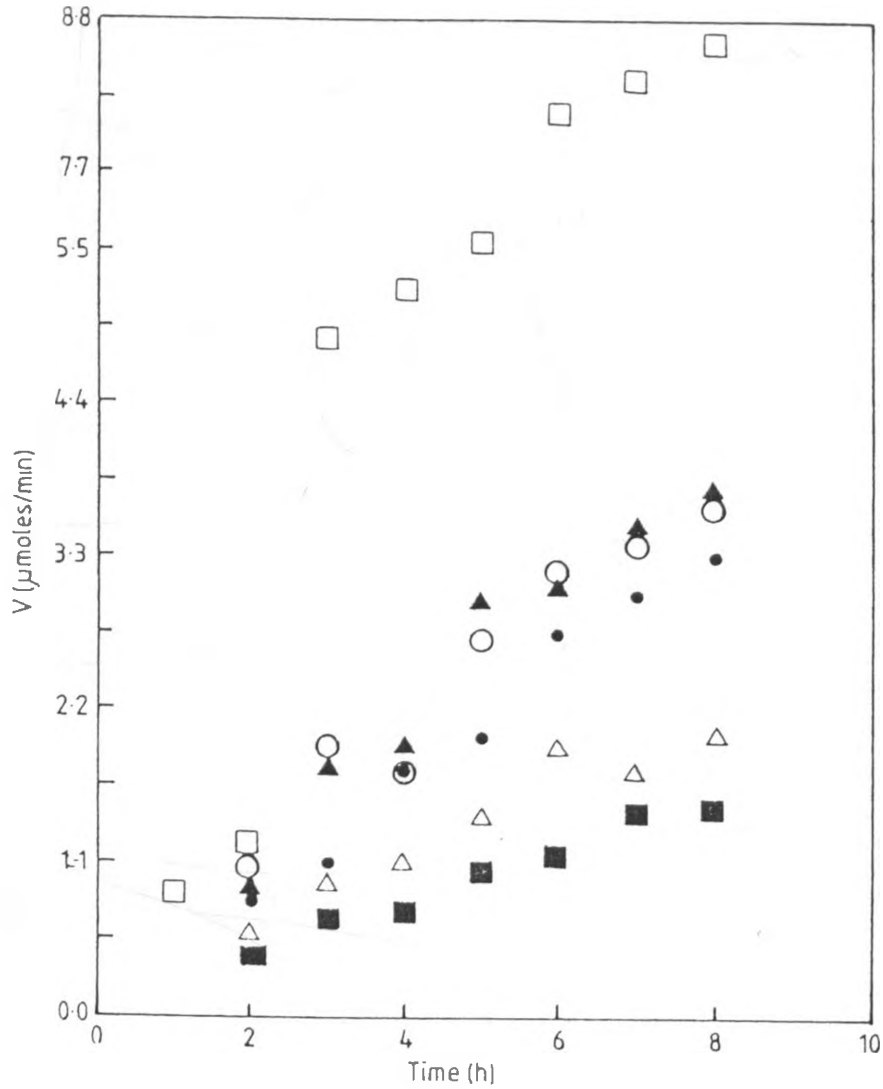

8·8
7·7
5·5
4·4
3·3
2·2
1·1
0·0
V (μmoles/min)
0
2
4
6
8
10
Time (h)

Fig. 8 Tsetse trypsin activity plotted as a function of time, for teneral (●) and non-teneral flies (○), *in vivo*. The flies were fed on rat blood and tsetse trypsin generated by gentle teasing following dissection.

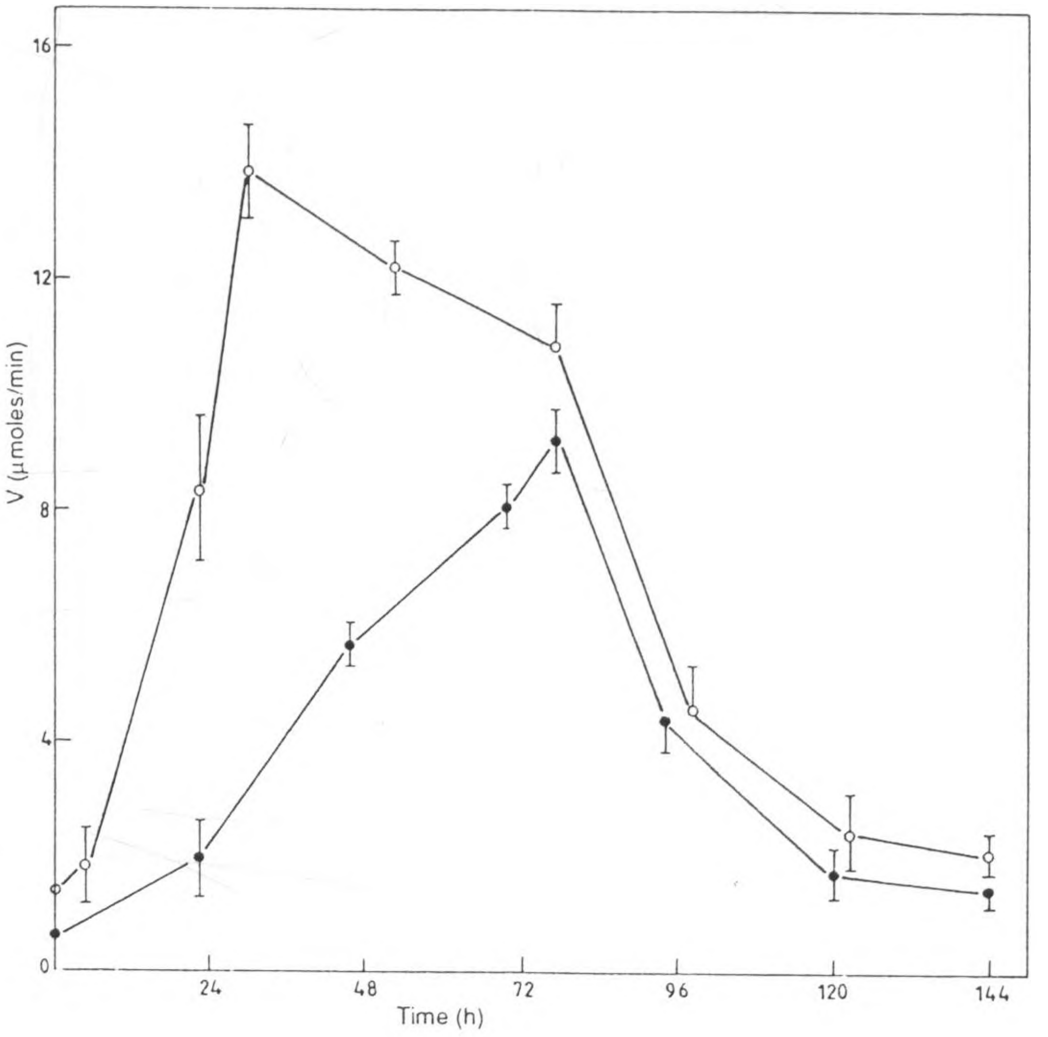

16
12
8
4
0
V (µmoles/min)
24
48
72
96
120
144
Time (h)

Fig. 9. **The effect of glucosamine on transformation of *T. b. brucei* bloodstream to procyclic forms in non-teneral flies (starved for 3 and 4 days), *in vivo*.** Non-tenerals starved for 3 days (▲), tenerals (□), non-tenerals starved for 4 days (□), control tenerals (●).

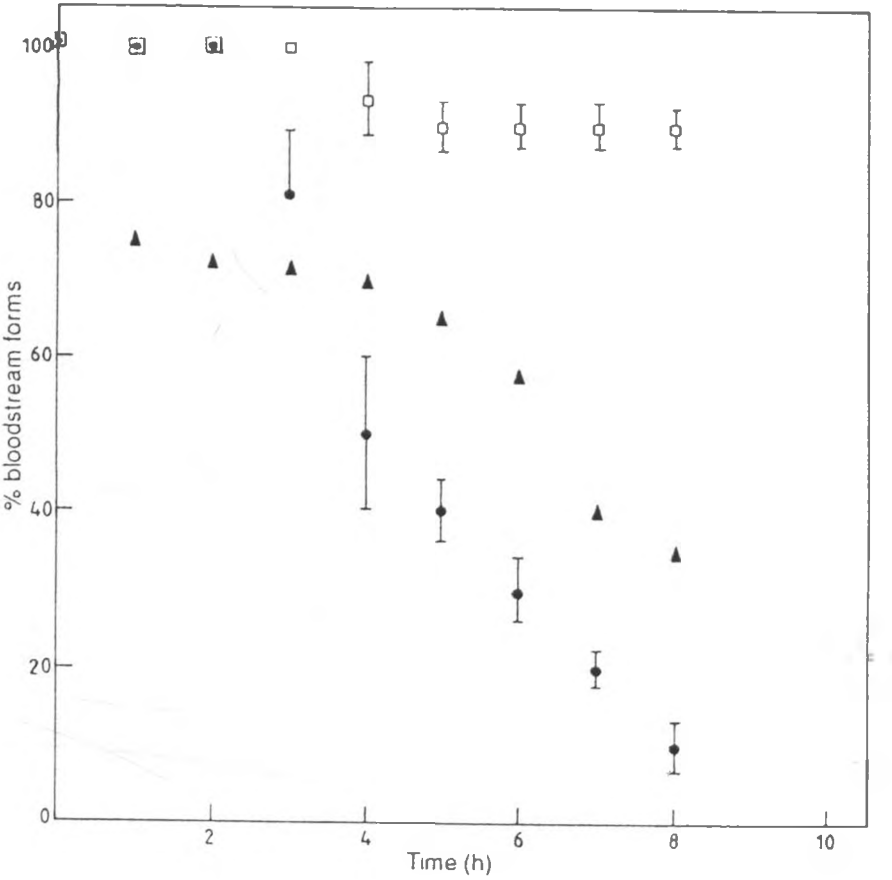
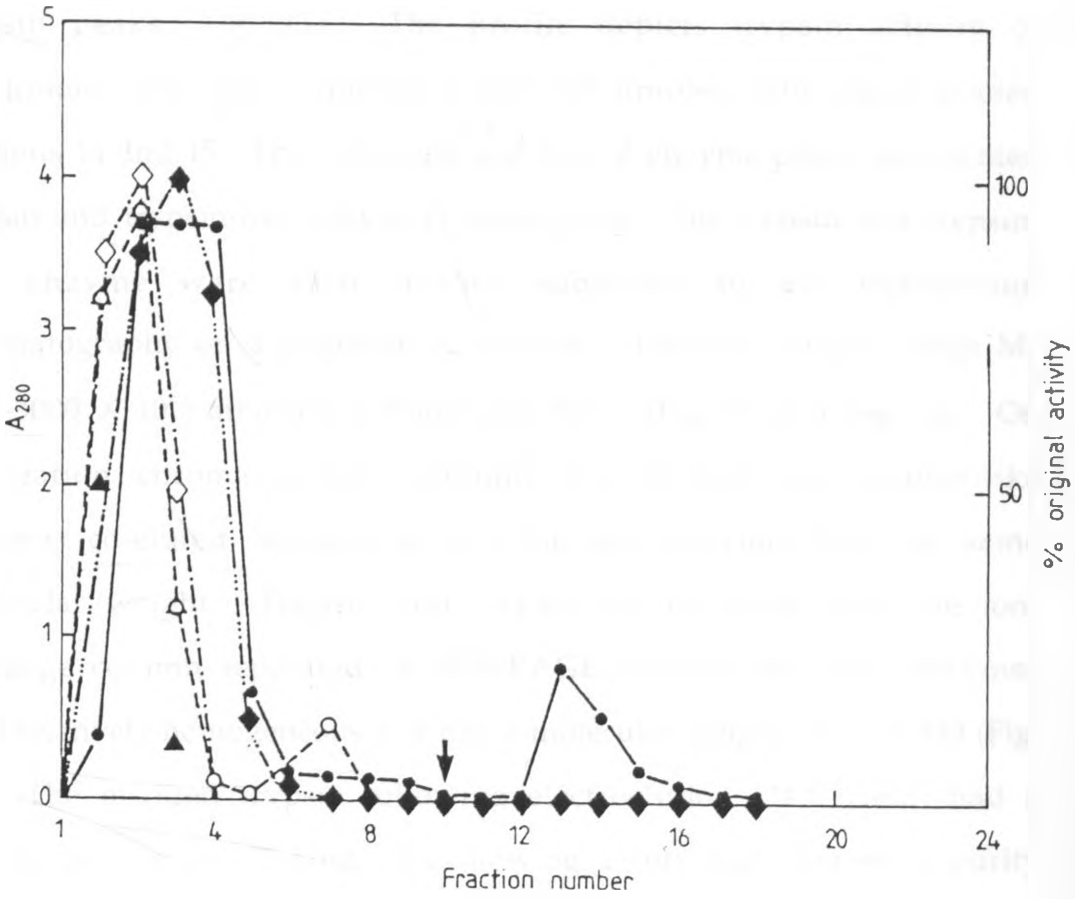

100
80
60
40
20
0
% bloodstream forms
2
4
6
8
10
Time (h)

Fig. 10 Elution profile for crude midgut homogenate on Con-A, Lentil and N, N' diacetyl chitobiose columns. The eluents were assayed for trypsin activity (◆), (◇), (▲) and protein content (●), (○), (△), respectively. The bound proteins were eluted from fraction 10 indicated by the arrow.


5
4
3
2
1
0
A_{280}
100
50
% original activity
1
4
8
12
16
20
24
Fraction number

3.5 Purification of tsetse midgut trypsin and partial characterisation

The purification of tsetse midgut trypsin utilized anion exchange, gel permeation and affinity chromatography, and electroelution. The anion exchange chromatography on a Mono Q column resulted in two trypsin peaks (Fig. 11). The profile depicts trypsin activity of 0.34 µmoles/min/mg in fraction 3 and 0.09 µmoles/min/mg in pooled fractions 14 and 15. The unbound and bound enzyme peaks represented trypsin and trypsin-like enzymes, respectively. The trypsin and trypsin-like enzyme were each further subjected to gel permeation chromatography on a Superose 12 column (Molecular weight range M_r 1000-3000 X 10^5) attached a Pharmacia FPLC (Fig. 12 and Fig. 13). On permeation chromatography column, the trypsin and trypsin-like enzymes co-eluted, suggesting that the two enzymes had the same molecular weight. Trypsin and trypsin-like enzymes from the ion-exchange column separated on SDS-PAGE showed that both enzymes were relatively homogeneous and had a molecular weight of ~ 24 KD (Fig. 14). The purified trypsin following electroelution (34% yield) had a specific activity of 375 Units/mg showing a very high degree of purity (Table 7). In a parallel experiment, trypsin and trypsin-like enzymes were stained for activity after separation by non-denaturing PAGE. The results showed that trypsin (unbound peak) had three bands which stained for enzyme activity (Mr ~ 128, 73 and 57) (Fig. 15). On the other hand, trypsin-

like enzyme peak had two bands which stained for enzyme activity (~ 73 and 58 KD) (Fig. 15). The trypsin had an activity that was 2-3 times more compared to trypsin-like enzyme. Both enzymes were similarly inhibited by D-glucosamine (Fig. 16). In another experiment, trypsin was subjected to SDS-PAGE and then stained for enzyme activity. This results showed only one band which stained for enzyme (Mr ~ 24) (Fig. 17). Further characterisation of the trypsin and trypsin-like enzyme, showed that activity was optimal between pH 7-8, and declined at pH 6 and 8.8 . In addition, activity was partially retained following boiling for 5 min in the presence of β-mecaptoethanol.

Fig. 11 Separation of trypsin and trypsin-like enzyme on anion exchange chromatography. The fractions were assayed for trypsin activity (---) and protein content, A_{280} (——) The % indicate salt gradient.

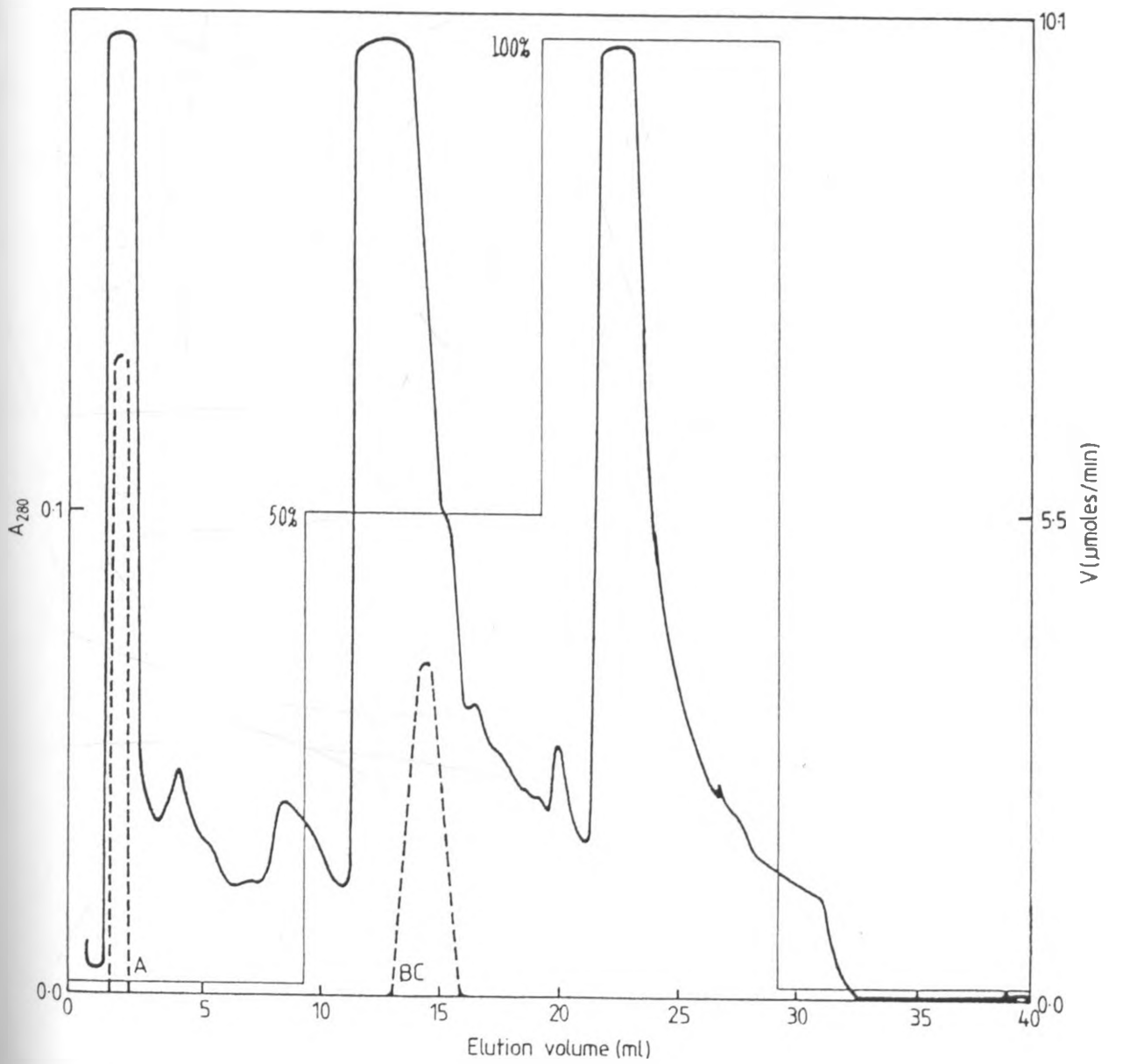

100%
50%
A
BC
A_{280}
0·1
0·0
10·1
5·5
0·0
V (μmoles/min)
0
5
10
15
20
25
30
35
40
Elution volume (ml)

Fig. 12 Gel permeation of trypsin (Mono Q fraction A) on the Superose 12 column. Protein content, A_{280} (—) and E, trypsin activity (---).

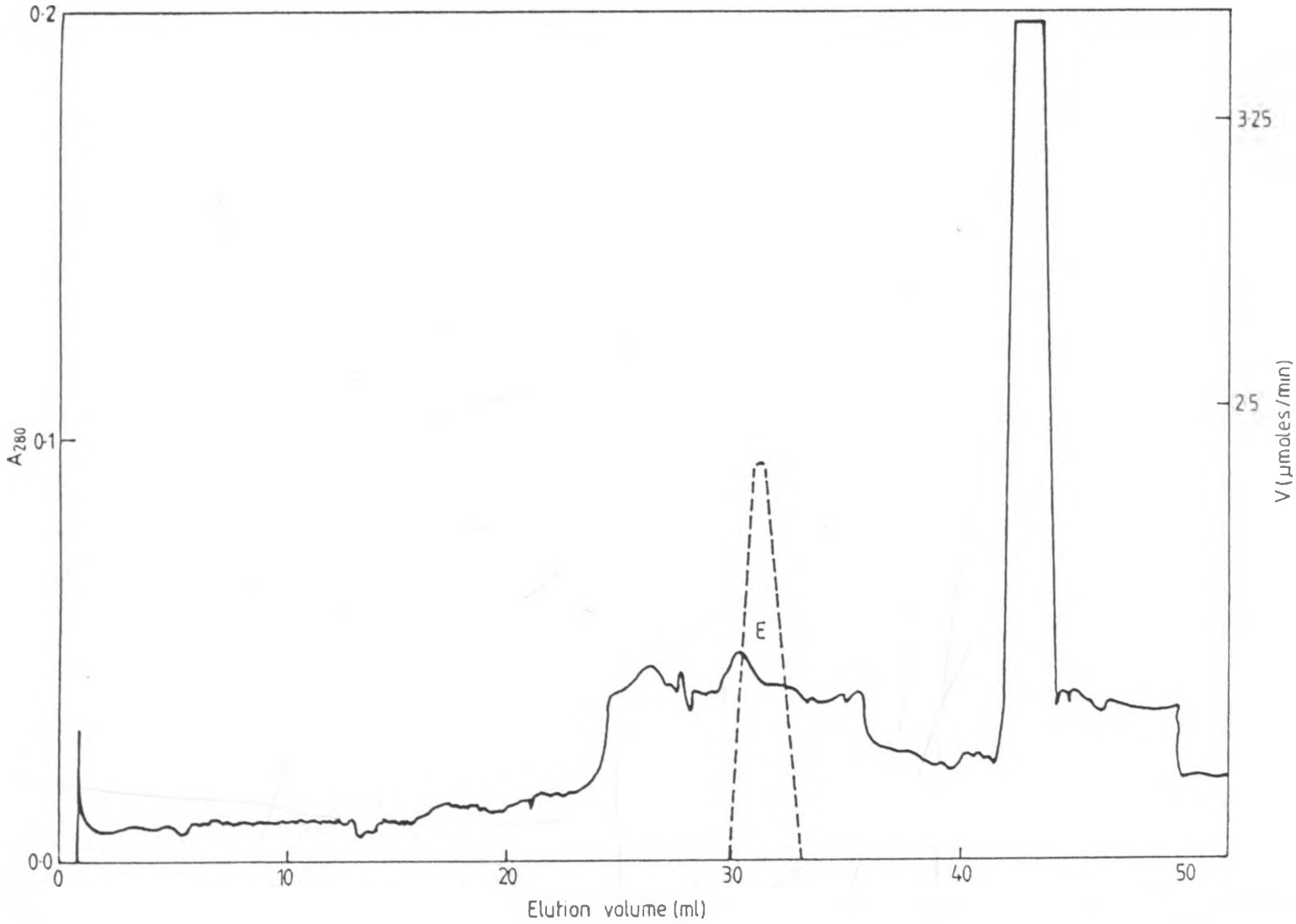
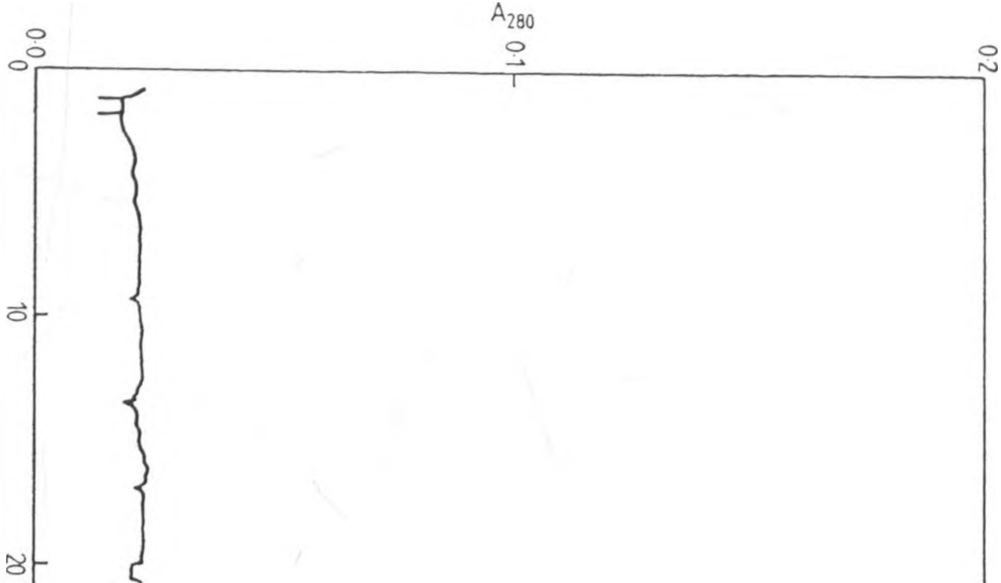

0·2
0·1
0·0
A_{280}
3·25
2·5
V (μmoles/min)
E
0
10
20
30
40
50
Elution volume (ml)

Fig. 13 **Gel permeation chromatography on the Superose 12 column (FPLC) of trypsin-like enzyme (Mono Q fraction BC).** A_{280} (—) and F, trypsin activity (---).


A_{280}
0.0
0.1
0.2
10
20

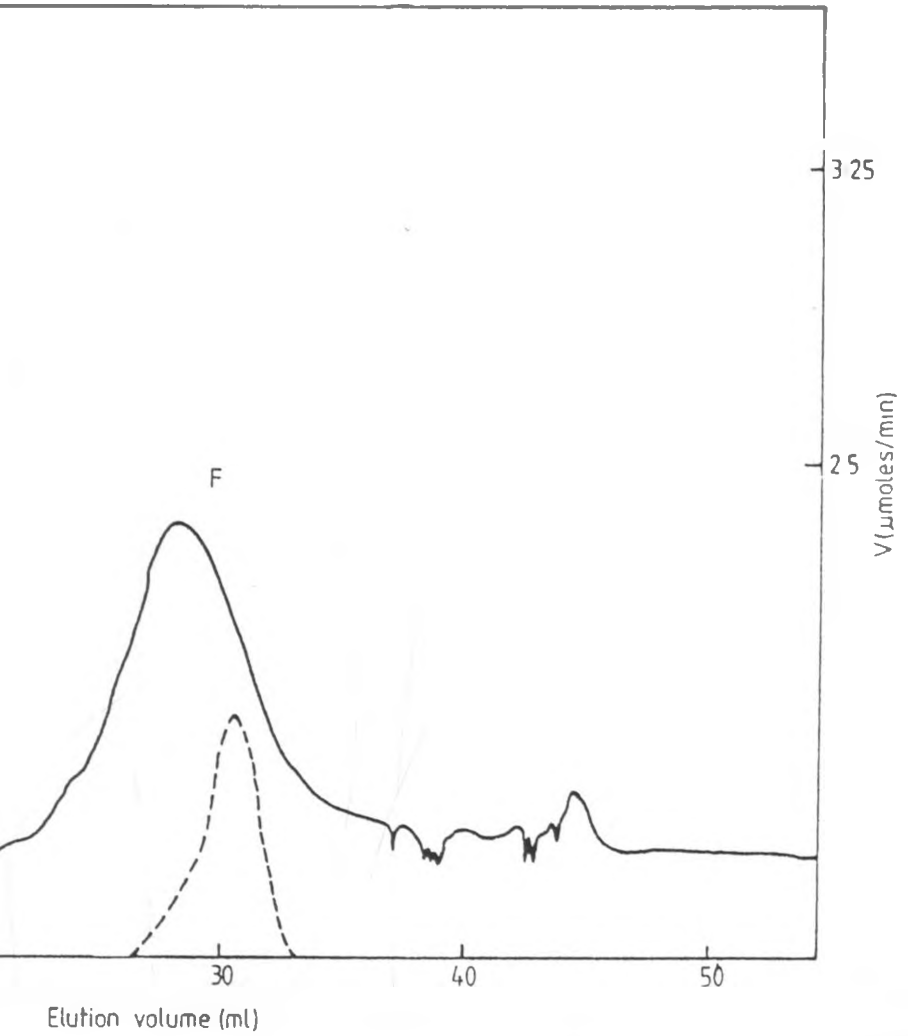

3.25
2.5
V(μmoles/min)
F
30
40
50
Elution volume (ml)

Fig. 14 SDS-PAGE of trypsin, trypsin-like enzyme and crude midgut homogenate.

1 Bovine pancreas trypsin (30 µg)

2 Tsetse trypsin (30 µg)

3 Tsetse trypsin-like enzyme (30 µg)

4 Crude midgut homogenate (10 µg)

5 Low molecular weight standard (Bio-Rad, 5 µl)

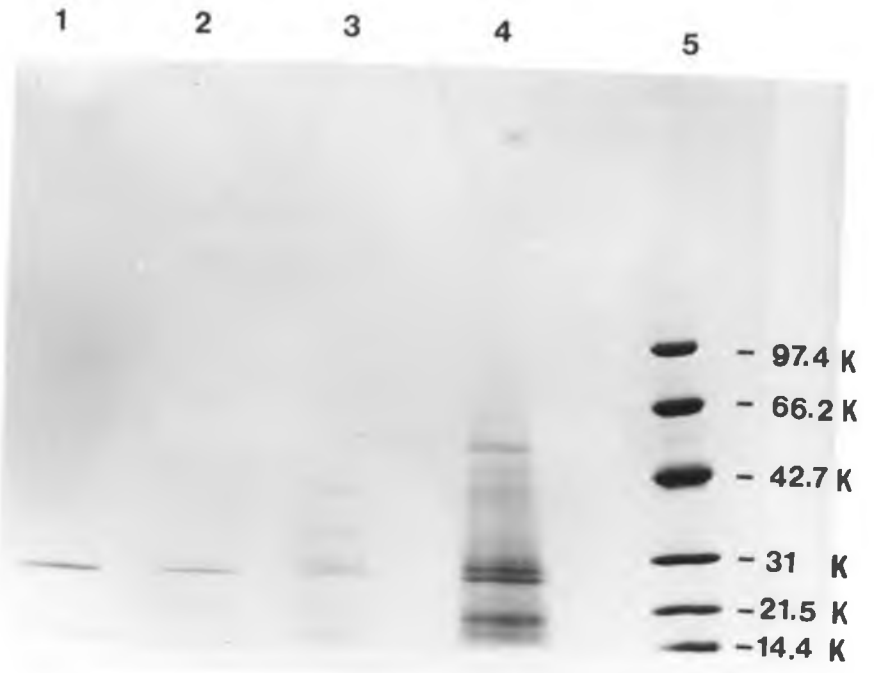

1
2
3
4
5
- 97.4 K
- 66.2 K
- 42.7 K
- 31 K
- 21.5 K
- 14.4 K

Table 7. Purification of *G. m morsitans* midgut trypsin

	Volume (ml)	Protein (mg)	Trypsin activity(U)	Specific activity(U/mg)	Yield %
Crude midgut	10	200	1360	7	100
Anion exchange	2	10	1280	128	94
Gel filtration	1.6	3	850	280	63
Electro-elution	1.2	1.2	460	375	34

The Table shows isolation of trypsin using the procedures shown. Samples were concentrated by acetone precipitation, activities are expressed as U (µmoles/min). Data are the average of 4 determinations.

Fig. 15 Trypsin activity stained on Native-PAGE (1, 2 & 3) and protein stain (4-7).

1 & 5) Crude midgut homogenate (2 µg)

2 & 6) Tsetse trypsin (5 µg)

3 & 7) Tsetse trypsin-like enzyme (5 µg)

4) Low M_r Standard (Pharmacia, 10 µl)

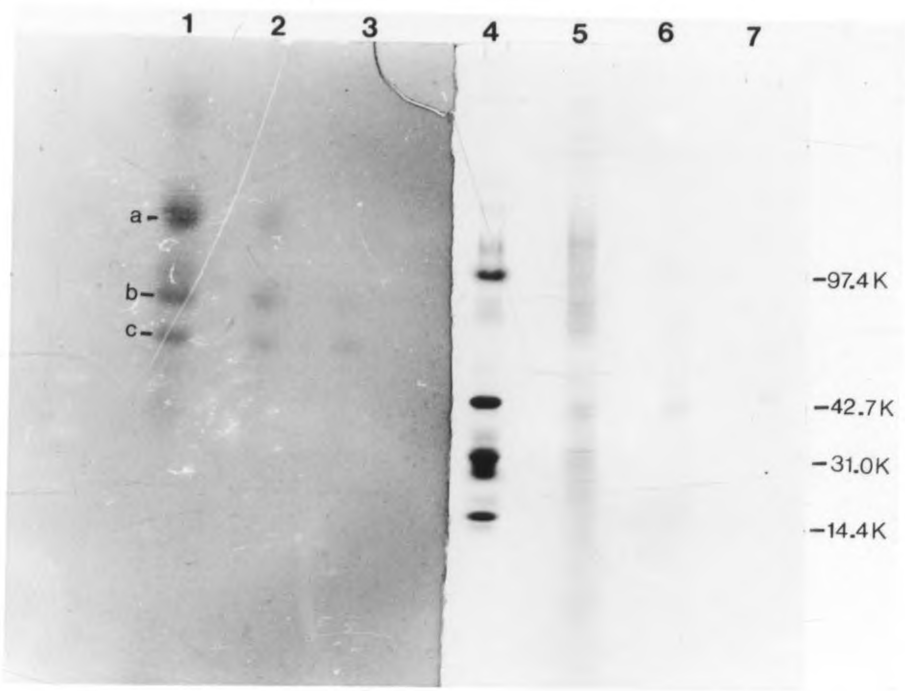

1
2
3
4
5
6
7
a
b
c
97.4K
42.7K
31.0K
14.4K

Fig. 16 Inhibition of trypsin and trypsin-like enzyme by D-glucosamine. Percentage original activity plotted against different D-glucosamine concentrations. Trypsin (•) and trypsin-like enzyme (○).

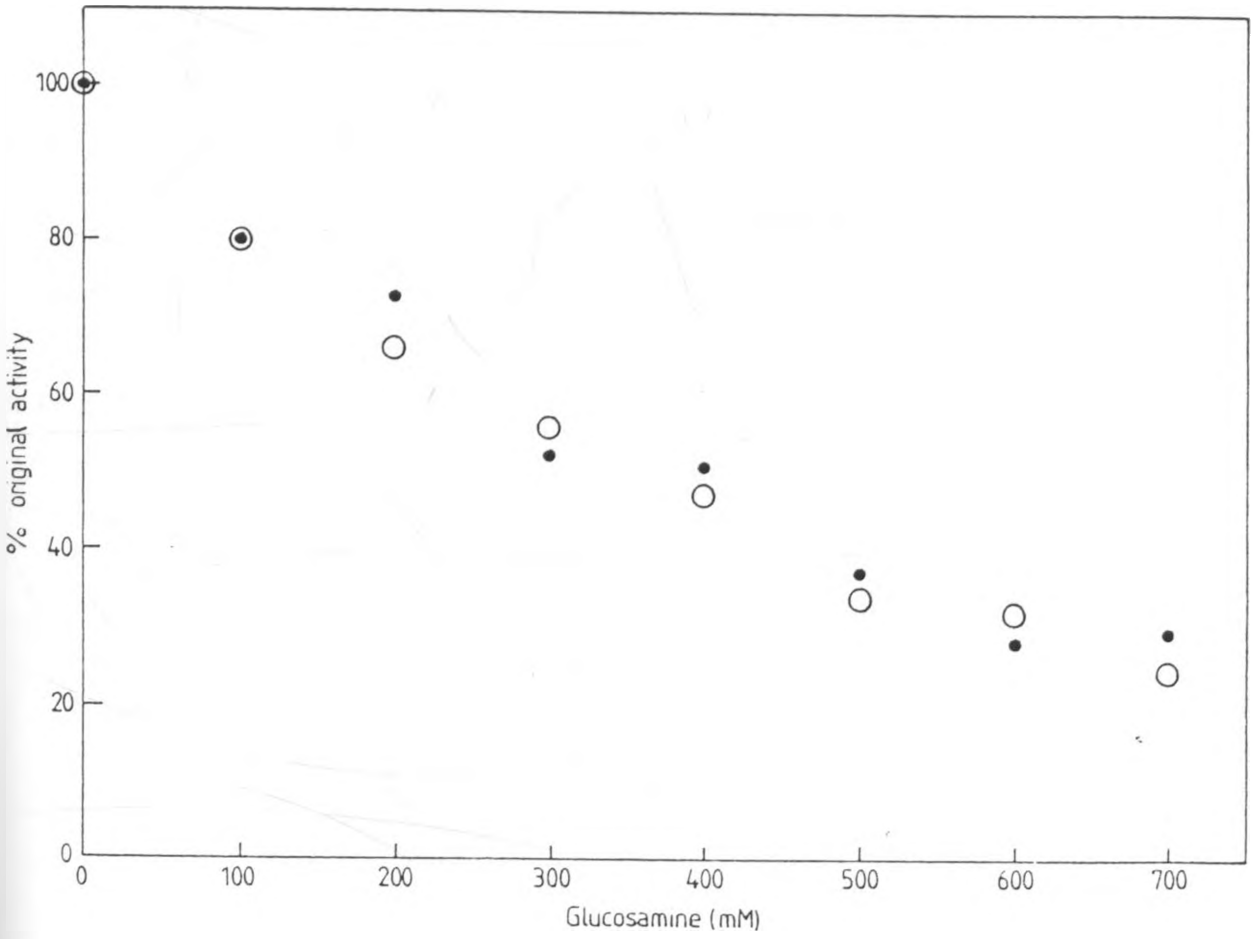

100
80
60
40
20
0
% original activity
0
100
200
300
400
500
600
700
Glucosamine (mM)

Fig. 17 Tsetse midgut trypsin stained for activity on SDS-PAGE.

1 Tsetse trypsin (60 µg)

2 Molecular weight standard (Pharmacia, 10 µl)

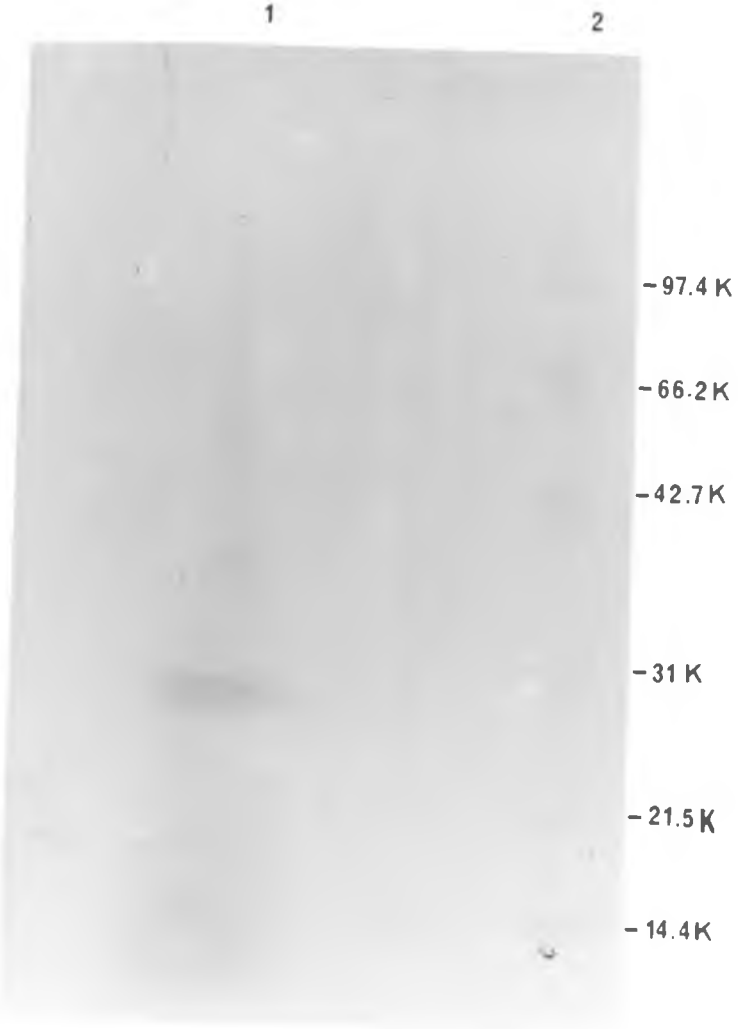
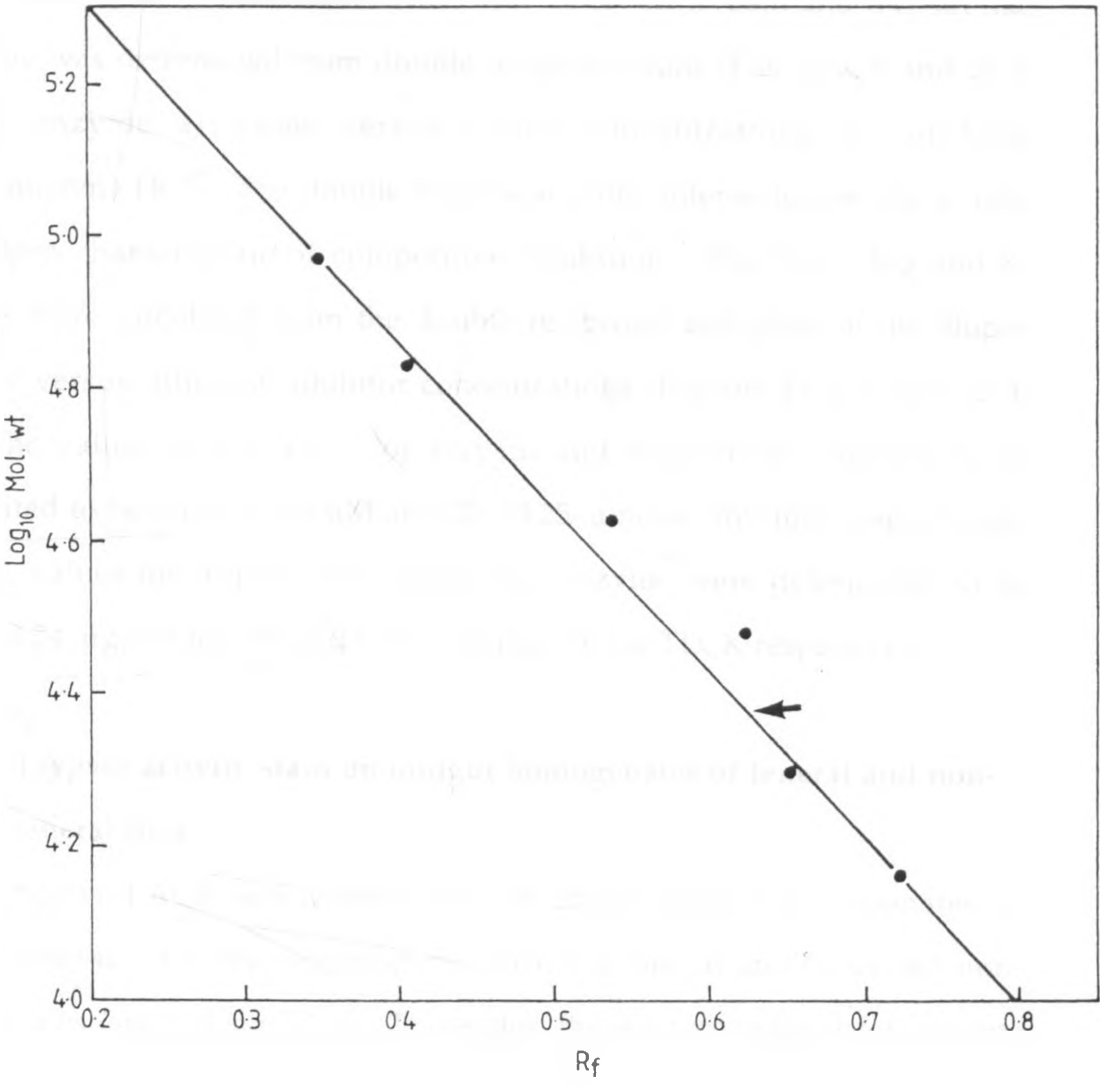

1
2
- 97.4 K
- 66.2 K
- 42.7 K
- 31 K
- 21.5 K
- 14.4 K

Fig. 18 Standard curve to molecular weight determination on SDS-PAGE. Log_{10} molecular weights of the protein standards Phosphorylase b (94 KD), Bovine serum albumin (64 KD), Ovalbumin (43 KD), Carbonic anhydrase (30 KD), Soybean-trypsin inhibitor (21 KD) and α-Lactalbumin (14 KD) were plotted against relative morbility. The arrow indicates trypsin-like enzymes (~ 24 KD) (30 μg).


5·2
5·0
4·8
4·6
4·4
4·2
4·0
Log10 Mol. wt
0·2
0·3
0·4
0·5
0·6
0·7
0·8
Rf

3.5.1 Kinetic studies on the effect of STI and TLCK on tsetse midgut trypsin and trypsin-like enzyme

The mode of inhibition by STI and TLCK on trypsin and trypsin-like enzyme was determined from double reciprocal plots (Fig. 19 a, b and 20 a, b) of enzyme activities versus varied concentrations of substrate (Chromozym-TRY). The double reciprocal plots, intersected on the y axis, a pattern characteristic of competitive inhibition. The V_{max}, K_M and K_i values were calculated from the double reciprocal and plots of the Slopes of 1/V versus different inhibitor concentrations (Figures 21 a, b and 22 a, b). The values of K_M, V_{max} for trypsin and trypsin-like enzyme were estimated to be 0.183, 0.184 µM and 25, 13.25 µmoles/ml/min respectively. The K_i values for trypsin and trypsin like enzyme were determined to be 0.295, 0.24 µg/ml for STI and 0.12, 0.15 mg/ml for TLCK respectively.

3.6 Trypsin activity stain on midgut homogenates of teneral and non-teneral flies

Native-PAGE was carried out on crude midgut homogenates of unfed teneral (24 h after emergence), once fed teneral and twice fed non-teneral flies (starved for 72 h). Thereafter, the gel was stained for enzyme

activity (Fig. 23). The results showed that once fed teneral flies lacked the 57 KD band and the twice fed non-teneral flies had an extra band 35 KD.

Fig. 19 a Lineweaver-Burk analysis of the effect of STI on midgut trypsin activity. Different concentrations of STI were used 0 (●), 0.2 (○) and 0.4 µg/ml (△).

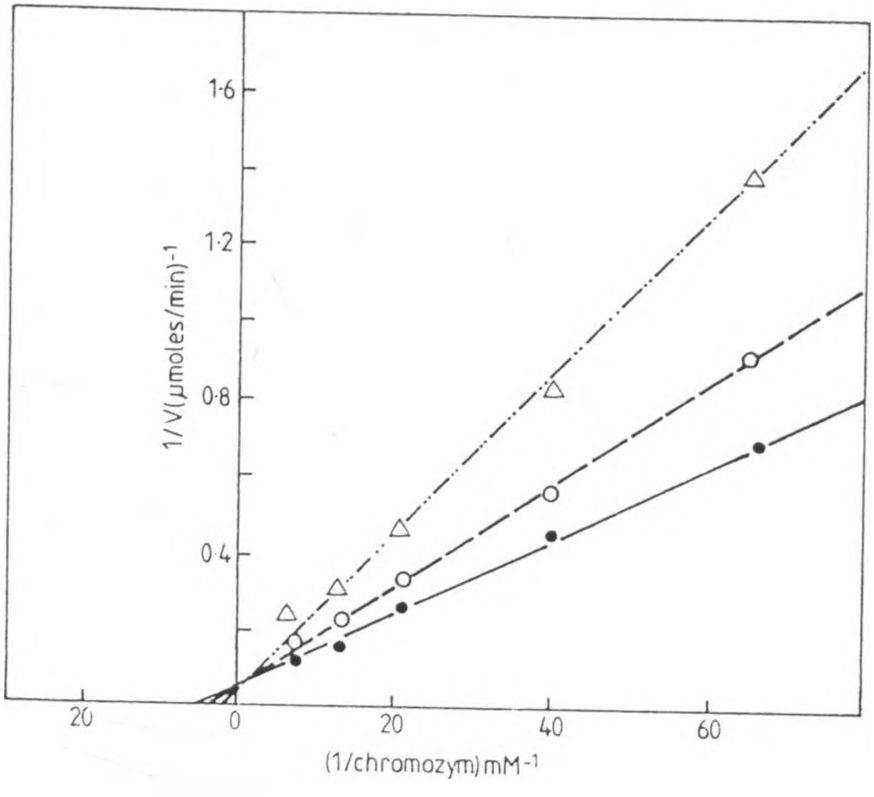

1·6
1·2
0·8
0·4
1/V(μmoles/min)⁻¹
20
0
20
40
60
(1/chromozym) mM⁻¹

Fig. 19 b Lineweaver-Burk plots showing the effect of TLCK on midgut trypsin activity. Chromozym-TRY was as substrate with different concentrations of TLCK 0 (●), 0.2 (○), 0.4 (△) and 0.6 mg/ml (▲).

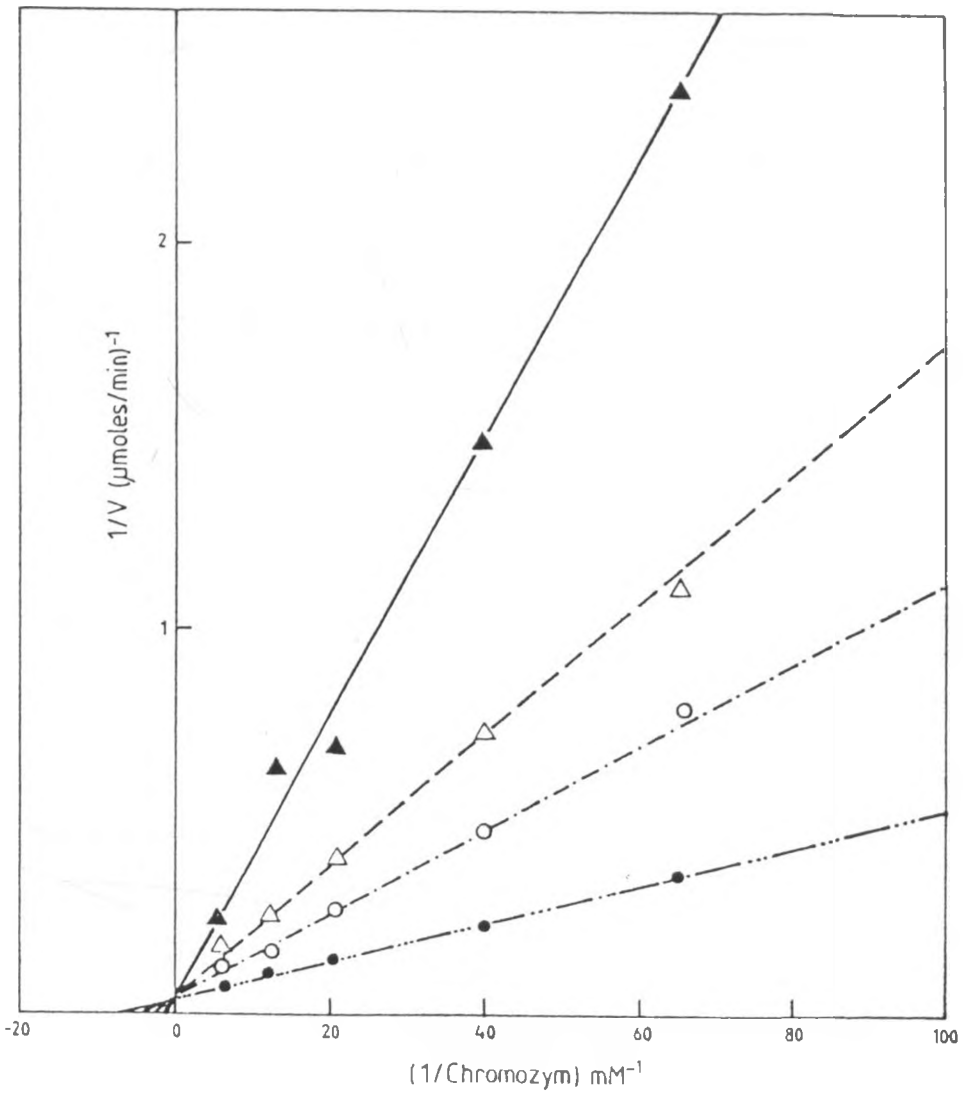

1/V (µmoles/min)$^{-1}$
2
1
-20
0
20
40
60
80
100
(1/Chromozym) mM^{-1}

Fig. 20 a **Double-reciprocal plot analysis of the effect of STI on midgut trypsin-like enzyme.** Chromozym-TRY was used as substrate, at various STI concentrations 0 (●), 0.2 (○) and 0.4 µg/ml (△).

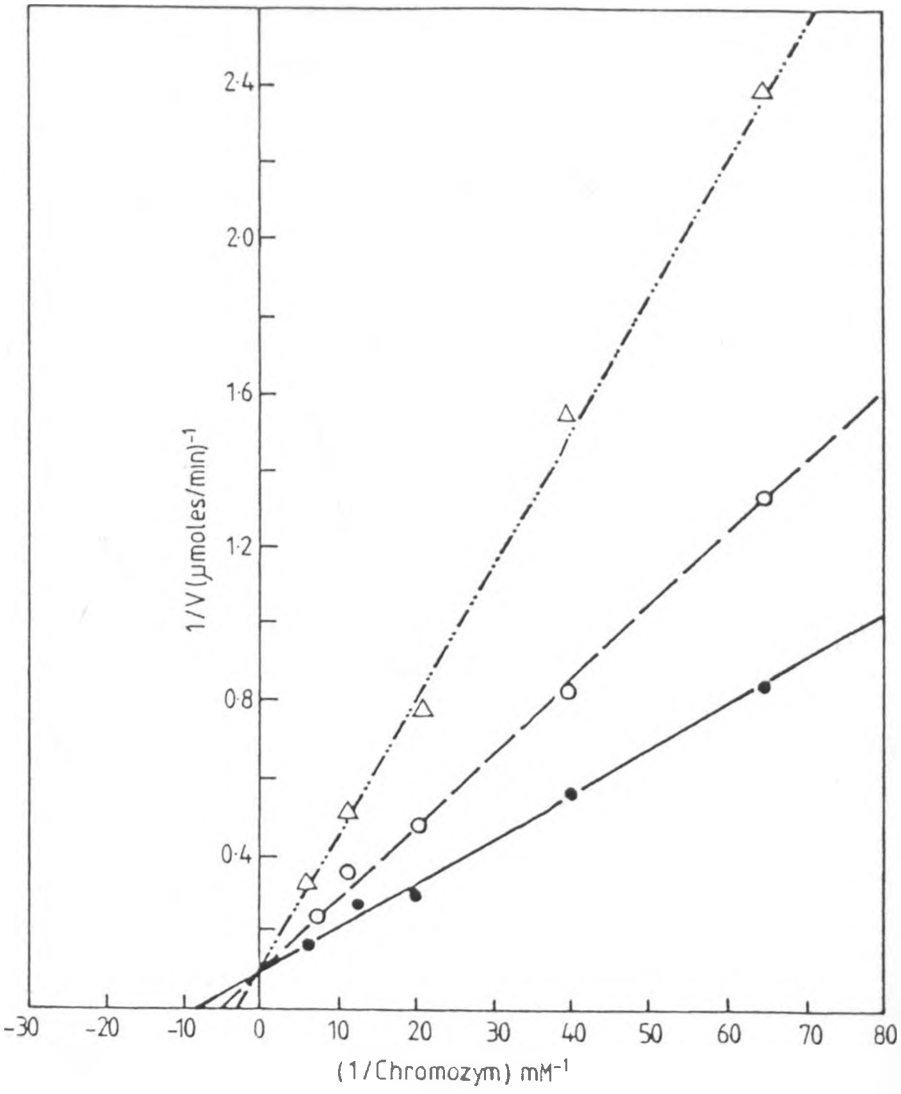

2·4
2·0
1·6
1·2
0·8
0·4
1/V (μmoles/min)⁻¹
-30
-20
-10
0
10
20
30
40
50
60
70
80
(1/Chromozym) mM⁻¹

Fig. 20 b **Double reciprocal plots for the analysis of effect of TLCK on midgut trypsin-like enzyme.** Chromozym-TRY was used as substrate with various concentrations of TLCK 0 (●), 0.2 (○) and 0.4 mg/ml (△).

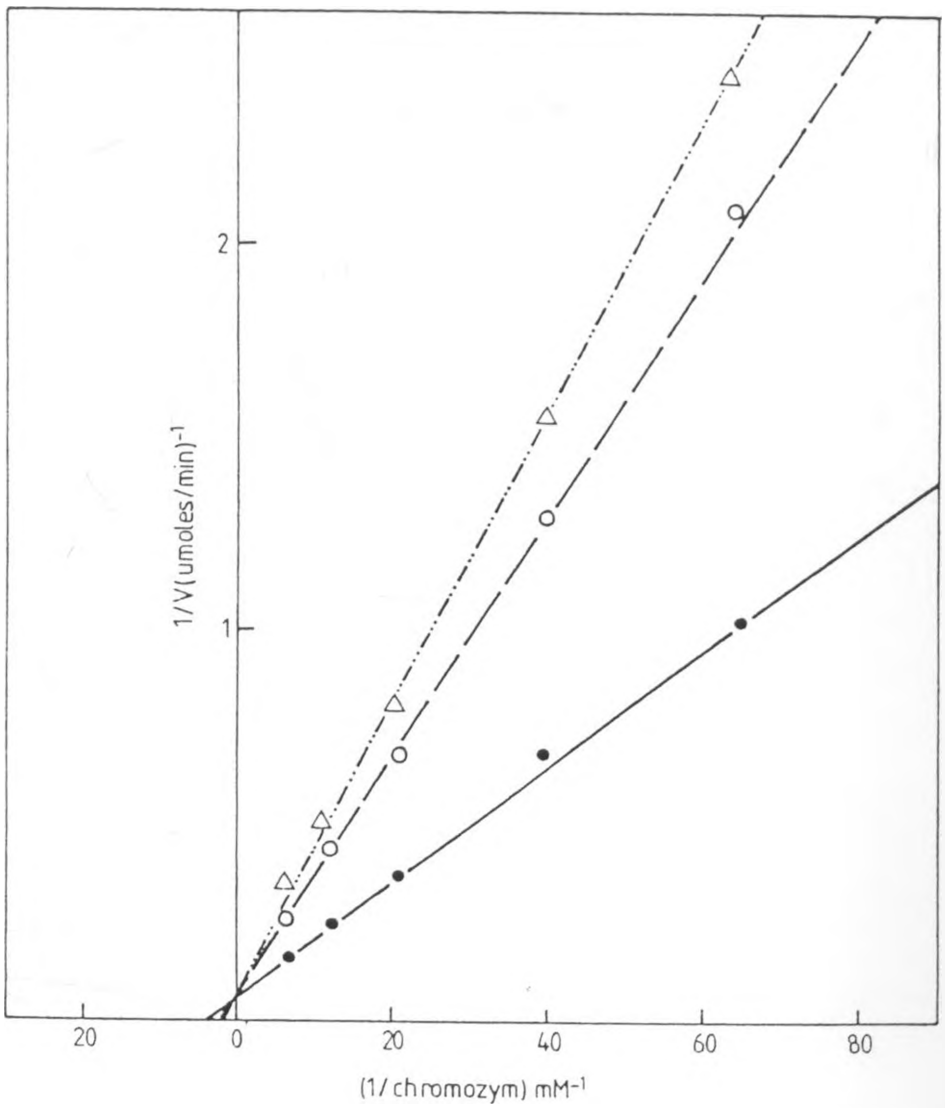

2
1
1/V(umoles/min)$^{-1}$
20
0
20
40
60
80
(1/chromozym) mM^{-1}

Fig. 21 a Slopes of Lineweaver-Burk plots (Fig. 19 a) were plotted against varied concentrations of STI.

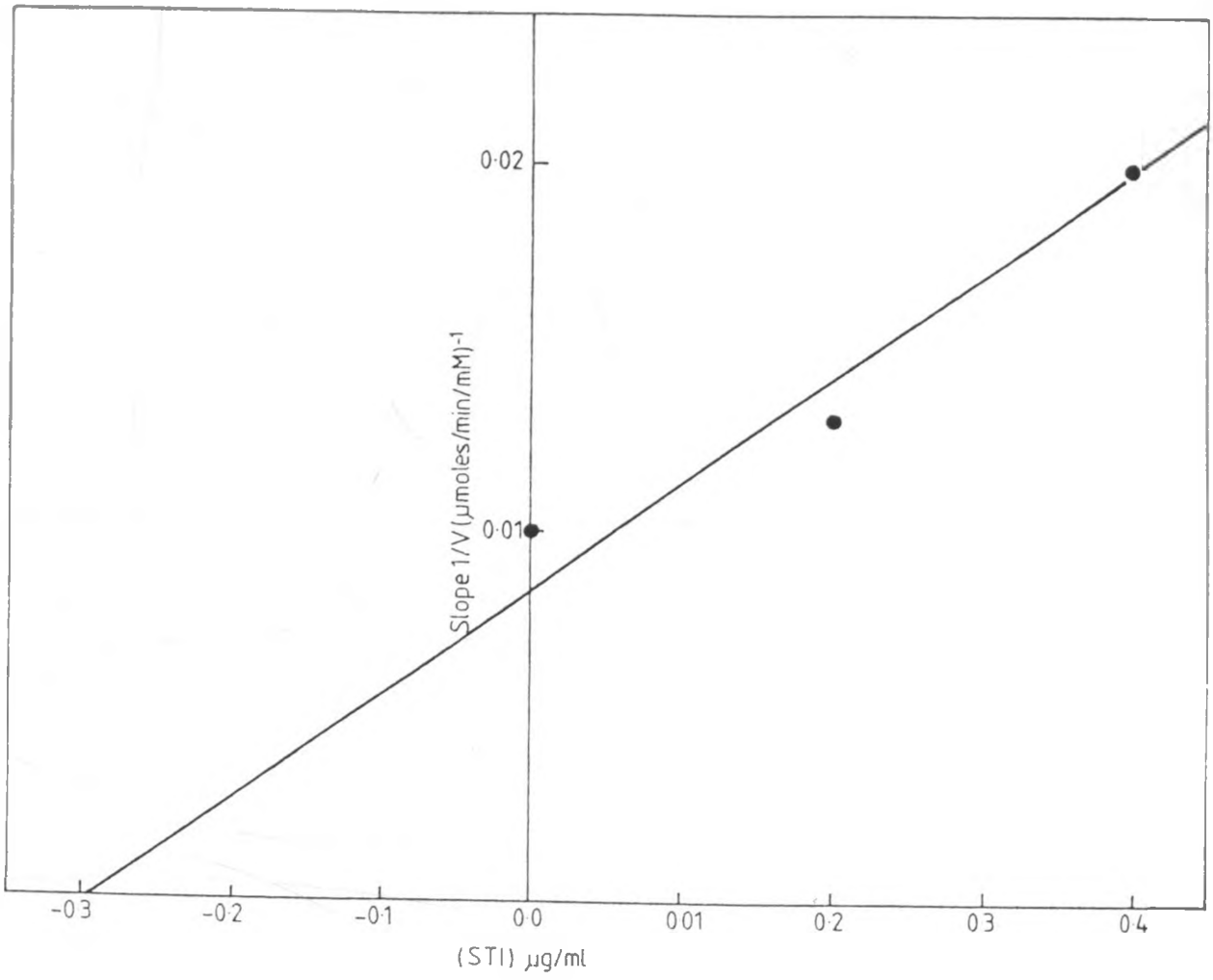

0·02
0·01
Slope 1/V (µmoles/min/mM)-1
-0·3
-0·2
-0·1
0·0
0·01
0·2
0·3
0·4
(STI) µg/ml

Fig. 21 b Slopes of double-reciprocal plots (Fig. 19 b) versus concentrations of TLCK.

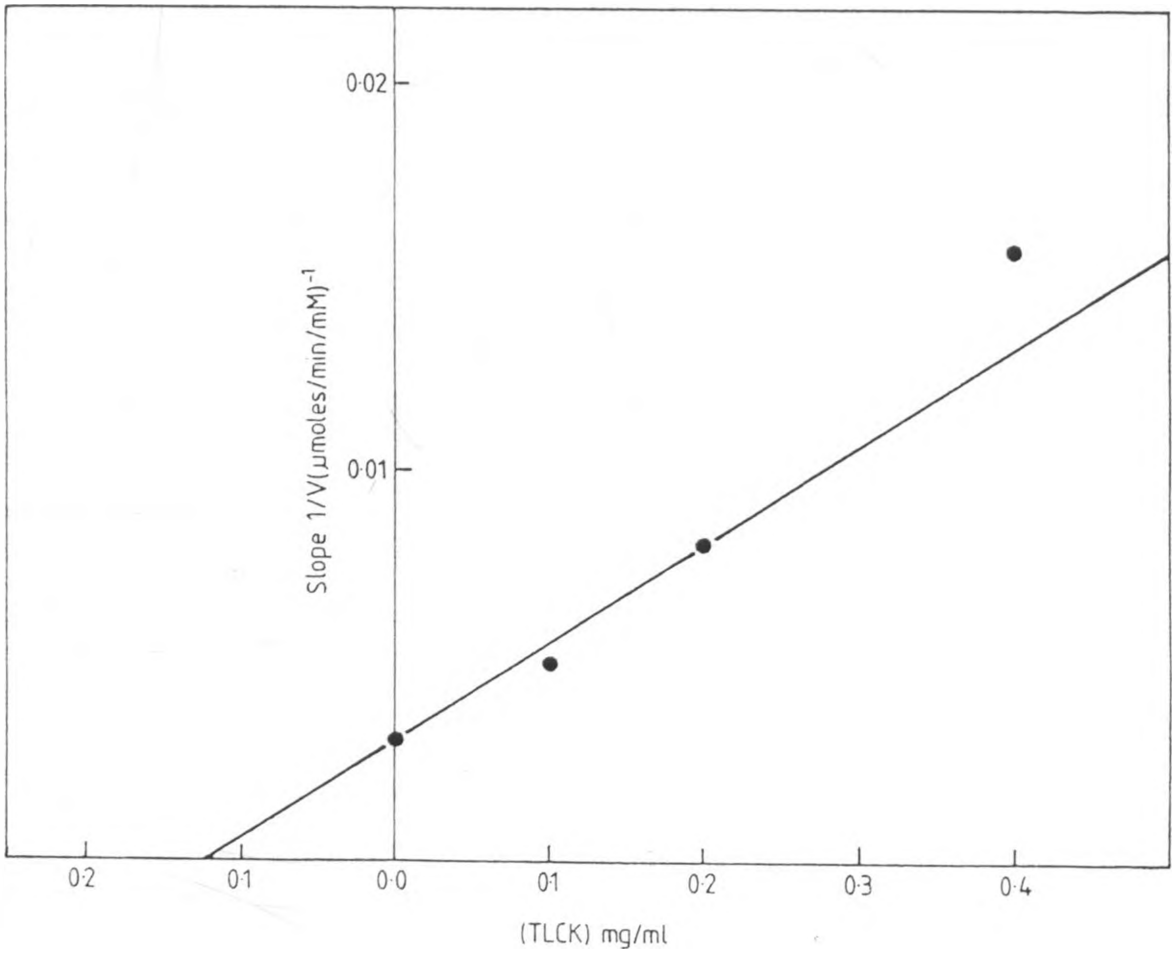

0·02
0·01
Slope 1/V(μmoles/min/mM)⁻¹
0·2
0·1
0·0
0·1
0·2
0·3
0·4
(TLCK) mg/ml

Fig. 22 a Slopes of 1/V versus various concentrations of STI. The values were obtained form the double-reciprocal plots (Fig. 20 a).

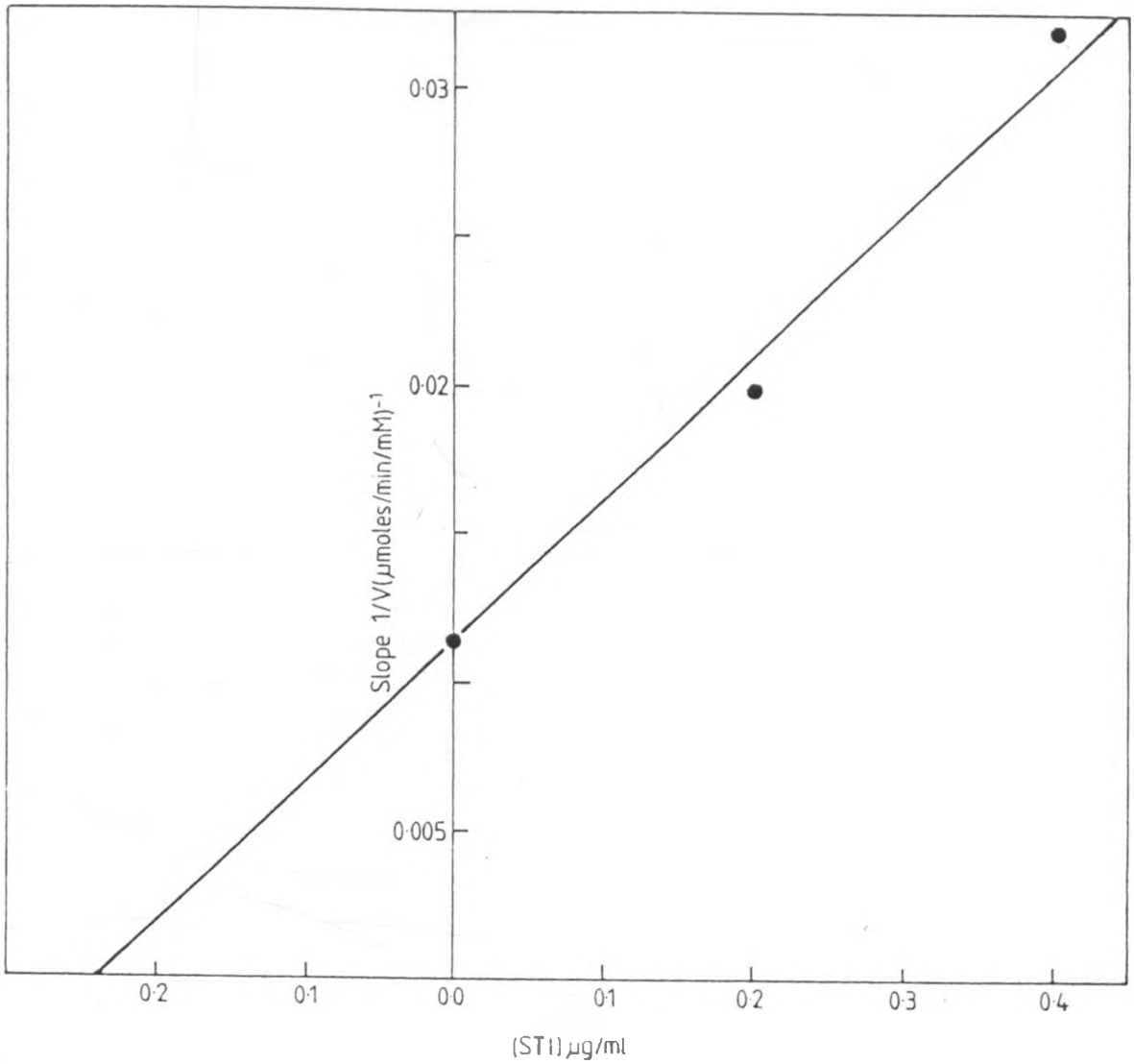

0·03
0·02
0·005
Slope 1/V(μmoles/min/mM)⁻¹
0·2
0·1
0·0
0·1
0·2
0·3
0·4
(STI) μg/ml

Fig. 22 b Slopes of 1/V were derived from Fig. 20 b and plotted against different concentrations of TLCK.

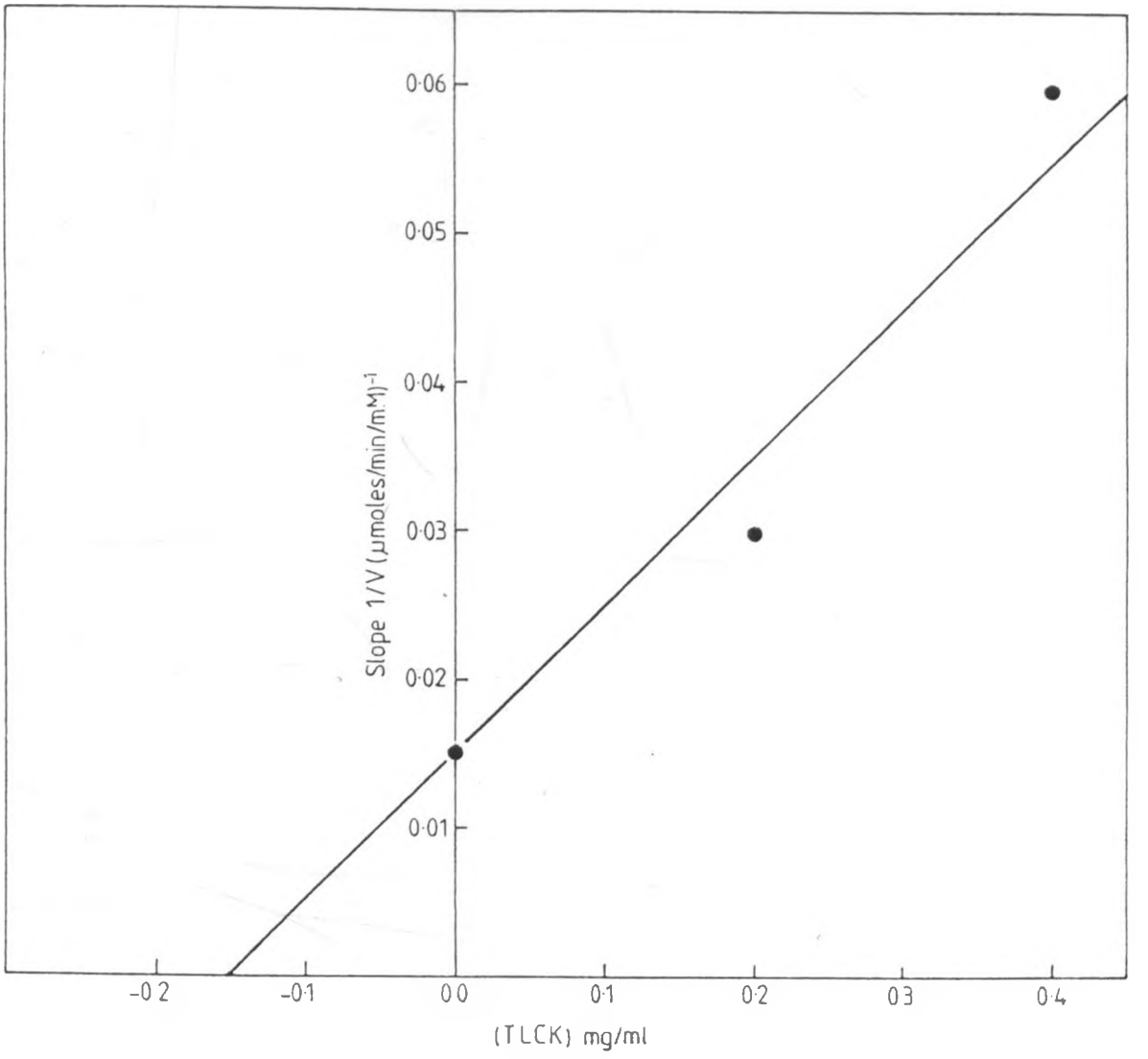
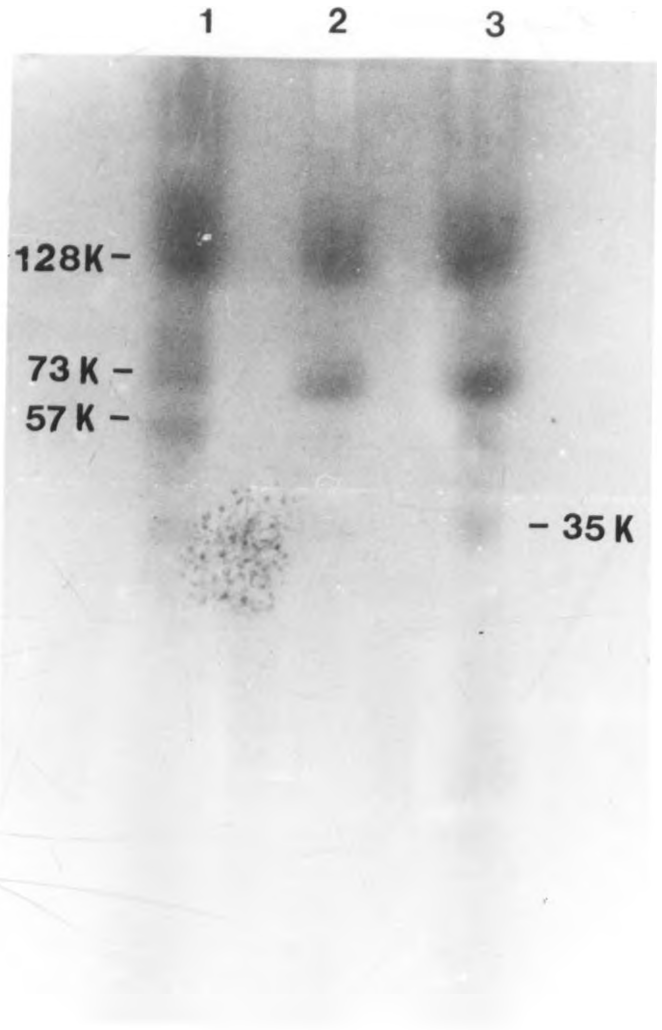

0·06
0·05
0·04
0·03
0·02
0·01
Slope 1/V (μmoles/min/ml.M)⁻¹
-0 2
-0·1
0 0
0·1
0·2
0 3
0·4
(TLCK) mg/ml

Fig. 23 Trypsin activity stain on Native-PAGE.

1 Unfed teneral homogenate (60 μg)

2 Once fed teneral homogenate (60 μg)

3 Twice fed non-teneral homogenate (60 μg)


1
2
3
128K –
73 K –
57 K –
– 35 K

CHAPTER 4

DISCUSSION

The amino sugar glucosamine, competitively, partially and reversibly inhibited tsetse trypsin activity. The other hexoses and glucosamine analogues (Scheme 2) showed no apparent inhibition on the trypsin activity. D-Glucosamine could also partially inhibit bovine pancreas trypsin activity. However, a higher concentration of glucosamine (300 mM) was required to reduce enzyme activity by half as compared to 200-250 mM required by tsetse midgut trypsin. None of the other sugars tested had any inhibitory effect on bovine pancreas trypsin. The K_M and K_i values were determined using Chromozym-TRY as substrate to be 4.2 X 10^{-5} M and 6.8 X 10^{-2} M, respectively. These parameters indicated that glucosamine, which is not a substrate for trypsin, had approximately a thousand fold less affinity for trypsin in comparison to Chromozym-TRY. The K_i is, therefore, very significant considering that there are no structural similarities between glucosamine and trypsin substrates like BApNA, TAME and Chromozym-TRY. Conversely, in an in vitro experiment, glucosamine had no inhibitory effect on tsetse midgut aminopeptidase activity. It would, therefore, appear that glucosamine inhibition is specific to trypsin or trypsin-like enzymes in tsetse midgut.

Feeding teneral flies on blood containing 100 mM glucosamine inhibited transformation of Trypanosoma brucei brucei bloodstream to procyclic forms. However, the process was only partially inhibited in non-teneral flies given a second feed after 72 h. Transformation of the parasites in teneral flies occurred normally in the absence of glucosamine with 50-60% of the parasites transformed after 5 h. However, in non-teneral flies transformation occurred normally, although 90% of the parasites were lysed within 2 h after feeding. The reduction in parasite numbers was attributed to the fact that trypsin levels in non-teneral flies were 2 times higher than in teneral flies. In contrast, non-teneral flies given a second feed after 96 h of starvation, behaved like teneral flies in terms of their ability to transform the parasites. The number of parasites in this group of non-teneral flies remained the same throughout the transformation period. Preliminary attempts to study the binding of tsetse midgut trypsin onto specific sugar immobilized columns, for example Con-A sepharose, lentil and N, N' diacetylchitobiose, were unsuccessful. These results were anticipated, in view of the fact that the respective specific sugars, that is, α-methylmannopyranoside, mannose and N, N' diacetylchitobiose, had no inhibitory effects on tsetse trypsin or trypsin-like enzyme activity. The tsetse midgut trypsin was purified and partially

characterized. The purification of the enzyme involved anion exchange and gel permeation chromatography. The trypsin and trypsin-like enzymes had the same molecular weights (~ 24 KD). However, the enzymes differed in charge, with the trypsin-like enzymes having a net negative charge at pH 8.0. The two enzymes were inhibited by glucosamine, although trypsin was found to be 2-3 times more active than trypsin-like enzyme when Chromozym-TRY was used as substrate. The trypsin inhibitors (STI and TLCK) inhibited the two enzymes. In addition, the values of K_I, K_M and V_{max} were determined for the two trypsin inhibitors (Section 3.5.1). The rate of hydrolysis of BApNA was 2-30 times more rapid for trypsin than trypsin-like enzyme. The tsetse midgut trypsin had optimal activity at pH 7-8, and declined at pH 6 and 8.8. Surprisingly, both trypsin and trypsin-like enzymes were very stable and regained partial activity after boiling (4 min) in the presence of β-mecaptoethanol. Specific substrate staining of non-denaturing polyacrylamide gels showed the presence of 3 bands with trypsin activity (~ 128, 73, and 57 KD). However, trypsin-like enzyme had two bands which stained for activity (~73 and 57 KD). On the other hand, staining for tsetse midgut trypsin activity on SDS-PAGE revealed only one band ~24 KD. In a different set of experiment, Native-PAGE was carried out on

crude midgut homogenates of unfed teneral flies, teneral flies once fed and non-teneral flies fed twice. These latter comparative study showed that the three sets of flies, had different trypsin activity bands on Native-PAGE. The interesting aspect in these activity banding patterns, was that the ~57 KD band was absent in once fed, and could only be faintly detected in non-teneral flies. The ~ 57 KD protein band with trypsin activity was probably inhibited by blood trypsin inhibitors. Moreover, a new distinct trypsin active band (~35 KD) could only be detected in the non-teneral flies. This new band when electroeluted was the only band that showed agglutination of procyclic *T. b. brucei*. It was interesting to note that ingestion of a bloodmeal stimulated the release of a qualitatively different trypsin. Furthermore, D-glucosamine (600 mM) completely inhibited all the trypsin active bands on Native-PAGE.

Lectins are multivalent proteins of non immune origin which bind carbohydrates non covalently and specifically (Sharon and Lis, 1989 a). Although believed initially to be confined to plants, lectins have now been demonstrated to be ubiquitous in nature (Sharon and Lis, 1989 b). For example, lectins have been shown to be present in the midguts of the tsetse flies, *Glossina austeni* and *G. m. morsitans* (Ibrahim et al., 1984). These lectins have been shown to immobilize and agglutinate

trypanosomes (Croft *et al.*, 1982; Ibrahim *et al.*, 1984; Abubakar, unpublished). 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). Furthermore, the *G. m. morsitans* midgut lectins are specifically inhibited by glucosamine (Ibrahim *et al.*, 1984; Welburn and Maudlin, 1989). Maudlin and Welburn, (1988 a, b) have 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). Thus, lectin levels have been hypothesized to be directly related to susceptibility, with the flies that have the highest lectin levels having the lowest infection rates (Welburn *et al.*, 1989). In addition, lectins provide the signal for established trypanosomes to proceed with maturation (Welburn and Maudlin, 1989). Establishment of midgut infection involves the complete differentiation of the parasites (Hoare, 1972). The removal of serum from infective feed, significantly increased trypanosome infection rates in tsetse flies (Gingrich *et al.*, 1985; Maudlin *et*

al., 1984). These observations led to the conclusion that serum wasessential in stimulating lectin release (Maudlin et al., 1984). The midgut of *G. m. morsitans* contains at least six proteolytic enzymes (Gooding and Rolseth, 1976). Of the six proteases in the posterior midgut, trypsin is the most predominant enzyme (Cheeseman and Gooding, 1985). The tsetse midgut trypsin and lectin have the following properties in common. The release of trypsin and lectins are induced following a bloodmeal by a component of the serum (Gooding, 1974; Maudlin *et al.*, 1984). Trypsin and lectins have been reported to cause lysis of trypanosomes, as well as differentiation of the surviving parasites (Imbuga et al., 1992 a; Welburn and Maudlin, 1989). In other insects like the mosquito, high levels of protease activity have also been reported to cause lysis of *Plasmodium* (Yeats 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 *L. major* to survive in Plebotomus papatasi (Borovsky and Schlein, 1987). Like lectins, trypsin is only active in the posterior midgut of the tsetse fly (Maudlin and Welburn, 1987; Gooding, 1976). However, a protease inhibitor isolated from the anterior midgut of *G. m. morsitans* effective on tsetse trypsin and trypsin-like enzymes could explain lack of trypsin-like activity in the

anterior midgut (Houseman, 1980). It is also possible that the inhibitor protects the gut cells from continuous low level release of proteases during starvation (Vogel *et al.*, 1968). In the present study it has been shown that glucosamine specifically inhibits trypsin activity, as is the case with the lectins. The specificity of D-glucosamine as tsetse trypsin inhibitor proved to be very exquisite, in that the configurations at positions 4, 2 and 1 were very essential. In this respect, galactosamine (position 4, hydroxyl group altered), mannosamine (position 2, amine altered) and methyl-α-glucosamine (position 1, altered by methylation) did not inhibit trypsin activity. In addition, the amine group had to be in the primary state since N-acetyl-glucosamine had no inhibitory effect. The mechanism of glucosamine inhibition on trypsin activity, which was determined to be reversible, partial and competitive, suggested that the inhibition is effected most probably in an allosteric manner. This would imply that glucosamine binds to a different site from the active site. More recently, it has been reported that trypanosomes or trypanosome membranes (*T. b. brucei*) inhibit *G. m. morsitans* midgut trypsin activity (Imbuga *et al.*, 1992 b). This suggested that in order to survive in the midgut, the trypanosomes have to inhibit trypsin and trypsin-like enzymes.

The success of trypanosome establishment in the tsetse midgut is dependent on transformation from bloodstream to procyclic forms as an essential initial step. Czichos *et al.*, (1986) reported that the transformation of *T. b. brucei* bloodstream to procyclic forms could be stimulated by a decrease in temperature from 37^0 C to 30^0 C and presence of cis-aconitate. On the other hand, *Triatoma infestans* intestinal homogenates, when incubated with *Trypanosoma cruzi*, caused differentiation of the epimastigotes to the metacyclic trypomastigotes (Isola *et al.*, 1986). Furthermore, electron microscopy work showed that *Trypanosoma congolense* bloodstream forms underwent morphological changes on the cell surface when treated with trypsin or pronase (Frevet *et al.*, 1986). Similarly, it has been shown that bovine pancreas and tsetse midgut trypsin, stimulate transformation of *T. b. brucei* bloodstream to procyclic forms (Yabu and Takayanagi, 1988; Imbuga *et al.*, 1992 a). The present work has demonstrated that feeding teneral and non-teneral flies (72 h after initial bloodmeal) on an infective feed containing glucosamine (100 mM) resulted into 90% and 35% inhibition of parasite transformation, respectively. The trypsin activity levels were 2 times higher in non-teneral compared to teneral flies. Maudlin and Welburn, (1987) noticed that maintaining flies on glucosamine (60 mM) resulted in 100% midgut

infection. They concluded that the D-glucosamine caused inhibition of lectin activity which could have otherwise lysed the parasites. In the present study, feeding non-teneral flies on D-glucosamine (100 mM) caused partial inhibition of transformation, therefore, 60 mM could have resulted in even less inhibition. Consequently, most of the parasites would have transformed. In other words, the lectins referred to above could possibly be the same as tsetse midgut trypsin or trypsin-like enzymes. 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 again be attributed to trypsin levels in teneral and non-teneral flies. Starving flies for 96 h after a bloodmeal, resulted in trypsin levels dropping to the initial levels prior to the first bloodmeal. This new finding would suggest that such flies can easily become infected with parasites. Furthermore, as in the case of tenerals, feeding this group of non-teneral flies on an infective feed containing D-glucosamine led to inhibition of the transformation process. These results confirmed an earlier finding that increasing the period of starvation led to increased susceptibility to trypanosome infection in non-teneral flies (Gingrich *et al.*, 1982 a, b; Makumyaviri *et al.*, 1984; Gooding,

1987; Welburn *et al.*, 1989). Welburn *et al.*, (1989) attributed the observation to be due to decreased lectin levels following periods of starvation. However, from this study their observations could be explained to be due to the lowered trypsin levels in these flies. On the other hand, Lehane and Msangi, (1991) suggested that the peritrophic membrane has a role to play in trypanosome maturation. These two workers supported the view that since the peritrophic membrane is continuously being secreted, its soft parts act as a gateway for trypanosome migration to the salivary glands (Freeman, 1973).

The affinity chromatography procedures used for the isolating pancreatic trypsins proved to be unsuitable for the tsetse enzymes. This demonstrated marked differences between trypsins from the two sources. For example, at pH 8.0, tsetse trypsin readily bound to Soybean trypsin inhibitor-agarose column. However, the enzyme-inhibitor complex could not be readily dissociated under conditions described by Abbeele and Decleir, (1991), Feinstein et al., (1974), Zwilling and Neurath (1981), and Gooding and Rolseth (1976). Under similar conditions bovine pancreas trypsin is quantitatively eluted from the column in active form using 0.1 M Na-Acetate buffer, pH 3.0 (Feinstein *et al.*, 1974). The affinity chromatography method has proved unsuccessful in purifying Cray fish,

tsetse midgut and human pancreatic trypsins (Zwilling and Neurath, 1981; Gooding and Rolseth, 1976; Feinstein *et al.*, 1974). Nevertheless, a combination of anion exchange, and gel permeation chromatography proved to be an efficient way of purifying the trypsins. The molecular weight of ~24 KD determined for trypsin and trypsin-like enzyme were very close to those reported by Gooding (1976) who estimated the molecular weights to be ~20 and 19 KD, respectively. The molecular weight values of trypsin from other sources like bovine pancreas (~24 KD), human pancreas (~21 and 20 KD) and *G. p. palpalis* (~24 and 26 KD) are very close to the one reported here (Feinstein *et al.*, 1974; Abbeele and Decleir, 1991). The trypsin and trypsin-like enzyme were similarly inhibited by glucosamine, TLCK and STI. Gooding and Rolseth, (1976) reported 1% hydrolysis of BApNA by trypsin-like enzyme 24 h after feeding, a finding which is in conformity with the current results. Therefore, it appears that trypsin and trypsin-like enzyme have several properties in common. Although, the tsetse midgut showed three bands with trypsin activity on non-denaturing gels, only one enzyme band (~24 KD) was observed on SDS-PAGE. These results suggest that the trypsin bands observed on non-denaturing gels could be dimers, trimers and pentamers of trypsin. Alternatively, the two bands (~ 73 and 57 KD) could aggregate to form ~128 KD band. Two multiple forms of trypsin have been

reported in *A. fluviatilis* and human pancreatic trypsin (Feinstein *et al.*, 1974; Zwilling and Neurath, 1981).

The trypsin band with molecular weight of 35 KD was electroeluted and found to be capable of agglutinating trypanosomes. This suggested that this trypsin could have a lectin-like activity. Interestingly, although high trypsin levels can be released by thoroughly homogenizing, the agglutination activity of such a homogenate is very low. However, upon feeding, there was an increase in agglutination activity. This became even more intense with a second bloodmeal. Assuming that trypsin and lectin are closely related or the same molecule, then the mechanism by which trypanosomes are lysed could be explained. Incubation of bloodstream trypanosomes with tsetse midgut trypsin prior to agglutination assays, resulted in enhancement of the process. In contrast, inhibition of trypsin activity in the homogenate using STI showed reduced agglutination capacity. These results suggested that trypsin cleaves off certain residues from the surface coat of bloodstream trypanosomes. Thus exposing sugar residues which are recognized by the lectin. The hypothesis that trypsin and the lectin could be the same molecule is not unique, since proteases with lectin-like activity have been reported (Mirelman, 1987). These proteases with lectin-like activities have been previously reported to be

toxic to colonic loops, by causing reduced thymidine and leucine incorporation into tissue cultured BHK-21. Other sugar binding enzymes like glycosidases, glycosyltransferases, glycosylkinases, glycosylepimerases and glycosylpermeases have also been reported (Barondes, 1981). Under certain conditions, the sugar specific enzymes with their multiple binding sites act like lectins by agglutinating cells (Barondes, 1981).

Further research need to be carried out in future to elucidate the following.

1. Determine the component of serum which induces trypsin/lectin release.
2. Study the molecular basis of the trypsin and lectin synthesis, with a view to cloning the gene(s) that code for the two molecules.
3. Elucidate the precise mechanism of lectin/trypsin action, what biochemical changes lead to lysis, transformation and maturation of the parasites.

CHAPTER 5

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