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ISOLATION OF GLOSSINA TRYPANOLYSIN/TRYPANOAGGLUTININ AND ASSESS OF THEIR USE IN THE INTERRUPTION OF TRYPANOSOME DIFFERENTIATION

BY

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DECLARATION

I certify that none of the material offered in my thesis has previously been submitted by me for a degree of this or any other university and that it represents work performed by me alone.

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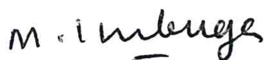

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ABBREVIATIONS

$AgNO_3$	Ammonium nitrate
Apo A-1	Apolipophorin A-1
Apo L-1	Apolipophorin L-1
Apo L-111	Apolipophorin L-111
ARQU	Animal Rearing and Quarantine Unit
BCA	Bicinchonic acid
Ca^{++}	Calcium ion
Chromozym Try	Carbobenzoxy-val-gly-arg-nitroamide acetate
DEAE	Diethyl aminoethyl
DEPC	Diethyl pyrcarbonate
DFP	Diisopropyl fluorophosphate
DNA	Deoxy-ribonucleic-acid
FAO	Food and Agriculture Organization
EATRO	East African Trypanosomiasis Research Organization
EDTA	Ethylenediamine tetra-acetic acid, disodium salt
FCA	Freund's complete adjuvants
FCS	Foetal calf serum
FPLC	Fast protein liquid chromatography
FRG	Federal Republic of Germany
HCl	Hydrochloric acid
HDL	High-density lipoprotein
H_2O_2	Hydrogen peroxide
ICIPE	International Center of Insect Physiology and Ecology
IGM	Immunoglobulin class M
ILRI	International Livestock Research Institute
LS	Long slender
Mg^{++}	Magnesium ion
NaCl	Sodium chloride

NaOH	Sodium hydroxide
Na_2PO_4	Sodium phosphate
O_2	Oxygen
OH	Hydroxide
OIE	International Office of epizootology
PAS	Periodic acid Schiff's reagent
PBS	Phosphate-buffered saline
PEG	Poly-ethylene glycol
PMSF	Phenylmethyl-sulphonyl-fluoride
PSG	Phosphate-salined glucose
PLs	Plasmatocytes
RNA	Ribonucleic acid
SDM-70	Semi-definined medium-70
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SS	Short stumpy
STI	Soybean trypsin inhibitor
TBS	Tris-buffered saline
TCA	Trichloroacetic acid
TLCK	N-α-p-Tosyl-L-Lysine chromethyl ketone
TLF	Trypanosome lytic factor
TPCK	Tosylamide-2-phenyl-ethyl chloromethyl ketone
Tris	Tris-(hydroxy methyl) aminomethane
TSF	Trypomastigotes soluble fraction
VSG	Variant surface glycoprotein

UNIT ABBREVIATIONS

°C	Degree (s) celsius (centigrade)
cm	Centimetre
e	Erlanger
Fig.	Figure, figures
g	Gramm
h	Hour
ha	Hectar
log	Logarithm
M	Molar concentration
mA	Milliamphers
mg	Milligramme
mg	Microgramme
min	Minute
min^{-1}	Revolutions per minute
ml	Millilitre
ml	Microlitre
mm	Millimeter
mM	Millimolar
mm	Micrometer
mmol	Micro molar
M_r	Relative molecular weight (mass)

Mw	Molecular weight
N	Normal
N°	Number
nm	nanometre
pH	Log_{10} hydrogen ion concentration
RH	Relative humudity
rpm	Revolutions per minute
sec	Second
v	Volt
%	Percent
<	Less than
>	Greater than
~	Approximately

ABSTRACT

An important step in the establishment of gut-adapted trypanosome infections in tsetse involves their differentiation from bloodstream into procyclic (midgut) forms. This complex process is mediated by a wide variety of factors, all of which are intrinsic to both the tsetse vector and the host blood. Most of the ingested trypanosomes are lysed by a wide variety of factors including various digestive enzymes, agglutinins,lectins, trypanolytic proteins and other factors that are yet ucharacterised factors. The small number of trypanosomes that survive lysis continue with the process of development theirby establishing themselves into infective forms within the tsetse. Of the many factors that have been implicated in tsetse-trypanosome interactions, trypsin or trypsin-like enzymes and lectin-like molecules have received the most attention. For example, at least six proteolytic enzymes, including trypsin, trypsin-like enzyme, chymotrypsin and carboxypeptidase amohave been identified. Similarly, the purification and characterization of a lectin-trypsin complex has been recently reported. Although it has suggested that this molecule plays an important role in the differentiation of trypanosomes, the actual mechanisms still remain unknown. Apart from the trypsins and lectin-like molecules, a molecule that is specifically involved in lysis of trypanosomes has been partially described in the midguts of Glossina palpalispalpalis. The role of trypanolysin in tsetse midgut has not yet been studied fully. Therefore, in the present study, midgut trypanolysin from G. m. morsitans was isolated and characterized. Midgut homogenates from twice-fed G. m. morsitans had the highest trypanolysin activity against bloodstream-form trypanosomes followed by those once-fed and unfed. The induction of trypanolysin activity by bloodmeal increased gradually reaching a peak at 72-120 h after the bloodmeal, and then decreased rapidly, with only 25% of the peak activity remaining after 192 h.

Tsetse membrane-fed with serum showed slightly higher trypanolysin activity compared to those fed on red blood cells (RBCs) and whole blood. Studies on different species of tsetse showed that flies with the highest trypanolysin activity might be the most refractory and The activity of trypanolysin was shown to be concentration dependent. Trypanolysin caused lysis of bloodstream-form trypanosomes, while the procyclics were unaffected. Divalent cations Ca^{++} and Mg^{++} had no effect on the activity of the trypanolysin. Trypanolysin was not affected by tosylamide-2-phenylethyl chromethyl ketone (TPCK), N-a-p-tosyl-l-lysine chromethyl ketone (TLCK), phenyl methyl sulphonyl fluoride (PMSF), diisopropyl fluorophosphate (DFP) and iodoacetamide. Soybean trypsin inhibitor (STI) not inhibited trypanolysin activity. Also, trypanolysin had no trypsin activity nor even affected by the enzyme. Moreover, all the sugars tested had no effect on trypanolysin activity. In contrast, pronase at a concentration of (10 mg/ml) rendered the molecule completely inactive. However, the activity of trypanolysin was completely inhibited by diethyl pyrcarbonate (DEPC) and partially by aprotinin. The trypanolysin was inactivated during storage at 27° C and 4° C after 15 and 32 days respectively. Similarly, heating the midguts to 60-80° C led to loss of the activity. The activity was also unstable when the molecule freez-thawed at -20, -70 or -80° C after 55, 43 and 31 days, respectively. The molecule was found to be very stable at -20, -70 and -80° C for almost 60 days of storage. When the midgut homogenate from G. m. morsitans was separated by a combination of an ion-exchange and affinity chromatography the trypanolysin activity was recovered in the bound fraction. The native molecular weight of the isolated trypanolysin was determined ~ 669 KDa against the molecular weight markers (Pharmacia) standard curve. Analysis of trypanolysin by SDS-PAGE revealed only one band of molecular weight ~14 KDa. The high molecular weight protein was lipidated and also glycosylated.

Antibodies raised against the protein showed that only tsetse of the genus Glossina gave positive reactions in western blots. In vitro study howed that trypanolysin was inhibited by the antiserum raised against the molecule. In the in vitro and in vivo transformation studies using trypanolysin showed that trypanolysin (amognst other factors) fed to tsetse was responsible for transformation blockage and lysis of trypanosoms. These results from this study suggest that the midgut trypanolysin plays an important role in the establishment of trypanosome infection in tsetse.

CHAPTER ONE

GENERAL INTRODUCTION

African trypanosomes are eukaryotic hemoflagellates that cause potentially fatal diseases in both man and livestock. Infections are caused by protozoan parasites that belong to members of the order Kinetoplastida. Organisms in this group are distinguished by the presence of unique structure called the kinetoplast. Trypanosomes are members of the parasitic familly Trypanosomatidae and the genus Trypanosoma. The familly Trypanosomatidae divided into two sections, Salivaria and Stercoraria. Each section is subdivided into subgenera, species and subspecies.

The mean length of Trypanosoma brucei sspp. is 17.0-20.0 mm. The morphology of the parasite shows marked polymorphism, the posterior end is pointed to blunt, the kinetoplast is small subterminal and has conspicuous modulating memberane with or without flagellum. The main morphological features distinguishing the developmental stages in trypanosomes are the position of the kinetoplast (specialized region of the mitochondrion containing a dense network of DNA) and the way in which the flagellum is attached to the body of the cell and the degree of its extension anteriorly.

Trypanosomes were first shown to be pathogenic to man and livestock at the beginning of the century. In 1895, Bruce found trypanosomes in the blood of a cow afflicted with the disease "Nagana", which was known to be associated with tsetse bite. "Nagana" in the Zulu language refers to as a "state of depressed spirits" (Fuller, 1924). "Nagana" is a wasting disease responsible for massive losses in animal productivity in tropical Africa.

In cattle, "Nagana" is caused by three species of trypanosomes, namely Trypanosoma congolense, Trypanosoma vivax and T. brucei. Trypanosomiasis also occurs in pigs where it is caused by Trypanosoma simiae (Stephen, 1966). Trypanosomes were shortly afterwards found in the blood (Dutton, 1902) and then in the cerebrospinal fluid of human beings suffering from sleeping sickness

(Castellaani, 1903). These trypanosomes are also the most important parasites from the medical and veterinary points of view, for they include the causative agents of human sickness. The more acute form of the human disease is caused by Trypanosoma brucei rhodesiense and classically the rhodesian sleeping sickness occurs in East and Central Africa. The milder and chronic form of the disease (the gambian sleeping sickness) is most commonly seen in West Africa and is caused by Trypanosoma brucei gambiense.

All three T. b. gambiense, T. b. rhodesiense and T. b. brucei were placed by Hoare (1964) in the subgenus Trypanozoon. All numbers of the T. brucei (Trypanozoon) complex share a commmon morphology, life cycle and major biochemical features, as well as antigenic components.

The main emphasis is placed on pathogenic trypanosomes which are cyclically transmitted by tsetse of the genus Glossina. "Tsetse", in the Setswana language of Botswana, means, "a fly destructive of cattle " (Fuller, 1924). Only one genus, Glossina, is included in the familly Glossinidae. The genus Glossina contains 30 living taxa, 22 species and 8 subspecies (Potts, 1973). The species are assigned to three subgenera (Glossina, Nemorhina and Austenina), which are also referred to, respectively, as the Morsitans (savannah), Palpalis (riveriene) and Fusca (forest) groups named after the commonest species in each group.

The colour of tsetse is non-descriptive but ranges from yellowish or greyish to dark or blackish brown with the upper side of the thorax marked with greyish brown stripes (Buxton, 1955; Liard, 1977). In resting postures, their wings lie closed flat over the proboscis horizontally over the abdomen in such a way that the apices of the two wings coincide, giving the tsetse a distinctive elongated appearance (Nash, 1969; Liard, 1977). The length of the tsetse together with the palp and proboscis is between 6 to 14 mm (Liard, 1977). The distinction between the sexes is the hypopygium, a cushion-

like projection on the underside of the tip of the abdomen. This is the male genitalia which is absent in females (Buxton, 1955). Both sexes feed on vertebrate blood.

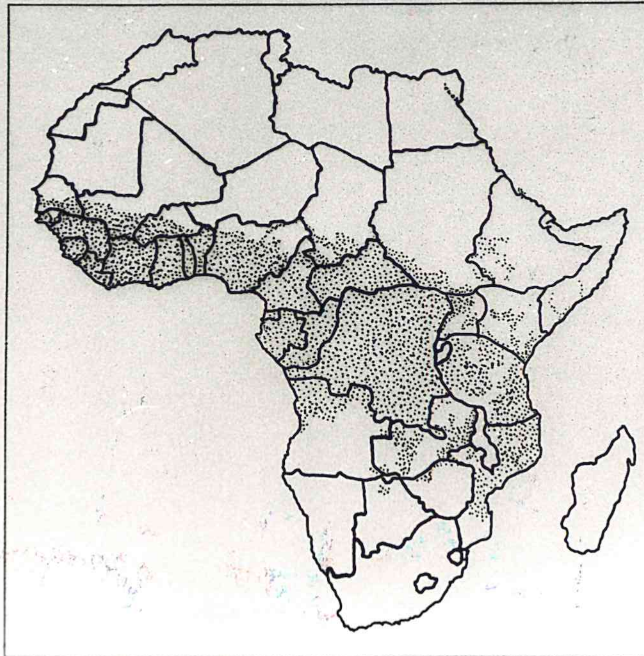
The females need only mate once during the course of its reproductive life (Pollock, 1970). This usually happens about 3 days after emergence which is normally after the first bloodmeal (Jordan, 1974). The sperm is stored in two spermathecae and suffices for fertilization of all eggs produced during her life. Tsetse are larviparous. The female does not lay eggs but gives birth at intervals to a single larva (Engelman, 1970). A fully grown larva is produced every 9-10 days (Saunders and Dodd, 1972). Other larvae are born at more or less regular intervals. The intervals before the first larva is born, and between subsequent births are regulated by temperature. The larva, which at birth attains a weight greater than that of its mother, quickly burrows beneath the soil on which it has been deposited. The pregnant female chooses sites for larviposition which vary according to season, but ensure that her offsprings are protected, during their further development, from extremes of climate. Birth takes place during the third larval instar. When burrowing is completed, usually at a half to one inch below the soil surface, the larval skin hardens to form the puparium. During pupariation, the cuticle darkens and hardens to provide a resistant covering capable of preventing desiccation (Nash, 1969). Within this shell the tsetse undergoes a fourth larval instar and then pupates and metamorphoses to become a pharate adult which in due course emerges to the open air and, as a teneral tsetse, begins within a few hours the same cycles of behaviour. The interval between larvipostion and emergence is 31 days at 24° C (Jackson, 1944 and Glasgow, 1963a). The duration of pupal stage, which begins with immobilization of the larva and lasts until the emergence of the adult tsetse, varies with species, sex and external conditions particularly temperature. Normally pupal stage has been found to last for 25-35 days (Shah-Ficher and Say, 1989), but durations as long as 50 days (Pollock, 1989) are possible. The

teneral tsetse emerge from the pupal shell by breaking it using its bladder-like ptillium (Nash, 1969). The duration of the tsetse life span varies inversely with maximum temperature in the environment (Nash, 1969; Liard, 1977). It is also species variant.

The tsetse-infested areas cover an estimated 37% of the whole African continent, involving 37 countries and since the 1950s, tsetse have continued to spread (Maclennan, 1980), with approximately 50 million cattle being at risk. Tsetse, which transmit trypanosomes, occur over 11 million square kilometers or about half of the non-desert area of Africa (Jordan, 1986; FAO, 1961; FAO/WHO/OIE, 1982). Trypanosomiasis is thus endemic over a vast region of Africa and its distribution is defined by the range of the tsetse.

African trypanosomiasis is restricted to central Africa, the vector being limited by the Sahara to the north, and by drier, colder areas to the south (Fig. 1). This is an area approximately equal to the size of the United States. Trypanosomiasis, however, also occurs outside the tsetse belt in Africa for example in South America. Furthermore, approximately 25,000 cases of human sleeping sickness occur each year (ILRAD Reports, 1987).

Fig. 1 The geographical distribution of tsetse in Africa (Based on Ford and Katondo, 1997 and Katondo, 1984; taken from a computerized database as reported by Lessard et al., 1990).



In their reports on the impact of trypanosomiasis control and eradication of tsetse in several agro-ecological zones of Africa, Jank et al. (1988) and Tacher et al. (1988) have assessed that within the 7 million km^2 of tsetse infested savannah, livestock numbers should be increased by approximately 120 million. In the past two decades, the increase in total food production in Africa has not kept pace with the growth in population, resulting in a decline in per capita food production (Pritchard, 1988; Mukhebi, 1990). The production of animal protein is lower in Africa than in any other continent due, in part, to tsetse-transmitted trypanosomiasis.

This disease complex causes the loss of million of dollars annually in livestock production, and milllions more must be spent on curative and prophylactic drug treatments and vector control programs. Thus, Trypanosomiasis is regarded as economically the most important disease of livestock on the continent (Jawara, 1990).

Therefore, there is a need for proper tsetse and trypanosomiasis control measures in order to realize the full socio-economic potential of livestock production on the continent. The benefits of tsetse and trypanosomiasis control are enormous especially when viewed, not only within the narrow context of animal production, but also, within the broader perspective that much of the tsetse infested land in the humid and sub-humid zones has a relatively high agricultural potential, particularly with respect to growing stable foods.

So far, the control of trypanosomiasis has been based on the control of the tsetse population through trapping systems, clearing the habitat or biological control.

Ground spraying and sequential aerosol application of residual insecticides by aircraft can be toxic to non target organisms including man.

Continued suppression of the disease through chemotherapy or vaccination has proved to be difficult, costly and bleak as the trypanosomes develop drug resistance and because of the phenomenon of antigenic variation. Also, the drug industry has lost interest in this field since it does not seem to offer much commercial returns.

The complete or even a partial elimination of animals believed to be reservoirs of the disease is a drastic step to take. Eradication is inconceivable in view of numerous animal reservoir hosts, and this has implications for the control strategy.

Attempts at releasing the sterile male insects has had some impact on the control of a number of insect pests of economic importance. The technique has been contested because, mutations brought about by irradiations could result in new strains of tsetse which might be impossible to handle. It has also been argued that the sterile males could be better transmitters of the disease (Stiles, 1991).

Other control strategies which include the use of pheromones or attractants, impregnated traps and use of pathogens and insects growth regulators (Itard, 1975; Abbeele and Declair, unpublished; Denlinger, 1975) have not been very successful.

Consequently, it is very important to develop alternative control methods especially these that are environmentaly safe, cheap, easily applied and affordable. Biological control using bacteria has been used successfully for the control of vectors of disease, for example, mosquitoes (Vandekar and Dulmage, 1982; Meyers and Yousten, 1978) and black flies (Lacey et al., 1982).

Bacillus thuringensis has successfully been used for control of mosquito larvae (Daiand Gill, 1992). The antibacterial activity in tsetse immune hemolymph been shown to be completely inhibited by preincubation with inhibitor-A, a proteolytic enzyme from B. thuringensis which specifically inactivates cecropins and attacins in immune hemolymph of Hyalophora cecropia (Dalhammar and

Steiner, 1984). Furthermore, injection of tsetse with cyclohexamine, an inhibitor of eukaryotic protein synthesis, completely inhibited production of the antibacterial activity, suggesting that the antibacterial factors are proteins and also confirming de novo synthesis (Kaaya, 1987). These factors were shown to be stimulated only by live and not by killed bacteria.

The recent improvements in bioassays and rigorous standardization of pathogenicity tests have allowed field experiments to be carried out in the tropics to show the effectiveness of different fungal species. In Brazil, Metarhizium anisopliae has been used to control spittle bugs, Mahanarva posticata, on sugarcane and more than 10,000 ha are treated annually, resulting in 40% of pest reduction (Ferron, 1981).

Glossina palpalis and G. m. morsitans (Jura et al., 1988) males can be infected by the DNA virus. The virus has been partially characterized (Odindo et al., 1986). Virus infection results in lesions in the tsetse, arrests spermatogenesis and causes severe tissue degeneration. Such males are completely sterile. The infection of male tsetse with the DNA virus does not appear to interfere with their flight activities. The infected males are as vigorous as the normal ones. They readily locate the females and, apart from their sterility, mate as efficiently as the normal males. The virus-infected tsetse males display a very high level of mating competitiveness and would be ideal in the sterile-male insect release program for the control of tsetse. However, methods for large scale infection of tsetse with the Fungus are yet to be developed.

Importance of the study

In Sub-Saharan Africa, sleeping sickness is still an important disease. In addition, "nagana" still impedes livestock production directly and crop production indirectly.

The control of trypanosomiasis which currently relies on the control of tsetse has not been able to curb the high rate of disease transmission. Intensive and repeated spraying of residual insecticides can have disastrous effects on the local fauna (Koeman et al., 1971).

Trypanosomiasis control through vector control has several interesting possibilities; but other approaches need to be explored so as to widen our arsenal for the control of tsetse population. Some of these novel approaches are:

(a) to produce an antibody in the mammalian host which inhibits the activity of the anticoagulant in the tsetse salivary secretion;

(b) discovery of a specific blocking agent for neuromuscular or neurosecretory activity in tsetse

(c) and study of the genetics of those tsetse populations that are susceptible to trypanosome infections.

Instead of trying to eradicate the insects, vector biologists hope to produce transgenic strains that are incapable of transmitting disease (Aldhouse, 1993).

However, for the objective to be achieved, it is imperative that there should be understanding of the mechanisms involved in the interaction between tsetse and the trypanosomes.

Gut adapted trypanosomes experience substantial immediate (Turner, Barry and Vickerman, 1988) and delayed (Dipeolu, 1975; Welburn, Maudlin and Ellis, 1989) mortality when bloodstream-forms transform to procyclic-forms in tsetse. For unknown reasons, some stocks of bloodstream-

forms thrive in this hostile gut environment, whereas others die out. The mechanism underling these processes have received considerable attention (Maudlin, 1990) but less effort has been extended to the role of host in transmission (Moloo, 1989, 1984; Mihok, et al, 1991).

Tsetse are susceptible to infection with trypanosomes, although infection rates vary considerably among the many combinations of tsetse and trypanosomes. For Trypanozoon and Nannomonas, establishment of infection in the tsetse gut is a complex process involving interaction between the tsetse, its symbionts and the stock of trypanosomes.

The tsetse peritrophic membrane also affects trypanosome development by preventing or restricting the passage of parasites and pathogens (Wigglesworth, 1972).

Although the involvement of lectins or lectin-like molecules in both the lysis and the differentiation of the trypanosomes has become widely accepted, the mechanism of their action remains a matter of conjuncture. For instance, it is unclear whether lysis and differentiation are the direct effects of the lectin activity. The whole picture is further complicated by recent findings that trypsin or trypsin-like enzymes might also be involved in the same functions (Imbuga et al., 1992).

Tsetse trypanosome interactions are mediated by trypanoagglutinin, trypanolysin and digestive proteases. The role of trypanolysin in the trypanosome development in the tsetse has not yet been studied in depth.

In order to understand the tsetse trypanosome interaction, some of the factors involved have to be studied in depth and their role in trypanosome transformation or destruction assessed fully. This knowledge can be exploited to interrupt the trypanosome transformation cycle. Hence the need to isolate, and characterize these molecules.

Consequently, the present study was undertaken with the following objectives:

Overall objective

Isolation of G. m. morsitans trypanolysin/trypanoagglutinin and assessment of their use in the interruption of trypanosome differentiation.

Specific objectives:

(a) isolation and purificatioin of trypanolysin/ trypanoagglutinin from G. m. morsitans;

(b) characterization of trypanolysin/trypanoagglutinin;

(c) study of the effect of trypanolysin/trypanoagglutinin on trypanosome differentiation in vitro and in vivo;

(d) raise antibodies against trypanolysin/trypanoagglutinin;

(e) assess of the effect of antibodies on trypanosome transmission cycle.

CHAPTER TWO

LITERATURE REVIEW

2.1 Vectors of the disease tsetse

Tsetse belong to the genus Glossina Wiedemann, the species of which are found in Africa, with some exceptions. The genus Glossina (Swynnerton, 1936; Buxton, 1955; Mulligan, 1970; Potts, W.H., 1974) are now well known to be the carriers of the pathogenic agents of certain virulent diseases in Africa. Thus, G. palpalis transmits T. gambiense, the causative agent of sleeping sickness, from man to man by means of its piercing mouthparts. Similarly, G. morsitans transmits T. rhodesiense which is responsible for the more local or Rhodesian form of the disease. G. morsitans is also the main vector of the trypanosomes which cause the disease known as "nagana" (Bruce, 1985) among domestic animals.

2.2 Trypanosomes

The trypanosomes of the "Brucei" group are digenetic (Hoare, 1972) with a complicated life cycle which alternates between two hosts: one, an invertebrate, representing the intermediate host or vector, in which the flagellates develop primarily in the gut; the other, a vertebrate, in which they inhabit the blood and/or the lymphatic tissue.

It has been proposed that the mammalian trypanosomes be divided into two sections: (1) stercoraria and (2) salivaria. Stercorarian trypanosomes undergo development in the Glossina but with the exception of Trypanosoma rangeli, the infective forms are deposited in the faeces of the vector. Salivarian trypanosomes either undergo cyclic development in the Glossina before being transmitted

with the saliva, or are transmitted mechanically. It is the trypanosomes in the salivary glands that are infective to man and his livestock. The African trypanosomes elicit pathogenicity in these animals.

Four subgenera are recognized within the salivarian species of Trypanosoma. They are: Trypanozoon, Nannomonas, Duttonella and Pycnomonas (Logan-Henfery et al., 1992).

Three stages (initial, established, mature) can be recognized in the development of an infection of T. brucei in Glossina. When G. morsitans was fed on infected blood, 50% of the tsetse developed initial infection in the midgut 3 days latter but only 9% developed established infection in the peritrophic space and foregut. Established infections were detectable 5 to 30 days after infective feed. However, the number of mature infections, that is, tsetse able to transmit metacyclics, was very low. Only 39 flies out of many hundreds examined successfully transmitted T. brucei by bite and only 15 of those were infections observed in the salivary glands (Dipeolu and Adam, 1974).

A characteristic feature of trypanosome infections in animals is that their abundance follows a wave-like pattern over time. In each wave of parasitaemia one variant will dominate and then be cleared as the host develops the appropriate antibody to the surface-coat protein (variant specific glycoprotein, VSG, or variant specific antigen, VSA) of the trypanosome. Following clearance, another variant will dominate. However, this switching from one variant to another is not due to exposure to the antibody because the phenomenon occurs in immunosuppressed animals. This phenomenon of antigenic variation is believed to be responsible for the trypanosome's ability to produce chronic infections in man and other vertebrate hosts. This characteristic plus the persistence of the vector as well as the long infection time in the vertebrate host are all factors contributing to the continuous infection in Africa. The variant specific antigen of metacyclic of the trypanosome forms are

similar and the infected populations may contain as many as 27 variant antigen types (Turner et al., 1988).

By application of techniques used in molecular biology, it had been demonstrated that trypanosomes may contain as many as 1,000 different genes for variant antigenes within their genome. The genetic process by which cells activate genes for variant antigenes allows considerable genetic varation to occur during trypanosome growth. The trypanosomes have a large reportopire of variant surface antigenes (VSAs). It is this large reportoire of genes, that enables the trypanosomes to escape the host's immune response and survive in their hosts (Inverso et al., 1988). The phenomenon of antigenic varation is complex. The very large numbers VSA genes and the unpredictability of the sequences of switches in VSA types are the main abstacles to the development of vaccine against the African trypanosomes (Cross, 1975). The possobility of developing a vaccine to prevent trypanosomiasis based on VSAs appears difficult, if not impossible, because of the large numbers of specificities of VSAs produced by the large numbers of genes coding for the VSAs, and the continuous changes in the nucleotide sequences of these genes as a result of genetic exchange during sexual and other processes.

Since the work of Cross (1975), who developed a simple biochemical procedure for the isolation of variant surface glycoprotein (which constitutes the variant specific antigen), our knowlege of the structure of the VSA has increased enormously. The surface coat of Trypanozoon species has several rather unusual characteristics.

The predominant variant antigen types in metacyclics will be the one that dominated in the infective blood meal. In the vertebrate host, infective metacyclic forms injected by a feeding Glossina

develop into rapidly dividing long slender (LS) trypomastigotes. At a latter stage there is a switch to an intermediate (I) form, which is rarely seen to divide, and then to a non-dividing short stumpy (SS) form which lacks a free flagellum. It has been suggested that the SS forms are not infective to the mammalian host and that they and the I forms are infective to Glossina (Fig. 2). The gut of an insect is composed of three main regions, foregut, midgut and hindgut. The mouth leads into the buccal cavity and hence to the pharynx, which may be developed as a muscular pump moving food down the narrow oesophagous to the storage organ or crop. In Diptera, the crop is blind diverticulm from the oesophagous (Fig. 3). The crop is purely storage and secrets no enzymes. Any digestion that occurs in the crop is due to the action of enzymes contained in the saliva.

The midgut of many insects is lined with a non-adherent, thin, transperent tube, the peritrophic memberane, secreted at the proventriculus. Its functions are to protect the midgut from abrasion by food particles and to prevent or restrict the passage of trypanosomes and pathogens (Wigglesworth, 1972). In dipteran larvae the peritrophic memberane is secreted at the anterior end of the midgut and moulded by an invagination of the oesophagous. It then forms a single continuous memberane which may extent into the hindgut. In the context of vaccinating mammalian hosts against parasitic arthropods, the peritrophic memberane may either restrict penetration of ingested immune components or serve as a target for immunological attack. In Glossina, it influences the development of trypanosomes. It is present in most adult Diptera (Peters, 1992). The ultrastructure of the midgut of the most important blood-sucking insects has been reviewed by Billingsley (1990). The midgut enzymes are adapted to the diet of the insects. Thus, the midgut of blood-feeding Glossina secretes very active proteases but little carbohydrases.

Fig. 2 Diagram of the life cycle of trypanosomes of the T. brucei group (Modified from Vickerman and Barry, 1982).

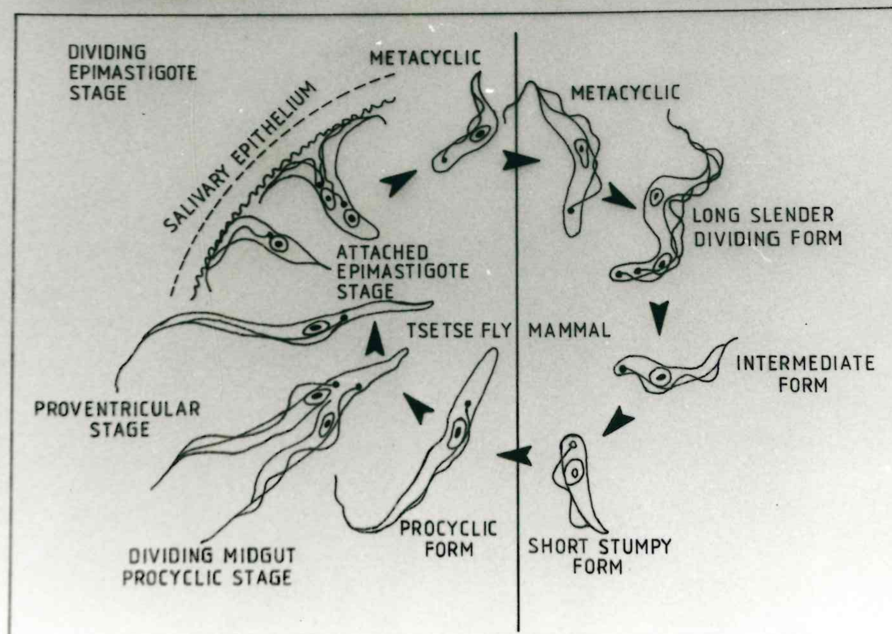
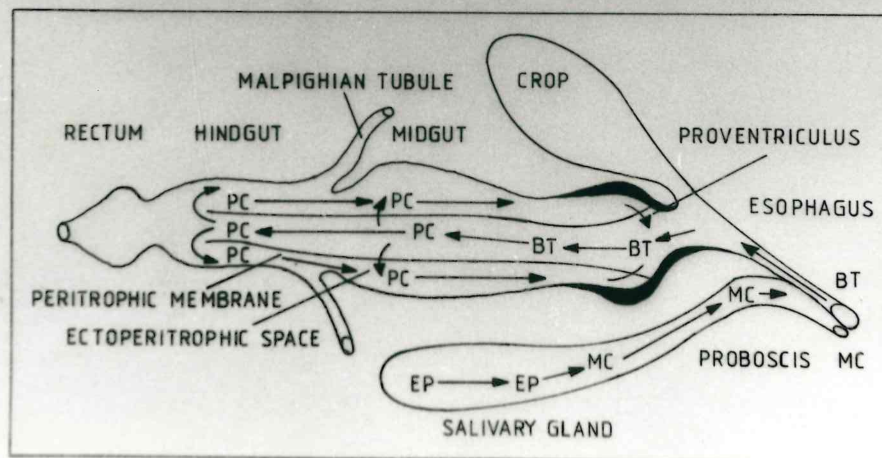


Fig. 3 The migration of trypanosomes of the T. brucei group in the tsetse. The arrows indicate direction of the migration through the tsetse. The morphogenic forms of the trypanosomes in the various locations in the tsetse are indicated by the following code: BT, blood trypanosomes; PC, procyclic-forms; EP, epimastigotes-forms and MC, infected metacyclic-forms (Molyneux and Ashford, 1993; Vickerman et al., 1988).



The main function of the hindgut is the absorption of water from the faeces and urine. Urine passes into the hindgut from the Malpighian tubules, which open just behind the midgut.

Trypanosomes pass with the ingested blood into the crop of Glossina from which they are passed via the proventriculus into the midgut where they are contained within the endoperitrophic space (Fig. 3). In the midgut the I and SS forms differentiate into procyclic trypomastigotes which develop and divide extensively (Figs. 2 and 3). In Glossina the procyclic trypomastigotes have no variant specific antigen coating but are uniformly coated with a new antigen, procyclin (Seed and Hall, 1992). It has been shown that a single ingested trypanosome can infect a tsetse. The trypanosomes then move into the ectoperitrophic space via the open free end of the membrane or by penetrating the membrane (Fig. 3). This process takes place in about 2 weeks after an infective feed.

In the ectoperitrophic space the trypanosomes move forward to the proventriculus where they pass through the soft newly secreted peritrophic membrane to become free in the oesophagus and move up the food channel to the opening of the salivary duct in the hyopharynx. They pass within the salivary duct to reach the salivary glands, where they develop into epimastigotes which attach to the epithelial cells of the salivary glands by means of a junction (hemidesmosome) between the flagellum and the micrrovilli. Extensive division takes place and finally metacyclic forms detach from the microvilli and develop a surface coat like that found in trypanosomes in the vertebrate host. Metacyclic forms injected into a susceptible vertebrate host develop into rapidly multipling LS trypanomastigotes (Figs. 2 and 3).

The probability of transmission occurring is enhanced by the fact that the first drop of saliva produced by the feeding tsetse is the largest and will presummably carry with it most metacyclics. If

feeding is interrupted another large drop of saliva is produced when the tsetse attempts to feed again (Youdeowei, 1975). This implies that a probing tsetse is almost as likely to transmit as one that feeds to repletion. The above account follows the classical description of the cycle of Trypanozoon in Glossina (Hoare, 1972), but recent observations raise doubt about its being the complete history (Evans and Ellis, 1983). It has been shown that T. rhodesiense can penetrate the peritrophic memberane in the central two-thirds of the midgut. Nine days after an infective feed all the trypanosomes were in the endoperitrophic space (Ellis and Evans, 1977). The trypanosomes then actively penetrated the midgut cells and undamaged trypanosomes were seen between the basement memberane of the midgut cells and the haemocoel memberane (Evans and ellis, 1975). It is claimed that the trypanosomes reached the haemocoel and infected the salivary glands, enabling normal transmission to occur when the tsetse feeds (Evans and Ellis, 1979). Certainly such a route would be considerably shorter than that involved in moving to the salivary gland via the salivary duct.

Another objection to the classical explanation has been the claim that the pH of the midgut at the open end of the peritrophic memberane was such as to be lethal to trypanosomes (Freeman, 1973). It has also been suggested that trypanosomes and red blood cells pass into the peritrophic space through the soft anterior part of the peritrophic memberane (Freeman, 1973).

The natural infection rates of tsetse have long been used by those concerned with epidemiology and control to assess the risk or "challenge" created by the disease. Until recently, these natural infection rates were thought to be determined largely by the biology of the tsetse. Clearly, an infective feed is an essential prerequisite for a tsetse infection. However, the number of trypanosomes ingested

by the tsetse during the infective feed does not influence the infective rate. Indeed, a single trypanosome has been found to be sufficient infecting a tsetse (Maudlin, 1989).

2.3 Tsetse trypanosome interactions

An important part of the life cycle of African trypanosomes occurs within the tsetse. These flies are not merely transport organisms which transfer trypanosomes from infected to non-infected vertebrate hosts, but are intermediate hosts of these disease-causing organisms and part of their life cycle occur within these tsetse. In order for the trypanosome to survive therefore, they must be able to live and defend themselves against two completely different immune systems, the vertebrate and the invertebrate. Bloodstream-form trypanosomes are covered by a dense surface coat consisting of the variant surface glycoprotein (VSG). The bloodstream-forms are ingested when the tsetse takes a parasitized bloodmeal from a vertebrate host. In most cases, T. brucei pass through the oesophagus to the crop. However, other Trypanosoma, the life cycle is restricted within the tsetse mouthparts. After feeding, the trypanosomes migrate to the peritrophic membrane lining the midgut where the blood is digested. In the midgut, the trypanosomes encounter a physiologically hostile environment that include various digestive enzymes (Cheeseman and Gooding, 1985), lectins (Welburn et al., 1989), agglutinins (Ingram and Molyneux, 1988); Stiles et al., 1990), trypanolytic proteins (Stiles et al., 1989), and possibly other yet unknown factors. Most trypanosomes that enter the midgut are killed by one or more of these factors, and those that survive transform into procyclic (midgut) forms (Maudlin and Welburn 1987; Stiles et al., 1990). These trypanosomes establish themselves in the ectoperitrophic space as midgut or "immature" infections. The successful establishment of the trypanosomes within the

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

Thus, after entering the tsetse midgut, the probability of the trypanosomes completing their life cycle within the vector is inversely dependent on the complexity of the changes elaborated. This in turn is related to the species of trypanosome involved. Within the tsetse, T. brucei seems to have the most complex life cycle compared to T. congolense and T. simiae and therefore should have the least chance for successful cyclical transmission (Buxton, 1955).

Tsetse species differ greatly in their abilities to transmit trypanosomes. Generally, it has been observed that the Morsitans group is more susceptible and therefore are the more efficient vectors of trypanosomes, while the Palpalis and Fusca groups appear to be poor vectors (Harley and Wilson,

1986; Harley, 1971; Roberts and Gray, 1972; Moloo and Kutuza, 1988b; Moloo et al., 1987, 1992a, b). It is possible that the differences in the infection rates reflect differences in the midgut environments of the tsetse species such that the trypanosomes, T. brucei, T. rhodesiense, T. gambiense, T. vivax and T. simiae, can become established more readily and undergo full cyclical development in some tsetse while arrested in the midguts of others (Molyneux, 1983; Moloo and Kutuza, 1988 b). The reasons for this are not fully understood.

2.4 Tsetse midgut environment

The midgut is situated between the distal end of the proventriculus and the junction of the two Malphigian tubules (Service, 1986). It is lined along its entire length by the peritrophic membrane (Service, 1986). The midgut is very long and convoluted. During feeding, blood is sucked up the proboscis, passes to the crop and later to the midgut. In case of an infected bloodmeal, the trypanosome encounter a physiologically hostile environment within the tsetse midgut. Here, they either migrate to regions of low anti-trypanosomal molecules (anterior midgut) or are lysed in the posterior midgut while others survive. The distribution of trypanosomes within the midgut is dependent on how efficient the destructive molecules can be avoided.

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

2.4.1 Peritrophic membrane

The involvement of peritrophic membrane in the trypanosome life cycle was demonstrated by Wigglesworth and Hoare (1972). In tsetse, this membrane, secreted by cells of the proventriculus, consists of annular sheath of chitin and associated protein. The membrane is absent in newly emerged flies but develops rapidly after eclosion, lining the entire length of the midgut after 80-90 h (Lehane and Msangi, 1991). The peritrophic memberane protects the midgut cells from abrasion by hard fragments in blood and acts as an ultrafilter but offers no hinderance to digestive enzymes and digestion products (Wigglesworth, 1972). More recently, it has been proposed that the peritrophic membrane may also act as a physical barrier to the penetration of trypanosomes (Lehane and Msangi, 1991). Bloodstream-form trypanosomes enter the gut inside the membrane (endoperitrophic space) and then transform into procyclic-form and establish themselves as midgut or "immature" infections in the ectoperitrophic space between the midgut cells and the bloodmeal. How trypanosomes pass from the endo-to the ectoperitrophic space is debatable.

It had been reported that, as tsetse grow older, they become more refractory to infection with both Brucei (Van Hoof et al., 1973; Wijers, 1958; Ward, 1968; Harley, 1970; Makumyaviri et al., 1984) and Congolense group trypanosomes (Distelmans et al., 1982; Mwangela et al., 1987). Attempts to explain this age-dependent variation in susceptibility has centered on the nature of the peritrophic membrane (Lehane and Msangi, 1991). Since newly emerged flies lack a peritrophic membrane, the inability of older tsetse to support trypanosome infection was thought to be due to the development of peritrophic membrane.

The observations of Ellis and Evans (1977) conflict with the attempts to explain the age-dependent variation to trypanosome infection rates by changes in the peritrophic membrane during the first days. There is also evidence that older tsetse can be infected with trypanosomes.

It has also been reported that older tsetse which are normally refractory, can be infected with trypanosomes if they are starved for 3-4 days prior to taking an infected meal (Gingrich et al., 1982 a; Makumyaviri et al., 1984), suggesting that fully developed peritrophic membrane is not a physical barrier to the penetration of trypanasomes but that other factors may be involved.

More recently it has been suggested that gut lectins and not the peritrophic membrane determine the age-dependent susceptibility of tsetse for trypanosomes (Maudlin and Welburn, 1987; 1988). These lectins are produced in response to stimulation from the serum content of the first bloodmeal thus explaining the age-dependent infectability of tsetse, as only trypanosomes ingested with the first bloodmeal will not encounter protective lectins. However, this association of lectins with the peritrophic membrane may partly explain the paucity of lectins in the newly emerged tsetse, where the peritrophic membrane is incomplete (Lehane and Msangi, 1991).

The establishment of trypanosomes in the midgut has also been associated with the presence of Rickettsia-like organisms (RLOs) in the midgut epithelial cells (Maudlin and Ellis, 1985).

2.4.2 Rickettsia-Like Organisms (RLOs)

Rickettsia is the generic name for a group of minute micro-organisms which may be said to be intermediate between the smallest bacteria and viruses. They are rod-shaped, coccoid and often pleomorphic gram-negative organisms. All Rickettsial are regarded as parasitic or mutualistic and are

associated with arthropods which may act as vectors or primary hosts. Rickettsia-like organisms are present in the midgut cells of several Glossina species. Susceptibility to trypanosome infection in G. m. morsitans has been shown to be a maternally inherited trait associated with the presence of tsetse symbionts, the RLOs, in the midgut cells (Maudlin and Ellis, 1985; Maudlin et al., 1986).

RLOs have also been shown to occur in lower levels of midgut lectins, especially in 'susceptible' tsetse. From studies using cultures RLOs established in vitro in mosquito cells, these cultures have been shown to produce the enzyme chitinase which can hydrolyses chitin to glucosamine. Glucosamine produced in this way in the larval/ pupal period in RLOs-infected teneral tsetse accumulate in the larval midgut and act as a lectin inhibitor when the emergent tsetse takes its first feed (Maudlin and Welburn, 1988b; Baker et al., 1990). The accumulated glucosamine render the tsetse susceptible to trypanosome infection, at least for the first feed. On the other hand, lectin levels in the midguts of non-teneral tsetse when switched on by the bloodmeal are too high to be affected by RLOs activity.

Moloo and Shaw (1989) dispute this theory. Having found RLOs in all G. m. centralis, both infected or uninfected, they concluded that susceptibility is not associated with these organisms. The RLOs were found to be numerous in older tsetse compared to younger tsetse, yet the former are more resistant to infection than the latter. The importance of the first feed being infective for the establishment of T. brucei infections has long been accepted but this factor has also been shown to affect the probability of a tsetse developing T. congolense infection. Maintaining the puparial stage of G. m. morsitans at 3° C lower than normal reduced the numbers of RLOs carried by emerging tsetse. The susceptibility of these tsetse to midgut infection with T. congolense was also significant with

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

2.4.3 Proteolytic enzymes

Digestion of the bloodmeal occurs in the posterior region of the midgut. As proteins are the most nutritive components of the blood, digestion is mostly proteolytic.

In Glossina species, at least six proteolytic enzymes, responsible for bloodmeal digestion, have been reported (Gooding and Rolseth, 1976; Cheeseman and Gooding, 1985). These proteases include trypsin, trypsin-like enzyme, chymotrypsin-like, carboxypeptidase A and B, aminopeptidase among others (Cheeseman and Gooding, 1985). Of these, trypsins have received more attention due to their role in trypanosome differentiation (Yabu and Takayanagi, 1988; Imbuga et al., 1992b). Trypsin and trypsin-like enzymes account for about 70% of midgut proteases (Abbeele and Decleir, 1991) and are therefore more important.

Gingrich et al. (1985) observed an increased midgut infection rate in tsetse fed on trypanosomes suspended in serum free blood. As a possible explanation they postulated that a relationship existed between the activity of midgut proteinases and the development of a procyclic infection. Proteinase-induced morphological changes in African trypanosomes have been reported by Frevert et al. (1986), who observed with the electron microscope that trypsin or pronase treatment of

T. congolense bloodstream-forms led to striking changes in the cell shape. However, the mechanisms underly the proteinase-induced structural alternations in trypanosomes are not yet understood. Similarly, the interaction between proteinases and the trypanosome has also been proposed for other hematophagous insect vectors. For example, trypsin-like proteinases in Aedes aegypti are responsible for destruction of ingested ookinetes of the fly (Gass and Yeats, 1979; Yeats and Steiger, 1981). In Phlebotomus paptasi, it has been suggested that a specific component of the trypsin-like activity prevents the survival of Leishmania donovani and modulation of this component by Leishmania major enables it to thrive (Borovsky and Schlein, 1987). The anterior midgut possess proteinase inhibitors which inhibit G. m. morsitans posterior midgut trypsin (Houseman, 1980). The inhibitor is subspecies specific, each anterior midgut lysate inhibits only the homologous lysate. The exact nature of the inhibition mechanisms is not known. It is likely that the inhibitor protects the gut cells from proteolytic activity during periods of starvation (Vogel et al., 1968).

The possibility that trypsin activity is involved in the elimination and/or transformation of bloodstream-forms has been shown by preliminary in vivo studies of the midgut trypsin of G. p. palpalis on T. brucei (Abbeele and Declair, 1991). Trypsin has been implicated as one of the factors important in the transformation of the trypanosome from bloodstream to procyclic-forms. The loss of surface coat has been attributed to proteolytic activity of midgut trypsin (Yabu and Takayangi, 1988; Imbuga et al., 1992 b). Imbuga et al. (1992 b) have also shown that tsetse midgut trypsin as well as bovine pancreas trypsin can stimulate bloodstream-form transformation. Furthermore, the in vivo inhibition of trypsin activity using protease inhibitors such as soybean trypsin inhibitors (STI), resulted in reduced transformation rates (Imbuga et al., 1992 b). In addition, different species of Glossina, when fed before

an infective feed could clear trypanosomes from their midguts faster than tsetse infected as tenerals (Welburn et al., 1989). This difference was attributed to trypsin activity levels which rise after a bloodmeal and peak at 72 h and 24 h in tenerals and non-tenerals, respectively (Osir et al., 1993). Subsequently, the activity drops but still remain higher than the initial levels prior to the first bloodmeal (Nguu and Osir, unpublished data). Thus the ability of non-teneral flies to clear trypanosomes after an infective feed is due to the trypsin activity. It has been reported that older G. m. morsitans can be infected with T. b. brucei if they are starved 3-4 days prior to an infective bloodmeal (Gingrich et al., 1982a; Makumyaviri et al., 1984). This can be attributed to the reduction in trypsin activity during starvation.

Although, trypsin has been shown to stimulate trypanosome differentiation, optimal concentration of this protease are crucial for this process. High trypsin levels lead to the destruction of trypanosomes from the tsetse midgut. For the trypanosomes to avoid this proteolytic destruction they have evolved means of inhibiting trypsin activity. Imbuga et al. (1992 b) found that bloodstream-form trypanosomes inhibited trypsin activity by upto 80%.

2.4.4 Effect of host blood

Host blood type at the time of infective feed alters infection rates in tsetse maintained subsequently on one host (rabbits). This effect was detected in both susceptible G. m. centralis and refractory G. m. morsitans (Maudlin et al., 1968; Shaw and Moloo, 1991). Some host blood contain factors that do not promote trypanosome development (Nguu et al., 1996). Effects of host blood on trypanosome infection in tsetse vectors were investigated by Moloo (1981). The results showed that,

rabbits and cows as maintenance hosts produced the highest tsetse infection rates of T. brucei and T. vivax while mice produced the lowest. In case of T. congolense, significantly higher tsetse infections were obtained when goat and rabbit served as maintenance hosts. These observations suggest that the host blood on which G. m. morsitans were maintained after an infective feed influenced infection rates. Similarly, Mihok et al. (1993), investigated the effects of blood from selected domestic and wildlife species on infection rates in G. m. morsitans and G. m. centralis. Infective blood was fed to tsetse through a membrane but were maintained subsequently on rabbit. It was suggested that species-specific factor (s) present in the host blood could have been responsible for the result.

Given the many biochemical changes that occur in the tsetse during trypanosome establishment, it is difficult to point out the mechanism through which host blood factors might act. These factors are neither known to be proteins, which are digested efficiently by the tsetse, nor analogs of glucosamine since sugars are not present in high concentration in blood. This then leaves lipids as possible candidates, since they are poorly digested (Langley et al, 1987), suggesting that they could remain intact for longer time. Levels of lipids are also known to differ in host blood that produce different infection rates, such as in cows and pigs (Kabayo et al, 1986). How lipids act in facilitating trypanosome survival is open to speculation. For example, Songa et al. (1983), reported that trypanosome lysis failed to occur in complement C6-deficent rabbit serum, suggesting that C6 is required for parasite lysis.

Traore et al. (1987) reported that West African cattle which are naturally resistant to trypanosomiasis have a higher trypanolytic activity in their serum than trypanosensetive cattle. Also, Traore et al. (1987) showed higher levels of serum polyamine oxidase in resistant cattle. It had been

found that oxidation of spermidine by polyamine oxidase led to the production of unstable aldehydes, acrolein, ammonia, O_{2-}, OH., and H_2O_2 and it was concluded that acrolein and H_2O_2 showed strong trypanolytic activity but the other products did not appear to be toxic for trypanosomes. The physiological importance of polyamine oxidase-mediated trypanolysis is unclear. Verducci et al. (1989) reported the presence of natural immunoglobulin class M (IgM) active against T. equiperdum in human serum and could represent one of the natural mechanisms of resistance of refractory hosts against trypanosome infections. The trypanolytic activity of human serum was found to be associated with the fraction containing 19-S antibodies. Funato et al. (1993) reported trypanolytic factor and its inhibitor in guinea pig serum against T. b. gambiense (Welcome strain), that was suppressed completley in normal serum, suggesting that the suppression might be caused by the binding of the inhibitor to the factor. Tytler et al. (1995) reported human high-density lipoprotein (HDL), termed trypanosome lytic factor (TLF), which is responsible for the cytotoxicity of human serum to T. brucei. It was suggested that the trypanosome lytic factor does not have a direct role in lysis but are necessary for the correct assembly of the lytic high-density lipoprotein particle. Apolipoprotein (Apo) A-1, apol-111 and apol-1 contribute to lysis in reconstituted particles but individually they are not cytotoxic. The formation of a lytic high-density lipoprotein particle required apo L-111, suggesting its potential role as a toxin. Thermal inactivation of trypanosome lytic factor activity correlated with the amount of denatured apol-1, indicating that apol-1 was involved in lysis of T. brucei by the native trypanolytic factor. Gillett et al. (1992) reported that high-density lipoprotein isolated from human and baboon plasma were trypanolytic (typically » 95% and 60% lysis, respectively, at 1.0 mg protein/ml), whereas bovine and sheep high-density lipoprotein were benign (< 8% lysis). Hajduk et al. (1995) reported the

involvement of high-density lipoproteins in normal and Tangier patients sera in T. b. brucei killing. When fractionated by gel filtration chromatography, both normal and Tangier sera displayed 2 peaks of trypanosome lytic activity; one at 150,000 to 600,000 MW and the other at > 1,000,000 MW (Hager et al., 1994). Leupeptin inhibition suggests that a thioprotease may be involved in the mechanism of trypanosome lytic factor-mediated lysis of T. brucei. A lytic mechanism involving cell surface binding, endocytosis and lysosomal targeting was proposed. That was followed by lysosomal disruption and subsequent autodigestion (Hager et al., 1994).

2.4.5 Lectins

During the life cycle of gut-adapted trypanosomes, the critical time is the initial establishment of a midgut infection in the tsetse. Within hours of entering the tsetse, most bloodstream-forms are lysed before transforming into gut-adapted procyclic-forms (Turner et al., 1988). Although multiple factors probably affect trypanosome establishment and maturation in the fly, tsetse "immune" processes mediated by lectins appear to be crucial.

Lectins are divalent or multivalent carbohydrate-binding proteins that are grouped together because they agglutinate cells or other materials that display more than one saccharide of sufficient complement. They were discovered in plants, but are also found in all other categories of living things.

Lectins are heat-sensitive proteins or glycoproteins and have been suggested to play a role in immune defense by enhancing clumping of the trypanosomes, bacteria and viruses and by enhancing phagocytosis due to the strong opsonizing activity. These effects, however, are inhibited by specific sugars, thus demonstrating the ability of carbohydrate-binding molecules to function as recognition

molecules. Lectins are widely distributed in invertebrates in which they have been reported to be synthesized in the haemocytes and fat body cells (Ratcliffe, 1986) and their role as recognition molecules in invertebrates was demonstrated by Renwrantz and Stahmer (1983).

In the tsetse, the involvement of lectins in transformation of trypanosomes was first proposed by Pereira et al. (1981) who suggested the lectins from the midgut of Triatoma bugs could stimulate the transformation of Trypanosoma cruzi in vitro. Isola et al. (1986) found that transformation of T. cruzi from epimastogotes to metacyclics could be stimulated by incubation with Triatoma infestans Klug intestinal homogenate. Hence the normal role of midgut lectins is to mediate the lysis of most trypanosomes entering the gut (Maudlin and Welburn, 1987). The midguts of teneral tsetse have been shown to have little lectin activity compared to non-teneral tsetse. This suggests as in the case of trypsin, that the production of lectin is induced by bloodmeal (Welburn et al., 1989; Imbuga et al., 1992). The rate of midgut killing of trypanosome varies both between and within species of tsetse and these differences correlate with lectin output measured by in vitro trypanoagglutination tests (Welburn et al., 1989). Thus, tsetse with the highest lectin output are the most successful at preventing trypanosomes establishment in the midgut (Welbuen et al., 1989). In addition, G. m. morsitans midgut lectin has also been implicated in providing the signal for established midgut trypanosomes to proceed with the process of maturation (Maudlin and Welburn, 1988a). Subsequently, it was shown that inhibition of lectin activity using glucosamine temporarily prevented maturation of trypanosomes, which could proceed once the inhibition was removed (Welburn and Maudlin, 1989). This suggested that midgut procyclic-form trypanosomes are not preprogrammed to directly complete their life cycle in the fly, but remain in the midgut stage until they receive the midgut signal to proceed with

maturation. However, it has been proposed that the lectin may act in two different ways. Firstly, it might act as a trigger by binding to individual procyclic trypanosome membrane and the trypanosomes differentiate (Welburn and Maudlin, 1990). Secondly, it might simply act as mitogen, a common property of lectins (Goldstein and Poretz, 1986).

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

2.4.6 Trypanoagglutinin

The humoral factors upon which much attention has been focused are the agglutinins, so termed because of their ability to agglutinate vertebrate erythrocytes in vitro (Bernheimer, 1952; Lackie, 1980), bacteria (Pauley et al., 1971; Pistole, 1978) and trypanosomatid flagellates (Ingram et al., 1983; 1984). It is now recognized that most of these agglutinins are lectins. Agglutinating activity has been found in the hemolymph, midgut and hindgut of G. austeni against calf, guinea pig and chicken erythrocytes (Ibrahim et al., 1984). More recently, Stiles et al. (1990) described a trypanolysin and a trypanoagglutin from the midguts of G. p. palpalis and G. p. gambiensis. The trypanolysin caused lysis of the trypanosomes while trypanoagglutinin resulted in the agglutination of the trypanosomes. As with midgut proteases (Cheeseman and Gooding, 1985; Imbuga et al., 1992; Osir et al., 1993), these two molecules were active only in the posterior midgut. Agglutinins were heat labile above 50° C and had a periodic cycle of activity in response to bloodmeal intake. Interestingly, these

molecules were not affected by pronase inhibitors or trypsin but were inactivated by pronase. The precise mechanisms of action of these molecules are still not clearly understood, hence the need for further research in this area.

2.5 Vectorial capacity of tsetse

Vectorial capacity of tsetse refers to picking up of the parasites from an infected vertebrate host, their development within the the tsetse vector and subsequent transmission to the the next vertebrate host. There capacities are dependent on:

(a) endegenous factors of the tsetse (such as species, sex, age, physiology and host preference);

(b) the trypanosome itself (that is, the infectivity of the trypanosomes, the various strains and forms and the population) and

(c) ecological factors (climate, presence of hosts, etc.). These factors are important, and studies have shown that temperature determines the duration of the life-cycle and the infection rate in tsetse.

Tsetse species differ greatly in their abilities to transmit trypanosomes of whatever species. G. pallidipes and G. m. centralis appear to have similar vector competences for T. vivax but differ greatly with regard to susceptibility to T. congolense and T. brucei, the former having a lower vector competence than the latter (Moloo et al., 1992). Studies on the vectorial capacity of the tsetse using different hosts have shown that irrespective of the host species used, T. congolense infections rate are highest in G. m. centralis and lowest in the Palpalis and Fusca group tsetse (Moloo et al., 1992).

Generally, it has been observed that the Morsitans group of tsetse is the most susceptible and therefore efficient vectors of trypanosomes while the Palpalis and Fusca groups appear to be poor vectors (Harley et al., 1968, 1971; Roberts and Gray, 1972; Moloo and Kutuza, 1988b; Moloo et al., 1987, 1992). It is possible that there are differences in the gut environment of the tsetse studied such that the trypanosomes of the subgenera Trypanozoon and Nannomonas can become established more readily and undergo full cyclical development in some tsetse while arrested in the gut of others (Molyneux, 1983; Moloo and Kutuza, 1988b). Many studies have shown that infection rates of T. congolense and T. brucei in Glossina species are generally low (Buxton, 1955; Harley and Wilson, 1968; Harley, 1971; Jordan, 1974; 1976). These marked differences in susceptibility to trypanosome infections observed both in the field and the laboratory suggest that tsetse may possess certain intrinsic refractoriness to trypanosome infections. Maudlin (1982) showed that lines of G. m. morsitans highly susceptible or refractory to T. congolense and T. brucei could be selected in the laboratory. These observations reaffirmed the supposition that genetics could determine susceptibility of refractoriness to trypanosome infections.

2.6 Insect immunity

Like all other higher organisms, insects have a very effective immune system comprising of both cellular and humoral components and can defend themselves against invading microorganisms as well as protozoan parasites.

In adult tsetse, bacteria injected into the haemocoel are quickly phagocytized by the plasmatocytes (PLs) and within 1-3 h post-injection, several plasmatocytes containing intracellular

bacteria are observed in the hemolymph (Kaaya et al., 1988). However, phagocytosis of infected T. brucei was not observed at any time and blocking of phagocytic haemocytes with India ink or sheep erythrocytes did not result in increased parasitemia, thus suggesting that phagocytosis might not play any direct role in defending tsetse against trypanosome infection (Kaaya et al., 1986 b).

Haemocoelic infections of tsetse by T. brucei have been reported by several authors (Mshelbwala, 1972; Otieno, 1973; Kaaya et al., 1986 b) and the trypanosomes appear quite healthy and active in the tsetse hemolymph (Kaaya et al., 1986 b). Furthermore, 2-3% of G. m. morsitans support development and maturation of bloodstream-form of T. b. brucei, when inoculated in their haemocoels (Otieno et al., 1976; Kaaya et al., 1986 b). Thus in a given population, the majority are refractory to trypanosome infection, while a few "odd" individuals are susceptible. It would be interesting to study the factors that render these few individuals susceptible to infection and to act as vector of trypanosomes.

Indeed, there is increasing evidence to suggest that lectins protect insect vectors against infection by the parasites transmitted by them (Pereria et al., 1980; 1981; Ibrahim et al., 1984; Walbanks et al., 1986; Maudlin et al., 1988). Although in recent years several investigators of vector immunity have reported the role of lectins in defense against parasite infection, it is most unlikely that an insect vector would rely only on one mechanism for defending itself against infection by any particular parasite.

These observations clearly show that an insect vector can be defended against parasitic infection by several factors e.g. sandflies against Leishmania by peritrophic membrane (Orihel, 1975) and by lectins (Walbanks et al., 1986); mosquitoes against malarial parasites by peritrophic membrane

(Orihe, 1975) and phagocytosis (Weathersby and McCall, 1968). The roles played by these different defence mechanism in protecting insect vectors against infection are therefore not well understood and merit further investigations.

Availability of large quantities of purified proteins of disease vectors will allow more elaborate experiments to be conducted so as to broaden our current understanding of the interactions that occur between the vectors, their parasite and pathogens. It must be emphasized that immunity in vectors to disease parasites which they transmit would be of great advantage to man and animals. Knowledge of vector immune mechanisms might help to develop ways of strengthening the immunity in vectors, thus might assessed in the interruption of the disease cycle.

CHAPTER THREE

3. PROPORETIES OF TRYPANOLYSIN IN CRUDE HOMOGENATES

3.1 Materials and Methodos

3.1.1 Experimental insects and animals

Tsetse (G. m. morsitans, G. m. centralis, G. pallidipes and G. f. fuscipes) were reared on a 12 h: 12 h light: dark photoperiod at 75% relative humidity (RH) and 27° C and supplied by the Animal Rearing and Quarantine Unit (ARQU) of the International Center of Insect Physiology and Ecology (ICIPE). G. longipennis Corti, were supplied by the Tsetse Vector Laboratory of the International Livestock Research Institute (ILRI). Male Wistar rats (2-4 months old) were used for maintaining serial passages of trypanosomes and New Zealand white rabbits were obtained from ARQU for immunization purposes.

3.1.2 Preparation of insect midgut homogenates

Three hundred teneral tsetse (24 h after emergence) were allowed to feed on rats for 30 min and then maintained (72 h, 27° C). The tsetse were immobilized by brief chilling (4° C) after which their midguts were carefully dissected, and suspended in 3.0 ml ice-cold 20 mM Tris-HCl, pH 8.0. The midguts were then homogenized for 45 sec using a Virtis homogenizer (Gardiner, USA) and the homogenates centrifuged two times (12,000 g, 15 min, 4° C) in a Heraeus Minifuge (Ostrode, Germany). The supernatant solution was filtered through a 0.2 mm Millipore filter and stored at -20° C.

3.1.3 Trypanosome isolation

3.1.3.1 Bloodstream-form trypanosomes (T. b. brucei)

Pleomorphic T. b. brucei of a stock derived in 1969 from East African Trypanosomiasis Research Organisation (EATRO) was used. The stock was isolated as previously described (Otieno et al., 1983). The trypanosomes were maintained by passaging in rats from stabilates that had been cryopreserved in liquid nitrogen. Before obtaining parasitized blood from an infected rat, peak of parasitaemia was determined by piercing the tip of the tail with a disposable lancet to obtain a drop of blood which was placed on a slide, covered with a cover slip and the thin wet blood films examined using a phase-contrast microscope. Blood was obtained by cardiac puncture, and the trypanosomes isolated (Fig. 4) by ion-exchange chromatography on a diethyl aminoethyl-cellulose (DEAE) column (Fig. 5) (Lanhan and Godfrey, 1970) and then suspended in phosphate saline glucose (PSG) containing 0.15 M $NaPo_4$, 0.1 M NaCl and 1% glucose, pH 7.2. The trypanosomes were then counted using a hemocytometer equipped with an improved Neubauer ruling and the final concentration adjusted to » 5 x 10^6 trypanosomes/ml with phosphate-buffered saline (0.15 M $NaPo_4$ and 0.1 M NaCl, pH 7.2.

3.1.3.2 Procyclic-form trypanosomes (T. b. brucei)

Procyclic-form trypanosomes (T. b. brucei) were obtained by transferring the bloodstream-forms into an SDM-70 medium (Brun and Jenni, 1977; Brun and Schonenberger, 1970) containing 10% foetal calf serum (FCS). The trypanosomes were cultivated (27° C) with twice weekly passages. No antibiotics were included in the medium. Prior to use, the trypanosomes were pelleted from the

medium by centrifugation (3,000 rpm, 10 min, 4° C) and washed once in PBS in order to remove serum components.

Fig. 4 Isolated bloodstream-form trypanosomes (T. b. brucei) by DE-Cellulose 52

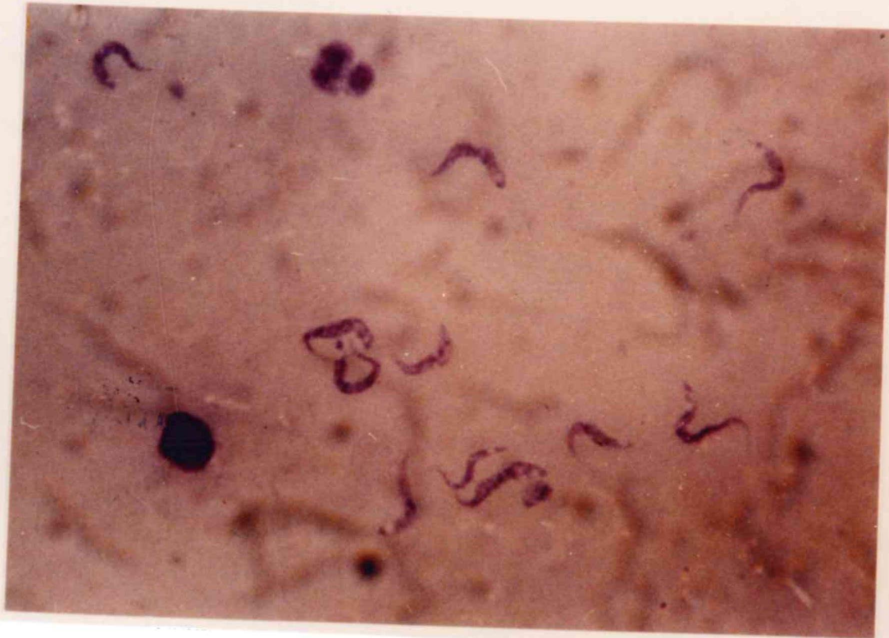
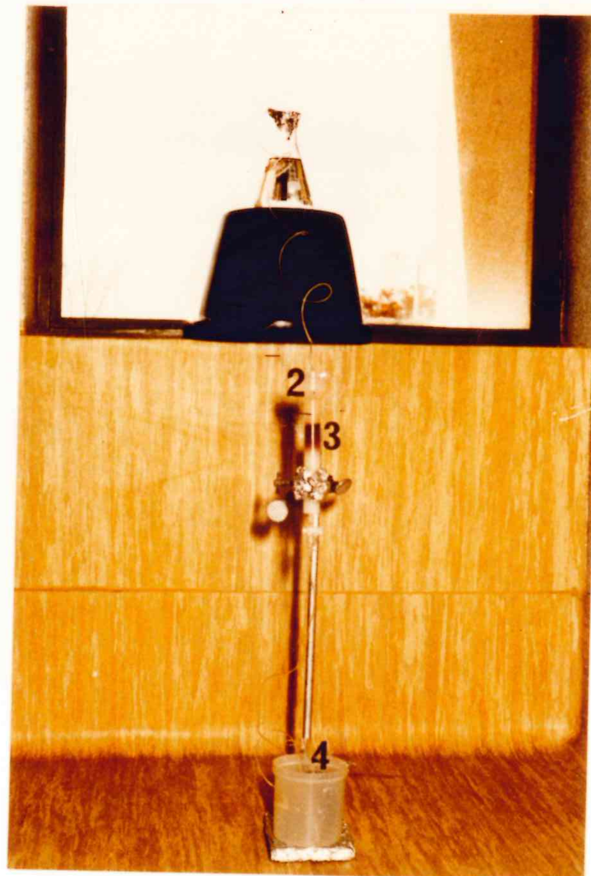


Fig. 5 DE-Cellulose 52 column for isolation of trypanosomes

1. Phosphate buffered saline- glucose
2. DE-CEllulose 52 column
3. Infected rat blood
4. Isolated trypanosomes (T. b. brucei)



3.1.4 Estimation of protein Concentrations

Protein estimations were carried out by the Bicinchoninic acid (BCA) method (Pierce, Rockford, 111. USA) using instructions supplied by the manufacturer. Bovine serum albumin (BSA) (Pierce) was used as the protein standard. Working reagents were prepared by mixing 50 parts of Reagent A (1.0 ml of base reagent which contains; sodium carbonate, sodium bicarbonate, bicinchonic acid detection reagent and sodium tartarate in 0.2 N NaOH) with 1 part of Reagent B (25 ml of 4% copper sulphate solution). 2.0 ml of the working reagent was added to each tube and mixed well. Samples from G. longipennis, G. fuscipes, G. pallidipes, G. m. morsitans, G. m. centralis and isolated trypanolysin were pipetted into appropriately labelled test tubes to determine protein concentrations. For blank, 0.1 ml of 20 mM Tris-HCl, pH 8.0 was used. All tubes were incubated (30 min, 37° C) in water bath. After incubation, tubes were cooled to 27° C. The absorbance of each tube was measured at 562 nm. The standard curve was prepared from BSA with protein concentrations.

3.1.5 Trypanolysin assays

All trypanolysin assays were carried out in triplicates in Microtitre plates (Nunc, Denmark). Double serial dilutions of the different midgut homogenates from G. longipennis, G. fuscipes, G. pallidipes, G. m. morsitans, G. m. centralis and isolated trypanolysin were prepared using PBS. An equal volume of the trypanosome that contained » 5 X 10^6 trypanosomes/ ml was added to each dilution. After mixing, the plates were incubated for 2 h at 27° C . Lysis of the trypanosomes was checked after every 30 min using an inverted microscope (Leitz Dialux 600, Germany). The controls consisting of: (1) trypanosomes and PBS and (2) trypanosomes and 20 mM Tris-HCL, pH 8.0. Data is presented as reciprocals of end point dilution titres (the least dilution that caused lysis of the

trypanosomes). Protein estimations were also, carried out for each of the end point dilution as an estimation of the minimum trypanolysin concentration that gave lysis for each one of the species (mentioned above) and the isolated trypanolysin.

3.1.5.1 Trypanosomes lysis by trypanolysin

The midgut homogenates from unfed, once-fed, twice-fed G. m. morsitans and the isolated trypanolysin were serially diluted as described in section 3.1.5 and the starting concentration was 14.34, 16.25, 5.75 and 4.70 mg/ml, respectively. Aliquots of procyclic and bloodstream-form trypanosomes containing » 5 x 10^6 trypanosomes/ml were added to each well and incubated (2 h, 37° C). Lysin titres were determined as described in section 3.1.5.

3.1.6 Time course and induction of trypanolysin by bloodmeal

The trypanolysin activities of midgut homogenates obtained from G. m. morsitans of different feeding states were assessed as follows: the unfed tsetse had their midguts dissected 24 h after emergence. The once-fed were given a bloodmeal 24 h after emergence and the midguts dissected after 72 h. The twice-fed tsetse were fed 24 h after emergence, starved for 72 h, fed again and midguts obtained after 72 h. The homogenates from these groups of tsetse were assayed for their abilities to lyse bloodstream-form trypanosomes as described in section 3.1.5. In another experiment, the time course of trypanolysin induction was assayed as follows: 200 teneral G. m. morsitans were given a bloodmeal 24 h after emergence. Of these, 20 were dissected immediately after feeding (0 h) and

another 20 flies 5 h after feeding. The rest of the tsetse (160) were dissected at 24 h intervals and trypanolysin activities assayed as described in section 3.1.5.

3.1.6.1 Induction of trypanolysin by serum and red blood cells (RBCs)

Healthy rat was bled by cardiac puncture. The blood was allowed to clot (2 h, 27° C), and then held (4° C) for not longer than 24 h. Serum was separated by centrifugation (1,500 rpm, 10 min). In order to prepare red blood cells, rat blood was collected in heparin and centrifuged (1,000 x g, 10 min) in a Heraeus 2 Minifuge. After removing the plasma and the "buffy coat", the pellet (containing the red blood cells) was washed five times in PBS by centrifugation (1,000 x g, 10 min 27° C). One group of 50 tsetse were membrane-fed on serum, the second group on red blood cells and the third group (control) on whole blood. After 72 h, the midguts were dissected from each group and assayed for trypanolysin activity using bloodstream-form trypanosomes as described in section 3.1.5.

3.1.7 Potency of trypanolysin as function of time

A separate study was carried out to determine the trypanolysin titre as a function of time. Double serial dilutions of midgut homogenates were prepared in 20 mM Tris-HCl, pH 8.0. An equal volume of the trypanosomes (» 5×10^6 trypanosomes/ml) was added to each dilution. After mixing, the plates were incubated (2 h, 27° C). The control consisted of trypanosomes in 20 mM Tris-HCl, pH 8.0. Lysis was checked after every 10 min using an inverted microscope (Leitz Dialux 600, Germany). Data is represented as reciprocals of dilution that gave complete lysis occurring as a function of time. Protein estimation was also determined for each dilution.

3.1.8 Trypanosome infection rates in tsetse

50 teneral tsetse from each species (G. longipennis, G. pallidipes, G. m. morsitans, G. fuscipes and G. m. centralis) were membrane-fed on infected goat blood infected with T. b. brucei stock EATRO (1969) (» 5 x 10^7 trypanosomes/ml). Control flies were fed on non-infected blood. All the tsetse were maintained (27° C) and a record of tsetse that died during the experimental period was made. Three days after the infective feed, the tsetse were memberane-fed on non-infected goat blood and all surviving tsetse were dissected on day 6 (section 3.1.2). The midguts were homogenized individually in 10 ml PBS. A total of 2 ml of two-fold serial dilution of each homogenate was spotted on to a slide glass, stained with Giemsa and examined for trypanosomes using phase-contrast microscopy (x 400 magnification). The morphology of the trypanosomes was also noted.

3.1.9 Effect of Ca^{++} and Mg^{++} on trypanolysin activity

The effect of divalent cations (Ca^{++} and Mg^{++}) on trypanolysin activities was assessed. Isolated trypanolysin was incubated (overnight, 4° C) with an equal volume of 16 mM ethylenediamine tetra-acetic acid, disodium salt (EDTA), pH 7.2 and then dialysed against PBS (48 h, 4° C). Bloodstream-form trypanosomes were added and trypanolysin activity was assayed as described in section 3.1.5.

3.1.10 Effect of temperature on trypanolysin activity

In this study, the midgut homogenates from tsetse fed once on clean rat blood were incubated (20 min) in a water-bath maintained at 27, 37, 40, 50, 60, 70 and 80° C. The samples were subsequently allowed to attain room temperature (27° C) and the trypanolysin assays carried out as described in section 5.1.5. A sample maintained at 27° C served as the control.

3.1.11 Effect of storage on the stability of trypanolysin

In a separate study, the trypanolysin assays were carried out under standard conditions except that the temperature of the incubations was 27° C, +4, -20, -70 and -80° C.

3.1.12 The effect of freezing and thawing on trypanolysin activity

The effect of freezing and thawing on the activity of isolated trypanolysin was assessed. Samples were frozen at -20° C, -70 and -80° C and thawed at 27° C. The activity was assayed and estimated by the method described in section 3.1.5.

3.2 Results

3.2.1 Determination of the minimum protein concentrations that gave lysis

Proteins estimation for the experimental samples included in the bioassay system for tenerals, once-fed, twice-fed tsetse and the isolated trypanolysin, the concentrations were found to be as follows: 3.16, 14.34, 16.25, 5.75 and 4.70 mg/ml, respectively (Tab. 1).

Table 1 Effect of number of feeds on trypanolysin activity
(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Type of midgut homogenate	**Protein concentration (mg/ml)**	**Trypanolysin titre**
Unfed	3.16	4
Once-fed	14.34	16
Twice-fed	16.25	32
Isolated lysin	5.75	
Isolatd lysin	4.70	

Midgut homogenate from Unfed G. m. morsitans gave trypanolysin titre 4 and protein concentration determined as 3.16 mg/ml. The titre increased four fold (from 4 to 16) and protein concentration estimated as14.34 mg/ml with once-fed tsetse. The trypanolysin titre increased further by 50% with twice-fed, however protein concentration was estimated as 16.25 mg/ml. Protein concentration of isolated trypanolysin by ion-ion-exchange and FPLC was found as 5.75 and 4.70 mg/ml. However, trypanolysin titres were not determined.

Also, the protein contents of midgut homogenates from the different species were estimated to be: G. longipennis (45 mg/ml), G. pallidipes (34 mg/ml), G. fuscipes (22.2 mg/ml), G. m. morsitans (14.34 mg/ml) and G. m. centralis (17.5 mg/ml) (Tab. 1).

The results showed that the minumun protein concentrations that lysed trypanosomes were as follows: G. longipennis (0.18 mg/ml), G. pallidipes (0.55 mg/ml), G. fuscipes (0.71 mg/ml), G. m. morsitans (1.76 mg/ml) and G. m. centralis (1.07 mg/ml) of protein (Tab. 1)

3.2.2 Trypanolysin titres in different tsetse species

Trypanolysin was found in all Glossina species. The results of trypanolysin titre showed that, G. longipennis has the highest titre (512) followed by G. pallidipes (64), G. fuscipes (32) and G. m. centralis (16). However, G. m. morsitans gave the lowest (8) compared to the other species included in the test (Tab. 2).

3.2.3 Effect of trypanolysin on bloodstream and procyclic-form trypanosomes

Sample of crude midgut homogenate obtained from once-fed G. m. morsitans tested for its ability to lyse bloodstream-form of T. brucei demonstrated that bloodstream-form trypanolysin gave a titre of 16. Samples of midgut homogenate and isolated trypanolysin tested for their ability to lyse procyclic-forms of T. b. brucei showed that neither midgut homogenates from once-fed (14.34 mg/ml), twice-fed tsetse (16.25 mg/ml) and nor isolated trypanolysin (5.75 mg/ml and 4.70 mg/ml) was able to lyse the trypanosomes. In contrast, lysis of bloodstream-form trypanosomes were observed.

Table 2 Protein contents, trypanolysin titres and the protein concentrations that lysed trypanosome

(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Tsetse	Midgut protein contents (mg/ml)	Lysin titre	Protein content (mg/ml)
G. m. morsitans	14.34	8	1.76
G. m. centralis	17.5	16	1.07
G. longipennis	45	512	0.18
G. pallidipes	34	64	0.55
G. f. fuscipes	22.2	32	0.71

The protein contents of G. m. morsitans midgut homogenates was the lowest (14.34 mg/ml), the lowest trypanolysin titre (8) and the minimum protein concentration that lysed trypanosome was found to be (1.76 mg/ml). G. longipennis protein concentration was the highest (45 mg/ml) with the highest trypanolysin titre (512) and the minimum protein concentration that lysed trypanosome was found to be (0.18 mg/ml).

3.2.4 Time course and induction of trypanolysin by bloodmeal

Trypanolysin activity was induced by bloodmeal. In this case, the activity increased gradually with time reaching peak level 72-120 h post feeding. Thereafter, the activity decreased to only 25% of the peak activity after 192 h (Fig. 6). The activity as also increased with the number of times that the tsetse were fed. For example, at 72 h, tsetse fed twice had the highest trypanolysin titre (32) with the highest protein concentration (16.25 mg/ml) followed by those fed only once with trypanolysin titre (16) and protein estimation as (14.68 mg/ml) and then the unfed which gave the lowest trypanolysin activity (4) and and the lowest protein concentration as (3.16 mg/ml) (Tab.1).

3.2.5 Induction of trypanolysin by serum and red blood cells (RBC)

Tsetse membrane-fed with serum displayed slightly higher trypanolysin activity (32) compared to those fed on red blood cells (16) and whole blood (16) (Tab. 3). However, tsetse fed on red blood cells gave the same titre as those fed on whole blood.

3.2.6 T. b. brucei midgut infection rates in tsetse

On day 6, out of 50 infected tsetse, the surviving were found to be as follows: G. longipennis (48), G. pallidipes (45), G. fuscipes (37), G. m. morsitans (26) and G. m. centralis (29) which corresponds to the following infection rates: 4.17%, 11.1%, 35.1%, 92.3% and 80.8%, respectively (Tab. 4). The bloodstream-forms of T. b. brucei transformed into long and slender procyclic-forms in the midgut of tsetse (except G. longipennis) by day six. A free flagellum was observed in some

Fig. 6 Time course trypanolysin activiy in *G. m. morsitans*

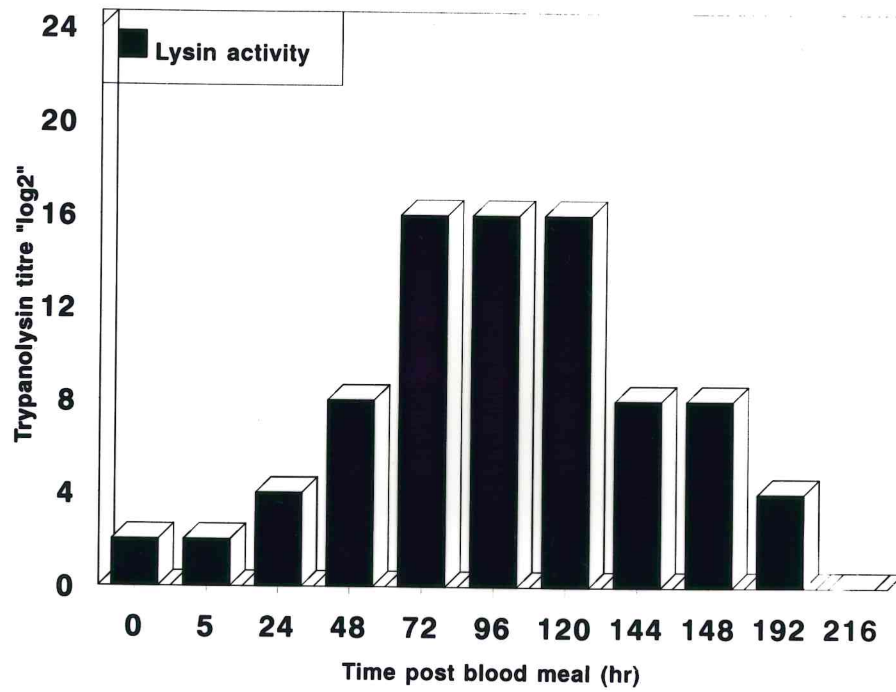


Table 3 Induction of trypanolysin activity by serum, red blood cells and whole blood

(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Type of midgut homogenates	Trypanolysin titre
Whole blood (control)	16
Serum meal	32
Red blood cells meal	16

G. m. morsitans were fed on different meals (whole blood, serum and RBCs) and then the gut homogenates assayed for trypanolysin activity. Whole blood meals presented trypanolysin titre 16. The titre increased two fold (from 16 to 32) with serum meal. However, the titre with red blood cells was the same as that of whole blood meal (16).

Table 4 Midgut infections rate in different tsetse species

Tsetse fly species	No of tsetse fed	No of tsetse died in 24 h intervals						No of tsetse disse.	Inf. rate (%)
		24	48	72	96	120	144		
G. longipennis	50	1	0	0	1	0	0	48	41.7
G. m. morsitans	50	4	6	5	0	3	6	26	92.3
G. m. centralis	50	3	9	6	2	1	0	29	80.8
G. pallidipes	50	1	1	1	1	1	0	45	11.1
G. f. fuscipes	50	1	1	3	1	6	6	37	35.1

G. longipennis gave the lowest midgut infection rate (4.17%) followed by G. pallidipes (11.1%), G. f. fuscipes (35.1%) and G. m. centralis (80.8%). However, G. m. morsitans gave the highest midgut infection rate at 92.3%.

individuals whereas it was absent in others. The kinetoplast of many of these procyclics was situated between the nucleus and posterior end (Fig. 7). However, the bloodstream-forms failed to transform in midgut of *G. longipennis* and they were lysed (Fig. 8).

Fig. 7 Trypanosomes (T. b. brucei) transformed by G. m. morsitans

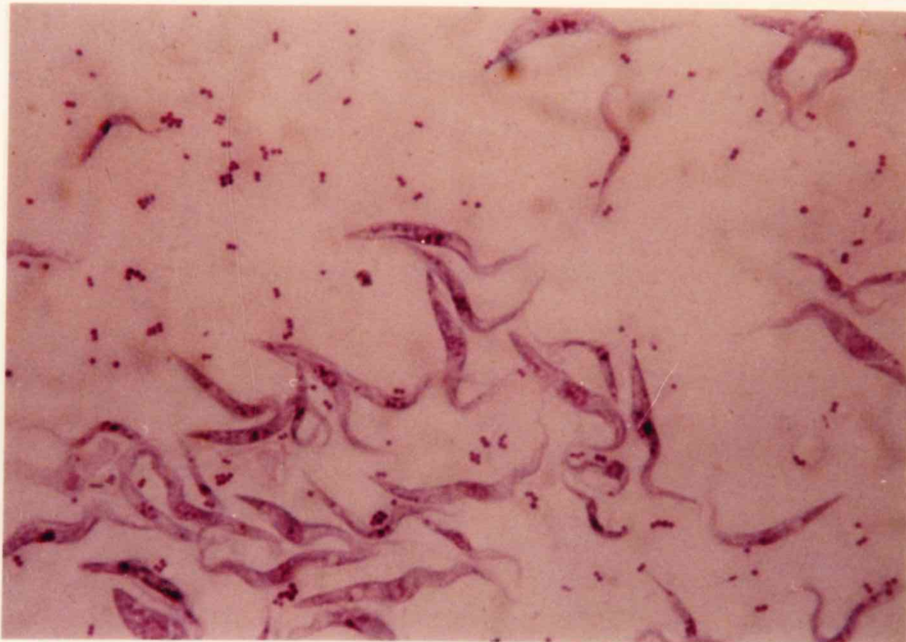
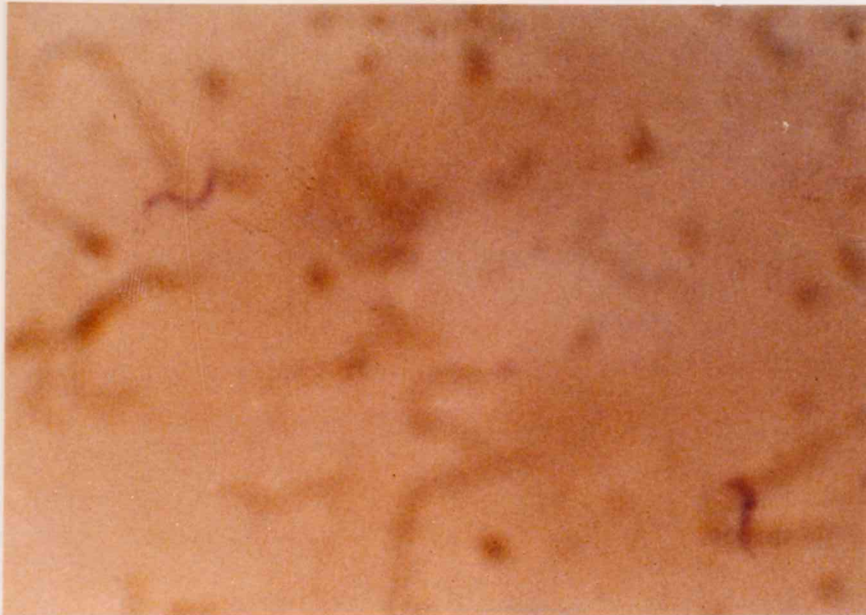


Fig. 8 Lysis of trypanosomes (T. b. brucei) by G. longipennis midgut homogenates



3.2.7 Potency of trypanolysin as function of time

A separate study was carried out to determine the trypanolysin titre as a function of time. It was observed that trypanolysin concentration (27.24 mg/ml) with trypanolysin titre (64); trypanolysin concentration (13.58 mg/ml) with trypanolysin titre (32); trypanolysin concentration (6.75 mg/ml) with trypanolysin titre (16) trypanolysin concentration (3.39 mg/ml) with trypanolysin titre (8) and trypanolysin concentration (1.77 mg/ml) with trypanolysin titre (4) completly lysed the trypanosomes in 10, 20, 30, 50, and 120 min, respectively. However, protein concentration (1,18 mg/ml) had no effect on the bloodsteam-forms (Tab. 5).

3.2.8 Effect of Ca^{++} and Mg^{++} on trypanolysin activity

This experiment was carried out to determine the effect of divalent cations on trypanolysin activity. Divalent cations, Ca^{++} and Mg^{++}, had no effect on the activity of the trypanolysin.

3.2.9 Effect of temperature on trypanolysin activity

The experiment was carried out to determine the sensetivity of increasing temperatures (27, 37, 40, 50, 60, 70 and 80° C) on the activity of trypanolysin. Temperatures ranging from 27 to 50° C have no effect on trypanolysin activity. Temperature of about 60° C reduced the trypanolysin activity by 50% and the activity declined further by 50% at 70° C, whilst at 80° C, virtually all the activity was lost (Tab.6). From this experiment, 50° C was found to be the optimum temperature for trypanolysin activity.

Table 5 Potency of trypanolysin

(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Protein concentration (mg/ml)	Trypanolysin titre	Lysis time (in min)
27.24	64	10
13.58	32	20
6.75	16	30
3.39	8	50
1.77	4	120
1.18	0	0

Protein concentration 27.24 mg/ml with titre 64 lysed trypanosome in 10 min. The following protein concentrations 13.58, 6.75, 3.39 and 1.77 mg/ml gave the following corresponding trypanolysin titre 32, 16, 8, and 4 lysed trypanosome lysed trypanosomes at 20, 30, 50 and 120 min, respectivelly. However, protein concentration of 1.18 mg/ml with trypanolysin titre 0 was found to has no effect on trypanosomes.

Table 6 Heat stability of the trypanolysin

(Trypanolysin titres are expressed as resiprocals of end point dilutions)

Temperature (° C)	Trypanolysin titre
27	64
37	64
40	64
50	64
60	32
70	16
80	0

Trypanolysin titres remained constant from 27° C to 50° C and declined two fold (from 64 to 32) at 60° C. At 70° C, the trypanolysin titre declined further by 50% and a value of zero was recorded at 80° C.

3.2.10 Stability during storage

The experiment was setteled to determine the suitable temperature (27, 4, -20, -70 and -80° C) preserving the activity of trypanolysin. The effect on trypanolysin activity exposed to different temperatures indicated that the activity was completly lost by storage both at 27° C (15 days) and 4° C (32 days). However, the activity of the molecule remained stable at -80, -70 and -20° C up to the end of the experiment (60 days) (Tab. 7).

3.2.11 The effect of freezing and thawing on the activity of trypanolysin

The experiment was carried out to determine the effect of freezing and thawing on trypanolysin activity by using different temperatures (-20, -70 and -80° C). After the 30th freeze-thaw cycle of trypanolysin, the sample was found to be very stable with no appreciable loss of activity. The activity, however, progressively decreased after the 33th, ^{41}th and 55th cycle at -80, -70 and -20° C, respectively (Tab. 8).

Table 7 Effect of storage state on trypanolysin activity

(Trypanolysin titres expressed as reciprocals of end point dilutions)

Days of storage	Trypanolysin titres Temperature ° C				
	27	+4	-20	-70	-80°C
2	64	64	64	64	64
5	64	64	64	64	64
7	32	64	64	64	64
10	16	64	64	64	64
15	0	64	64	64	64
20	0	64	64	64	64
25	0	32	64	64	64
30	0	8	64	64	64
32	0	0	64	64	64
60	0	0	64	64	64

Trypanolysin titres remained constant from 27, +4, -20, -70 and -80° C up to day 5 of storage. The titre declined two fold (from 64 to 32) on day 7 at (27° C). On day 10, the titre declined further by 50% and a value of zero was recorded on day 15 at (27° C). The titre remained constant at +4° C up to day 20 of storage and on day 25 the activity declined two fold (from 64 to 32). Also, the titre declined further by 75% on day 30 and a value of zero was recorded on day 32 at +4° C. However, the activity remained constant at -20° C, -70 and -80° C up to the end of experiment, on day 60.

Table 8 Effect of freezing and thawing on trypanolysin activity

(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Freeze-thaw cycle	Trypanolysin titres		
	Temperature ° C		
	-20	-70	-80
1	64	64	64
30	64	64	64
31	64	64	16
32	64	64	2
33	64	64	0
38	64	32	0
39	64	8	0
40	64	4	0
41	64	0	0
51	16	0	0
52	8	0	0
53	4	0	0
54	2	0	0
55	0	0	0

Trypanolysin titre remained constant from -20, -70 and -80° C up to the 30th cycle and declined four fold (from 64 to 16) at the 31st cycle at -80° C. At the 32nd cycle, the titre declined further eight fold from (16-2) and a value of zero was recorded at -80° C at the 33rd cycle. Also, trypanolysin titre declined two fold (from 64 to 32) at the 38th cycle at -70° C. At the 40th cycle, the titre declined further eight fold from (32 -4) and a value of zero was recorded at 41st cycle at -70° C. In addition, trypanolysin titre declined eight fold (from 64 to 4) at 53rd cycle at -80° C. The titre declined further by 50% at 54th cycle and a value of zero was recorded at 55th cycle at -20° C.

3.3 Discussions

In the present study, it was established that teneral tsetse had very low titres of midgut trypanolysin, suggesting that trypanolysin is secreted in response to bloodmeal. This finding might explain the increased susceptibility of teneral tsetse to trypanosome infection. The midguts of fed tsetse present a far more hostile midgut environment to invading trypanosomes than does the teneral gut due to the presence of trypanolysin.

It was noticed that midgut homogenates from 72 h once-fed tsetse lysed bloodstream-form T. b. brucei suggesting that some component of bloodmeal is responsible for stimulating the release of trypanolysin. Stiles et al. (1991) reported that, in Glossina species, bloodmeal stimulates the release of different molecules including proteolytic enzymes, lectins and trypanolysins.

This study also established that trypanolysin activity from the midguts of G. m. morsitans increased with number of times that the tsetse were fed, that is, twice-fed tsetse had the highest activity followed by those fed once and the unfed. This suggests that regular feeding increased trypanolysin activity since twice-fed tsetse showed a higher trypanolysin activity compared to once-fed and unfed. Stiles et al. (1990) reported that regular feeding help to reduce the probability of infection due to the increased lysin activity.

It was noted that trypanolysin activity increased gradually after 48 h reaching a peak from 72-120 h after the bloodmeal. The activity decreased to 50% after 144 h. Van den Abelle and Declair (1992) reported that bloodmeal induced agglutination activity in G. m. morsitans showed a similar trend to that of the trypsin. Starting immediately after the bloodmeal, the activity increased and reached a peak 48-72 h later. Similar findings have been reported by Stiles et al. (1990) in G. palpalis

where, peak agglutination activity followed a cyclical pattern commencing with the lowest activity immediately after the bloodmeal, and returning to the baseline level at 120 h post-bloodmeal. Abubakar et al. (1995) also reported that in G. m. morsitans agglutination activity increased gradually reaching a peak 48-72 h. Thereafter, the activity decreased rapidly with only 36% of the peak activity remaining after 144 h. In the present research, peak trypanolysin activity more or less followed a similar pattern to that reported for trypanoagglutinin and trypsin.

It was as well demonstrated that tsetse fed on serum had a higher trypanolysin titre than those fed on red blood cells and whole blood. This implies that serum has a component (s) that stimulates trypanolysin activity. Possibly the same situation occurs with lectins which are secreted in response to stimulation by serum (Maudlin and Welburn, 1987, 1988; Gooding, 1974 and Maudlin et al., 1984)

Traore (1987) reported that West African cattle which are naturally resistant to trypanosomiasis have a higher trypanolytic activity in their serum than trypanosensetive cattle and serum trypanolytic activity of individual naturally resistant animals remained stable when tested over a period of 18 months and was not modified by trypanosome infection.

Rifkin (1984) reported that normal human serum induced irreversible acute damage to the normal permeability properties of the T. brucei plasma membrane. Ferrante (1984) had mentioned that trypanolytic activity and agglutinins for T. muscli were demonstrated in sera from refractory hosts (man, cattle, rabbit, guinea pig and rat) and the trypanolytic activity was a result of the ability of the trypanosomes to activate complement in these normal sera.

Tytler et al. (1995) purified apolipoproteins (apo) from the subclass of human high-density lipoprotein (HDL), termed trypanosome lytic factor (TLF), which is responsible for the cytotoxicity of

human serum to T. brucei. Hajduk et al. (1995) demonstrated that in normal serum most of the trypanolytic activity was found in a high-density subclass of high-density lipoprotein.

Gingrich et al. (1982); Maudlin et al. (1984) have shown that removal of serum from infective feeds can significantly increase trypanosome infection rates in tsetse. This proposes that trypanolysin induction might be due to blood serum component, however, the component of serum not yet known.

It was observed that neither crude midgut homogenate from once-fed, twice-fed tsetse nor semi-purified trypanolysin lysed procyclic-form trypanosomes. Trypanolysin as well as trypsin were involved in the lysis of the bloodstream-form trypanosomes but not procyclics. In contrast, trypanoagglutinin agglutinates procyclic-form trypanosomes. Imbuga et al. (1992) provided evidence for the involvement of trypsin or trypsin-like enzymes in trypanosome differentiation and lysis. Osir et al. (1993) also reported close relationship between lectins and trypsins.

The results showed that the minimum protein concentration that lyses trypanosomes were found with G. longipennis and the highest with G. m. morsitans.

Infection rate studies showed that the rate of midgut killing by trypanolysin in G. m. morsitans, G. m. centralis and G. fuscipes was lower than in G. pallidipes and G. longipennis. It is possible that the highest concentration in gut trypanolysin output of tsetse studied such as G. longipennis and G. pallidipes appeared to be more resistant while G. -m. orsitans, G. m. centralis and G. fuscipes susceptible.

Tsetse species differ greatly in their ability to transmit salivarian trypanosomes (Duke et al., 1936). One notes that effects of species are confounded by the feeding preferences of different tsetse species (Duke et al., 1936). Similarly, Duke et al (1936) infected two species of tsetse with different

stocks of Brucei group trypanosome and concluded that for all stock G. m. centralis was a significantly better vector than G. fuscipes.

Moloo et al. (1988) also, found that G. fuscipes was a poor vector of T. brucei when compared with G. m. centralis. It was reported that the Morsitans group of tsetse (with the exception of G. austeni) are good vectors of all trypanosome species (Maudlin, 1991). It is possible that trypanolysin secreted in the tsetse midgut, in response to the bloodmeal serum, is normally responsible for lysing trypanosomes that enter the guts of refractory tsetse, susceptible tsetsesimply dont secrete enough trypanolysin to remove the invading trypanosomes.

In this work it was noted the molecule was found to be not affected by EDTA or chellating agents Ca_{++} and Mg_{++}. Up to some extent, Kasschau et al. (1986) reported that homogenates of Schistosoma mansoni lysed human red blood cells was not affected by the addition of Ca^{2+}.

In contrast, Mullan et al. (1985) reported lytic enzyme that had an apparent pH optimum of 6.5-6.9. Ward (1975a, b) also, identified a complex of endo-and exopeptidases in the larval digestive tract of the webbing clothes moth, Tineola bisselliella , which of particular interest is the presence of two distinct metallo-proteases that were sensitive to EDTA. These proteases complement the bond specificity of the conventional tryptic and chymotryptic activities present and were active at highly alkaline pH 9.5 and consistent with the normal midgut pH of T. bisselliella that is common to many other lepidopteran larvae.

It was observed that temperatures above 60° C decressed G. m. morsitans trypanolysin activity against T. b. brucei 50%. Similarly, Stiles et al. (1990) mentioned FPLC fractions of midgut extracts of G. p. palpalis that were heat labile above 50° C. Abubakar et al. (1995) reported that

trypanoagglutinin was sensitive above 40° C. In addition, it was noted that, isolated trypanolysin was completely inactivated at temperatures above 80° C. Alao, Abubakar et al. (1995) reported trypanoagglutinin that was inactivated 100% at temperature above 60° C. Ferrante et al. (1985) reported a trypanolytic activity in bovine, sheep and rabbit serum that was inactivated by heating (56° C, 30 min). Also, Funato et al. (1992) reported trypanolytic factor of human serum against T. b. gambiense (Wellcome strain) that was inactivated by same conditions. In contrast, Gregorio et al. (1991) reported trypanolytic activity from tissue extracts of T. infestans salivary glands that was heat sensitive. It is possible that midgut trypanolysin activity was consistent with the fact that, trypanolysin, being proteinaceous are denatured by heat, just as was the case with lectins (Abubakar et al. (1995).

It was observed that incubation (27° C) and storage (4° C) rendered trypanolysin inactive after 15 and 32 days, respectively. Also, the molecule remained stable when stored at -20 and -80° C. Kasschau et al. (1986) monitored that incubation of haemolytic activity from S. mansoni (22 h, 38° C) showed little effect. Rifkin (1984) remarked that the binding of high-density lipoprotein to T. brucei was instantaneous at 4° C and readily reversible.

It was noticed that after the 30th freeze-thaw cycle of trypanolysin, the sample was found to be stable with no apprecible loss of activity. The activity, however, progressively decreased after the 33th, 41th and 55th cycle at -80, -70 and -20° C, respectively, suggesting that several freeze-thaw from the lowest temperature to 27° C denatured the molecule.

CHAPTER FOUR

PURIFICATION OF TRYPANOLYSIN

4. Materials and Methods

4.1 Isolation and purification of trypanolysin

Isolation of the molecule was carried out using a combination of ion-exchange and gel permeation chromatography. Midguts from teneral G. m. morsitans were dissected 72 h after feeding and homogenized. The homogenate was centrifuged twice (12,000 x g, 15 min, 4° C) in a Heraecus Minifuge and the resulting supernatant solution was dialysed overnight with three changes against 20 mM Tris-HCl buffer, pH 8.0 and then filtred through a 0.2 mm Millipore filter (Nalge, Rochester, New York). In the first step, separation was carried out on an ion-exchange chromatography column K 16 (Pharmacia, Uppsala, Sweeden) type K 16/20, diameter 1.6 cm, length 20 cm and bed volume 40 ml diethyl aminoethyl (DEAE) Sephacel. The column was pre-equlibrated using 20 mM Tris-HCl buffer, pH 8.0. Crude midgut homogenate (3.0 ml) was then loaded and the column washed with the same buffer at the rate of 2.0 ml/min^{-12}. The absorbance was continuously monitored at 280 nm. Elution of the bound proteins was carried out using a salt gradient (0.1-0.5 M NaCl) and fractions of (37-50) collected. The eluted fractions were then assayed for trypanolysin activity as stated in section 3.1.5. and the active fractions pooled, concentrated to 0.5 ml using polyethylene glycol (PEG-20,000, Serva, Westbury, NY, USA). After overnight dialysis with three changes against 20 mM Tris-HCl, pH 8.0, the sample was frozen until required.

The second step of isolation was carried out using a fast protein liquid chromatography (FPLC) system. The sample (0.5 ml) from the ion-exchange chromatography was loaded via a 500 ml

loop in to a Mono Q HR 5/5 an ion-exchange column (Pharmacia, Uppsala, Sweeden) attached to a FPLC system equipped with a model gradient programmer (GP-250). The flow rate was maintained at 1.0 ml/min^{-1} and the absorbances continuously monitored at 280 nm. The column was first washed with buffer A (20 mM Tris-HCl, pH 8.0) and the bound fractions eluted using a linear gradient of buffer B (20 mM Tris-HCl in 0.0-500 mM NaCl). Fractions (10-14) were collected and each tested for trypanolysin activity. The active fractions were pooled, concentrated to 0.5 ml and dialysed against buffer A as described above.

The third step of isolation, was carried out using epoxy-activated Sepharose 6-B. The basic procedure for using epoxy was to weigh 2.0 g freez-dried material (about 6.0 ml final gel volume). The gel was washed and reswolled on a sintered glass filter (G-3) using distilled water (100 ml/g). For coupling, the ligand (1.5 g glucosamine) was dissolved in 50 ml distilled water. The ligand solution was mixed with the gel suspension and incubated (16 h, 35° C) in a shaking water bath. The excess ligands was washed away using the coupling solution followed with distilled water, bicarbonate buffer (0.1 M, pH 8.0), acetate buffer (0.1 M, pH 4.0) and the remaining excess groups blocked with 1.0 M ethanolamine for 4 h. Finally, the treated FPLC sample was loaded onto the epoxy column. The flow rate was maintained at 0.5 ml/min^{-1} and the absorbance was continuously maintained at 280 nm. The column was first washed with 20 mM Tris-HCl, pH-8.0 and the fractions (19-22) were collected, assayed for trypanolysin activity as described in section 3.1.5. The active fractions were pooled, concentrated 0.5 ml, dialysed against 20 mM Tris-HCl, pH 8.0 as above and frozen until required.

4.1.1 Polyacrylamide gel electrophoresis (PAGE)

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Gradients (4-15%) were cast using a gradient maker (BRL, Gaithersburg, MD., USA). Samples were mixed in an equal volume of sample buffer (130 mM Tris-HCl, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1% ß-mercaptoethanol, pH 6.8) and boiled for 5 min in a water bath, prior to application on to the gel. Running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) was used and electrophoresis was performed at 27° C at a constant current of 30 mA.

Electrophoresis under non-denaturating conditions was carried out at 27° C with a constant voltage (V) of 70 as described for SDS-PAGE except that the buffers did not contain SDS and ß-mercaptoethanol and the samples were also not heated.

After electrophoresis, the gels were stained overnight for proteins with Coomassie Brilliant Blue (0.6%) (Weber and Osborn, 1969) in a solution of acetic acid, methanol and distilled water in ratios of 9.2: 50: 40.8, respectively, overnight. The gels were then soaked for 12-20 h at 27° C with several changes of destaining solution that contained acetic acid, methanol, distilled water in ratios of 9.2: 50: 40.8. The gel was also silver stained.

4.1.1.1 Silver staining

The gel was fixed in 50% methanol and 10% acetic acid for 2 min, rinsed twice with 50% methanol for 10 min each and then washed with distilled water for 5 min. The gel was rehydrated twice with 50% methanol for 10 min each, and 10% of 25% aqueous glutaraldehyde was added for 30

min then washed with distilled water for 30 min. The gel was then stained in Silver stain (containing two solutions, A: 0.8 $AgNO_3$ in 2.5 distilled water; B: 1.0 ml of 2 M NaOH in 20 ml distilled water, and while shaking, solution A was added drop-wise, slowly and carefully not to form a precipitate and then topped with distilled water up to 100 ml) for 15 min and the stain removed by washing in distilled water for 5 min. For colour development, the gel was covered by the devoloper [2.5 ml of citric acid (1% w/v) in to 250 ml cylinder, plus 125 ml of 37-40% formaldehyde solution and then topped up to 250 ml with distilled water] and swirled around in the hands as satisfactory colour stain observed. The colour development was stopped with 5% acetic acid and the gel stored in 7% acetic acid in a plastic bag (Wray et al., 1981).

4.1.1.2 Estimation of molecular weight

Both native and SDS-PAGE were used to determine the molecular weight of the isolated trypanolysin. For estimation of native molecular weight, Pharmacia protein standards were used: a-lactal albumin (M_r » 14,400), trypsin inhibitor (M_r » 20,100), carbonic anhydrase (M_r » 30,000), ovalbumin (M_r » 43,000), albumin (M_r » 67,000), phosphorylase b (M_r » 94,000), lactase dehydrogenase (M_r » 140.000), catalase (M_r » 232,000), ferritin (M_r » 440,000), thyroglobulin (M_r » 669,000). For SDS-PAGE, Bio-Rad protein standards were used: lysozyme (M_r » 14,400), trypsin inhibitor (M_r » 21,500), bovine carbonic anhydrase (M_r » 31,100), ovalbumin (M_r » 45,000), BSA (M_r » 66,200), phosphorylase b (M_r » 97,400). The molecular weight of the trypanolysin was estimated from the plots of log. molecular weights versus the relative migration of the standards.

4.1.1.3 Staining for carbohydrates

Staining of gels for covalently-bound carbohydrates was carried out according to the method of Kapitany and Zebowski (1973). Samples were first separated by sodium dodecyl sulphate (SDS) or native-PAGE. Before staining, the gels were fixed in 12.5% (w/v) trichloroacetic acid (TCA) for 1 h or more, and rinsed thoroughly in distilled water. Oxidation of the glycoproteins was carried out by soaking the gels in 1% (w/v) periodate for 2 h in dark at 27° C and then washing extensively with 4 changes of 15% (v/v) acetic acid with. Staining of the gel with Schiff periodic acid (PAS) (2 h, 27° C) in the dark. Destaining was carried out in 7% (v/v) acetic acid with at least 4 changes with shaking in the dark.

4.1.1.4 Staining for lipids

Staining for lipoproteins in gels was carried out according to Narayan (1975). Protein samples were separated by SDS-PAGE (4-15%). This was followed by soaking in Sudan Black B solution overnight. Destaining was carried out in acetone: acetic acid: distilled water (of 3: 4: 13).

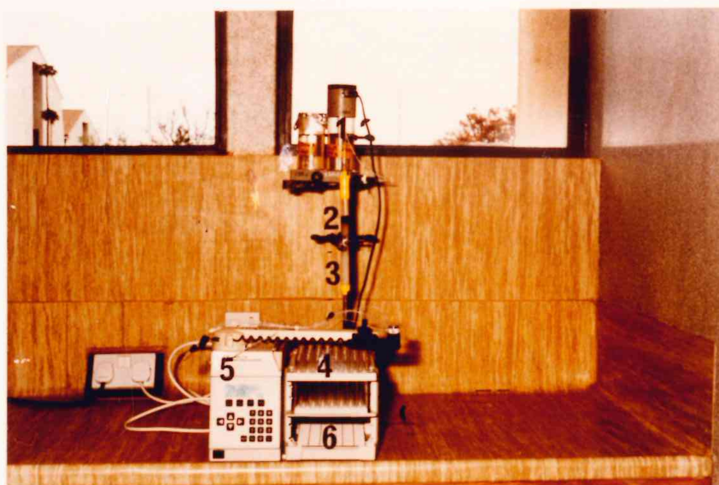
4.2 Results

4.2.1 Isolation and purification of trypanolysin

Trypanolysin was isolated in three steps. The first step was achieved by separation of the midgut homogenates on a conventional anion-exchange chromatography column (Fig. 9) using gradient procedure and elution of the bound proteins with increasing concentrations of NaCl (0.1-0.5 M) in 20 mM Tris-HCl, pH 8.0). The highest trypanolysin activity was recovered in

Fig. 9 Ion-exchange chromatography column

1. Gradient mixer
2. Crude midgut homogenate
3. Ion-exchange column
4. Tubes
5. Recorder
6. Fraction collector



the bound fractions (95%, 0.5 M NaCl) (Fig. 10).

In the second step of isolation, fraction with trypanolysin activity was isolated on a Mono Q anion-exchange column. Elution of the bound proteins was carried out using a NaCl gradient (0.1-0.5 M) in 20 ml Tris-HCl, pH 8.0. The first peak was eluted at 45% while the second peak eluted at 70-80% 1.0 M NaCl (Fig. 11).

The third step of isolation was achieved by using epoxy-activated Sepharose 6-B column (Fig. 12). In this case, the trypanolysin was eluted using 20 mM TrisHCl, pH 8.0 (Fig. 13).

4.2.2 Characterization of trypanolysin

4.2.2.1 Molecular weight estimation

The corresponding fractions of semi-purified trypanolysn from ion-exchange and fast protein liquid chromatography columns are shown in Figs. 14 and 15, respectively. The purity of the isolated trypanolysin was ascertained by non-denaturing PAGE. A single band with a molecular weight of Mr » 669 KDa was observed (Fig. 16).

Analysis of trypanolysin by SDS-PAGE revealed one band of Mr » 14 KDa suggesting the presence of one subunit type (Fig. 17).

4.2.2.2 Staining for carbohydrates and lipids

The molecule was stained for carbohydrate with periodic acid Schiffs stain (PAS). The results showed that the high molecular weight protein was glycosylated (Fig. 18).

Fig. 10 Profile of trypanolysin:
First step of purification

Approximately 14.34 mg/ml of protein was loaded onto a conventional ion-exchange Chromatography column K 16 equilibrated with 20 mM Tris/HCl), pH 8.0. The flow rate was 2.0 ml/12 min and elution was by linear NaCl gradient, 0.5 mM. Fractions (2 ml) were assayed for trypanolysin activity.

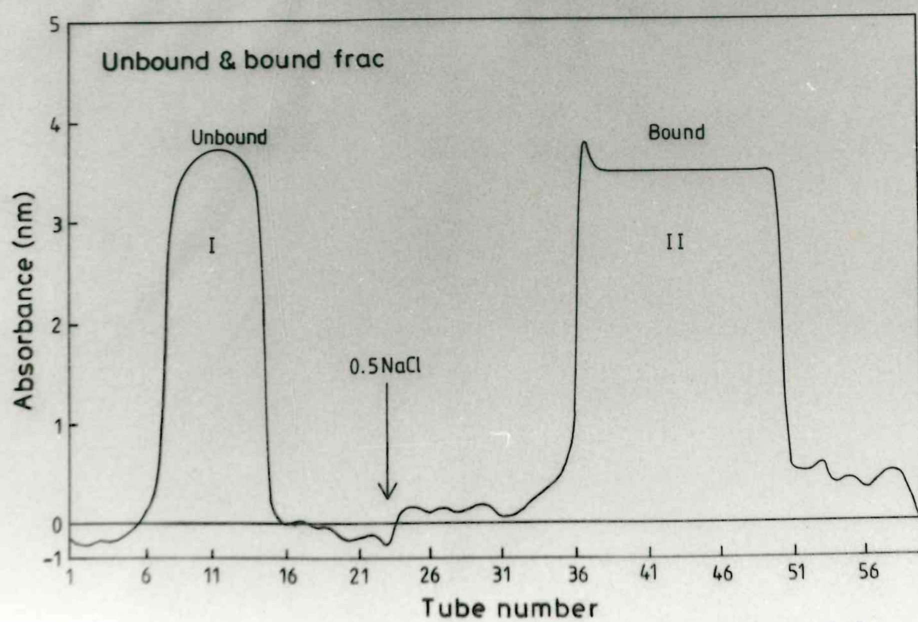


Fig. 11 Semi-purification of trypanolysin by Fast Protein Liquid Chromatography

Fractions 37–50 (Fig.10) which showed trypanolysin activity were pooled, concentrated to a volume of 0.5 ml and dialysed against Tris/HCl, pH 8.0. The sample was loaded to a FPLC. Elution was by NaCl gradient, 0.0-500 mM at a flow rate of 1.0 ml/2 min.

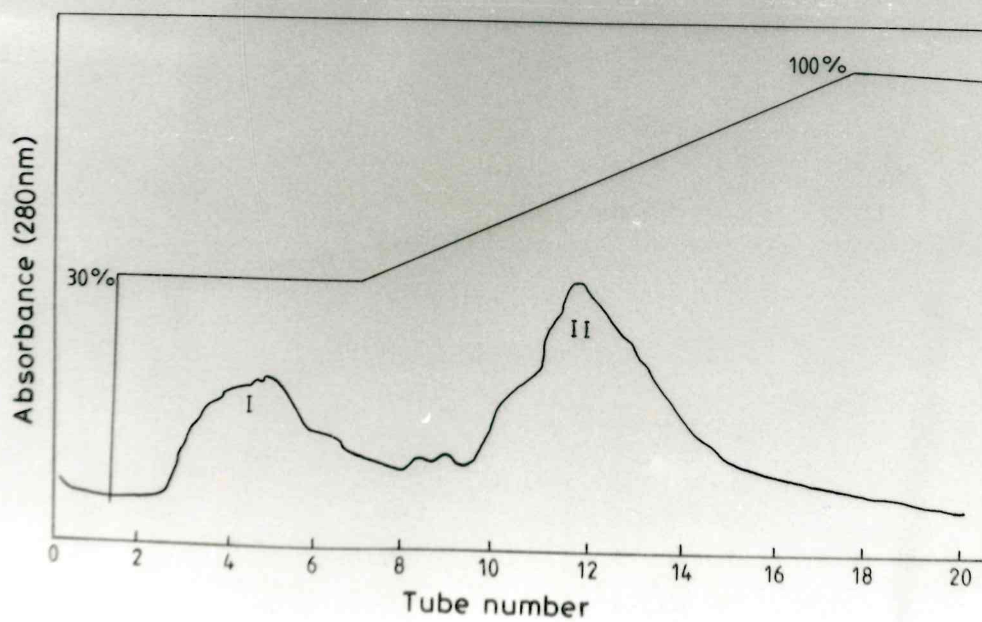


Fig. 12 Affinity chromatography column

1. 20 mM Tris/HCl, pH 8.0
2. Affinity column
3. Peristaltic pump
4. UV-monitor
5. Tubes
6. Fraction collector
7. Chart recorder

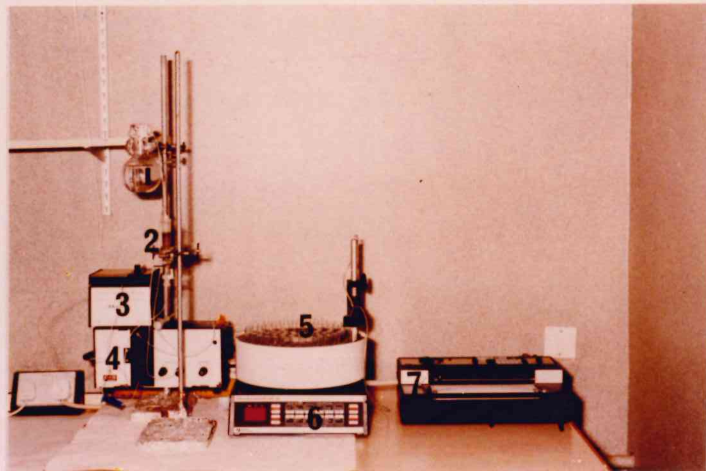


Fig. 13 Profile of trypanolysin:
Third step of purification

Fractions 10–14 (Fig.12) which showed trypanolysin activity were pooled, concentrated to a volume of 0.5 ml and dialysed against Tris/HCL, pH 8.0. The sample was loaded to an Epoxy-activated Sephanose 6-B. Elution was with 20 mM Tris/HCl, pH 8.0 at a flow rate of 0.5 ml/min.

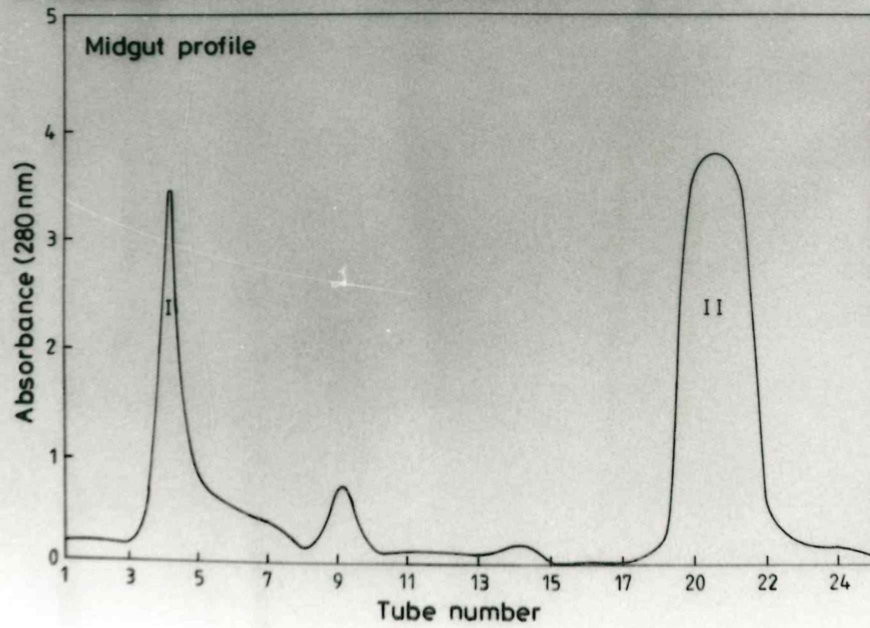


Fig. 14. Molecular weight estimation on non-denaturing–PAGE

Crude midgut homogenate and semi-purified trypanolysin were subjected to non-denaturing–PAGE (4-15%)
1. High molecular weight markers (6 µl) (Pharmacia)
2. Crude midgut homogenate (60 µg)
3. Semi-purified trypanolysin (40 µg)

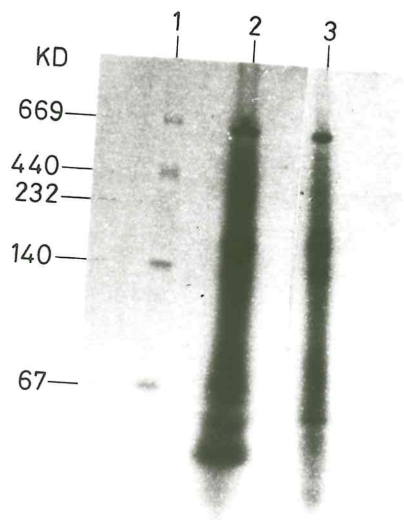


Fig.15. Molecular weight estimation on non-denaturing–PAGE
Crude midgut homogenate and semi-purified trypanolysin were subjected to non-denaturing–PAGE (4-15%)

1. High molecular weight markers (6 µl) (Pharmacia)
2. Crude midgut homogenate (60 µg)
3. Semi-purified trypanolysin (40 µg)

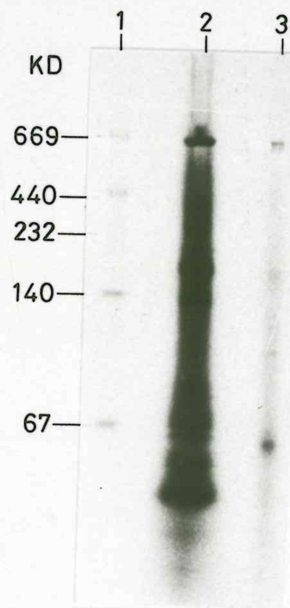


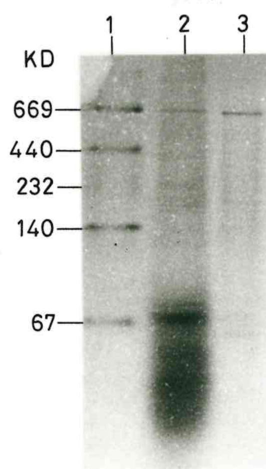
Fig. 16 Molecular weight estimation on non-denaturing-PAGE

(A) Crude midgut homogenates and purified trypanolysin were subjected to non-denaturing-PAGE (4-15%)

Lane: 1. High molecular weight markers (6 ml) (Pharmacia)
2. Crude G. m. morsitans midgut homogenates (30 mg)
3. Purified trypanolysin (60 mg)

(B) 1. Native-PAGE standard curve molecular weight markers were:

1. Thyroglobulin	(Mr » 669,000)
2. Ferritin	(440,000)
3. Catalase	(232,000)
4. Lactase dehydrogenase	(140,000)
5. Bovine serum albumin	(67,000)



Standard curve for native–PAGE using HMW Pharmacia protein markers.

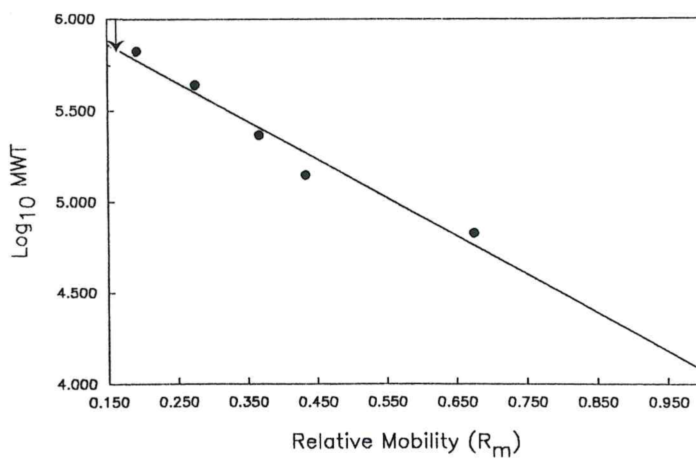


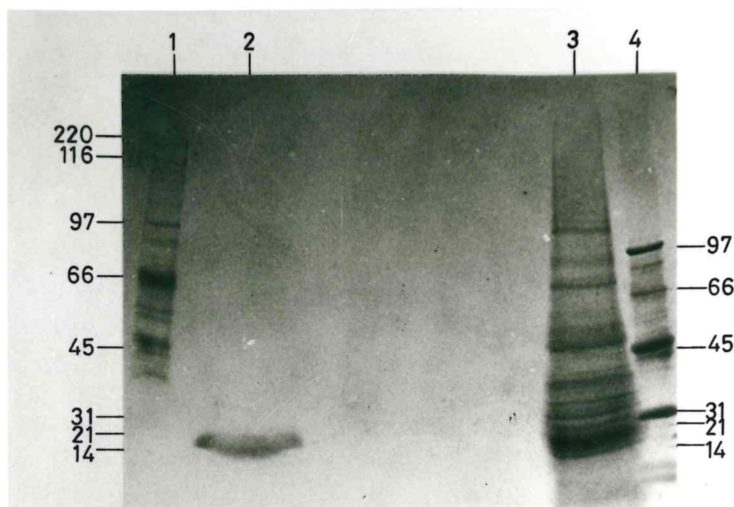
Fig. 17 Molecular weight estimation on SDS-PAGE

(A) Crude midgut homogenates and purified trypanolysin were subjected to SDS-PAGE (4 -15%) and silver staining

Lane: 1. High molecular weight markers (2 ml) (Pharmacia)
2. Purified trypanolysin (30 mg)
3. Crude G. m. morsitans midgut homogenates (2 mg)
3. Low molecular weight markers (2 ml) (Pharmacia)

(B) 1. SDS-PAGE standard curve molecular weight markers were:

1. Myosin	(Mr » 200,000)
2. ß-galactosidase	(116,000)
3. Phosphorylase b	(97,000)
4. Albumin	(66,000)
5. Ovalbumin	(45,000)
6. Carbonic anhydrase	(31,000)
7. Trypsin inhibitor	(21,000)
8. a-lactalbumin	(14,000)


1
2
3
4
220
116
97
66
45
31
21
14
97
66
45
31
21
14

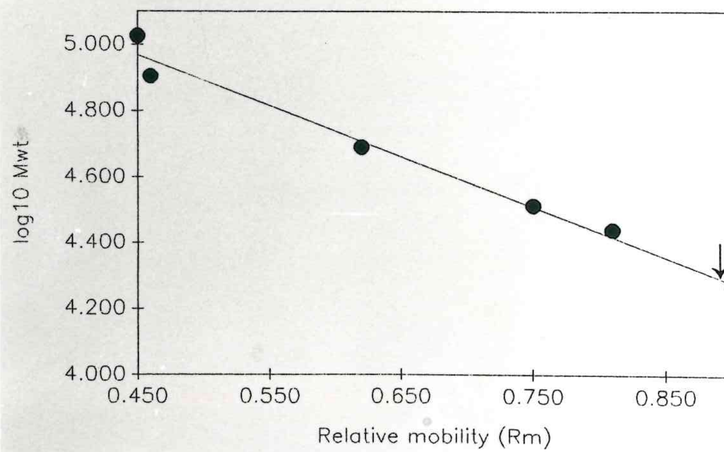
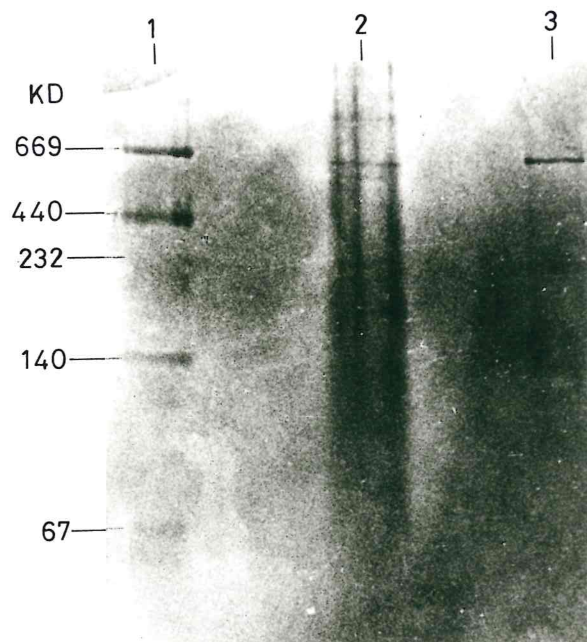

Standard curve for SDS-PAGE using HMW Pharmacia
5.000
4.800
4.600
4.400
4.200
4.000
log10 Mwt
0.450
0.550
0.650
0.750
0.850
Relative mobility (Rm)

Fig. 18. Staining for carbohydrates
Non-denaturing–PAGE (4-15%)
Stained with Periodic Acid
Schiff Stain

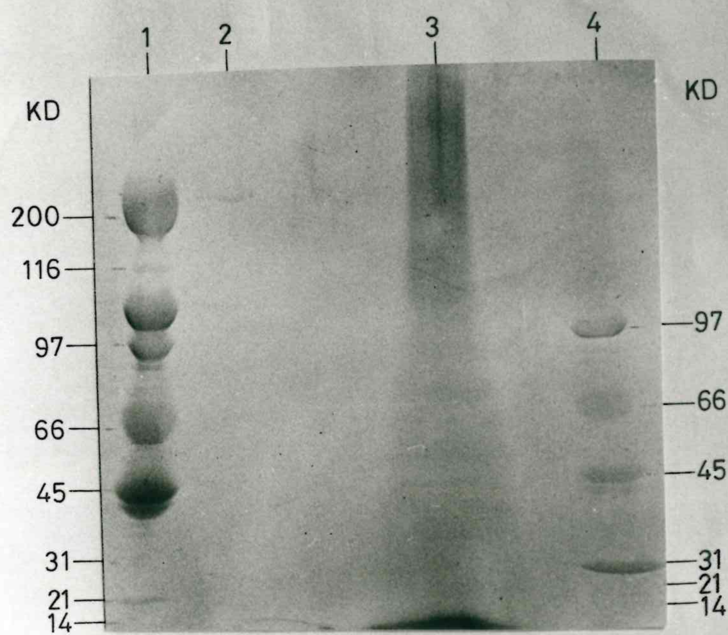
Lane:
1. High molecular weight markers (10 µl) (Pharmacia)
2. G. m. morsitans crude midgut homogenate (60 µg)
3. Purified trypanolysin (30 µg)



The presence of lipids in the trypanolysin was confirmed by staining with Sudan Black B. The high molecule weight protein was found to contain lipids (Fig. 19).

Fig.19. Staining for lipids
SDS–PAGE (4-15%) stained with Sudan Black–B Stain

Lane:
1. High molecular weight markers (10 µl) (Pharmacia)
2. Purified trypanolysin (30 µg)
3. G. m. morsitans crude midgut homogenate (60 µg)
4. Low molecular weight markers (10 µl) (Pharmacia)



4.3 Discussions

In this study, a combinatiion of ion-exchange and affinity chromatography was used to purify trypanolysin. The activity was recovered only in the bound fractions on the anion-exchange column. Similar findings using fast protein liquid chromatography were reported for G. p. palpalis and G. p. gambiensis by Stiles et al. (1990). In other studies on G. m. morsitans and G. longipennis, also reported that trypanoagglutinin activities were recovered in the bound fractions only (Abubakar et al., 1995).

The purified trypanolysin was found to be of high molecular weight (M_r » 669 KDa). It had only one unit of about M_r » 14 KDa. Furthermore, the trypanolysin was found to be lipidated and glycosylated. Lipophorin is the principal haemolymph protein throughout the life cycle of many insects (Chino et al., 1981; Beenakkers et al., 1985; Shapiro et al., 1988). Synthesis of lipophorin occurs in the fat body from where it is released into the haemolymph (Prasad et al., 1986b, 1987; Venkatesh et al., 1987).

All lipophorins so far studied consist of two apoproteins, apolipophorin-1 (apolp-1; M_r » 210 - 250 KDa) and apolipophorin-11 (apolp-11; M_r » 70 KD-85 KDa). Both apoproteins are glycosylated with mannose rich oligosaccharide chains (Ryan et al., 1984; Shapiro et al., 1988). The lipid moiety is predominantely composed of phospholipids and diacyglycerides. Ryan et al. (1984) also, reported that a third protein, apolipophorin-111 (apolop-111, M_r» 18-20 KDa) associates reversibly with lipophorin of certain insects especially those that utilize lipids for flight. The involvement of apolp-111 in flight-related lipid mobilization from the fat body has been studied in Manduca sexta (Shapiro and Law, 1983) and Locusta migratoria (Mwangi and Goldsworthy, 1977, Chino et al., 1986).

In L. migratoria, Goldsworthy et al. (1985) reported the existence of two glycosylated apolp-111 species (M_r » 16 and 20 KDa). Other workers also, have since identified two or three isoform of apolp-111 in L. migratoria which are indistinguishible by SDS-PAGE but seperable by either non-denaturing PAGE or by ion-exchange chromatography (Chino and Yazawa, 1986; Van Heusden et al., 1987; Van der Host et al., 1991). Chino and Yazawa (1986) have proposed that the isoforms may be due to variations in the phosphorylation of the oligosaccharide chains. Beenakers (1988) also, reported that most of the haemolymph proteins have lipids moiety.

Tytler et al. (1995) suggested that the trypanolysin lytic factor lipids do not have a direct role in lysis of trypanosomes but are necessary for the correct assembly of the lytic high density particle. Apolipoprotein A-1 (Apo A-1), apolipoprotein L-111 (apol-111) and apolipoprotein L-1 (apol-1) contribute to lysis in reconstituted particles but individually they are not cytotoxic. Similarly, Hajduk et al. (1995) fractioned trypanolytic activity from normal and Tangier of high-density lipoprotein by gel filtration chromatograohy and both normal and Tangier sera displayed two peaks of trypanosome lytic activity: one at M_r » 1,500 to 600 KDa and the other at > 100 KDa.

In addition, Funato et al. (1992) purified trypanolytic factor of human serum which was estimated to be of high molecular weight (M_r » 3 x 1,000 KDa). Moreover, Mullan, et al. (1985) isolated lytic enzyme by means of ion-exchange chromatography and further purified by gel filtration and ultrafiltration, might be it is the same case of most haemolymph proteins which had been found to have a lipid moiety (Beenakers, 1988).

CHAPTER FIVE

BIOLOGICAL ACTIVITY OF TRYPANOLYSIN

5 Introduction

The digestion of ingested plant material by animals/insects is markedly affected by the presence of naturally occuring inhibitors of digestive enzymes.

Usually protease inhibitors, that are postulated to have evolved as defence mechanisms against insects (Applebaum, et al., 1972). Protease inhibitors, themselves proteins, exhibit high affinity to specific enzymes and strongly bind at or near the active site. However, they are not cleaved as natural substrates, at least not at the same velocity, nor do they subsequently dissociate to the same extent, if at all. Because of the basic similarity to enzyme-substrate complexes, at the active site, protease inhibitors elicit specificity by causing changes in configuration of the substrate thereby affecting the potential enzyme inhibitor binding kinetics. Protease inhibitors are also present in mammalian blood and affect digestion by haematophagous insects.

Proteases are grouped according to the structure of their active centres and according to amino acid specificity. Endopeptidases belong to one of the following groups: SH-proteases, having a cysteine in the active centre; carboxyl proteases, in which an acidic residue is involved in the catalytic process and serine proteases; typical tryptic and chymotryptic activities have been identified in numerous insects as major components of the digestive fluids. In other words, insect endopeptidases are, loosely defined as proteolytic enzymes, cleave the internal peptide bonds away from amino-or carboxyl-terminal and exhibits various degrees of amino acid specificities.

In most cases these activities are described within the context of the complete digestive protease complement and their identification is based on hydrolysis of specific substrates and their inhibition by protease inhibitors exhibiting various degrees of specificity to known serine proteases. Identification of proteases is usually accomplished by the use of inhibitors, which may be synthetic products or natural origin. The synthetic inhibitors commonly used such as: diisopropyl fluorophosphate (DFP) and paraphenyl methyl sulphonyl fluoride (PMSF), are general inhibitors of serine proteases. Tosyl-L-lysine chloromethyl ketone (TLCK) is specific for tryptic activity and tosyl-L-Phenyalanine chloromethyl ketone (TPCK) is specific for chymotryptic activity. Iodoacetamide is a specific inhibitor for thiol groups.

Naturally occuring protease inhibitors are proteins that interact and block the active centre of protease. Those commonly used include, for an example, soybean trypsin inhibitor (STI). Aprotinin is a basic polypeptide which is active against kalikrein and plasmin. Aprotinin also inhibits many serine proteases.

Pronase is a mixture of endopeptidases and exopeptidases (including carboxypeptidases) which has non-specific protease activity. Diethyl pyrcarbonate (DEPC) is an effective inhibitor used as a reagent for carbethoxylation of exposed N-7 groups of unpaired adenine residues in DNA or RNA and also modifing histidine and tyrosine residues in proteins. -

In using the above protease inhibitors in each of the following experiment, the assay was replicated three times.

5.1 Materials and Methods

5.1.1 The effect of diisopropyl fluorophosphate (DFP) on trypanolysin activity

Isolated trypanolysin was incubated (30-45 min, 27° C) with increasing concentrations of diisopropyl fluorophosphate (DFP) solution (0.0-1.0 mg/ml) prior to addition of the isolated trypanosomes (» 5 x 10^6 trypanosomes/ml). Control consisted of trypanosomes and DFP. The activity of trypanolysin was assessed as described in section 3.1.5.

5.1.1.1 The effect of iodoacetamide on trypanolysin activity

The experiment was conducted to assess the effect of iodoacetamide on the activity of trypanolysin. Isolated trypanolysin was incubated (30-45 min, 27° C) with increasing concentrations of iodoacetamide solution (0.0-18.5 mg/ml) prior to addition of the isolated trypanosomes (» 5 x 10^6 trypanosomes/ml). Control consisted of trypanosomes and iodoacetamide.

5.1.1.2 The effect of trypsin on the activity of isolated trypanolysin

Isolated trypanolysin was incubated (30-45 min, 27° C) with increasing concentrations of trypsin (0.0-1.0 mg/ml) prior to addition of the isolated trypanosomes (» 5 x 10^6 trypanosomes/ml), the residual enzyme activity was determined at each inhibitor concentration. Controls consisted of trypanosomes and trypsin. The activity of isolated trypanolysin was checked as described in section 3.1.5.

5.1.1.3 The effect of pronase on trypanolysin activity

Trypanolysin was incubated (30-40 min, 27° C) with increasing concentrations of pronase solution (0.0-10 mg/ml) prior to addition of the isolated trypanosomes (» 5 x 10^6 trypanosomeses/ml). Two sets of controls were used. The first consisted of trypanosomes and trypanolysin and the second of trypanosomes and pronase.

5.1.1.4 The effects of sugars on trypanolysin activity

The effects of the following sugars: D-glucosamine hydrochloride, D-mannose, D-glucose anhydrate, +-N-Acetyl-D-glucosamine, Methyl-a-D-glucopyranoside, a-D+glucose, D-ribose, Methyl-b-D-glucopyranside, D+Trehalose dihydrate, Maltose, D-galactosamine and Sucrose on the activity of trypanolysin were seperately assessed as follows. Final concentrations (700 mM) of the sugars were prepared in PBS. Isolated trypanolysin was pre-incubated (30-45 min, 27° C) with the sugars and an equal volume of isolated trypanosomes (» 5 x 10^6 trypanosomes/ml) were then added and trypanolysin activity determined as described in section 3.1.5. Control assays consisted of the same mixture but without the sugars.

5.1.1.5 The effect of protease inhibitors on trypanolysin activity

The effect of soybean trypsin inhibitor (STI, Millipore Corp, Freehad, USA) on trypanolysin activity was assessed as follows. Increasing concentrations of STI (0.0-1.0 mg/ml) were mixed with trypanolysin prior to addition of the isolated bloodstream-form trypanosomes (» 5 x 10^6 trypanosomeses/ml). The lysis of the trypanosomes was assessed as described in section 3.1.5. The

effect of the protease inhibitors phenyl methyl sulphonyl fluoride (PMSF), aprotinin, diethyl pyrcarbonate (DEPC), N-a-p-Tosyl-L-Lysine chromethyl ketone (TLCK) and Tosylamide-2-phenylethyl chloromethyl ketone (TPCK) were assessed as described above except that PMSF was pre-dissolved in isopropanol. Similarly, TLCK and TPCK were dissolved in absolute ethanol and aprotinin in PBS. Increasing concentrations of PMSF (0.0- 17.0 mg/ml), TLCK (0.0-1.0 mg/ml), TPCK (0.0-1.0mg/ml), aprotinin (O.O-2.5 mg/ml) and double serial dilutions of diethyl pyrcarbonate (DEPC) were used to determine the inhibitors end point. Control consisted of: (1) trypanosomes and trypanolysin and (2) trypanosomes and the various inhibitors.

5.1.1.6 Enzyme assay

Trypsin activity of the isolated trypanolysin was assayed using a chromogenic substrate, carbobenzoxy-val-gly-arg-4-nitroamide acetate (Chromozym-Try; Boheringer-Mannhim, FRG). The reactions were initiated by the addition of 80 mmol substrate in 50 mM Tris-HCl, pH 8.0. The total assay volume was fixed in 1.0 ml. Changes in absorbances were monitored at 410 nm using a Beckman model DU 50 spectrophotometer fitted with a thermostat control. The change in molar extinction at 410 nm (e_{410}=8800; Erlanger et al., 1961) was used to calculate the amount of the substrate hydrolysed.

5.2 Results

5.2.1 The effect of diisopropyl fluorophosphate (DFP) on trypanolysin activity

Trypanolysin activity was not affected by increasing concentrations (0.0-1.0 mg/ml) of the inhibitor. DFP also had no effect on the bloodstream-form trypanosomes in the control experiment since the trypanosomes were intact at end of incubation period.

5.2.1.1 The effect of iodoacetamide on trypanolysin activity

The inclusion of iodoacetamide in the trypanolysin assay system had no effect on the activity of trypanolysin. Iodoacetamide also had no effect on the bloodstream-form trypanosomes in the control experiment.

5.2.1.2 The effect of trypsin on the activity of isolated trypanolysin

Trypanolysin activity was found to be not affected by trypsin. However, concentrations of trypsin ranging from 0.8 to 1.0 mg/ml had an effect on trypanosomes in the control experiment.

5.2.1.3 The effect of pronase on trypanolysin activity

Pronase at lower concentrations (0.0-1.0 mg/ml) and (2-4 mg/ml) partially inactivated trypanolysin activity i.e by 25% then 50%, respectivelly. However, pronase concentration at 10 mg/ml was completely inactivated trypanolysin activity (Tab. 9).

Table 9 The effect of pronase on trypanolysin activity

(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Pronase (10 µg/ml)	Trypanolysin titre
0.0	64
2.0	16
4.0	8
6.0	8
8.0	8
10.0	0

Trypanolysin activity declined four fold (from 64 to 16) i.e, when the pronase concentration was increased from 0.0 -2.0 µg/ml. At 4.0 µg/ml, trypanolysin titre decreased further by 50% and remained constant at that value pronase concentration of up to 8.0 µg/ml. However, a value of zero was recorded at 10.0 µg/ml.

5.2.1.4 The effect of sugars on trypanolysin activity

A final concentration of 700 mM of the following sugars: D-glucosamine hydrochloride, D-mannose, D-glucose anhydrate, +-N-acetyl-D-glucosamine, Methyl-b-D-glucopyranoside, a-D+glucose, D-ribose, Methyl-b-D-glucopyranoside, D+-Trehalose, Maltose, D+-galactosamine and Sucrose had no effect on trypanolysin activity.

5.2.1.5 The effect of protease inhibitors on trypanolysin activity

Increasing concentrations of soybean trypsin inhibitor (0.0-1.0 mg/ml) had no effect on trypanolysin activity. Trypanolysin was not affected by Tosylamide-2-phenyl chloromethyl ketone (0.0-1.0 mg/ml), N-a-p-Tosyl-L-Lysine chromethyl ketone (0.0-1.0 mg/ml) or phenyl methyl sulphonyl fluoride (0.0-17.0 mg/ml). On the other hand, while aprotinin partially inhibited trypanolysin activity (Tab. 10), diethyl pyrcarbonate completely inactivated the activity.

5.2.1.6 Enzyme assay

A synthetic chromogenic substrate, carbobenzoxy-val-gly-arg-4-nitroamide acetate (Chromozym-Try; Boheringer-Mannhim, FRG) was used to assess whether trypanolysin had any tryptic activity. The isolated trypanolysin showed no detectable trypsin activity compared to » 12.0 x 10^{-2} mmoles/min/ml which was found in the crude midgut homogenates of G. m. morsitans.

Table 10 The effect of aprotinin on trypanolysin activity

(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Aprotinin(2.5 µg/ml)	Trypanolysin titre
0.0	64
0.2	16
0.4	8
0.6	8
0.8	8
1.0	8

Trypanolysin activity declined four fold (from 64 to 16) when the approtinin concentration was increased from 0.0 to 0.2 µg/ml. At 0.4 µg/ml, trypanolysin titre decreased further by 50% However, the activity remained constant at that value up to 1.0 µg/ml.

5.3 Discussions

In this study, it was established that pronase at a concentration of 10 mg/ml completely abrogated the G. m. morsitans trypanolysin activity. Similar findings were reported by Stiles et al. (1991) in trypanolysin from G. p. palpalis and G. p. gambiensis. Since pronase is a general protease, it is possible that trypanolysin could have proteolytic activity.

Birk et al. (1963) reported that Tribolium castaneum larval trypsin was unaffected by soybean trypsin inhibitor (STI) which classically inhibited bovine trypsin. Stiles et al. (1990) reported that trypanoagglutinin and trypanolysin were not affected by protease inhibitor. Similarly, in the present investigation, it was noticed that upto 1.0 mg/ml soybean trypsin inhibitor had no effect on trypanolysin activity.

On the contrary, two midgut proteases, found in Tenebrio molitor adults, have been found to be, the first, a-protease, inhibited by soybean trypsin inhibitor and the second, ß-protease, is inhibited by natural trypsin inhibitor, respectively (Zwilling, R., 1968; Zwilling, R. et al., 1972). Similarly, Gibel et al. (1971) reported that trypsin and chymotrypsins from the adult worker honey bee midgut are inhibited by STI. Also, Kunz. (1987b) reported that STI inhibited midgut proteases of the mosquito A. aegypti and of the tsetse fly Glossina austeni (Hawkins, 1966). Moreover, Ahamad et al. (1989) reported that STI partially inhibited three enzymes in the midgut content of Lepidopteran larvae and bollworm Spodoptera littoralis.

Gooding (1974b) reported that high concentrations of trypsin inhibitors are found in the anterior midgut of the tsetse fly G. morsitans where no digestion or apparent alteration of ingested erythrocytes occurs. Presumably these inhibitors originate from the host blood and initially hinder

erythrocyte degradation. In addition, Sonneborn et al. (1969) reported that the midgut of queen bees larvae lack trypsin and chymotrypsin activities.

The midgut of Glossina contains proteolytic enzymes lectins/trypanoagglutinins and trypanolysins among others. Out of six proteases present in the posterior midgut, trypsin is the most predominant enzyme (Cheesman and Gooding, 1985). Previous investigations have suggested a close relationship between lectins and trypsin or trypsin-like enzymes in the tsetse midgut. The release of lectins and trypsin are induced following a bloodmeal (Gooding, 1974; Maudlin et al., 1984). The study showed that trypanolysin has no trypsin activity, nor is it affected by the enzyme. This suggests that either the trypanolysin has no active sites for trypsin or that the sites are hidden within the tertiary structure. Alternatively, trypsin may be cleave off segments that are important in the function of this molecule. However, Abubakar et al. (1995) was observed that 1.0 mg/ml soybean trypsin inhibitor completely inhibited trypanoagglutinin activity of G. m. morsitans midgut indicating the close relation of trypanoagglutinin to trypsin (Osir et al., 1993).

Miller et al. (1974) reported that trypsin from the midgut of Manduca sexta was not inhibited by TPCK. Sasaki and Suzuki (1982) also reported that proteases from the regurgitated digestive juice of fifth instar silkworm larvae neither inhibited by TLCK nor TPCK demonstrating the complete absence of trypsin and chymotrypsin, respectively. In addition, Ahamad et al. (1989) reported that no inhibition was observed with the chymotrypsin inhibitor, TPCK, on the midgut content of S. littoralis. It had been noticed that midgut a-protease from T. molitor adults was not inhibited by either TLCK or TPCK (Zwilling, R., 1968; Zwilling, R. et al., 1972). Similarly, in the present work, trypanolysin was not affected either by TLCK or TPCK.

On the contrary, Yang and Davies (1971) reported that chymotrypsin in A. aegypti was inhibited by the a-globulin fractions of human and horse sera. Also, Giebel, et al. (1971) separated proteases from adult worker honey bee midgut, one is easily classified as trypsin and two others as chymotrypsins on the basis of ester and amide substrate specificity and by inhibition with either TLCK for the trypsin or TPCK for the chymotrypsins. In addition, Ahamad et al. (1989) reported a unique feature of protease action encountered in the midgut content of lepidopteran larvae and bollworm S. littoralis and these alkaline proteases had been resolved into three trypsins inhibited by TLCK. Moreover, Pritchett et al. (1981) had demonstrated tryptic and chymotryptic activity in larval digestive fluid Trichoplusina ni and these enzymes were inhibited typically by TLCK and TPCK on the appropriate substrates. Miller et al. (1974) purified a trypsin exhibiting typical substrate specificity by affinity chromatography from the midgut of the tobacco hornworm, M. sexta and inhibition studies were found to be consistent with its identification as trypsin. Finally, midgut protease of the dominant T. molitor imaginal trypsin studied by Golan (1981) was inactivated by TLCK. Since, trypanolysin was found to be not affected by either TPCK nor TLCK, this implies that the molecule has neither tryptic nor chymotryptic activity.

Sasaki and Suzuki (1982) isolated two classes of alkaline proteases from the regurgitated digestive juice of fifth instar silkworm larvae. The first class (P 11) and the second (P 111) were typically inhibited by DFP. Also, Carty (1979) isolated T. molitor larval chymotrypsin from the midgut which was inhibited by DFP. In this study, however, it was established that trypanolysin was not affected by DFP. This implies that, the molecule does not have serine as an essential amino acid at its active site that could be blocked by DFP. Similar findings have been reported by Stiles et al. (1991) on

G. palpalis trypanolysin. Zwilling, R., (1968) and Zwilling, R. et al. (1972) reported that a-protease from T. molitor adults was inhibited by PMSF. Also, Ahamad et al. (1989) reported the inhibition of alkaline proteases from the midgut content or digestive fluid of lepidopteran larvae and bollworm by PMSF. However, in this study, trypanolysin was not affected by PMSF. Similar observations were made by Stiles et al. (1991), in which the Palpalis trypanolysin and trypanoagglutinin were unaffected by PMSF. Consequently, this implies that trypanolysin does not belong to the serine protease group.

Houseman (1987) reported that the gut wall of Rhodnius prolixus contains less than 0.5% of the thiol protease present in the gut lumen, and concluded that the cathepsin-B present in the lumen is digestive protease.

In this study, iodoacetamide was found not to affect the trypanolysin activity. Since the inhibitor reacts with the free-SH groups on the protein, this suggests that trypanolysin has no SH-groups at its active site, or does not lyse the trypanosomes by utilizing this amino acid.

In this work, it was noticed that aprotinin partially activated trypanolysin activity. In contrast, Barr et al. (1996) reported that aprotinin did not affect Trypanosoma cruzi trypomastigotes soluble fraction (TSF).

However, diethyl pyrcarbonate completely inhibited the activity. Diethyl pyrcarbonate is a known inhibitor of histidine and tyrosine residues that are essential for biological activity of most proteins. It is therefore possible that trypanolysin lyses the trypanosomes by binding to its histidine and tyrosine residues.

It was further observed that none of the different sugars tested had an effect on the activity of trypanolysin. This is unlike the case of agglutinin where the presence of glucosamine-binding lectin

was first reported by Ibrahim et al. (1984). Also, Welburn et al. (1980) have reported that G. m. morsitans haemolymph showed a high agglutination activity for human B group red blood cells and that this agglutination was inhibited with galactose/ N-acetyl galactosamine. In addition, it has been reported that haemolymph lectin of infected tsetse was inhibited by a- D-melibiose (Welburn et al., 1990). It has also been reported that the maturation of both T. b. rhodesiense and T. congolense in G. m. morsitans was significantly reduced when the above sugars were included in the bloodmeal (Welburn et al., 1990).

However, Andrea et al. (1995) reported that trypanolytic lytic factor from human haptoglobulin caused lysis of T. b. brucei and its activity was inhibited by catalase. On the other hand, Abubakar et al. (1994) reported that trypanoagglutinin from G. m. morsitans midgut was D-glucosamine specific. Pereira et al. (1981) also reported that lectins in the crop, midgut and haemolymph of R. prolixus are specific for N-acetyl-D-mannose, a and b-D-galactose, respectively. The results in the present study show that trypanolysin has no specificity for carbohydrates and therefore, the molecule is unlikely to be a lectin/trypanoagglutinin or acts as one. It is possible that the molecule has no active sites for these sugars and thus does not exhibit carbohydrate binding.

CHAPTER SIX

IMMUNOLOGICAL CHARACTERIZATION OF TRYPANOLYSIN

6 Materials and Methods

6.1 Immunological studies

6.1.1 Raising antibodies against the tpypanolysin

Antibodies against trypanolysin were raised in a New Zealand white rabbit using a previously described protocol (Osir et al., 1986). Prior to immunization, the rabbit was bled from the marginal ear vein for pre-immune serum. A primary injection (300 mg of the purified trypanolysin in 1.0 ml 20 mM Tris-HCl, pH 8.0) was emulsified in an equal volume of Freund's complete adjuvant (FCA) and administered subcutaneously at different sites. Two booster injections (200 mg protein in 0.5 ml 20 mM Tris-HCl, pH 8.0 in incomplete Freund's adjuvant) were administered intramascularly on the 8th and 24th day, respectively. The rabbit was then bled one week after the last booster through the main ear artery. To obtain the antiserum, the blood was left to stand (27° C, 4-6 h) and then kept overnight at 4° C. The serum was then separated from the clots by centrifugation (1000 x g, 30 min, 4° C). The antiserum was stored at -20° C.

6.1.2 Double radial immunodiffusion

Double radial immunodiffusion was carried out as described (Ouchterlony, 1968; Osir et al., 1989). Molten agarose 1% (w/v) in PBS was carefully poured onto a glass plate (10 cm x 10 cm) on a level stand and left to set. Six wells were punched peripherally around a central one. To check for the presence of antibodies against trypanolysin (the antigen), antiserum was placed in the central well and

the antigen (trypanolysin) in the peripheral wells. To test for cross-reactivity, the midgut samples from G. palpalis, G. m. morsitans, G. fuscipes, Phlebotomus dubosci, Aedes aegipti and purified trypanolysin were pippeted in the peripheral wells with the antiserum in the central well. Pre-immune rabbit serum was used as control. Diffusion was allowed to take place in a humid chamber (27° C, 24 h). The plates were washed extensivelly with PBS to remove unprecipitated proteins on gel, then dried by blotting with 3.0 mm filter papers. The gel was then stained with Coomassie Brilliant Blue and destained as described in section 4.1.1.

6.1.3 Immunoblotting

To check for immunological reactivity of the antiserum raised against the trypanolysin, midgut homogenates from various insect species (G. m. morsitans, G. fuscipes, P. dubosci and A. aegypti) and isolated trypanolysin were used in immunoblotting experiments (Towbin et al., 1979; Burnette, 1981). Samples were separated by non denaturing-PAGE (4-15%) and electrophoretically transferred [constant current of 0.8 milliamper/centimeter^2 (mA/cm^2) of nitrocellulose for 2 h, 27° C] onto a nitrocellulose memberane (Schleicher and Schuelle Company Service)) in Towbins transfer buffer [48 mM Tris-HCl, 29 mM Glycine, and 20% (v/v) methanol] using an LKB 2117-NOVABLOT electrophoretic transfer kit. The success of the transfer was ascertained by staining with Rouge Ponceau [0.05% (w/v) in 3% (w/v) trichloroacetic acid (TCA)]. The blot was destained by washing in distilled water and then rinsed briefly in Tris-buffered-saline (TBS) (20 mM Tris/HCl, pH 7.5, 500 mM NaCl) containing 5% fat-free milk powder. Non-specific binding sites on the blots were blocked in the same solution TBS-milk 5%). The blots were then washed with TBS for 5 min and incubated overnight with antiserum solution (diluted x 50 in TBS-milk-1%) at 27° C with shaking. The blots

were then washed 4 times for 15 min each in TBS-milk 1% and then washed once with TBS. After washing, the blots were immersed in horse-radish peroxidase labelled anti-rabbit IgG diluted 1000 x in TBS-milk 1%, and incubated (2 h, 27° C) with shaking. Unbound secondary antibody was washed off with TBS-milk 3 x for 10 min each with constant shaking. The blot was then rinsed once in TBS to remove milk and then in 10 mM Tris/HCl buffer, pH 6.8 (TB). The blot was then incubated in substrate solution [0.3% (w/v) 4-chloro-1-naphthol in methanol (diluted x 5 with TB) and containing 0.33 ml of hydrogen peroxidase solution (H_2O_2) per ml of final substrate solution] until the bands became visible (5-10 min). The excess substrate was washed off with distelled water and the blot preserved by keeping it between filter papers in the dark.

6.1.4 Immunoinhibition

6.1.4.1 In vitro inhibition of trypanolysin activity by antisera

The effect of antiserum raised against trypanolysin on the lysis of bloodstream and procyclic-form trypanosomes was studied in vitro. Double serial dilutions of the purified trypanolysin were pre-incubated with the antiserum (30 min, 27° C) prior to the addition of bloodstream and procyclic-form trypanosomes. The controls consisted of serial dilutions of: (1) trypanolysin only (2) trypanolysin and non-immune serum. Trypanolysin assay was then carried out as described above (section 3.1.5).

6.1.5 Transformation studies using trypanolysin

6.1.5.1 In vitro studies

Teneral G. m. morsitans (100) were allowed to feed on trypanolysin and maintained for 72 h at 27° C. The tsetse were immobilized by brief chilling, after which the midguts were carefully dissected,

teased gently and mixed with freshly isolated trypanosomes (» 5 x 10^7 trypanosomeses/ml). All incubations of such mixtures were carried out at 27° C. At different times, two hourly interval, the incubation mixture was vortexed and 20 ml aliquots withdrawn for the preparation of wet smears to observe the progress of transformation process over 24 h. For the control experiments, the tsetse were fed on an infected blood only.

The wet smears were fixed for 10 min in absolute ethanol, stained for 1.0 h with Giemsa's stain, rinsed several times with distilled water and examined using a Dialux compound microscope (Leitz Wetzlar, FRG). The trypanosomes were counted and classified as being typical bloodstream-forms, midgut or transition-forms described on the basis of their morphological characteristic (Lloyd and Johnson, 1924; Ghiotto et al., 1979).

6.1.5.2 In vivo studies

100 teneral G. m. morsitans were allowed to feed on trypanolysin. After 72 h at 27° C, the tsetse were fed through an artificial silicone membrane. The medium consisted of freshly isolated trypanosomes > 5 x 10^7 trypanosomes/ml. At various times, two hourly intervals up to 24 h, the midguts were removed as described (Lloyd and Johnson, 1924; Ghiotto et al., 1979) and transformation assessed. Control experiments were involved tsetse fed on an infected blood only.

6.1.6 Trypanolysin activity in different tsetse species after trypanolysin feeding

Teneral tsetse (100 of each species; G. longipennis, G. m. morsitans, G. fuscipes, G. m. centralis and G. pallidipes) were allowed to feed on trypanolysin in PBS. After 72 h at 27° C, the

tsetse were fed on an infected rat with high parasitaemia (» 5 x 10^7 trypanosomes/ml) and maintained (72 h, 27° C). The tsetse were refed again on an uninfected rat and maintained for further 72 h at 27° C. Trypanolysin assays from 50 different tsetse species (described above) were carried out using bloodstream-form trypanosomes (section 3.1.5). In another set of experiments, the rest of flies (50) from each different species were dissected, their midguts homogenized individually in 10 ml PBS, then 2 ml aliquots of 2 fold serial dilutions of the gut homogenates were spotted onto a glass slide and stained in Giemsa. These preparations were then examined by phase-contrast microscopy at (x 400 magnification). Tsetse fed on infected blood were kept as control. Dead tsetse during the experiment were recorded.

6.2 Results

6.2.1 Immunologica characteristic of trypanolysin

6.2.1.1 Double radial of immunodiffusion analysis

The presence of antibodies against the trypanolysin was detected by double radial immunodiffusion (Fig. 20). A precipitin line was observed when crude midgut homogenates from G. m. morsitans, G. fuscipes, and G. p. palpalis were tested for cross-reactivity with antisera raised against trypanolysin (Fig. 21 A). This was also the case when homogenates from P. dubosci and A. aegypti were tested (Fig. 21 B).

Fig. 20 Double radial immunodiffusion of isolated trypanolysin against antiserum to trypanolysin

The central well contained antiserum (20 μl)
The peripheral wells contained (1) antigen (5 μg)

Fig. 21 A Double radial immunodiffusion of midgut samples from other insects species rather than Glossina against antiserum to trypanolysin

The central well contained antiserum (20 µl)
The peripheral wells contained:

(1) Aedes aegypti crude midgut homogenates (10 µg)
(3) Stomoxis calcitrans crude midgut homogenates (10 µg)
(5) Clean rabbit serum (10 µg)

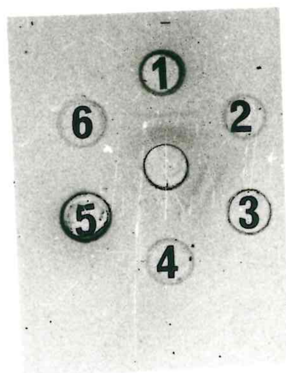
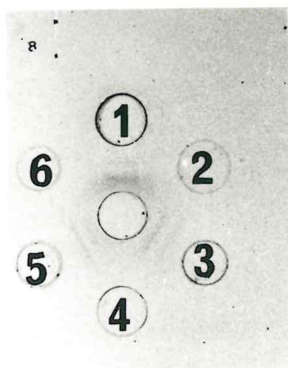


Fig. 21 B Double radial immunodiffusion of midgut samples from tsetse against antiserum to trypanolysin

The central well contained antiserum (20 µl)
The peripheral wells contained:

(1) G. fuscipes crude midgut homogenates (4 µg)
(3) G. m. morsitans crude midgut homogenates (5 µg)
(5) G. palpalis crude midgut homogenates (3 µg)



6.2.1.2 Immunoblots analysis

The immunological cross-reactivity between anti-trypanolysin antibodies and samples from G. m. morsitans, G. fuscipes and isolated trypanolysin was tested by immunoblotting. The antibodies detected protein bands with apparent molecular weights of about M_r » 669 KDa which corresponded to molecular weight of G. m. morsitans trypanolysin. No cross-reactivity was detected with samples from P. dubosci or A. aegypti (Fig. 22).

6.2.1.3 Immunoinhibition

6.2.1.3.1 Effect of antiserum on trypanolysin activity

The effect of antiserum raised against the trypanolysin on trypanolysin activity was detected in vitro using bloodstream and procyclic-form trypanosomes. The results showed that the antiserum had virtually inhibitory effect on the trypanolysin.

6.2.1.4 Transformation studies using trypanolysin

The results of transformation studies **in vitro** and **in vivo** on G. m. morsitans fed on trypanolysin showed that by 2 h all the trypanosomes had been lysed (Fig. 23). In contrast, bloodstream-form trypanosomes were transformed to procyclic-forms in the control after 6 h (Fig. 24).

6.2.1.5 Trypanolysin activity in tsetse species after trypanolysin feeding

The results of the trypanolysin assay showed that G. longipennis fed on trypanolysin showed an increased titre elevated from 2048 compared to a titre of 256 which was obtained when the

Fig. 22. Immunoblot analysis of isolated trypanolysin G. f. fuscipes midgut homogenates (30 µg), isolated trypanolysin (5 µg), G. m. morsitans (30 µg), Aedes aegypti (60 µg), Stomoxis calcitrans (60 µg) samples were first separated by non-denaturing–PAGE and then electrophoretically transferred to nitrocellulose paper. The blot was then reacted with the antiserum.

1. High molecular weight markers (5 µl) (Pharmacia)
2. Stomoxis calcitrans crude midgut homogenate
3. Aedes aegypti crude midgut homogenate
4. G. m. morsitans crude midgut homogenate
5. Isolated trypanolysin
6. G. f. fuscipes crude midgut homogenate

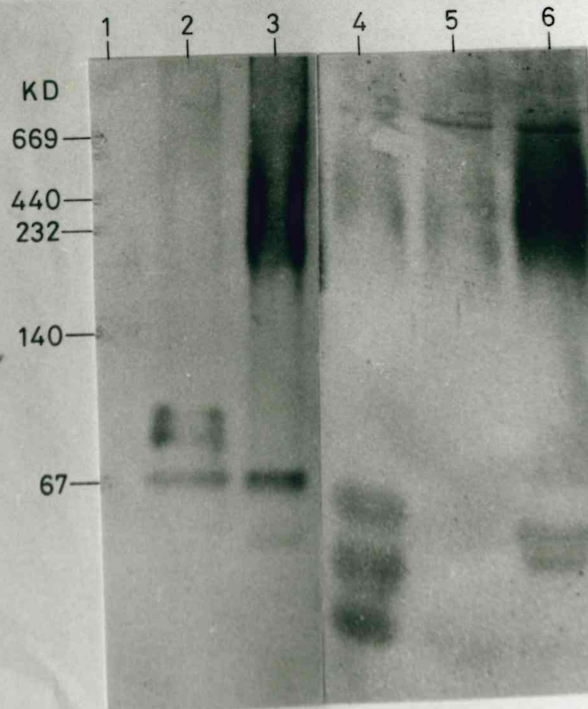


Fig. 23 Trypanosomes (T. b. brucei) lysed by G. m. morsitans midgut homogenates

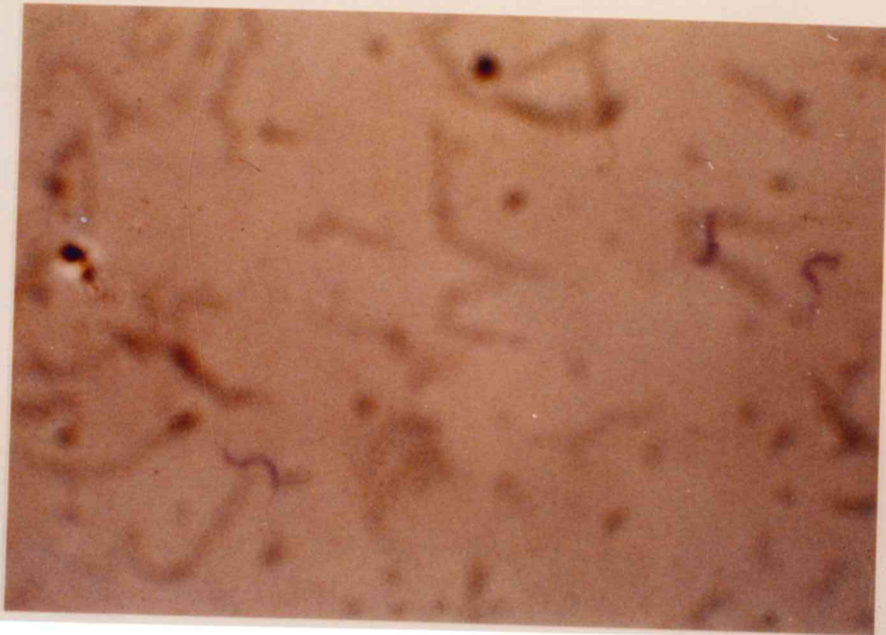
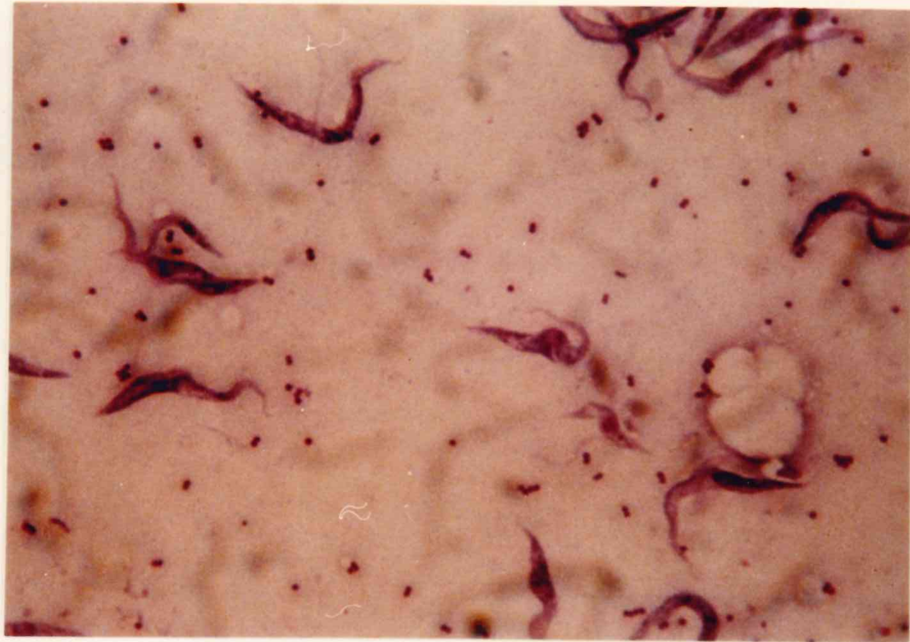


Fig. 24 Trypanosomes (*T. b. brucei*) transformed *in vivo* by *G. m. morsitans*



tsetse were fed on clean rat. Increase in trypanolysin activity was also observed in G. m. morsitans, from 128 when fed on trypanolysin to 32; G. m. centralis, from 256 to 32; G. pallidipes, from 1024 to 128 and G. fuscipes, from 256 to 64 (Tab. 11). On the other set of experiments, the midgut homogenates from the different tsetse species, described above, were showed lysis. In contrast, tsetse fed on clean rat as control supported transformation.

3 Disscussions

Immunological methods have long been used to check for protein with similar antigenic determinants. A positive reactivity is always expected when antigen shares epitopes with the protein under investigation.

In double radial immunodiffusion studies using rabbit antibodies against the purified trypanolysin, a single precipitin band was observed with purified trypanolysin, P. dubosci, A. aegypti, G. fuscipes, G. m. morsitanss and G. p. palpalis midgut extracts.

In this study, out of the species tested for immunological cross-reactivity using the antibodies raised against the purified trypanolysin, only tsetse of the genus Glossina gave positive reactions in western blots. The bands detected in G. m. morsitans and G. fuscipes all had apparent molecular weight of » 669 KDa which corresponded to the molecular weight of the purified trypanolysin. No reaction was detected with P. dubosci nor Aedes aegypti samples. It is possible that the bands with lower molecular weight that the antibody recognised in western blot experiments of A. aegypti and P. dubosci shared some epitopes with the tsetse midgut trypanolysin. This suggests that this trypanolysin is present only in the Glossina spp.

Bloodstream and procyclic-form trypanosomes were not lysed in the in vitro by trypanolysin

Table 11 Trypanolysin activity in different tsetse species after trypanolysin meal

(Trypanolysin titres are expressed as reciprocals of end point dilutions)

Species of Glossina	Trypanolysin titre	
	Clean blood meal	**Trypanolysin meal**
G. longipennis	256	2048
G. m. morsitans	32	128
G. m. centralis	32	256
G. pallidipes	128	1024
G. f. fuscipes	64	256

Tsetse were fed on different meals (trypanolysin meal and rabbit clean blood meal) and then the gut homogenates assayed for trypanolysin activity. Trypanolysin meal fed to different tsetse species compared to clean blood meal gave an increased titre. For example, G. longipennis from (2048) to (256); G. m. morsitans, from (128) to (32); G. m. centralis, from (256) to (32); G. pallidipes, from (1024) to (128) and G. f. fuscipes, from (256) to (64). However, G. longipennis gave the highest trypanolysin titre (2048) while, G. m. morsitans gave the lowest (128).

in presence of antiserum raised against trypanolysin suggesting that trypanolysin was completly inhibited by the antiserum.

An important step in the establishment of the trypansomes (Trypanozoon and Nannomonas) within the tsetse vector involves transformation from bloodstream in to procyclic-(midgut) forms (Vickerman, 1985). This process is accompanied by high trypanosomes mortality mediated by several midgut factors that include lectins, trypsin or trypsin-like molecules and lysin (Maudlin, 1991; Osir et al., 1993). Since the midgut factors are bloodmeal-induced, the infection prevelance would be expected to be influenced by the type of the host blood at the time of an infected bloodmeal (Mihok et al., 1993). This point has already been demonstrated by Moloo (1981). It is presently unclear exactly how host blood influence the types and quanties of midgut factors released. Since the blood samples that gave low rates of trypanosome transformation are the same ones that gave lower infection prevelance, it may be assumed that the two process are closely linked. Those trypanosomes that transform efficiently in the tsetse midgut are more likely to reach maturation than those that do not.

In this study, the species (G. m. morsitans, G. longipennis, G. fuscipes, G. m. centralis and G. pallidipes) fed on trypanolysin showed an increased trypanolysin titres and also cleared trypanosomes from the midgut after 6 days of infection.

In the in vitro and in vivo transformation studies using trypanolysin, showed that by 2 h all the trypanosomes had been lysed. In contrast, bloodstream-form trypanosomes were transformed to procyclic-forms in the control after 6 h. It can be concluded that trypanolysin fed to the tsetse was responsible for lysis of trypanosomes, amongst other factors.

CHAPTER SEVEN

GENERAL DISCUSSIONS

Lectins, trypsins or trypsin-like enzymes and trypanolysins have attracted considerable interest among parasitologists due to the realization that they may be involved in host-parasite-vector inteactions, in such important disease vectors as Glossina (Maudlin and Welburn, 1987) and Rhodnius prolixus (Pereira et al., 1981).

For example, the establishment of trypanosome infections in tsetse vector is influenced by several factors, including lectins (Maudlin and Welburn, 1988a, b), trypsins or trypsin-like enzymes (Imbuga et al., 1992), trypanolysin (Stiles et al., 1990) as well as the type of host blood involved (Mihok et al., 1993).

Most of the trypanosomes entering the tsetse are lysed and those that escape continue with development. Indeed, it appears that tsetse are poor vectors of trypanosomes since infection in field-caught tsetse rarely exceeds 2% (Jordan et al., 1965). In order to gain insight into mechanisms involved in innate refractoriness, we have studied a molecule, trypanolysin, which plays an important role in lysis of trypanosomes in tsetse midgut. In the present study, a midgut trypanolysin from G. m. morsitans was isolated by a combination of ion-exchange and affinity chromatography. The activity of trypanolysin was detected only in the bound fractions. Osir et al. (1995) purified trypanoagglutinin from the midguts of G. longipennis by anion-exchange chromatography and the agglutination activity was also detected in the bound fractions. Similar findings using FPLC were reported for trypanoagglutinin and trypanolysin from G. p. palpalis and G. p. gambiensis (Stiles et al., 1990).

The native molecular weight of the isolated trypanolysin was determined by non-denaturing PAGE. A single band with molecular weight of 669 KDa was observed. Analysis of trypanolysin by

SDS-PAGE revealed only one band of molecular weight » 14 KDa, indicating the presence of a single subunit type.

It was further noticed that the trypanolysin was lipidated. The molecule was also found to be glycosylated. However, Hajduk et al. (1995) isolated high-density lipoproteins in normal and Tangier patients by gel filtration chromatography. Both normal and Tangier sera displayed two peaks of trypanosome lytic activity; one at 150 -600 KDa and the other at > 1,000 KDa.

Beenakers (1988) reported that most haemolymph proteins contain lipid moiety and are also glycosylated. Generally apoproteins lipophorins are glycosylated and the olygosaccharides are of the high mannose type (Ryan et al., 1984; Nagao et al., 1987). Ryan et al (1984 a) reported that apolipophorin-111 found in some adult insect species, that rely on lipids as flight fuel, are glycosylated

In Locusta migratoria, the lipophorin is glycosylated and associates with very high (12.5%) carbohydrate content (Wheeler and Gold, Sworthy, 1983a, b; Van der Horst et al., 1984). A similar observation was reported for the protein-4 (P-4) from immune haemolymph of diapaussing H. cercopia and M. sexta (Hulbert et al., 1985).

The presence of oligosaccharide chains in haemolymph proteins has also been exploited for the purification purposes (Ryan et al., 1986, 1989; Kawooya and Law, 1983). High mannose oligosaccharides bind to concanavalin A (Benziger and Fiete, 1979). The function of carbohydrate moieties found in haemolymph proteins has not been clēarly established but a recognition role either at secretory site or at the metabolic site has been suggested (Beenakkers et al., 1985). Osir et al (1986) showed that deglycosylation of Manduca sexta vitellogenin did not affect its uptake by the oocytes.

So far, in the present study, we do not have an explanation for the role of lipid and carbohydrate moieties found in tsetse midgut proteins. Therefore, their role have to be further investigated.

The present results clearly show that, soybean trypsin inhibitor (STI), upto 1.0 mg/ml had no effect on trypanolysin activity. Similarly, Stiles et al. (1990) reported trypanoagglutinin and trypanolysin were not affected by protease inhibitor. On the other hand, the same concentration of STI completely abrogated agglutinin activity of G. m. morsitans midgut (Abubakar et al., 1995) suggesting that, either trypanolysin has no sites for trypsin to cleave or the sites are hidden within the tertiary structure and are not exposed, Alternatively, trypsin cleave off segments that are not important in the function of this molecule.

None of the different sugars assessed had an effect on the activity of trypanolysin. Abubakar et al. (1994) reported that trypanoagglutinin from G. m. morsitans was D-glucosamine specific. Also, Pereira et al. (1981) reported that lectins in the crop, midgut and hemolymph of Rhodnius prolixus are specific for N-acetyl-D-mannose, a and b-D-galactose, respectively. It could be that, the molecule has no specific binding sites for these sugars and thus, does not exhibit different carbohydrate binding specificities and is thus, unlikely to be a lectin or to act as one.

Diethy pyrcarbonate completely inhibited the trypanolysin activity. Diethyl pyrcarbonate is a known inhibitor of histidine and tyrosine residues essential for biological activity of most proteins. It could be that trypanolysin lysis the trypanosomes by binding through its histidyl and tyrosyl residues. However, it is not clear whether these residues are on the protein itself or on the trypanosome membrane.

It was noted that teneral tsetse had very low titre of midgut trypanolysin, suggesting that trypanolysin was secreted in response to bloodmeal. Midgut homogenate from 72 h once-fed tsetse was able to lyse bloodstream-form T. b. brucei, suggesting that some component of bloodmeal is responsible for initiating trypanolysin production. Similarly, it had been observed that the trypanolysin activity from midguts of G m. morsitans increased with number of times that they were fed. For example, 72 h twice-fed tsetse had the highest activity followed by those fed once and then the unfed which had the least activity suggesting, that regular feeding induced trypanolysin production. Stiles et al., (1990) reported that regular feeding reduced the probability of infection. Probably this might be due to the increased trypanolysin activity induced by regular feeding.

This work confirms that trypanolysin secreted in the tsetse midgut in response to bloodmeal serum, is normally responsible for killing trypanosomes that enter the guts of refractory tsetse. Susceptible tsetse simply do not secret enough trypanolysin to remove the invading trypanosomes. Possibly, the same situation occurs as that of lectins which could be produced in response to stimulation by serum content of the first bloodmeal (Maudlin and Welburn, 1987, 1988). Also, Gooding 1974; Maudlin et al (1984) reported that, the release of trypsin and lectins are induced followed a bloodmeal by a serum component. Gingrich et al. (1982) and Maudlin et al.(1984) similarly, have shown that removal of serum from an infective feeds can significantly increase trypanosome infection rates in tsetse, suggesting that some component of serum is necessary to induce trypanosome killing. This phenomenon may explain in vitro trypanolysin experiments. Nnonetheless, those components in question of serum are not yet known.

Generally, Morsitans group is more susceptible and therefore, efficient vectors of trypanosomes whereas, the Palpalis and Fusca tsetse appear to be poor vectors (Harley and Wilson,

1986; Harley, 1971; Roberts and Gray, 1972; Moloo and Kutuza, 1988b; Moloo et al., 1987, 1992a, b). This variation in susceptibility between species and across subspecies groups of tsetse could be related to differences in midgut trypanolysin activity. The interspecific differecences appear to result from variation in the ability of trypanosome to establish a midgut infection. This phenomenon may also apply to trypsin output. High trypsin levels play a major role in the destruction of trypanosomes from the tsetse midgut. In other words, tsetse with the highest trypanolysin output are the most successful in preventing trypanosomes establishment in the midgut and hence more refractory.

For example, in vivo studies of infection rates for different tsetse species by T. brucei, it was noticed that, G. longipennis, showed the least infection rates while, G. m. morsitans showed the highest. In addition, it was also observed that, most of T. b. brucei bloodstream-forms that had not been lysed by G. m. morsitans midgut factors were transformed to procyclic-form trypanosomes with the kinetoplast located in the middle whereas, those in G. longipennis did not show any transformation but on the contrary, showed lysis. Moreover, we noticed that G. longipennis had the highest trypanolysin concentration while, G. m. morsitans had the lowest concentration among the different tsetse tested. Usually, after entering the tsetse, the probability of trypanosome completing their life cycle is inversely dependent on the complexity of the changes eloborated. This, in turn, is related to the species of trypanosome involved. For example, T. brucei, seems to have the most complex life cycle within the tsetse and therefore should have the least chance for successful cyclical transmission (Buxton, 1955).

Maudlin (1989) reported that susceptibility to trypanosome infection in laboratory-bred G. m. morsitans was a maternally inhirited character. This maternal pattern of inheritence of susceptibility in tsetse has since been independently confirmed using a parent stock of wild G. m. morsitans.

Makunyaviri et al. (1984) using the sex-linked "salmon" eye mutation in G. m. morsitans found that tsetse carrying this allele were more susceptible to midgut infection with T. brucei. Maudlin et al (1986) had reported earlier that the establishment of midgut infections of both congolense and brucei is a maternally inherited character in teneral G. m. morsitans, whereas the maturation of brucei group infections is probablly controlled by a sex-linked recessive allele. It is possible that the differences in the infection rates reflects differencies in the gut environments of the tsetse studied. It had been reported that the trypanosomes of the subgenera Trypanozoon and Nannomonas can become established more readily and undergo full cyclical development in some tsetse flies while, arrested in the gut of others (Molyneux, 1983; Moloo and Kutuza, 1988b).

A study on a horse trypanosome, T. equiperdum, which is non-infective in humans, indicates that normal human serum causes lysis and agglutination of the trypanosomes, this was not observed with sera from animals susceptible to such infections (horse, mouse etc). Also, a passive transfer of normal human serum into mice infected with T. equiperdum decreases the number of circulating trypanosomes and protects mice from lethal infection, but transfer of sera from susceptible animals confers no protection suggested that natural immunity related to the host specificity of trypanosomes is accounted for, in part, by the presence of natural antibodies in the refractory hosts. In addition to combinations of host and trypanosome where natural immunity is strong enough to block infection, trypanocidal activity has been found in combinations of a human host and trypanosome.

Indeed there is increasing evidence suggesting that lectins protect insects vectors against parasite infection (Pireria et al., 1980; 1981; Ibrahim et al., 1984; Walbanks et al., 1986; Maudlin et al, 1988). Previous observations clearly show that an insect vector can be defended against parasitic infection by several factors. For example, sandflies can be protected against leishmania by peritrophic

memberane (Orihel, 1975) and by lectins (Walbanks et al., 1986) and mosquitoes against malarial parasites by peritrophic memberane (Weathersby and McCal, 1986). Although, in recent years, several investigators of vector immunity have reported the role of lectins in defence against parasite infection, it is most unlikely that an insect vector could rely only on one mechanism for defending itself against infection by any particular parasite. Hence, we decided to investigate this phenomenon further.

Kaaya et al., 1986b; Otieno et al., 1976 reported that, 2-3% of G. m. morsitans support development and maturation of bloodstream-form of T. b. brucei when inoculated in to their haemocoels. In these study with G. m. morsitans, it has been observed that, even the tenerals of unfed tsetse secreted a little trypanolysin. There is a possibility that, this is a factor of refractorinness of these "odd" individuals.

Maudlin (1989) suggested that these marked differencies in susceptibility to trypanosome infections observed both in the field and the laboratory, would mean that the tsetse may possess certain intrensic refractoriness to trypanosomes infections. Maudlin (1989) showed that lines of G. m. morsitans highly susceptible or refractory to T. congolense and T. brucei could be selected in the laboratory. These observations reaffirmed the suppostion that genetics could determine susceptibility of refractoriness to trypanosomes infection.

After feeding, the bloodstream-form trypanosomes migrate to the peritrophic membrane lining the midgut where the blood is digested. Olubayo et al. (1993) reported that critical events in the fly occur shortly after a bloodmeal between about day 3 and day 6. At this time, infections are lost due to several factors (Dipeolu et al., 1975). Similarly, in this study, it was observed that trypanolysin peaks around day 3 and day 6 post-feeding.

Therefore, it is possible that destruction of trypanosomes from the tsetse midgut could thus be as a result of trypanolysin activity in addition to the other factors mentioned. Most bloodstream-form trypanosomes that enter the midgut are killed by one or more of the mentioned factors, and those that survive transform into procyclic midgut forms (Maudlin and Welburn, 1987; Stiles et al., 1990). These procyclic-form trypanosomes establish themselves in the ectoperitrophic space as midgut or "immature" infections.

The probability of the procyclic-form trypanosomes completing their life cycle within the vector depends on their transformation into metacyclic-form trypanosomes and their ability to withstand the radical midgut environment. Gingrich et al. (1985) observed an increased midgut infection rate in tsetse fed on trypanosomes suspended in serum free blood. As a possible explanation postulated a relationship between the activity of midgut proteinases and the development of procyclic-form trypanosomes infection.

It had been reported that commercially available lectins agglutinate procyclic-form trypanosomes in vitro as did midgut homogenates from tsetse. Gingrich et al. (1985) also reported that procyclic-form trypanosomes in the posterior midguts of flies 72 h post feeding were being killed by lysis, presumably as a consequence of lectin action following adhesion to cell surface, although lysis may not be the direct effect of lectin activity.

So far, by in vitro and in vivo transformation studies on G. m. morsitans fed on trypanolysin, it has been discovered that at 2 h after feeding, the trypanosomes were found to be intact but not transformed. However, all the trypanosomes were lysed after 2 h. It is possible that tsetse yfed with trypanolysin are the most refractory since they can eliminate trypanosomes from their midguts before they could be established to subsequent developmental form. Since trypanolysin blocks transformation,

It is therefore possible that the release of enough trypanolysin could lyses trypanosomes by binding to its histidyl and tyrosyl resudues. Hence, the site of trypanolysin secretion within the tsetse is to be investigated for the minimum release of trypanolysin that gives lysis. Thus, could be amongst one of the efficient ways of interrupting trypanosomiasis cycle within the vector.

SUMMARY

The aim of this study was to isolate, purify and characterize trypanolysin, induced by component of bloodmeal, in the midgut of tsetse G. m. morsitans and to determine whether such molecule could be used to interrupt trypanosome's life cycle within the vector.

1. Midgut trypanolysin from G. m. morsitans was purified by a combination of an ion-exchange and affinity column chromatography.
2. Trypanolysin had a high molecular on native PAGE (» 669 KDa) and consists of one unit type (» 14 KDa) on SDS PAGE.
3. Trypanolysin had glycosyl and lipid moieties.
4. Trypanolysin had no trypsin activity, and neither could its activity be affected by the enzyme.
5. All the sugars tested had no effect on trypanolysin activity.
6. Trypanolysin was inhibited by diethyl pyrcarbonate.
7. Trypanolysin caused lysis of bloodstream-form trypanosomes, while the procyclics were unaffected.
8. Different tsetse species fed on trypanolysin blocked transformation, cleared trypanosomes within the tsetse midgut after 6 days of infection and was found to eliminate subsequent developmental cycle of trypanosomiasis.
9. Immunologically, trypanolysin was good immunogen and antibodies against the protein detected similar proteins in other members of Glossina familly. The immune sera inhibited trypanolysin activity in vitro.

SUGGESTIONS FOR FUTURE WORK

The study has given considerable insight into the biochemistry of trypanolysin in tsetse midgut, an area of tsetse physiology which though important, had hitherto not been thoroughly investigated. However, further work remains to be done to elucidate fully the impact of trypanolysin status and how such effects could be used in the design of novel control stratagies.

The trypanolysin described in G. m. morsitans was not secreted in enough concentrations and therefore, incapable of lysing all trypanosomes, entering the midgut, on their own. Although, the minimun protein concentration, that give lysis, from different tsetse species was established in vitro and the success of the trypanolysin to immunize tsetse in vivo, suggests further studies to be carried out targeting the site of biosynthesis of the molecule.

A new approach has to be developed in tackling tsetse borne-trypanosomiasis. Instead of trying to eradicate tsetse vector, biologists hope to produce transgenic strains that are incapable of transmitting trypanosomiasis. However, for this to be achieved, it is important that there should be detailed understanding of the mechanisms involved in the interaction between tsetse and the trypanosomes.

The obsevation that there is substrate inhibition of trypanolysin by diethyl pyrcarbonare is very intriguing and it would be interesting to establish the nature of the inhibition and how it relates to the in vivo levels of the substrate.

Further biochemistry work will be needed and in conjuction with the results within epidemiological data, the influence of these factors on trypanosome transmission could be assessed fully.

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