PURIFICATION AND CHARACTERIZATION OF TRYPANOLYSIN FROM THE MIDGUT OF DESERT LOCUST (*Schistocerca gregaria*), A NON-VECTOR OF TRYPANOSOMES

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DECLARATION AND RECOMMENDATION

Declaration

This thesis is my original work and has not been presented for a degree in any other University or any other award.

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DEDICATION

This work is dedicated to my dear daughter Ciru, my loving wife Agnes and my understanding and caring mum Jennifer. You people are so dear to me.

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ABSTRACT

African trypanosomosis, caused by protozoan parasites of the genus *Trypanosoma,* is a serious threat to human and animal health and thus a major hindrance to economic development in sub-Saharan Africa. *Trypanosoma* undergoes complex life cycle that is marked by differentiation and multiplication in the tsetse (*Glossina spp.*) vector and in the vertebrate host. Under field and experimental conditions, most flies are refractory to infection by trypanosomes. The establishment of infection in the tsetse midgut is an important first step in the disease transmission. However, the tsetse midgut presents a hostile environment that kills most of the trypanosomes ingested during an infective blood meal. Some of the tsetse midgut factors involved in this interaction include lectins, trypsins, trypsin-like molecules and trypanolysins among which trypanolysins have received least attention. Trypanolysins' presence and functions in haematophagous and non-haematophagous non-vector insects is not well documented. The present study was initiated to study trypanolysin properties in non-vector insect locust (*Schictocerca gregaria).* Trypanolysin was isolated from the midgut of locust *S. gregaria* through several consecutive chromatographic steps namely; gel filtration on Sephadex G-150, DEAE anion exchange and Concanavalin A Sepharose affinity chromatography. Further purification was achieved by electro-elution from native Polyacrylamide gel electrophoresis (PAGE). Trypanolysin eluted at the major peak on the gel filtration column, bound and eluted at about 260mM salt concentration on DEAE anion-exchange column but eluted in the unbound fraction in Con A affinity column. The native molecular weight was estimated to be about 28 KDa while under SDS-PAGE a single band of approximately 29 KDa was obtained. The proteineous nature of the trypanolysin was evidenced by the denaturation by temperatures above 50°C. Trypanolysin activity was not significantly affected by divalent cations (Mg^{2+} and Ca^{2+}) suggesting non-metallo co-factor dependence. The locust midgut trypanolysin is not glycosylated as evidenced by its failure to stain in periodic acid Schiff stain. The midgut trypanolysin elicited weak immunogenic response in a naïve New Zealand White rabbit. Western blot analysis using polyclonal antisera against the trypanolysin showed no cross reactivity with midgut protein extracts from haematophagous (tsetse, mosquitoes and sand fly) and non-haematophagous (cockroach, housefly) insects indicating that its *S. gregaria* specific. The findings of this work indicate that *S. gregaria* midgut trypanolysin exhibits significant differences in physical and chemical characteristics from trypanolysins in the vector *Glossina spp.*

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LIST OF ABBREVIATIONS

AAT	Animal African trypanosomosis
Con A	Concanavalin A
FAO	Food and Agriculture Organization
HAT	Human African trypanosomosis
IEF	Isoelectric focusing
KARI	Kenya Agricultural Research Institute
KDa	Kilo dalton
M	Molar concentration
mA	Milliampere
min	Minute
nm	Nanometer
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBSG	Phosphate buffered saline glucose
pH	Negative log of hydrogen ion concentration
pI	Isoelectric pH
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide gel electrophoresis
TEMED	N'N'N'N' tertramethylethylenediamine
TRC	Trypanosomosis Research Institute
Tris	Tris (hydroxymethyl) amino methane
µl	Microlitre
2D PAGE	Two dimensional Polyacrylamide gel electrophoresis

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information

African trypanosomosis is a serious threat to human and animal health in Sub-Saharan Africa. The causative agent of African trypanosomosis is a protozoan parasite of the genus *Trypanosoma* that is transmitted by tsetse (*Glossina spp*). Trypanosomes are unicellular eukaryotic protozoans that infect and ploliferate in wild and domestic animals, and in humans. The human infective forms are *Trypanosoma brucei rhodesiense,* which causes the acute form (East African type of trypanosomosis) and *Trypanosoma brucei gambiense* which causes the chronic form (West Africa type of trypanosomosis) (Smith, 1994; Aksoy, 2003;). It is estimated that over 60 million people living in 250 foci are at risk of contracting the disease with estimated 500,000 cases (WHO, 1998; FAO, 2004). Earlier reports had estimated the mortality to be 55,000 deaths annually (WHO, 1995).

Animal trypanosomosis (nagana) remains a massive hindrance to African agricultural development. It is estimated that 3 million herds of cattle die annually from nagana. Tsetse flies, the vectors of African trypanosomosis, infest over 9 million square kilometres of suitable grazing land in the sub-Saharan Africa. This puts over 60 million cattle at risk of contracting the disease leading to US $ 1.2 billion loss annually in direct losses from cattle deaths, reduced meat and milk production and control costs (Kristjanson *et al.*, 1999; FAO, 2004). *Trypanosoma brucei brucei, Trypanosoma congolense and Trypanosoma vivax* are the main species affecting livestock. The most important domestic animal parasites are *T. b. brucei* whose natural vector is *Glossina morsitans.*

Several approaches towards the control of trypanosomosis have been used. These include: vector control (Schofield and Maudlin, 2001), immunization, chemotherapy (Peregrine, 1994) and selective animal breeding for trypanotolerance (Ole Moiyoi *et al.*, 1997). Various vector control methods have been applied with varying degree of success and include: use of traps/ baits, sterile male release (Leak 1999; Vreysen *et al.*, 2000) insecticides and bush clearing. Bush clearing is hampered by high costs of clearing and maintenance due to rapid regeneration, depletion of wildlife and ecological imbalance (Hargrove *et al.*, 2001). Control of insect vector by use of chemical-based strategies is frustrated by the emergence of insecticides resistance (Rita *et al.*, 2004), environmental impacts of insecticides, inaccessibility of transmission sites, the

highly mobile and vastly spread population of the tsetse flies which prevent effective application of insecticides and lack of sustainability (Aksoy, 2000). The sterile insect technique (SIT) is expensive and requires geographical isolation or area-wide participation for it to be effective (Aksoy 2000; Vryesen *et al.,* 2000). Re-invasion is a major drawback in SIT, furthermore the non-targeted tsetse species continues to pose a threat even after the targeted species is eliminated.

Rearing of trypanotolerant animals in tsetse infested areas has been frustrated by the perception that their small size result in poor meat and milk production hence do not find acceptance among the farming communities (Holmes, 1997). It has also been demonstrated that domestic animals (including the trypanotolerant ones) can act as reservoirs for *T. b. gambiense* which causes human African trypanosomosis (Biteau *et al.,* 2001; Mac Leod *et al.,* 2001 Waiswa *et al.,* 2003). Since these animals are kept near human habitation, this may increase the incidences of human African trypanosomosis (HAT) in the farming communities. The ability of these animals to withstand infection in high tsetse challenge is questionable (FAO, 1991).

The extensive antigenic variation of the surface glycoprotein on the parasite membrane precludes the development of an effective vaccine (Jacobs-Lorena and Lemos, 1995; Aksoy, 2000; 2003). Vector management, chemotherapy and chemoprophylaxis are currently the most widely used control methods (Day and Sjogren, 1994; Aksoy, 2000).

The polymorphic nature of the parasite genome has led to development of drug resistance, lowering their effectiveness as control methods (Peregrine, 1994). The drugs currently available are highly toxic, have adverse side effects, costly, associated with drug resistance and sporadically available in areas of high fly challenge (Gray and Roberts, 1971; Jordan, 1986; Connor, 1994; Makumyaviri, 1998; Aksoy, 2000; Anene *et al.,* 2001). This coupled with the lethargy of pharmaceutical companies to produce new and more effective drugs (Jordan, 1986; Mc Neil, 2000) lowers the efficacy of chemotherapy as a control method. It is therefore imperative that more affordable, efficient, environmentally acceptable and sustainable disease management strategies are needed. Greater hope lies in integrated control programs. Alternative genetic-based strategies are being sought that can be included in the battery of tools available for integrated control programs. Transgenesis is being explored with an aim to modulate vector competence by expressing foreign genes with antipathogenic properties that can interfere with viability, development and / or transmission of the parasites (Aksoy, 2000).

Tsetse flies are the only biological vectors for African trypanosomosis; therefore, the disease transmission can effectively be curtailed by rendering the tsetse incapable of transmitting the parasite. The interaction between the vectors and the disease-causing pathogens starts at the midgut (Vickerman, 1985; Yan *et al.*, 2002) thus the midgut environment is very crucial in determining the success of disease transmission. By altering the midgut environment of susceptible tsetse species, the establishment and / or the maturation of the parasite in the vector can be interrupted culminating in transmission blocking. This integrated with other methods would contribute to the management of trypanosomosis.

Glossina morsitans morsitans is the natural vector for *T. b. brucei,* which causes Nagana. The vector's midgut contains several factors that determine whether the vector becomes infective or refractory (Woolhouse *et al.*, 1994; Msangi *et al.*, 1998; Lehane *et al.*, 2000). For transmission to take place, infection of the tsetse is paramount. A clear understanding of the biochemical properties of the factors determining refractoriness or infectivity may offer a leeway for their utilization in modulating the tsetse vectorial capacity. Such would include the introduction and expression of foreign genes in the insect whose products could confer pathogen refractory phenotypes (Rita *et al.*, 2004). Tsetse midgut endosymbionts, which have a strong presence in the tsetse midgut, are ideal candidate carriers of the foreign gene into the tsetse. The engineered refractory flies would then be released to gradually replace the parasite susceptible vector populations (Rita *et al.*, 2004) by virtue of mechanisms that confer reproductive advantages to the engineered insects (Beard *et al.*, 1994).

The complete exclusion of trypanosomes in non-vector insects is not well understood. The failure of the parasite to establish and mature in non-vector insects may be attributed to either the inability of parasite to circumvent the various general or specific barriers or the interaction may lack the necessary stimuli/signal for further development (Aksoy, 2003; Ogoyi *et al.*, 2003). A preliminary study on non-vector insect showed that crude *S. gregaria* midgut homogenate contains factor(s) able to lyse trypanosomes *in vitro* (Ogoyi *et al.*, 2003). This study also showed that crude *S. gregaria* midgut had the highest trypanolytic activity among the insects used (locust, coackroach, mosquito and body lice). Several attempts to purify and characterize trypanolysins in tsetse have been successfully undertaken (Stiles *et al.*, 1990; Osir *et al.*, 1995; Grubhorfer *et al.*, 1997; Osir *et al.*, 1999). However, complete purification and characterization of trypanolytic factors from midguts of non-vector insects have not been reported. The current

study was therefore designed to purify to homogeneity and characterize trypanolysin from the midgut of *S. gregaria,* a non-vector insect.

1.2 Statement of the Problem

Though a lot of efforts have been directed towards understanding trypanolytic factors in tsetse flies and their role in trypanosome differentiation and lysis, there is limited information about the presence and characteristics of these factors in non-vector insects such as *S. gregaria.* Such information would enhance our current knowledge of vector-parasite interactions and possible search for sustainable disease management strategies.

1.3 Objectives of the study

1.3.1 Overall objective

To isolate and characterize the physico-chemical properties of trypanolytic factor(s) from *S. gregaria* midgut.

1.3.2 Specific objectives

1. To isolate trypanolytic factor(s) from *S. gregaria* midgut.
2. To carry out physical and chemical characterization of the trypanolytic factors from *S. gregaria* midgut.
3. To establish the effect of sugars and divalent cations (Ca^{2+} and Mg^{2+}) on *S. gregaria* midgut trypanolytic factor(s).
4. To compare the homology of trypanolysins from vector and non-vector *S. gregaria* midgut through immuno-cross-reactivity.

1.4 Hypotheses

a. Trypanolytic factor(s) in *S. gregaria* midgut and *Glossina morsitans* midgut have the same physical and chemical characteristics.

b. Divalent cations (Ca^{2+} and Mg^{2+}) enhances the trypanolytic activity of locust midgut trypanolysin.

c. Trypanolysin is conserved across the various insect orders.

1.5 Justification

African trypanosomosis is a disease of immense medical, agricultural and economic importance in sub-Saharan Africa. The control methods applied so far have not effectively contained the disease with some having adverse effects on the environment, non-target organisms as well as the host animal. Most studies have focused on vector-host and parasite-host interaction in an attempt to control the problem. However research interest is now shifting towards understanding the parasite vector interaction and the factors behind this interaction. This is because without infected vectors, disease transmission would not occur. It is not clear why most tsetse flies are refractory to trypanosome infection. Similarly the lack of development of the infection in non-haematophagous non-vectors insects and haematophogous non-vectors even those fed on the same infected host is not clearly understood (Boulanger *et al.*, 2002).

The complete exclusion of trypanosomes in non-vector insects coupled with the finding that midgut homogenates of some non-vector insects contains trypanosome lysing factors may indicate that these factors may be associated with this exclusion. Recent preliminary findings showed that non-haematophagous non-vectors insects such as *S. gregaria* and *Periplaneta americana* midguts also contain trypanolytic activity with the former having higher activity Ogoyi *et al.*, (2003). The characterization of trypanolysins from the midgut of non-haematophagous non-vectors insects such as *S. gregaria* could open a new avenue for further research at molecular level which could lead to a better understanding of trypanosome-vector interaction. Furthermore, this could lead to their being engineered into the natural vector *Glossina spp.* leading to modulation of the vectorial capacity of tsetse flies and thus enhancing the control of trypanosomosis.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Economic importance African trypanosomosis.

African trypanosomosis is an infectious disease unique to Africa and caused by a protozoan parasite of the genus *Trypanosoma*. It is a severe disease which is always fatal if left untreated. The disease affects both humans [Human African Trypanosomosis (HAT) or sleeping sickness] and animals [Animal African Trypanosomosis (AAT) or Nagana] and occurs in 37 sub-Saharan countries covering more than 9 million km^2, an area which corresponds approximately to one-third of the Africa's total land area (WHO, 2006). The disease occurs only in Africa south of the Sahara where poverty, social instability, insecurity and weak health systems are widespread (WHO, 2006). The situation is made worse by the fact that the disease competes with pandemic diseases consigning sleeping sickness to a low ranking in health priorities thus receiving little support from national health authorities and donors. The disease is a threat to an estimated 50 million head of cattle (FAO, 2004). Every year, AAT causes about 3 million deaths in cattle while approximately 35 million doses of trypanocidal drugs are administered (FAO, 2004). The economic losses in cattle production alone are in the range of US$ 1.0-1.2 billion and an estimated US$ 4.75 billion per year in agricultural Gross Domestic Product (WHO, 2006). The disease in domestic animals and particularly cattle is a major obstacle to the economic development of the rural areas affected (WHO, 2006).Trypanosomosis is a daily threat to more than 60 million men, women and children in the world. However, disease surveillance currently covers only 3-4 million people. About 45,000 cases have been reported but it is estimated that 300,000-500,000 people are infected (WHO, 2006). Earlier estimates indicated that 55,000 people die annually from this disease (WHO, 1995).

2.2 Tsetse vector

Tsetse flies are the obligatory vectors for African trypanosomosis with both sexes being exclusively haematophagous (FAO, 1998). Tsetse flies belong to the genus *Glossina* which is made up of 31 members; 23 species and 8 sub-species (Service, 2001). Based on morphology, geographical distribution and behaviour, these species are divided further into three groups; the morsitans, palpalis, and fusca groups (Mira and Raph, 1989). The fusca group mainly occupies

the rainforests, the palpalis group mainly inhabits the riverine regions and savannah woodlands while the morsitans are restricted to the savannah (Colvin and Gibson, 1992; Jordan, 1986). A large variability in vectorial competence has been shown between species of *Glossina*. Tsetses belonging to the morsitans group are good vectors of *T. congolense* whereas those of the palpalis group are poor vectors of the same (Moloo and Kutuza, 1988). On the other hand, the fusca group are more important vectors of nagana than the HAT (Mira and Raph, 1989; Service, 2001). *Glossina* are best known for cyclic transmission of trypanosomosis though mechanical transmission has also been reported (Jordan, 1986) especially among the sub genera *Dutonella* (Molyneux and Ashford, 1983). But it is the tabanids and stomoxys that have been shown to be the most important mechanical transmitters of *T. vivax* to cattle (Molyneux and Ashford, 1983; Desqesnes and Dia, 2003; 2004).

2.3 Life cycle of *Trypanosoma brucei*

Trypanosomes are parasites with a 2-host life cycle: mammalian and arthropod. The life cycle starts when the tsetse takes an infected blood meal. The vector picks up a pleomorphic population of trypomastigotes consisting of long slender, intermediate and short stumpy forms of the parasite (Fig.1). The short stumpy bloodstream forms of the parasite are metabolically pre-adapted for transformation into procylic forms found in the invertebrate vector (Vickerman, 1965). They rapidly differentiate into procyclic forms in gut lumen, lose their variable surface glycoprotein (VSG) coat and express a new coat composed of procyclin proteins (Ruepp *et al.*, 1997). Transformation into procyclic form is mandatory for successful establishment of midgut infection. The role of the procyclin is not clear but it either protects the trypanosome from the harsh midgut environment or plays an important role in establishment (Roditi and Pearson, 1990).

Bloodstream forms are more susceptible to trypanolysin than the procyclic forms as very high concentrations of trypanolysin is required to lyse the later compared to the former (Osir *et al.*, 1999). *In vitro* studies have linked transformation from bloodstream forms to procyclic forms to temperature shift from 37 °C to 27 °C (Bienen *et al.*, 1981), cis-aconitate levels (Brun and Schonenberger, 1981), mild acidic conditions (Rolin *et al.*, 1998), trypsin levels (Imbuga *et al.*, 1992a) and level of glucose in the incubation medium (Milne *et al.*, 1998). However, only less than 10% of the bloodstream trypanosomes in the pleomorphic population transforms into

procylic forms (Gibson and Bailey, 2003). Upon entry to the vector gut the trypanosomes experiences a biochemically and physiologically hostile environment that is responsible for massive attrition leading to complete elimination of the trypanosomes from the gut of refractory species. This attrition has been linked to the action of trypanocidal lectins (Gibson and Bailey, 2003), agglutinins (Ingram and Molyneux, 1988), trypanolytic proteins (Stiles *et al.*, 1990; Osir *et al.*, 1999) and proteolytic enzymes (Cheeseman and Gooding, 1985). The procyclic trypomastigotes then migrate anteriorly to the paired salivary glands where they differentiate into attached epimastigote forms which undergoes transformation to yield the infective free living metacyclic forms which are secreted in saliva and transmitted to the next host when the vector takes a blood meal (Vickerman *et al.*,1988). *T. brucei* takes a minimum of two weeks to complete its life cycle in the tsetse fly but the fly continues to produce infective metacyclics for the rest of its life once infected (Gibson and Bailey, 2003) hence it can infect a new host every few days when it feeds or just pierces the skin with its proboscis (Aksoy *et al.*, 2001).

2.4 Establishment of midgut infection of tsetse flies

It has been reported that *T. b. gambiense* and *T. b. rhodisiense* can develop in any species of *Glossina* under experimental conditions. However, under field conditions only *G. morsitans* and *G. palpalis* are capable of acting as vectors (Smith, 1994). For tsetse flies to transmit African trypanosomosis they must first become infected themselves. However, infection rates under natural conditions are generally very low (Moloo, 1981; Moloo and Kutuza,1988). Infections with *T. brucei spp.* are detected in less than 1-5% of the fly population in the field (Woolhouse *et al.*,1994; Msangi *et al.*, 1998; Lehane *et al.*, 2000). Even for those that acquire midgut infection, less than a third develop salivary gland infection hence due to this attrition far less than 1% of wild flies have salivary gland (mature) infections (Hao *et al.*, 2001). Midgut infection rates are determined by a complex interaction between factors originating from the vector, host and trypanosomes species involved (Maudlin, 1991; Hunt *et al.*, 1994; Nguu *et al.*, 1996; Welburn and Maudlin, 1999). Levels of lectins in the midgut during parasite uptake, fly species, sex, age, and symbiotic associations in the tsetse fly (Imbuga *et al.*, 1992a; Hao *et al.*, 2001; Welburn and Mauldrin 1999) and nutritional stress (Kibui *et al.*, 2006) are some of the important vector-associated factors which plays an important role in determining success of parasite infection. The

low infectivity of tsetse may be attributed to the killing of the parasite by innate immune response in the fly

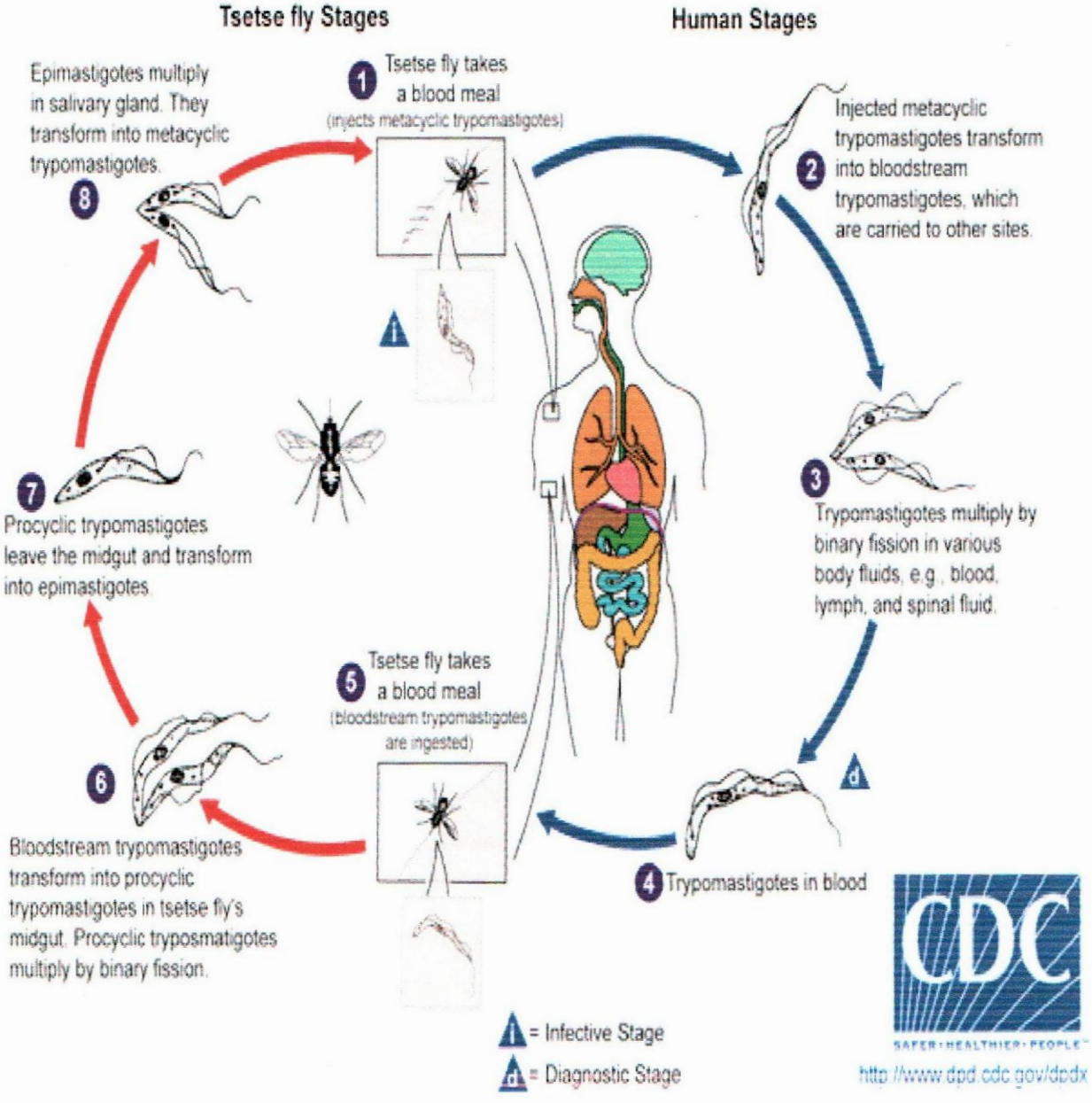


Figure 1: The life cycle of African trypanosomes (Adapted from CDC, 2004).

(Croft *et al.*, 1982; East *et al.*, 1983). The innate immune response involves phenooxidase cascade (Nigam *et al.*, 1997), lectins (Ingram and Molyneux, 1993; Abubakar *et al.*, 1995; Osir *et al.*, 1995), antimicrobial peptides (Boulager *et al.*, 2002; Hu and Aksoy, 2006) trypanolysins (Stiles *et al.*,1990) and haemocyte types (East *et al.*, 1980). However, a lot is still unknown about the molecular basis of refractoriness.

2.5 Factors influencing infection in tsetse flies

Glossina morsitans morsitans is the natural vector for *T. b. brucei,* which causes nagana in cattle. The vector's midgut contains several factors that determine whether the vector becomes infective or refractory. Some of these factors includes: lectins, trypsins, trypsin like molecules and trypanolysins (Woolhouse *et al.*, 1994; Msangi *et al.*, 1998; Lehane *et al.*, 2000; Boulanger *et al.*, 2002; Nayduch and Aksoy, 2007). Infectivity is also influenced by number of lectin binding sites on the trypanosome which is controlled by the trypanosome genotype (Leak, 1999).

2.5.1 Lectins

Lectins are glycoproteins of non-immune origin, which binds specifically to carbohydrates, and therefore agglutinates cells or complex carbohydrates (Abubakar *et al.*, 1995; Leak, 1999). Lectins are highly specific in their saccharide binding sites (Barondes, 1981). They interact with cells through their carbohydrates binding sites triggering a number of important biological phenomena in both vertebrate and invertebrates such as lymphoblast transformation and cell division, activation of T-suppressor cells and insulin-like stimulation of fat cells. Lectins also alter the movement of receptors on the cell membrane producing capping (Raper *et al.*, 2001). Attempts to characterize lectins from different insects have shown them to be related in a number of their physical and chemical characteristics such as molecular weight and effects of inhibitors (Stiles *et al.*, 1990; Osir *et al.*, 1995; Grubhoffer *et al.*, 1997).

Tsetse flies have lectins in their crops, midguts as well as their haemolymph (Aksoy, 2003). These lectins have been shown to exhibit some similarities across the species but display tissue specificity.

Immunobloting studies have shown that midgut extracts from *Glossina* species (*G. longipennis, G. morsitans* and *G. pallidipes*) contain cross-reacting epitopes while haemolymph extracts do not cross-react with midgut extracts (Osir *et al.*, 1995).

Midgut lectins have been shown to play an important role in the establishment of midgut infections in tsetse flies and are vital in determining whether tsetse becomes refractory or infective (Maudlin, 1988a). Lectins from *Glossina morsitans morsitans* midgut are specific for D-glucosamine (Osir *et al.*, 1995) while lectins from haemolymph of the same are specific to galactose and its derivatives (Ingram and Molyneux, 1990). *G. palpalis* and *G. pallidipes* have both galactosyl and glucosyl lectins while *G. morsitans* only have glycosyl lectins in their midguts (Leak, 1999). *Glossina morsitans morsitans* flies maintained on a glucosamine-containing diet throughout their lives show marked reduction in the number of parasites that establish themselves within the flies (Maudlin and Welburn 1987; 1988 a; b). Glucosamine is known to inhibit tsetse midgut lectins (Abubakar *et al.*, 2003). However, lectins are also implicated in killing of trypanosomes entering the gut of refractory flies (Pereira and Kabat, 1979). Tsetse flies possesses midgut lectins capable of killing trypanosomes *in vivo* by a process resembling programmed cell death (Moloo, 1981; Imbuga *et al.*, 1992a; Welburn and Maudlin,1999). Refractory tsetse secretes specific lectins that binds to trypanosomes leading to lysis and cell death (Maudlin,1991; Grubhoffer *et al.*, 1997; Raper *et al.*, 2001). Tsetse midgut lectins therefore have a dual function; to eliminate parasites from the gut as well as to promote differentiation of bloodstream trypanosomes. It is not clear how lectins or lectin-like molecules are involved in both lysis and promotion of differentiation of parasites (Maudlin and Welburn, 1988 a; b; Osir *et al.*, 1995). However the levels of lectins in the tsetse gut during the infective meal plays a major role in determining the success of infection (Maudlin, 1991). Variation in susceptibility in tsetse populations and species has been attributed to the levels and specificity of lectins (Maudlin and Welburn, 1987; Mihok *et al.*, 1992). Refractory tsetse species such as *G. pallidipes and G. p. palpalis* shows an un-induced baseline expression of midgut lectins hence are more apt in clearing trypanosomes (and possibly other microbes) present in first blood meal (Nayduch and Aksoy, 2007).

Factors affecting lectin levels in tsetse

Several factors affect the lectin levels in the tsetse midgut. Such factors includes the presence of maternally inherited bacterial endosymbionts and age of the fly (Welburn and Maudlin, 1999). These factors have been shown to exert a direct effect on the infection or refractoriness of tsetse. Some tsetse harbour three phylogenetically different bacterial endosymbionts; *Wigglesworthia*

glossinidius, Sodalis glossinidius and *Wolbachia spp.* that are maternally inherited in their midguts (Beard *et al.*, 1993; Haines *et al* 2002). Of these, *Wigglesworthia* and *Sodalis* have a strong presence in the midgut. These endosymbionts have been shown to play a major role in potentiating midgut infections in the tsetse fly (Welburn and Maudlin, 1991; 1999). *Sodalis glossinidius* is thought to influence lectin activity through production of lectin inhibitory sugar N-acetylglucosamine which accumulates during pupal development due to the bacteria's endochitinolytic activity (Welburn *et al.*, 1993). Presence of bacterial endosymbionts thus reduces the amounts of lectins available in the midgut of the tsetse flies making the flies to be more susceptible. Elimination of *S. glossinidius* has been found to increase refractoriness to trypanosome infection in tsetse (Dale and Welburn, 2001).

The levels of lectins are also influenced by the age of the fly with teneral flies having lower levels than older flies. Tsetse flies are more succeptible to infection in their teneral state when the peritophic membrane is not fully formed (Lehane and Msangi, 1991) or when antitrypanosomal lectin levels are low (Welburn and Maudlin, 1999). Older flies have lectin levels 100-200 times higher than teneral flies (Jacobson and Doyle, 1996) hence it is easier to infect teneral flies than older flies. This has been attributed to the fact that newly emerged flies lack a peritrophic membrane and lectins are found in association with the peritrophic membrane (Lehane and Msangi, 1991; Gibson and Bailey 2003).

2.5.2 Trypanosome genotype

Differences in susceptibility with different trypanosome species can reflect differences in lectin-biding sites as determined by their genotype (Leak, 1999). Genetically different trypanosomes have different number of lectin binding sites. Trypanosomes with few lectin-binding sites will mature even when lectin levels are very low (Maudlin and Welburn, 1988a). The levels of lectins in the gut at the time of trypanosome intake and the trypanosome genotype influence the outcome of transmission (Aksoy *et al.*, 2001). Lectins provide a signal that stimulates the maturation process of trypanosomes (Welburn and Maudlin, 1999).

2.5.3 Trypsin levels

Tsetse midgut also contains trypsin and trypsin-like enzymes, which have been demonstrated to be necessary for successful establishment of midgut infection. However, the role of these proteolytic enzymes in tsetse-trypanosome interaction is poorly understood. They however present a major barrier to trypanosome development within the tsetse fly (Cheesman and Gooding, 1985). High levels of trypsin kill the trypanosomes (Yeates and Steiger, 1981; Imbuga *et al.*, 1992b). Osir *et al* (1995) isolated a lectin-trypsin complex from *G. longipennis* and postulated that the enzyme may be required to cleave off specific surface molecules from the bloodstream form trypanosomes thus exposing lectin-binding sites thus facilitating agglutination. The abrogation of agglutination of bloodstream forms and not procyclic forms in the presence of trypsin inhibitors supports this position since procyclic forms may lack these molecules that may require to be cleaved off to expose the lectin binding sites (Osir *et al.*, 1995). Trypsin also inhibits differentiation of bloodstream trypanosomes into procyclic forms (Osir *et al.*, 1995). This indicates how close these two molecules work (Abubakar *et al.*, 1995).

2.5.4 Trypanolysins

Transmission of trypanosomosis has also been shown to be limited by the presence of midgut trypanolysins (Gooding, 1992). Trypanolysin is a protein found in the midgut of tsetse flies that is able to cause lysis of trypanosomes. The production of trypanolysin is bloodmeal-induced and its titres increases proportionally with the number of blood meals (Osir *et al.*, 1999). The levels of trypanolysin has also been shown to increase gradually after 48 hrs and peaks between 72 and 120 hours post bloodmeal and gradually decreases thereafter (Osir *et al.*, 1999). In tsetse flies, it has been shown that trypanolysin production is localised to the posterior midgut (Stiles *et al.*, 1990). Most of the trypanosomes entering the tsetse gut are killed by trypanolysin and those that survive remain in the anterior midgut until the trypanolysin levels falls to a concentration allowing for posterior migration (Nyambega, 2002).

Isolated trypanolysin have been shown to have a lytic activity against bloodstream forms of *T. b. brucei* but none on procyclic forms of *T. b. brucei* implying that bloodstream forms have certain binding domains for trypanolysin apparently absent in procyclic forms (Osir *et al.*,1999). Binding studies have shown that the variable surface glycoprotein (VSG) present on bloodstream

forms of *T.b.brucei* contains or is the binding protein to which trypanolysin binds to initiate lysis (Nyambega, 2002).

Stiles, (1990) detected the presence of a trypanolysin in the posterior midgut of *G. p. palpalis* but its apparent absence in *G. p.gambiense* appears to be correlated to the higher infectivity towards *T. b. brucei* and *T. congolense* compared to *G. p. palpalis*. Similarly trypanolysins activity has also been detected in other *Glossina* species such as *G. m. morsitans, G. m. centralis, G. pallidipes, G fuscipes fuscipes* and *G. longipenis* (Osir *et al.*, 1999). Tsetse midgut trypanolysins have been isolated and partially characterised; *G. austeni* trypanolysin was found to have a native molecular weight of 669 kDa and consists of two sub-units of molecular weights ~55 kDa and 29 kDa (Nyambega, 2002). *G. fuscipes* trypanolysin is a heat labile molecule that is not inhibited by chelating agents like EDTA, divalent cations like Ca^{2+} and Mg^{2+}, but is inhibited by diethyl pyrocarbonate and high concentrations of pronase (Osir *et al.*, 1999).

2.6 Refractoriness in non-vector insects

Epithelial immunity especially the production of anti-microbial peptides (AMPs) has been shown to play an important role in preventing the establishment of infections in insects (Boulanger *et al.*, 2002). Mosquitoes respond to plasmodium infection by production of AMPs, some of which have been shown to be trypanocidal (Lehane *et al.*, 2001). Although microbial immune challenge induces attacin expression, refractory tsetse shows an uninduced, baseline level of systemic (fat body) attacin, whereas the susceptible flies do not (Hao *et al.*, 2001). Furthermore, refractory species have a higher level of attacin expression in the proventriculus and midgut (Boulanger *et al.*, 2002). Blood feeding is found to up-regulate attacin expression in refractory species but not in the susceptible flies (Nayduch and Aksoy, 2007). It is worth noting that expression of AMPs concides with parasite attrition in the midgut of refractory tsetse (Gibson and Bailey, 2003). *Glossina* spp. and *Stomoxys calcitrans* feed on the same vertebrate hosts and have a very similar digestive physiology and midgut anatomy. Although *Stomoxys* is constantly exposed to trypanosomes, it kills them in the midgut within 2-4 days of ingestion. *Stomoxys* is not a biological vector of African trypanosomes. It is not clear why *Stomoxys* is not a biological vector of African trypanosomes. There is a possibility that the distinct vector and non-vector nature between *Glossina* and *Stomoxys* may lie in the differences in immune mechanisms (Boulanger *et al.*, 2002).

Initial evidence for the presence of factors that lyse bloodstream form trypanosomes in non-haematophagous non-vector insects was obtained by incubating bloodstream form trypanosomes with crude midgut homogenates *in-vitro* (Ogoyi *et al.* , 2003). Presence of trypanolytic activity in crude midgut homogenates of both haematophagous and non-haematophagous non-vector insects such as body louse (*Pediculs humanus humanus*), mosquito (*Aedes aegypti),* desert locust (*Schictocerca gregaria*) and cockroach (*Periplaneta americana)* was also reported with the desert locust showing the highest activity (Ogoyi *et al.* , 2003). The presence and characterisation of midgut trypanolysins in non-vector insects has however not been well documented. This study attempted to fill this gap by studying the properties of purified *S. gregaria* trypanolysin.

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Experimental Animals and Insects

Adult desert locusts, *Schistocera gregaria,* were obtained from the Zoology Department, University of Nairobi. They were maintained at 28–34°C, 40 –50 % relative humidity and photoperiod of 12 hrs: 12 hrs (light: dark).

Adult albino rats were obtained from the Department of Biochemistry, University of Nairobi and maintained on rat pellet. Male New Zealand white rabbit was obtained from the Department of Biochemistry, University of Nairobi and maintained on normal pellet diet throughout the course of experiment.

3.2 Parasite Maintenance and Isolation

The parasite, *Trypanosoma brucei brucei* strain 3397 derived from stock 2710 was obtained from Kenya Agricultural Research Institute-Trypanosomosis Research Centre (KARI-TRC) and passaged severally in adult albino rats within the Department of Biochemistry, University of Nairobi. Rats were infected by injecting them with 200 µl of parasitised blood containing about $5x\ 10^6$ of the parasites. The parasite levels were monitored regularly and at peak parasitaemia they were harvested from the rat blood drawn by cardiac puncture and isolated from the infected rats' blood by Diethyl Ethyl Amino Ethyl (DEAE) ion exchange chromatography as described by Lanham and Godfrey (1970). Briefly, heparinised blood was first centrifuged at 3,000 x *g* for 5 minutes at room temperature and the buffy coat applied to the DEAE column. The column was washed with cold Phosphate Buffered Saline containing 1% glucose (PBSG) and the eluate monitored by taking a drop and checking under the microscope until trypanosomes appeared which coincided with the appearance of turbid eluate. The eluate containing trypanosomes was collected in a 50 ml beaker placed on ice. This was then centrifuged at 1000 x g for 10 min at 4°C and the resulting trypanosome pellet re-suspended in fresh PBSG.

3.3 Counting of trypanosomes

Counting of the trypanosomes was done using an improved Neubauer ruled haemocytometer (Lumsden *et al.*, 1973; Paris *et al.,* 1982). Briefly, the haemocytometer was placed under a phase contrast microscope x40 objective and the trypanosomes counted in four large squares. The

number of trypanosomes was estimated by using the formula given below, and the final concentration adjusted to $1x10^7$ trypanosomes per ml with PBSG.

$N = n/4 \times (10^4 \times D \times V)$

Where:-

N = the number of trypanosomes in the undiluted buffer.

n = the number of trypanosomes counted in the 4 large squares of the haemocytometer.

D = Dilution factor

V = Volume of the undiluted trypanosomes suspension.

3.4 Insect midgut preparation

Insect midgut preparation was carried out according to Ogoyi *et al.*, (2005). Before the midguts were obtained, the locusts were starved overnight. The locusts were then briefly chilled and their midguts dissected in ice-cold phosphate buffered saline (PBS, pH 7.2). The midguts were then cut open to remove any remnants of the midgut content (undigested food) and rinsed twice in ice-cold PBS, pH 7.2. The midguts from 50 locusts were then pooled and homogenised using a hand-held glass homogeniser maintained on ice. The homogenate was then centrifuged at 12,000 *g* for 15 minutes at 4°C and the supernatant stored at –20°C until used

3.5 Trypanolysin assay

Trypanolysin assay was conducted by the method of Ogoyi *et al.*, 2003. Briefly, the midgut homogenates or purified trypanolysins were serially diluted on a micro-titre plate using 20µl of PBSG, pH 8.0. Equal volumes of parasite suspension (20µl) were then added to each well to make a final concentration of $5x10^6$ parasites per ml and incubated at room temperature for a maximum of 90 minutes. Lysis of trypanosomes was checked at different time points under a phase contrast microscope at a magnification of x40. Control experiment was set up in adjacent wells using PBSG, pH 8.0 and parasites. Percentage lysis was estimated by obtaining the

difference between the number of live parasites in the control and the experimental wells and expressing it as a percentage of the number of live parasites in the control well.

3.6 Protein concentration estimation

Estimation of protein concentration was done using Lowry method (Lowry *et al.*, 1951). Bovine serum albumin was used as the protein standard. The concentration of the trypanolysin was adjusted to 1 mg/ml for subsequent use.

3.7 Purification of trypanolysin from crude midgut homogenate

3.7.1 Gel permeation chromatography

Sephadex G-150 was prepared by boiling 8 g of the dry gel in distilled water for 30 min in a water bath and left to swell overnight in distilled water at room temperature. The slurry was then re-suspended in PBS buffer and then allowed to pack into a column of internal diameter 1.42 cm to a bed height 85 cm by passing elution buffer (0.1M PBS, 0.02%(w/v) NaN_3, pH 7.2) at a flow rate of 0.3 ml/min for 12 hrs by gravity.

The void volume was estimated by determining the retention time for Blue dextran 2000. This was done by loading 1.5 ml of 1% blue dextran in PBS, pH 7.2 in the column and eluting with PBS, pH 7.2 then collecting 3 mls fractions until the column was clear.

The column was then washed several times with PBS, pH 7.2 before loading 1.5 ml of standard molecular weight marker MW-GF-200 kit (Sigma) for calibration. Elution was carried out using PBS, pH 7.2 and 3 ml fractions were collected. The elution volume was determined by reading the absorbance at 280 nm using a Beckman Du 530 spectrophotometer.

The column was again washed several times with PBS, pH 7.2 then loaded with 1.5 ml (10 mg protein) of the crude midgut homogenate. Elution was carried out using PBS, pH 7.2 (devoid of NaN_3) collecting 3 ml fractions. The elution of the proteins was monitored by reading the absorbance at 280 nm. The fractions obtained were used for lytic assay and the fractions showing trypanolytic activity were pooled and concentrated using polyethylene glycol-8000 (Sigma) then dialysed against several changes of PBS and stored at -20°C until used.

3.7.2 Ion exchange chromatography

Anion exchange chromatography was performed using a modified protocol of Osir *et al.*, 1989 using Diethyl Ethyl Amino Ethyl (DEAE) cellulose (Sigma) bed volume 2 ml. Briefly, the column was pre-equilibrated with 20 mM PBS, pH 8.0. The lytic fraction (0.5 ml containing 5 mg protein) obtained from gel filtration was then loaded and left to bind at 4°C overnight. The column was then washed with starting buffer collecting 0.5 ml fractions. The absorbance of the eluant was monitored by reading the absorbance at 280 nm on a Beckman Du 530 spectrophotometer. The bound fraction was then eluted using a linear salt gradient (0.0-0.350 M NaCl in PBS, pH 8.0). The peak fractions were pooled, concentrated, dialysed and assayed for lytic activity as described in section 3.5.

3.7.3 Affinity chromatography

Trypanolytic fraction obtained from DEAE-cellulose column were further purified by affinity chromatography using a concanavalin (Con A)-sepharose column by the method of Ogoyi *et al.*,1995. Briefly the column was pre-equilibrated with 'Con-A buffer (0.01M $MgCl_2$, 1.0 mM$CaCl_2$, 0.5 M NaCl, pH 7.5). Equal volumes (1 ml) of 50% packed Con-A Sepharose and protein (about 1 mg/ml) were mixed in small tubes and gently shaken for 1hr at room temperature or overnight in the cold room to allow for maximum binding of the proteins. This was then re-packed in to the column and the column washed with the Con-A buffer. The bound fraction was eluted using 500 mM α–D methyl pyranoside in con–A buffer. The protein elution was followed by measuring the OD at 280 nm using a Beckman Du 530 spectrophotometer. The resulting fractions were concentrated and extensively dialysed against PBS before being used for lysis assay as previously described.

3.7.4 Electro elution

Gradient native PAGE gels (5-15%) were cast using a gradient former (BioRad) and run at 70 V at 4° C for about 8 hrs until the tracker dye (Bromophenol Blue) just ran off the bottom. Side strips from the same gel were cut and silver stained (Wray *et al.*, 1981) while the rest of the gel was maintained at 4°C. The side strips were then swollen to the original size of the gel then aligned on the unstained gel and the gel portion containing the band of interest sliced out. The

gel slice was chopped into smaller pieces and packed into a glass tube connected to a membrane cap with a dialysis membrane (molecular cut off of 12,000-15,000 Daltons) containing buffer (25 mM Tris/HCl, 192 mM glycine pH 8.3). This was then immersed into a tank (Transblot cell, Bio-Rad) containing tank buffer (25 mM Tris/HCl, 192 mM glycine pH 8.3) and the upper buffer chamber filled with the same buffer. Electro elution was carried out using a model 422 Electro-eluter (Bio-Rad) for four hours at 10 Amps per tube at 4°C. The resulting protein sample was concentrated by centrifugation using centricon tubes (MWCO 10 KDa, Bio Rad), dialysed and assayed for trypanolytic activity as previously described.

3.8 Physio-chemical Characterisation

3.8.1 Electrophoresis

Native Polyacrylamide gel (PAGE) was used to determine the native molecular weight of the purified trypanolysin. Samples were mixed with equal volumes of sample buffer (0.125 M Tris/HCl pH 6.8, 10% glycerol and 0.002% bromophenol blue) and applied onto a 5-15% native-PAGE gel. Molecular weight standards (Sigma) were run alongside the samples. The gels were run at a constant voltage (70 V) in 25 mM Tris-glycine buffer, pH 8.3 until the bromophenol tracking dye reached the end of the gel. Native PAGE was also used to follow the extent of purification of the midgut trypanolysin.

Sodium dodecyl sulphate (SDS)-PAGE was used for determination of molecular weight of the purified trypanolysin. This was carried out using the method described by Leammli (1970) in a vertical electrophoretic unit using 25 mM Tris-glycine buffer, pH 8.3 containing 0.1% SDS. Gradient gels (10-20% acrylamide) were used for the separation while 3.5% acrylamide gel was used as stacking gel. The samples plus the molecular weight markers were denatured by boiling for two minutes in equal volumes of sample buffer (0.125 M Tris/HCl pH 6.8, 5% SDS, 10% β-mercaptoethanol, 10% glycerol and 0.002% Bromophenol blue) prior to loading onto the gel. The gels were run at constant current of 20 mA until the Bromophenol tracking dye reached the end of the stacking gel then increased to 25 mA.

The gels were then silver stained as described by Wray *et al.* (1981). Briefly the proteins protein fixation was done by soaking the gels in 50% methanol, 10% acetic acid in distilled water for two minutes. The gels were then rinsed twice in 50% methanol for twenty minutes each then incubated for 30 minutes in 2.5% glutaldehyde. solution A (0.8g $AgNo_3$ in 2.5ml distilled

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water) was used to titrate solution B (1ml of 2M NaOH and 1.6ml NH_4OH adjusted with distilled water to a volume of 20ml). The gels were placed in the solution for fifteen minutes and later rinsed thoroughly in distilled water. Colour development was achieved by immersing the gels in a color developer (2.5ml of 1% citric acid (w/v) and 125µl of 38% formalin adjusted to 250ml with distilled) until a clear coloration was achieved. This reaction was stopped using a 5% acetic acid and the gels stored in 7% acetic acid prior to photographing

3.8.2 Two-dimensional gel electrophoresis

Isoelectric focusing was done on mini protein II system (Bio-Rad) according to the method of O'Farrel (1975). Briefly, casting of IEF gel was done using a gel solution 5% acrylamide, 3% ampholytes and 15% (w/v) glycerol. Samples were dissolved in equal volumes of first dimension buffer (9.5 M urea, 2% Triton X-100, 5% β-Mercaptoethanol, 1.2% ampholyte (pH 3.5-10)). Ammonium persulfate and TEMED were added just before loading the gel into the capillary tubes. The carrier ampholytes in the IEF gel were pre-focused for 10 min at 200 volts, 15 min at 300 volts and 500 volts to generate a pH gradient. 10 µl of the protein was then applied to each tube, overlaid with 25 µl of sample buffer (9 M urea, 0.8% 3/10 ampholyte (Sigma), 0.0025% bromophenol blue) and focused at 500V for 10 min and 750 volts for 3.5 hrs. The tube gels were then extruded from the tubes and run on a 10-20% SDS-PAGE and silver stained as previously described. The isoelectric point of midgut trypanolysin was estimated by comparing the relative mobility to that of pI markers run under similar conditions.

3.8.3 Thermal stability of *S. gregaria* midgut trypanolysin

Aliquots of the midgut trypanolysin were separately incubated for 20 minutes in water baths maintained at different temperatures (30, 40, 50, 60, 70°C). The samples were then cooled to room temperature (27°C) and 20µl of each sample used for trypanolysin assay as previously described. The control was maintained at room temperature.

3.8.4 Staining for Carbohydrate

To establish whether the *S. gregaria* midgut trypanolysin is glycosylated, staining for glycoproteins was carried out. This was done by running SDS-PAGE as previously described followed by periodic acid schiff staining by the method of Trivedi *et al.*, 1983. The gels were

fixed in a solution containing 40% methanol and 7% acetic acid for 1 hr then oxidised in 1% periodic acid and 3% acetic acid at room temperature for 2 hrs. The gels were then incubated in schiff reagent for 50 min in the dark and rinsed in 0.5 % sodium metabisulphite for 1 hr. The gels were then rinsed in large volume of distilled water.

3.8.5 Effect of sugars

The effect of sugars (N-acetylglucosamine, D-glucosamine, D-galactose and D-glucose) on the trypanolytic activity was assayed by separately pre-incubating 20µl of 1M sugar solutions with a 20µl of the trypanolysin for 30minutes at 27°C. Controls were set up consisting of sugars and buffer in the place of the trypanolysin. Lytic assay was then carried out as described in section 3.5 above.

3.8.6 Effect of divalent cation on the lytic activity

The effect of divalent cations (Ca^{2+} and Mg^{2+}) on trypanolysin's activity was assayed by incubating equal volumes of test fractions with 0.016 M EDTA, pH 7.4 overnight at 10°C. The mixture was then dialysed against PBS at 4°C for 48 hours. To 20 µl of the EDTA treated sample, 20µl of increasing concentrations of $MgCl_2$ and $CaCl_2$ (0.1-10 mM) from stocks prepared in PBS and adjusted to pH 7.4 were then added (Stiles *et al.*, 1990). Lytic activity was then carried out as previously described.

3.9 Immuno-cross reactivity study of the trypanolysin

3.9.1 Preparation of anti-trypanolysin antibody

Anti-trypanolysin antibodies were raised in a naive New Zealand white rabbit. Purified trypanolysin (500 µl of 0.56 mg protein) was completely emulsified with Freund's complete adjuvant (Sigma) in the ratio of 1:1 (v/v) and injected intramuscularly at multiple sites on the hind thigh of the rabbit using a 1ml syringe and 23 ½ G needle. After three weeks a booster consisting of 0.28 mg of the trypanolysin in Freud's incomplete adjuvant (Sigma) was administered as described and a second booster 0.28 mg of the trypanolysin in Freud's incomplete adjuvant after the fifth week. The rabbit was bred after a fortnight and each time before immunization from the marginal ear vein collecting 3 ml of blood each time. The fresh blood obtained was left to stand at room temperature for 30min and then overnight at 4°C.

Serum was then obtained by centrifuging at 1,200 x *g* for 30 min at 4°C and kept at –20°C until used. A dot blot check was performed to check for the presence of the anti-trypanolysin antibodies using the procedure described in section 3.9.2 below except that 2 µl of the serum samples were loaded directly on to the nitrocellulose paper rather than being transferred from a gel.

3.9.2 Western blot analysis

The purified trypanolysin was electrophoresed under denaturing conditions on a 10-20% gradient SDS-PAGE mini gel and transferred onto a nitrocellulose paper by semi dry blotting using a Bio-Rad transblot apparatus. The transfer was carried out at a current 3 mA/cm^2 of the membrane for 2 hrs. To ascertain transfer, the blot was stained using Rouge Ponceau S solution [5% (w/v) in 1% acetic acid] and destained in distilled water until the protein bands were clear. Further washes with distilled water were performed to wash off all the ponceau. The nitrocellulose paper was then incubated overnight with the blocking solution (20 µg/ ml BSA in coating buffer containing 1% gelatin) and then washed (3 x 10 min) in PBS-Tween (0.05%Tween 80 in PBS, pH 7.4). It was then incubated in a 1:400 dilution of immune sera in blocking buffer for 1 hr and washed as above before incubating with horse radish peroxidase-conjugated goat anti-rabbit secondary antibody (1:2500 dilution in coating buffer (0.05M bicarbonate buffer pH 9.9)) for 45 minutes on a rocking shaker. This was then washed (3 x 10 min) in PBS-Tween and bands revealed by incubating with the substrate, 4 - chloro-1-Napthol (1 part diluted in 6 parts of 0.05M Tris/HCl, pH 7.4) from a stock solution containing 3 mg/ml of 4 - chloro-1-Napthol in absolute methanol and 10 µl of 30% Hydrogen peroxide. Further colour development was stopped by rinsing in distilled water.

CHAPTER FOUR

4.0 RESULTS AND DISCUSSION

4.1 RESULTS

4.1.1 Purification of trypanolysin.

Purification of the locust midgut trypanolysin was achieved through three chromatographic steps involving gel filtration using Sephadex G-150, anion exchange using DEAE cellulose and affinity chromatography using Con A Sepharose and electro elution from a Native PAGE gel.

A Sephadex G-150 gel filtration column of internal diameter 1.42 cm X 85 cm bed height was set up as described under materials and methods and calibrated before loading the midgut homogenate. Blue dextran 2000 (Sigma) was used to estimate the void volume of the column. The void volume was estimated to be 43.5 ml. β-amylase carbonic anhydrase, alcohol dehydrogenase and bovine serum albumin (Sigma) were used to calibrate the column. Separation of crude locust midgut homogenate on a Sephadex G-150 column yielded four protein peaks (Fig. 2). The first and third protein peak did not show any activity while in the second protein peak, agglutination was noted while the fourth (main peak) gave a relatively impure trypanolytic fraction. The molecular weight of the trypanolysin was estimated to be about 29 kDa from the gel filtration column.

The trypanolytic fraction collected from gel filtration was concentrated and extensively dialysed before being loaded onto the DEAE anion exchange column. The locust midgut trypanolysin bound onto DEAE anion exchange column. The bound fraction was eluted using a linear ionic strength gradient (0-350 mM NaCl) giving three peaks (Fig. 3). Screening for lytic activity revealed that the trypanolysin eluted at about 260 mM salt concentration (peak 3).

Fractionation of the lytic fraction from anion exchange column using affinity chromatography on a concanavalin A column yielded one peak in the unbound and one peak in the bound fraction (Fig. 4). Trypanolytic activity was recovered in the unbound fraction indicating that locust midgut trypanolysin does not bind to a Con A affinity column. Further purification of the midgut trypanolysin was achieved via electro-elution from a native PAGE gel. This resulted in a pure protein showing one band on native PAGE gel.

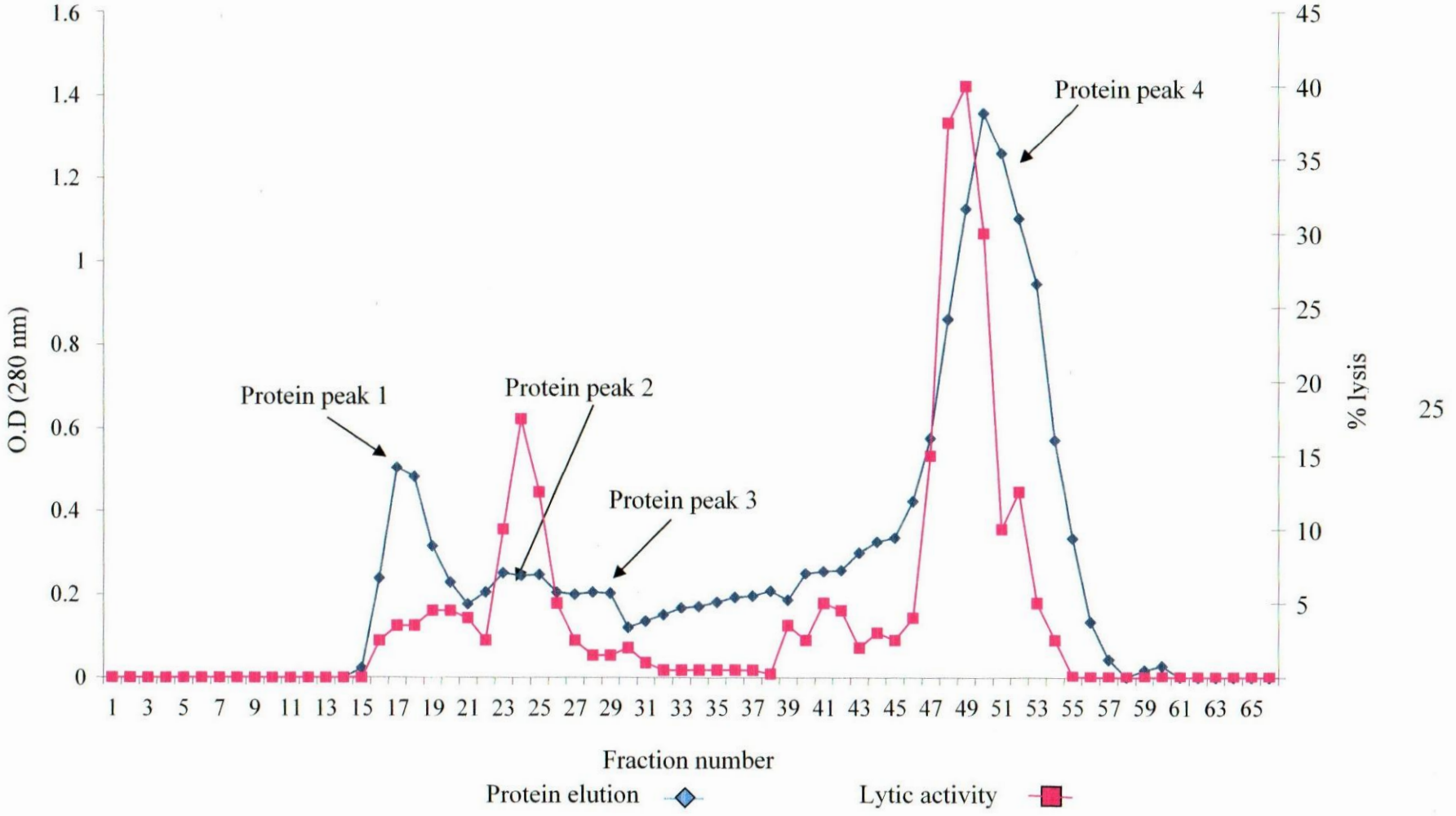


Figure 2: Separation of crude midgut homogenate on a Sephadex G-150 column. The column was eluted with PBS, pH 7.2 and fractions (3 ml) collected and assayed for trypanolytic activity. Trypanolytic activity was recovered in peak 4.
Peak 2 showed agglutination activity.

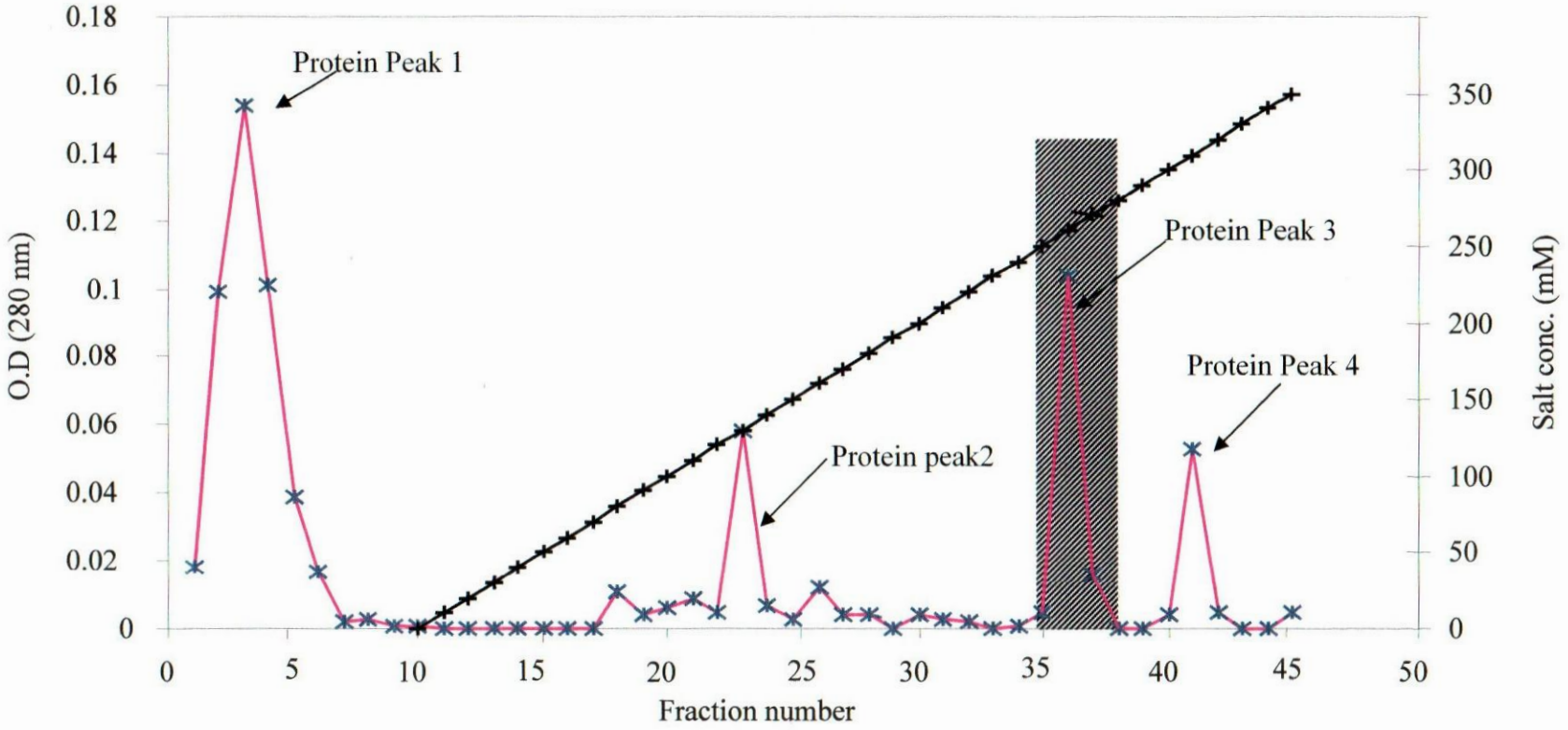


Figure 3: DEAE anion exchange chromatography profile of the trypanolytic fraction obtained from gel filtration. The unbound fraction was eluted using 20 mM PBS, pH 8.0 while the bound fraction was eluted using a 0-350 mM salt gradient (—×—). The lytic activity (▨) was recovered at 260 mM salt concentration (Peak 3). Peak 1 represents the unbound fraction. The (—×—) represents the protein elution profile.

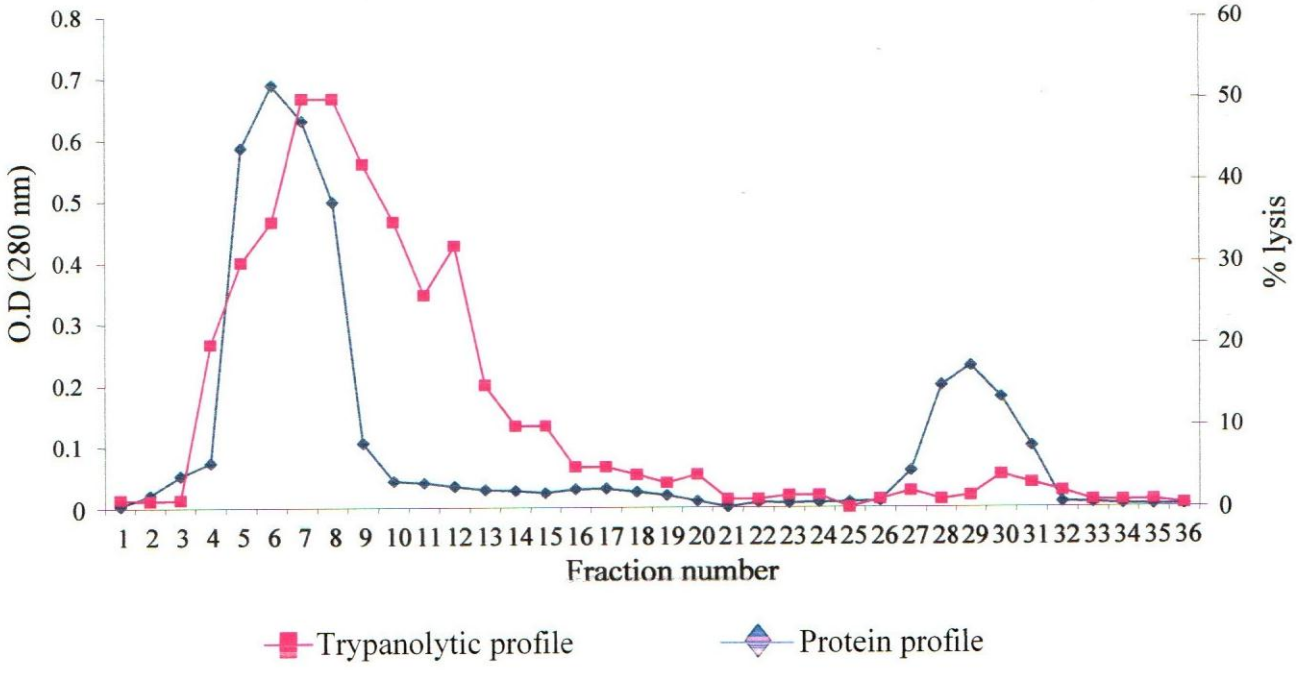


Figure 4: Fractionation of the trypanolytic fraction from anion exchange column on a Concanavalin–A sepharose affinity column. The unbound fraction was eluted using 20 mM Tris HCl, pH 7.2 while the bound fraction was eluted using 500 mM methyl-α D-manopyranoside in 20 mM Tris HCl, pH 7.2. The fractions (0.5 ml) collected were assayed for trypanolytic activity as described in section 3.5. Trypanolytic activity was recovered in the unbound fraction.

4.1.2 Physico-chemical characterization

The purified trypanolysin obtained was used for the characterization of the locust midgut trypanolysin. The Native molecular weight of the purified trypanolysin was determined by use of native PAGE to be about 28 KDa (Fig. 5). A plot of Kav against molecular weight also gave an estimate of the molecular weight of the peak showing trypanolysin molecule to be about 28 KDa. The molecular weight under reducing conditions was found to be about 29 KDa as depicted by SDS-PAGE (Fig. 6). Locust midgut trypanolysin is therefore a single polypeptide chain since it gave approximately the same molecular weight under native and denaturing PAGE.

Locust midgut contains hundreds of proteins as depicted on two dimensional (2D) Electrophoresis (Fig. 8). Most of these proteins have pIs in the acidic range. Analysis of purified locust trypanolysin on 2D electrophoresis (isoelectic focusing followed by SDS PAGE) yielded a single protein spot (Fig. 9). The isoelectric point of midgut trypanolysin was determined by comparing the relative mobility of the protein to relative mobility of pI markers from an identically run gel (Fig. 7). From the plot of pI against Rf, the pI of the trypnolysin was estimated to be 6.3. This suggests that *S. gregaria* trypanolysin has a bias towards acidic amino acid in composition.

The midgut trypanolysin is not glycosylated. This is evidenced by the fact that it failed to stain using Periodic Acid Schiff stain (Fig. 10). This augmented the fact that locust midgut trypanolysin did not bind onto a Con-A sepharose affinity column (Fig. 4).

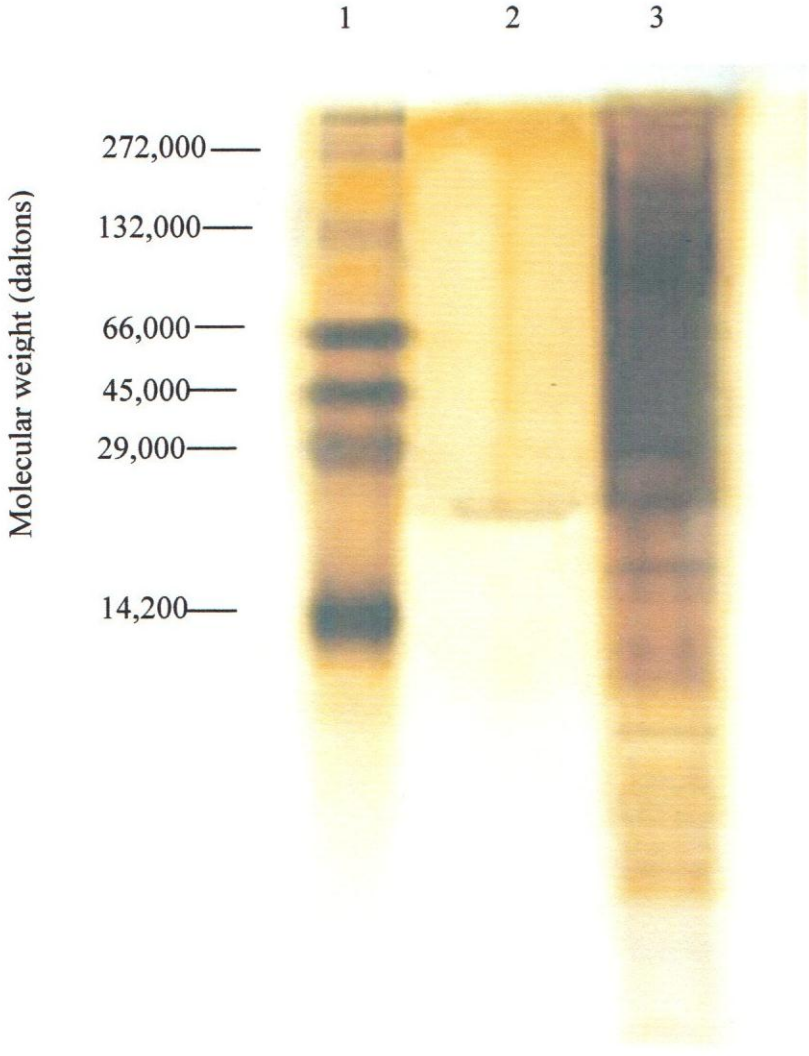


Figure 5: Analysis of midgut trypanolysin on a 5-15% native-PAGE. The gel was run at a constant voltage (70 V) and silver stained.

Lane 1 - Crude homogenate (20µg)

2 - Purified trypanolysin (10µg)

3 - Molecular weight standards (Sigma) (15µg)

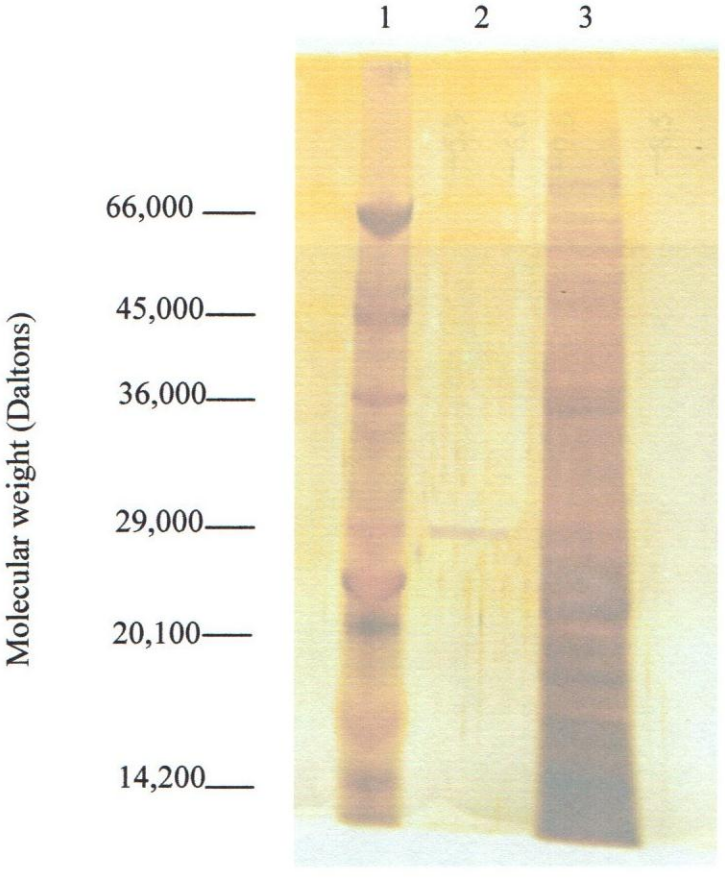


Figure 6: Analysis of midgut trypanolysin on a 10-20% SDS-PAGE. The gel was run at a current (25 mA) and silver stained.

Lane 1. Low molecular weight markers (Sigma) (15µg)

2. Purified trypanolysin. (10µg)

3. Crude midgut homogenate (20µg)

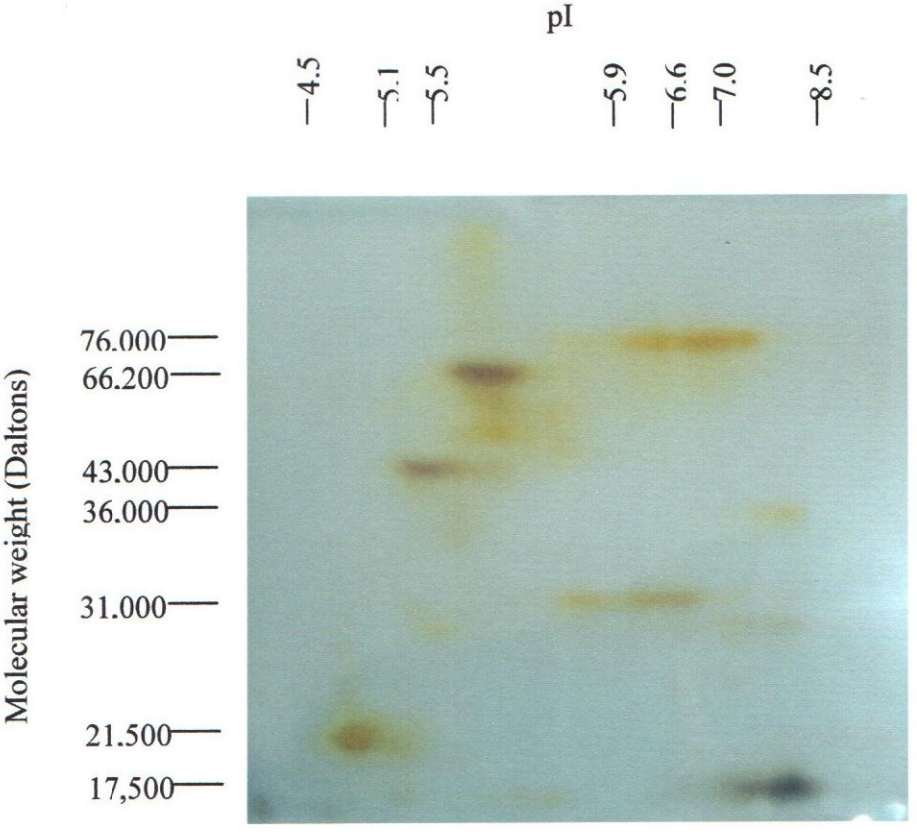


Figure 7: Estimation of pI of trypanolysin. 10 µl of 2D SDS-PAGE standards (Bio-Rad) were run in a capillary isoelectric focusing gel (pH 3.5-10) then laid on a 10-20% SDS-PAGE and run at 25 mA. A plot of Rf against pI was constructed and used for pI estimation.

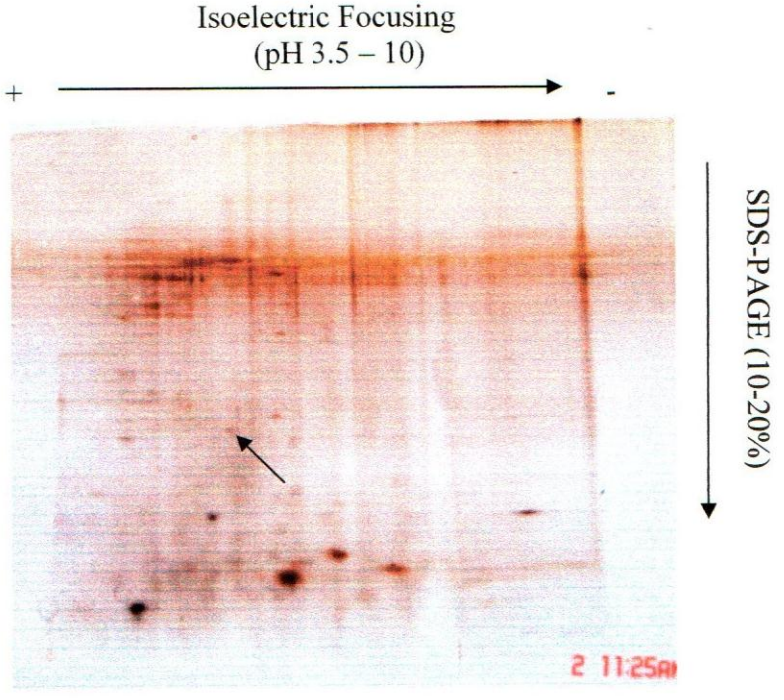


Figure 8: 2D electrophoresis of crude midgut homogenate. In the first dimension 10µg of crude midgut homogenate were separated by Isoelectric focusing (pH 3.5-10) in a capillary tube. The tube gel was extruded and then loaded onto a 10-20% SDS-PAGE for the second dimension. The gel was run at a constant current (25 mA) and silver stained. The position of the trypanolysin is indicated by the small arrow based on the established properties and by superimposition of gels in figures 8 and 9 using the Flicker® program.

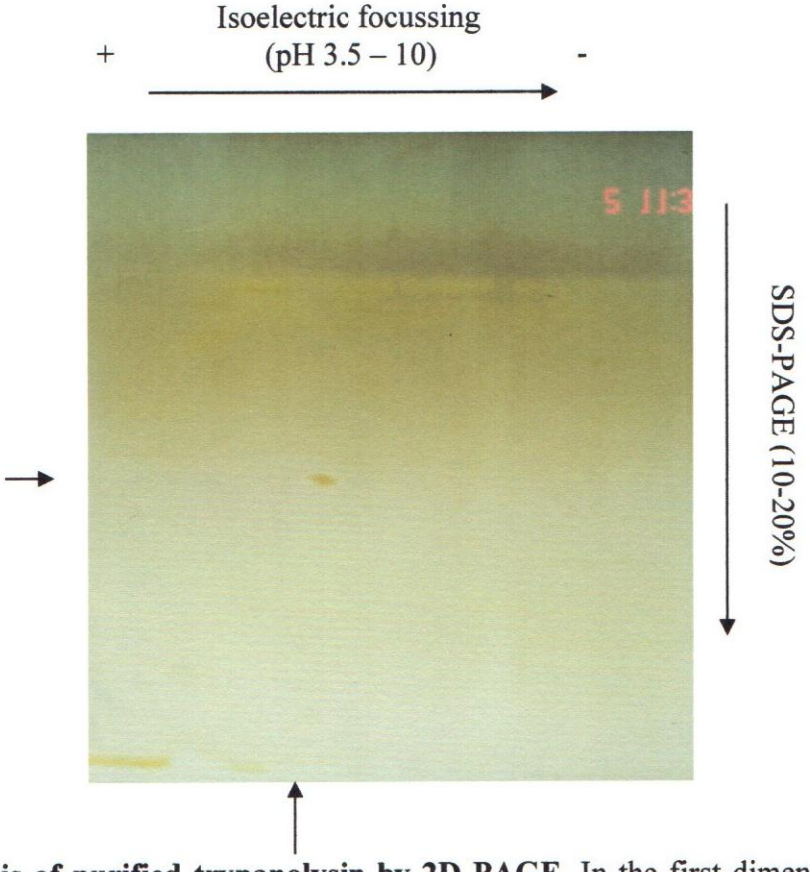


Figure 9: Analysis of purified trypanolysin by 2D PAGE. In the first dimension 2µg of the trypanolysin was separated by Isoelectric focusing (pH 3.5-10) then loaded on to a 10-20% SDS-PAGE for the second dimension. The position of the trypanolysin is indicated by the small arrows.

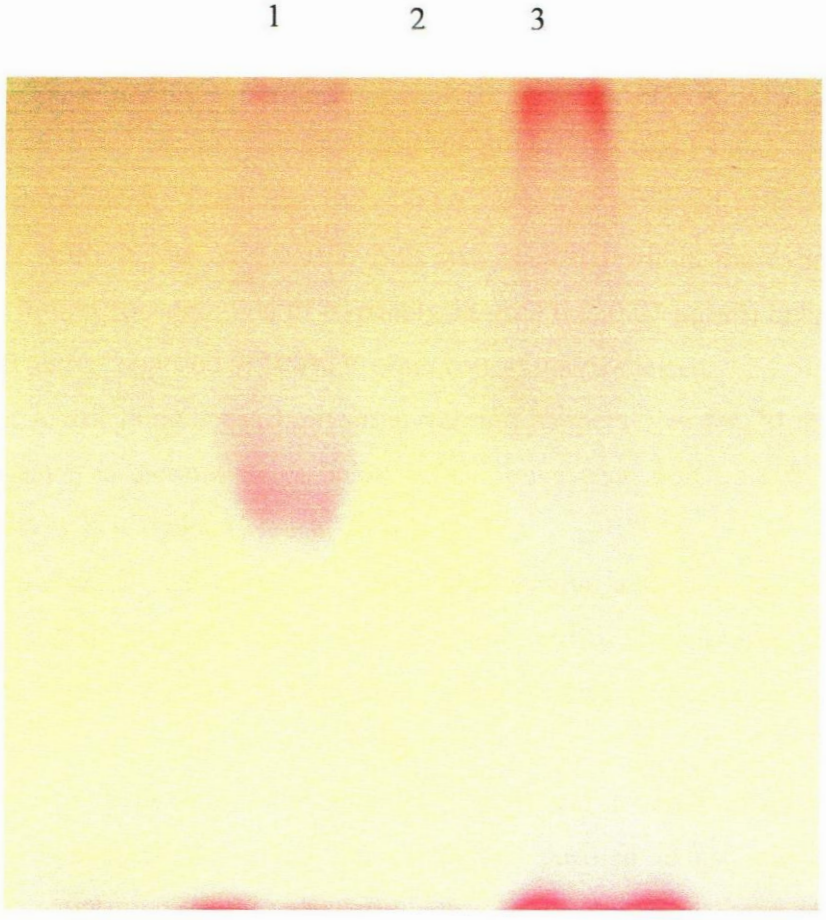


Figure 10: Analysis of midgut trypanolysin for glycoprotein by Periodic Acid Schiff staining.

Lane 1- Positive control. (Egg albumin) (20 µg)

Lane 2- Purified midgut trypanolysin (10µg)

Lane 3- Crude midgut homogenate (20µg)

4.1.3 Activity of midgut trypanolysin.

The effect of variation of the concentration of trypanolysin on the rate of trypanosome lysis was studied to establish the optimum concentration for use in subsequent experiments.

When doubling serial dilutions of purified trypanolysin was incubated with isolated bloodstream trypanosomes *in vitro* the rate of lysis decreased rapidly initially and leveled off with time. The higher the concentration the faster the lysis. Total lysis was achieved at concentrations 0.26 µg/ml and above while below only partial lysis was achieved within the experimental period (Fig. 11). At the lowest concentration of trypanolysin used (i.e. 0.07 µg/ml) only 70 % lysis was achieved after 90 min as opposed to 100% in other concentrations tested.

Incubation of locust midgut trypanolysin at varying temperatures for 20 min prior to lytic assay indicated that it is thermal stable below 40°C. However at 50°C, a 60% loss of activity occurs and its activity is completely lost at 70°C (Fig. 12).

The sugars D-glucose, D- galactose, N- acetyl glucosamine and D-glucosamine at 500mM concentration did not significantly reduce the activity of purified trypanolysin. Only less than 10 % loss in activity was observed with each of the sugars compared to the controls (without the sugars) (Fig. 13).

The presence of divalent cations Ca^{2+} and Mg^{2+} singly and in combination at concentrations ranging from 0-10 mM did not affect the activity of purified locust midgut trypanolysin. Incubation of trypanosomes with trypanolysin pre-incubated with these salts at varying concentrations and dialysed extensively before lytic activity assay gave results that were not significantly different from the one devoid of the salts (Fig. 14). This was true when the salts were used singly (Fig. 14) and when used in combination (Fig. 15).

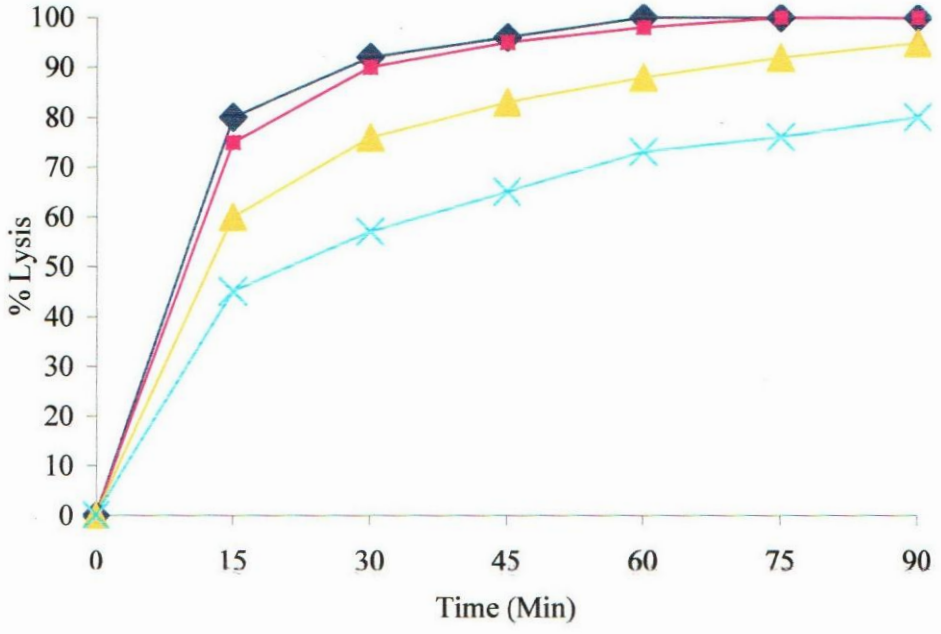


Figure 11: Trypanosome lysis by varying concentrations of purified trypanolysin. 20µl of purified trypanolysin (◆) 0.52 µg/ml; (■) 0.26µg/ml; (▲) 0.13µg/ml and (×) 0.07µg/ml were incubated with isolated bloodstream *T. b. brucei.* (~5.0 x 10^6 trypanosomes per ml) at room temperature. The number of intact parasites was monitored every 15 minutes and the mortality rate estimated by comparison to the control (without the trypanolysin).

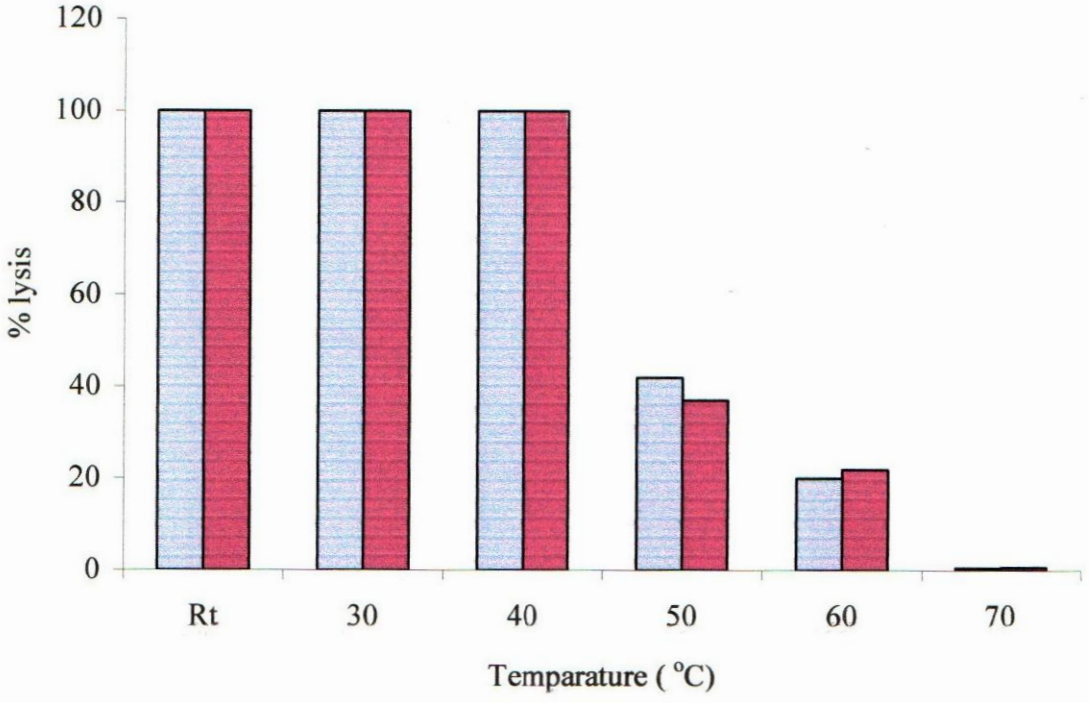


Figure 12: Analysis of trypanolysin for thermal stability. The purified trypanolysin ( ) and Crude midgut homogenate ( ) were separately incubated for 20 minutes at different temperatures (30, 40, 50, 60, 70°C) then used for lytic assay as described in section 3.5. Three determinations were carried out and the values averaged.

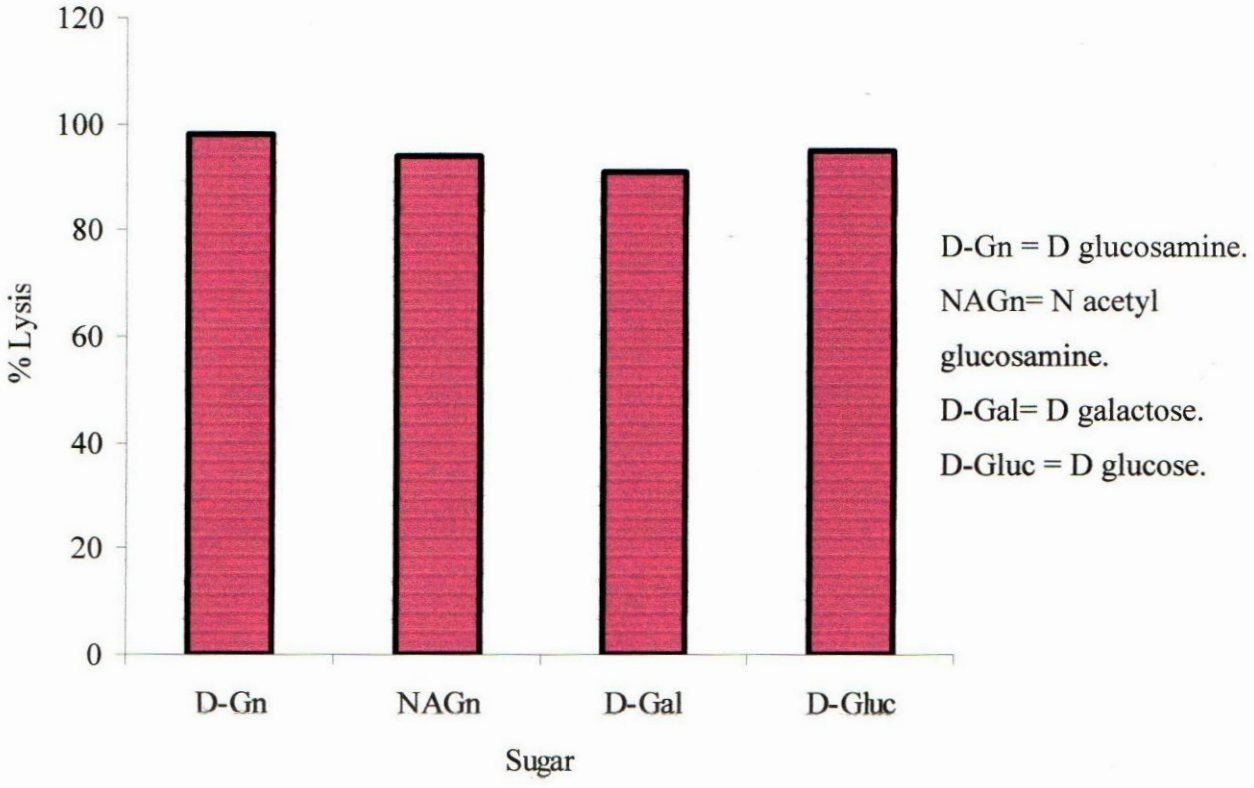


Figure 13: Effect of sugars on the lytic activity of purified locust midgut trypanolysin. The trypanolysin (20µl) was pre-incubated with 20µl of 1M sugar solutions for 30 minutes at 27° C before being used for lytic assay controls were set up consisting of sugars and buffer in the place of the trypanolysin. Three determinations were carried out and the values averaged.

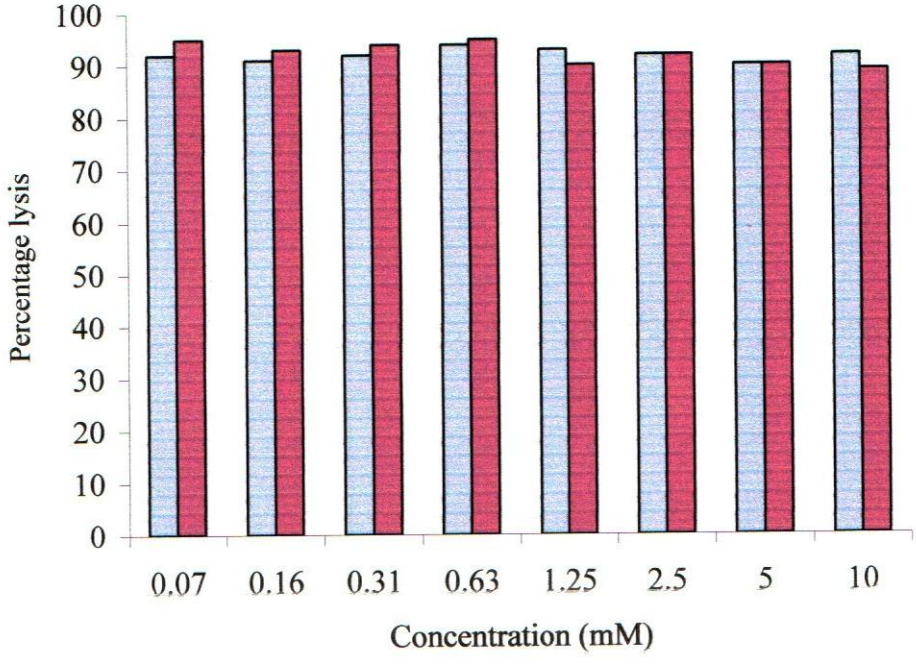


Figure 14: Effect of divalent cations Mg^{2+} and Ca^{2+} on the lytic activity of the trypanolysin. The trypanolysin was incubated with EDTA overnight dialysed for 48 h. To 20µl of the EDTA treated sample, 20µl of increasing concentrations of $MgCl_2$ ■ or $CaCl_2$ ■ (0.1-10mM) were added prior to lysis assay. Three determinations were carried out and the values averaged.

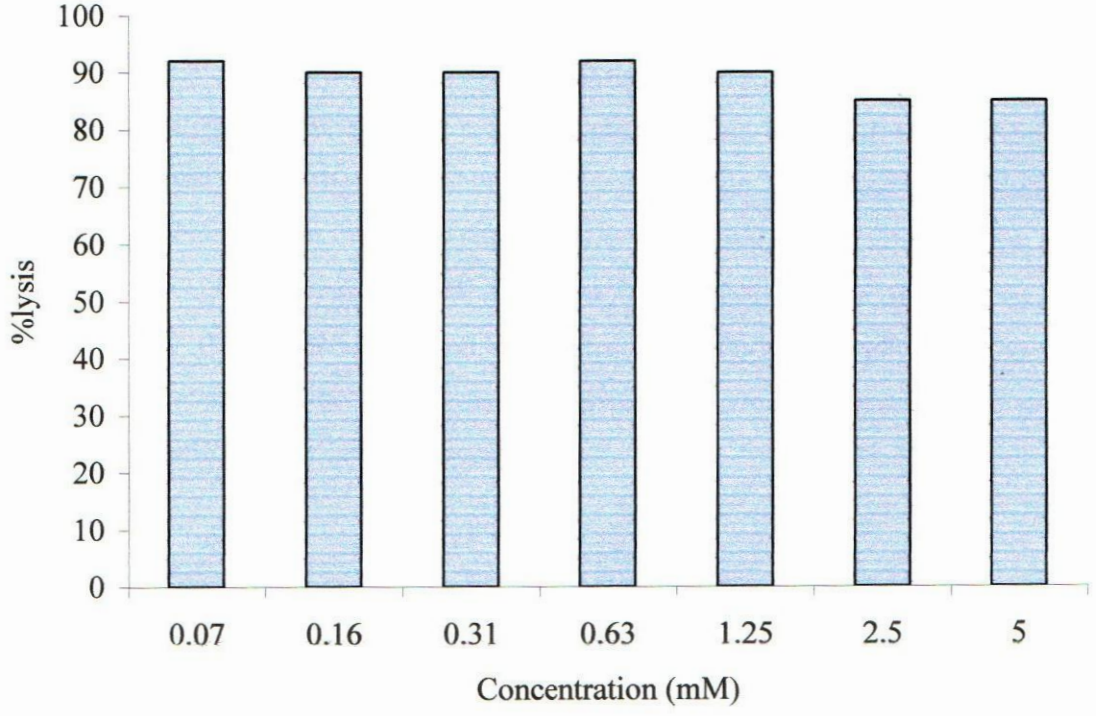


Figure 15: Effect of a combination of divalent cations Mg^{2+} and Ca^{2+} on the lytic activity of the trypanolysin. Details are as in Figure 14 except the salts were combined. Equal concentrations of the salts were used for each test.

4.1.4 Immuno-cross reactivity analysis.

Antibodies were raised against purified trypanolysin in a naïve New Zealand white rabbit. The antiserum was used to check for cross-reactivity with midgut extracts from haematophagous and non-haematophagous insects. The proteins were separated by SDS-PAGE before transferring to a nitrocellulose membrane for probing. Anti-trypanolysin antibodies cross reacted with the purified trypanolysin and to the single band corresponding to the trypanolysin in the crude locust midgut extracts. It however failed to cross react with crude midgut extracts from haematophagous (tsetse, sand fly and mosquito) and non-haematophagous (cockroach and housefly) insects used. Further, there was no evidence of cross-reaction with other proteins from S. gregaria midgut extract other than trypanolysin.

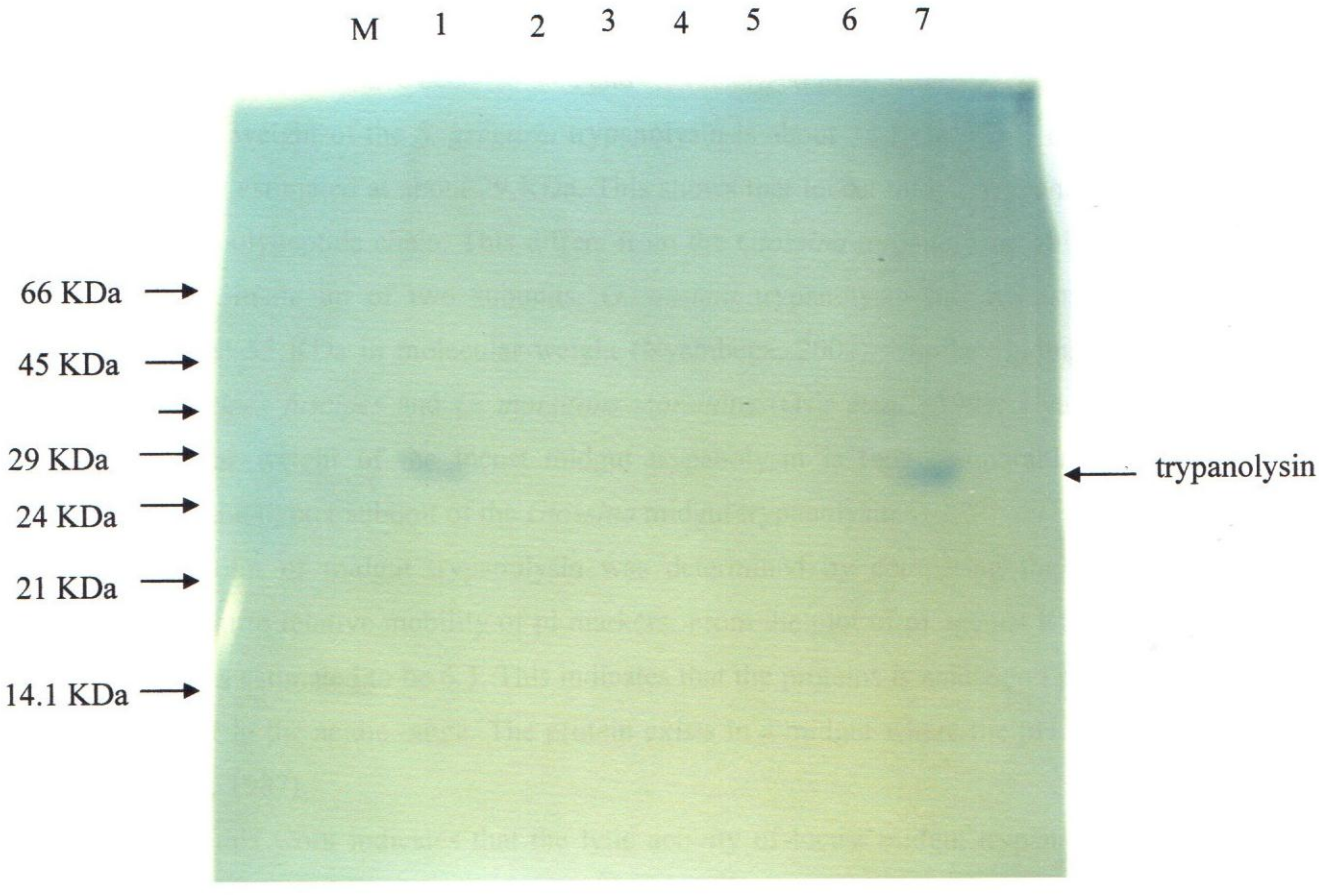


Figure 16: Immuno-cross reactivity analysis of anti-locust midgut trypanolysin antibodies.

Crude midgut extracts (20µg) from haematophagous and non-haematophagous insects were run on 10-20% SDS-PAGE, transferred onto nitrocellulose paper and incubated with anti-locust midgut trypanolysin antibodies.

Lane M. Molecular weight markers (Sigma)

1. Purified midgut trypanolysin
2. Crude tsetse midgut homogenate.
3. Crude sand fly midgut homogenate.
4. Crude cockroach midgut homogenate.
5. Crude housefly midgut homogenate.
6. Crude mosquito midgut homogenate.
7. Crude locust midgut homogenate

4.2 DISCUSSION

Tsetse midgut trypanolysins have been isolated and characterized in *G. fuscipes, G. morsitans* The native molecular weight of the *S. gregaria* trypanolysin is about 28 KDa, while denatured molecular weight was estimated at about 29 KDa. This shows that locust midgut trypanolysin is made up of a single polypeptide chain. This differs from the *Glossina* trypanolysin which have been reported to be made up of two subunits. *G. austeni* trypanolysin has two sub units approximately 29 and 55 KDa in molecular weight (Nyambega, 2002). Similar findings were observed for *G. fuscipes fuscipes* and *G. morsitans morsitans* (Osir *et al.*, 1999; Nyambega,, 2002). The molecular weight of the locust midgut trypanolysin is thus comparable to the molecular weight of the lighter subunit of the *Glossina* midgut trypanolysin.

The isoelectric point of midgut trypanolysin was determined by comparing the relative mobility of the protein to relative mobility of pI markers. From the plot of pI against Rf , the pI of the trypnolysin was estimated to be 6.3. This indicates that the proteins is acidic and probably have an optimum pH in the acidic range. The protein exists in a midgut where the pH is about 6.7-7.0 (Martin *et al.*, 1987).

The results from this work indicates that the lytic activity of locust midgut trypanolysin is independent of the presence of divalent cations Mg^{2+} and Ca^{2+} implying that the two ions are not necessary for its lytic activity. Purified locust midgut trypanolysin's lytic activity was also not inhibited by incubation with the amino sugar D-glucosamine. This implies that the trypanolysin's lytic activity is independent of D-glucosamine. D-glucosamine has been shown to specifically block the activity of *Glossina* proteolytic lectin (Abubakar 2003). Further, it was reported ealier that trypanolytic activity in crude midgut homogenates of *S. gregaria, P. americana, P. humanas humanas* was inhibited by D-glucosamine (Ogoyi *et al.*, 2003) . Glucosamine has also been shown to inhibit tsetse midgut proteolytic action of trypsin *in vitro* (Gibson and Bailey, 2003). D-glucosamine has also been reported to specifically and competitively inhibit trypsin in crude midgut homogenates of *G. morsitans morsitans in vitro* while glucose, fructose, mannose, inositol, galactose, galactosamine and *N*-acetyl-D-glucosamine had no significant effect (Osir *et al.*, 1992).

The inhibition reported earlier when crude midgut homogenate was used, (Ogoyi *et al.*, 2003) may therefore have been associated with the loss of lysis contributed by other factors such as trypsin, trypanocidal lectins and anti-microbial peptides. It is not clear at this juncture what

contribution to the overall trypanosome lytic activity emanate from factors other than the isolated trypanolysin. In tsetse fly for example Glossina proteolytic lectin (*Gpl*) which is trypanocidal, has been shown to have both lectin and trypsin component (Abubakar *et al.*, 1995). These molecules are also associated with lysis of trypanosomes in addition to that of trypanolysin and in crude midgut extracts they would have a contribution to the total lytic activity. It has been shown that lectins requires trypsin for their activity therefore in absence of trypsin, the activity of the molecule may be greatly compromised. Susceptibility of tsetse flies to trypanosome infection, which is associated with high midgut glucosamine levels, could be due to inhibition of trypsin or trypsin-like enzymes by this sugar (Osir *et al.*, 1992). Nyambega (2002) suggested that trypanolysins interacts with trypanosomes via the trypanosomes variable surface glycoprotein (VSG) and hypothesized that trypsin may be required for exposure of trypanolysin binding sites on the trypanosomes. However, results from this work shows that the activity of the locust trypanolysin in its purified form were comparable to the crude which indicates its independence from other molecules such as trypsin normally found in the insect gut. The time course for the release of trypanolysin in tsetse fly has been shown to coincide with that of anti-microbial peptides, lectins, trypsin and trypsin-like molecules and digestive enzymes and movement of food along the gut (Gibson and Bailey, 2003). Interestingly the elimination of trypanosomes coincides with digestion and movement of food within the gut. Osir *et al* (1999) attributed the destruction of trypanosomes in the midgut to trypanolysin activity in addition to other factors. Further studies therefore need to be carried out to establish the interaction between factors like lectins, trypsin/ trypsin like molecules and trypanolysins in trypanosome lysis.

Antibodies raised against the purified locust midgut trypanolysin failed to cross-react with crude midgut homogenates of non-haematophagous (housefly and cockroach) and haematophagous (tsetse fly, sand fly, and mosquito) insects tested. Similarly anti-*Glossina* proteolytic lectins antibodies did not show any cross reactivity with locust haemolymph proteins (Njagi, personal communication). This lack of cross-reactivity may point to the fact that this may be a unique protein found only in the locust *S. gregaria*. Earlier reports (Ogoyi *et al.*, 2003) indicated the presence of trypanolytic activity in crude midgut homogenates and haemolymph of both non-haematophagous and haematophagous insects albeit at varying levels. The results from the current study may therefore mean that the trypanolytic protein found in locust midgut is different from the trypanolytic proteins found in other insect species used in this study and thus

the difference in the level of activity earlier reported in these insects. The complete exclusion of development of trypanosomes in non-vector insects may therefore be due to the presence of specific trypanolysins which the parasite is unable to circumvent for successful development to occur.

Since locust midgut homogenate had shown the highest lytic activity, further work need to be carried out to establish the value and possible applications of this midgut trypanolysin in trypanosomosis control. Recent advances in molecular technologies and their application in insect science / technology have revolutionized the field of vector biology, and there is hope that such new approaches may form the basis for future tsetse and trypanosomosis control strategies.

Transgenesis is being explored with the aim of modulating vector competence by expressing foreign genes with anti-pathogenic properties that can interfere with viability, development and or transmission (Aksoy, 2000). With the advances in technology it is possible to prospect for such genes across the haematophagous vector divide and engineer them in appropriate vehicles for delivery to the natural vector. Tsetse, the vector for trypanosomosis harbors endosymbionts in their midgut of which genetic transformation systems have been developed so that foreign genes can be introduced and expressed in them (Beard *et al.*, 1993). One such bacterium is *Sodalis glossinidius* which is found in the midgut of tsetse. Since genetic transformation systems for the endosymbiont have been developed (Beard *et al,* 1993), engineered *S glossinidius* can therefore be used to deliver foreign genes to the vector midgut. Synthesis and secretion of anti-typanosomal gene products *in vivo* can provide a mechanism for disruption of parasite establishment in the gut thereby breaking the disease transmission cycle.

CHAPTER FIVE

5.0 CONCLUSIONS AND RECOMMENDATIONS

5.1 CONCLUSIONS

Purification of trypanolysin was successfully carried out from the midgut of a non-vector non-haematophagous insect (*Schistocerca gregaria).* This study shows that trypanolysins are also found in non-vector non-haematophagous insects. This study confirms presence of a trypanolysin in the midgut of the desert locust, *S. gregaria*, which is active in crude and purified forms.

The study also demonstrates that it is possible to purify this protein to homogeneity without compromising its lytic activity. From the results of this study, it is possible to infer that the lytic activity of this molecule is independent of other molecules commonly found in the midguts of insects such as trypsin and lectins. Activity of this protein was not significantly affected by presence of sugars such as D-glucosamine, N-Acetyl glucosamine and divalent cations like Calcium and Magnesium which have been shown to affect the activity of tsetse midgut trypanolysin.

Because of its inability to cross-react with proteins in crude midgut homogenates of the other insects used in this study, it is apparent that this protein may be unique to *S. gregaria*. It is therefore possible to deduce that the failure of developmemt of trypanosomes in non-vector insects may be due to the low levels and lack of specificity of their midgut trypanolysins.

5.2 RECOMMENDATIONS

This study has culminated with the purification of a protein that is clearly lytic to trypanosomes *in vitro*. Further studies need to be carried out to verify whether this molecule has the same activity in the insect midgut. This study did not investigate the interactions between the trypanolysin and other molecules like trypsin, trypsin-like molecules lectins and other digestive enzymes found in the insect gut. It would be interesting to investigate how these molecules influence the lytic activity of the trypanolysin in the *in vitro* and *in vivo* systems.

Locust midgut trypanolysin have comparable molecular weight to the lighter sub-unit of the trypanolysin from *Glossina* species It would be important to carry out N-terminal sequencing of this sub-unit as well as that of the locust midgut trypanolysin to determine whether the two could have sequence similarities. Further, the elucidation of the functions of each of the two sub-units of the *Glossina* trypanolysins could improve our understanding of tsetse trypanolysin interaction.

Further studies also need to be carried out to elucidate the gene coding for the locust midgut trypanolysin and carry out sequence homology alignment from DNA data banks to characterize its possible relationship with other genes coding for proteins with anti-trypanosomal activity.

REFERENCES

Abubakar, L. U., Bulimo, W. D., Mulaa, F. J. and Osir, E. O. (2003). Molecular analysis of a proteolytic lectin from the midgut of tsetse fly, *Glossina fuscipes fusipes*, that stimulates differentiation of African trypanosomes. *Insect Science and Its Application.* **23** (3): 197-205.

Abubakar, L., Osir, E. O. and Imbuga, M.O. (1995). Properties of blood-meal-induced midgut lectin from tsetse fly *Glossina morsitans. Parasitology Research.* **81**: 271-275.

Aksoy, S. (2000). Tsetse–A Haven for Microorganisms. *Parasitology Today.* **16** (3): 114 -118.

Aksoy, S., O'Neill S. L., Maudlin I., Dale C. and Robinson A. S. (2001). Prospects for control of African trypanosomosis by tsetse vector manipulation. *Trends in Parasitology.* **17** (1): 29-35.

Aksoy, S. (2003). Control of tsetse flies and trypanosomes using molecular genetics. *Veterinary Parasitology.* **115**: 125–145.

Anene, B. M., Onah, D. N. and Nawa, Y. (2001). Drug resistance in pathogenic African trypanosomes: what hopes for the future? *Veterinary Parasitology.* **96**: 83–100.

Barondes, S. H. (1981). Lectins: Their multiple endogenous cellular functions. *Annual Review of Biochemistry.* **50**: 207-231.

Beard, C. B., O'Neill, S. L., Mason, P., Mandelco L., Woese, C. R., Tesh, R. B., Richards, F. F. and Aksoy, S. (1993). Genetic transformation and phylogeny of bacterial symbionts from tsetse. *Insect Molecular Biology.* **1**: 123-131.

Beard, C. B., O'Neill, S.L., Tesh, R. B., Richards, F. F. and Aksoy, S. (1994). Modification of arthropod vector competence via symbiotic bacteria. *Parasitology Today.* **9**: 179–183.

Bienen, E., Hammadi, E. and Hill, G. C. (1981). *Trypanosoma brucei* biochemical and morphological changes during *in vitro* transformation of bloodstream to procyclic trypomastigotes. *Experimental Journal of Parasitology.* **51**: 408-417.

Biteau, N., Bringaud, F., Gibson, W., Truc, P. and Baltz, T. (2001). Characterisation of *Trypanosoma brucei* isolates using genetic microsatelite markers. *Molecular and Biochemical Parasitology.* **105**: 185-201.

Boulanger, N., Munks, J. L. R., Hamilton, J. V., Vovelle, F., Brun, R., Lehane, J.M., and Bulet, P. (2002). Epithelial innate immunity: a novel antimicrobial peptide with antiparasitic activity in the blood-sucking insect. *Stomoxys calcitrans. Journal of Biological Chemistry.* **277** (51): 49921-49926.

Brun, R. and Schonenberger, M. (1981). Stimulating effect of citrate and cis-aconitate on transformation of *Trypanosoma brucei* bloodstream forms to procyclic forms *in vitro. Z. Parasitenkd.* **66**: 17-24.

Centre for Disease Control (2004). Parasite and health. Trypanosomiasis, African fact sheet, http://www.dpd.cdc.gov/dpdx/HTML/Frames/S-Z/TrypanosomiasisAfrican, Date accessed 17/03/2008.

Cheeseman, M. T. and Gooding, R. H. (1985). Proteolytic enzymes from tsetse flies, *Glossina morsitans* and *Glossina palpalis* (Diptera: *Glossinidae*). *Insect Biochemistry.* **15** (6): 677-680.

Colvin, J. and Gibson, G. (1992). Host seeking behavior and management of tsetse. *Annual Review of Entomology.* **37**: 21-24.

Connor, R. (1994). African animal trypanosomiasis in infectious disease of livestock with special reference to southern Africa (Ed) Coetzer, J. A. W., Thompson, G. R. and Tustin R. C. 1: 167-205.

Crofts, S.,East, J. S. and Molyneux, D. H. (1982). Anti-trypanosomal factor in the haemolymph of *Glossina. Acta Tropica.* **39**: 239-302.

Dale, C. and Welburn, S. C. (2001). The endosymbionts of tsetse flies: manipulating host-parasite interactions. *International Journal of Parasitology.* **31** (5-6): 628-631.

Day, J. F. and Sjogren, R. D. (1994). Vector control by removal trapping. *American Journal of Tropical Medicine and Hygine.* **50**: (6) supplement 126-133.

Desqesnes, M. and Dia, M.L. (2003). *Trypanosoma vivax*: Mechanical transmission in cattle by one of the most common African tabanids *Atylotus agrestis. Veterinary Parasitology.* **119**: 9-19.

Desqesnes, M. and Dia, M. L. (2004). Mechanical transmission of *Trypanosoma vivax* in cattle by one of the most common African tabanids *Atylotus fuscipes. Experimental Parasitology.* **103**: 35-45.

East, J., Molyneux, D. H., Hillen, N. (1980). Haemocytes of *Glossina. Annals of Tropical Medicine and Parasitology.* **74**: 471-474.

East, J., Molyneux, D. H., Maudlin, I. and Dukes, P. (1983). Effects of *Glossina* haemolymph on salvarian trypanosomes *in-vitro. Annals of Tropical Medicine and Parasitology.* **77**: 97-99.

Food and Agriculture Organization (1991). Proceedings of the FAO expert consultation on the genetic aspects of trypanotolerance held in Rome, Italy, 3-4 September 1991. *FAO animal production and health paper* No. 94 pg 138.

Food and Agriculture Organization (1998). Integrated tsetse control and rural development. Joint meeting of the FAO panel of experts on technical ecological and development aspects of the programme for the control of African animal trypanosomosis and related developments. Accra Ghana, 7-9.

Food and Agriculture Organization (2004). The disease: Agriculture department animal production and health division-Programme Against African Trypanosomiasis, http://www.fao.org/ag/againfo/programmes/en/paat/disease.html, Date accessed 17/12/2007

Gibson, W. and Bailey, M. (2003). The development of *Trypanosoma brucei* within the tsetse fly midgut observed using green fluorescent trypanosomes. *Kinetoplastida Boilogy and Disease.* **2**: 1-6.

Gooding, R.H., (1992). Genetic variation in tsetse flies and implications for trypanosomosis. *Parasitology Today.* **8** (3): 92-95.

Gray and Roberts. (1971). The cyclical transmission of strain of *Trypanosoma congolense* and *T. vivax* resistant to normal therapeutic doses of trypanosomal drugs. *Parasitology.* **63**: 67-89.

Grubhoffer, L., Hypsa, V. and Volf, P (1997). Lectins' (hemagglutinins) in the gut of the important disease vectors. *Parasite.* **4** (3): 203-216.

Haines, L. R., Haddow, J. D., Aksoy, S., Gooding, R. H. and Pearson, T. W. (2002). The major protein in the midgut of teneral *Glossina morsitans morsitans* is a molecular chaperone from the endosymbiotic bacterium *Wigglesworthia glossinidius. Journal of Insect Biochemistry and Molecular Biology.* **32** (11): 1429-1438.

Hao, Z., Kasumba, I., Lehane, M. J., Gibson, W. C., Kwon, J., and Aksoy, S. (2001). Transmission: Implications for the development of tsetse-based strategies to reduce trypanosomosis. *Proceedings of the National Academy of Sciences.* **98** (22): 12648-12653.

Hargrove, J. W., Omolo, S., Msalilwa J. S. I. and Fox, B. (2001). Insecticide-treated cattle for tsetse control: the power and the problems. *Medical and Veterinary Entomology.* **14** (2):123 - 130

Holmes, P. H. (1997). New approaches to the integrated control of trypanosomes. *Veterinary Parasitology.* **71**: 121–135.

Hu, C. and Aksoy, S. (2006). Innate immune responses regulate trypanosome parasite infection of the tsetse fly *Glossina morsitans morsitans. Molecular Microbiology.* **60**: 1194–1204 .

Hunt, M., Brun, R., and Kohler, P. Studies (1994). Studies on compounds promoting the *in vitro* transformation of *Trypanosoma brucei* from bloodstream to procyclics forms. *Parasitology Research*. **80**: 600-606.

Imbuga, M. O., Osir, E. O., Labongo, V. L., Darji, N., Otieno, L. H. (1992a). Studies on tsetse midgut factors that induce differentiation of blood-stream *Trypanosoma brucei brucei in vitro*. *Parasitology Research*. **78** (1): 10-15.

Imbuga, M.O., Osir, E. O. and Labongo, V. L. (1992b). Inhibitory effect of *Trypanosoma brucei* on *Glossina morsitans morsitans* midgut trypsin *in vitro*. *Parasitology Research*.**78**: 273-276.

Ingram, G. A. and Molyneux, D. H. (1988). Sugar specificities of anti-human Abo (H) blood group erythrocyte agglutinins (lectins) and haemolytic activity in the hemolymph and gut extracts of 3 *Glossina* species. *Insect Biochemistry*. **18**: 269-279.

Ingram, G. A.and Molyneux, D. H. (1990). Haemagglutinins in haemolymph of *Glossina fuscipes fuscipes*: Isolation, partial characterization, selected physio-chemical properties and carbohydrate-binding specificities. *Insect Biochemistry*. **20**: 13-27.

Ingram, G. A.and Molyneux, D. H. (1993). Comparative study of haemagglutination activity in the haemolymph of three tsetse fly *Glossina* species. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*. **106**: 563-573.

Jacobs-Lorena and Lemos, F. J. A. (1995). Immunological strategies for control of insect disease vectors: a critical assessment. *Parasitology Today*. **11** (4): 144-147.

Jacobson, R. L. and Doyle, R. J. (1996). Lectin parasite interactions. *Parasitology Today*. **12**: 55-61.

Jordan, A. M. (1986). Trypanosomosis control and Africa rural development., pp 357 Longman Inc., New York.

Kibui, C., Van den Abbeele, J., De Deken R., Marcotty, T., Dorny, P and Van den Bossche, P. (2006). The effect of starvation on the susceptibility of teneral and non-teneral tsetse flies to trypanosome infection. *Medical Veterinary Entomology.* **20** (4): 388-392.

Kristjanson, P. M., Swallow, B. M., Rowlands, G. J. Kruska, R. L. and de Leeuw, P.N. (1999). Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. *Agricultural Systems.* **59**: 79-98.

Lanham, S. M. and Godfrey, D. G. (1970). Isolation of salvarian trypanosomes from man and mammals using DEAE-Cellulose. *Experimental Parasitology.* **28**: 521-534.

Leak, S.G.A. (1999). Tsetse biology and ecology their role in the epidiomiology and control of trypanosomosis. CABI publishing in association with ILRI.

Leammli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* **227**: 680-685.

Lehane, M. J. and Msangi, R. A. (1991). Lectin and peritrophic membrane development in the gut of *Glossina morsitans morsitans* and a discussion in their role in protecting the fly against trypanosome infection. *Medical and Veterinary Entomology.* **5**: 495-501.

Lehane, M. J., Msangi, A. R., Whitaker, C. J. and Lehane, S. M. (2000). Grouping of trypanosome species in mixed infections in *Glossina pallidipes*. *Parasitology.* **120**: 583-592.

Lehane, M. J., Kasumba, I. and Hoa, Z. (2001). Tsetse immune response and Trypanosome transmission: implication for the development of tsetse-based strategies to reduce Trypanosomosis . *Proceedings of the National Academy of Sciences of the United State of Amercia.* **98**: 12648-12653.

Lowry, O. H., Rosenberough, N. J., Farr, A. L. and Randall, R. J. (1951). A protein measurement with the folin phenol reagent. *Journal of Biological Chemistry.* **193**: 265-275.

Lumsden, W. H. R., Herbert, W. W. and McNeillage, G. J. C. (1973). Techniques with Trypanosomes. Churchill Livingstone, Edinburgh.

Macleod, A., Welburn, S., Maudlin, I., Turner, C.M.R. and Tait, A. (2001). Evidence for mini satellite variant repeat mapping. *Journal of Molecular Evolution.* **52**, 290-301.

Makumyaviri, A. M. (1998). The African trypanosomosis: a subject defying scientific research. *The Veterinary Book of Congo.* **1**: 102-104.

Martin J. S., Martin M. M. and Bernays E. A. (1987). Failure of tannic acid to inhibit the digestion or reduce digestibility of plant rotein in the gut fluids of insect herbivore: implications for theories of plant defense. *Journal of Chemical Ecology.* **13**: 605-621.

Maudlin, I. and Welburn, S. C. (1987). Lectin mediated establishment of midgut infections of *Trypanosoma congolense* and *Trypanosoma brucei* in *Glossina morsitans* . *Tropical Medicine and Parasitology.* **38**: 167-170.

Maudlin, I. and Welburn, S. C. (1988a). The role of lectins and trypanosome genotype in the maturation of midgut infections in *Glossina morsitans. Tropical Medicine and Parasitology.* **39**: 56-58.

Maudlin, I. and Welburn, S. C. (1988b). Tsetse immunity and the transmission of trypanosomosis. *Parasitology Today.* **4**: 109-111.

Maudlin, I. (1991). Transmission of African trypanosomosis : Interactions among tsetse immune systems, symbionts and parasite. In: Kerry, F.H. (Ed) *Advances in Disease Vector Research.* **7**: 117-148.

Mc Neil, D. (2000). Drug companies and third world: A case study in neglect. New York Times, NY, P. 1

Mihok, S., Otieno,L. H.,Darji, N., and Munyinyi, D. (1992). Influence of D-glucosamine on infection rates and parasite loads in tsetse flies (*Glossina spp.*) infected with *Trypanosoma brucei. Acta Tropica.* **5**: 217-228.

Milne, K., Prescott, A. R. and Ferguson, M. A. J. (1998). Transformation of monomorphic *Trypanosoma brucei* blood-stream form trypomastigotes into procyclic forms at 37°C by removing glucose from the culture medium. *Molecular and Biochemical Parasitology.* **94**: 98–112.

Mira, S. and Raph, S. R. (1989). Manual of tropical veterinary parasitology CAB international, UK.

Moloo, S. K. (1981). The effect of maintaining *Glossina morsitans* on different hosts upon the vector's subsequent infection rates with pathogenic trypanosomes. *Acta Tropica.* **38**: 125-136.

Moloo, S. K. and Kutuza, S. B. (1988). Comparative study on the infection rates of different laboratory strains of *Glossina* species by *Trypanosoma congolense. Medical and Veterinary Entomology.* **2**: 253-257.

Molyneux, D. H. and Ashford, R. W. (1983). The biology of *Trypanosoma* and *Leshmania* parasites of man and domestic animals. Taylor and Francis Ltd, London . pg.294.

Msangi, A. R.,Whitaker, C. J. and Lehane, M. J. (1998). Factors influencing the prevalence of trypanosome infection of *Glosssina pallidipes* on the Ruvu flood plain of eastern Tanzania. *Acta Tropica.* **70** (2): 143-155.

Nayduch, D. and Aksoy, S. (2007). Refactoriness in tsetse flies (Diptera: Glossinidae) may be a matter of timing. *Journal of Medical Entomology.* **44** (4): 660-665.

Nguu, E. K., Osir, E. O., Imbuga, M. O. and Olembo, N. K. (1996). The effect of host blood in the in vitro transformation of bloodstream trypanosomes by tsetse midgut homogenates. *Medical and Veterinary Entomology.* **10**: 317-322.

Nigam, Y., Maudlin, I., Welburn, S. and Ratcliffe, N. A. (1997). Detection of phenoloxidase activity in the hemolymph of tsetse flies, refractory and susceptible to infection with *Trypanosoma brucei rhodesiense. Journal of Invertebrate Pathology.* **69**: 279-281.

Nyambega, B. C. O. (2002). The mechanism of trypanolysin-mediated lysis of *Trypanosoma brucei brucei* (Kinetoplastida: Trypanomastidae). *MSc. Thesis*. Jomo Kenyatta University of Agriculture and Technology.

O'Farrel, P. H. (1975). High resolution two dimensional electrophoresis of proteins. *Journal of Biochemistry.* **250** (5): 4007-4021.

Ogoyi, D. O., Osir, E. O. and Olembo, N. K. (1995). Lipophorin and apolipophorin-III in solitary and gregarious phase of *Schistocerca gregaria. Comparative Biochemistry and Physiology.* 112B: 441-449.

Ogoyi, D. O., Achieng' D., Nguu, E. K. and Ochanda, J.O. (2003). Partial characterization of trypanosome lysing factor from the midgut of the desert locust, *Schistocerca gregaria. East African Medical Journal.* **80**: 43-48.

Ole Moiyoi, O. K., Jaye, A. B., Majiwa, P. A. O., Nantulya, V. M. and Masake R. A. (1997). Approaches to control of African trypanosomosis. *FASEB Journal.* **11** (9): A1132.

Osir, E. O., Labongo L. V. and Unnithan, G. (1989). A high molecular weight diapause associated protein from the stem borer *Busseola fusca*: purification and properties. *Archives of Insect Biochemistry and Physiology.* **11**: 173-187.

Osir, E. O. , Imbuga, M. O. and Onyango, P. (1992). Inhibition of *Glossina morsitans* midgut trypsin activity by D –glucosamine *Parasitology Research.* 79 (2): 93-97.

Osir, E. O., Abubakar, L. and Imbuga, M. O. (1995). Purification and characterization of a midgut lectin-trypsin complex from the tsetse fly *Glossina longipennis. Parasitology Research.* **81**: 276-281.

Osir, E. O., Abakar, M. and Abubakar, L. (1999). The role of trypanolysin in the development of trypanosomes in tsetse. Proceedings of the 25th ISCTRC meeting. Mombasa, Kenya. PP 417- 421.

Paris, J., Murray M. and McOdimba, F. (1982). A comparative evaluation of the parasitological techniques currently available for the diagnosis of African trypanosomiasis in cattle. *Acta Tropica*. **39**: 307-316.

Peregrine, A. S. (1994). Chemotherapy and delivery systems: haemoparasites. *Veterinary Parasitology*. **54**: 223-248.

Pereira, M. E. A. and Kabat, E. A. (1979). Immunochemical studies on lectins and their application to the fractionation of blood group substances and cells. *Critical Review of Immunology*. **4**: 33-78.

Raper, J., Portela, M. P., Lugli, E., Frevert, U. and Tomlinson, S. (2001). Trypanosome-lytic factors: novel mediators of human innate immunity. *Current Opinion in Microbiology*. **4** (4): 402-408.

Rita, V. M. R., Youjia, H. and Aksoy, S. (2004). Strategies of the home-team: Symbiosis exploited for vector-borne disease control. *Trends in Microbiology*. **12** (7): 325-336.

Roditi, I. and Pearson, T. W. (1990). The procyclin coat of African trypanosomes (or the not-so-naked trypanosomes). *Parasitology Today*. **6**: 79-82.

Rolin, S., Honoc-Quertiezer, J., Paturiax, H. F., Nolan, D. and Pays, E. (1998). Mild acidic stress as a differentiation trigger in *Trypanosoma brucei*. *Molecular and Biochemical Parasitology*. **93**: 251-262.

Ruepp S., Firger, A., Kurath, U., Renggli, C., Hemphill, A., Brun, R. and Roditi, I. (1997). Survival of *Trypanosoma brucei* in the tsetse fly is enhanced by the expression of specific forms of procyclin. *Journal of Cell Biology*. **37**: 1369-1379.

Schofield, C. J. and Maudlin, I. (2001). Trypanosomiasis control. *International Journal of Parasitology*. **31**: 615-620.

Service, M. W. (2001). Encyclopedia of arthropod transmitted infections of man and domesticated animals CABI Publishing, Wallingford, UK pg 533.

Smith, S. C., Harris, E. G. and Wilson, K. (1994). Effect of temperature regime on the toxicity of endosulfan and deltamethrin to tsetse flies, *Glossina morsitans morsitans*. *Tropical Science*. **34**: 391-400.

Stiles, J. K., Ingram, G. A. Wallbanks, K. R., Molyneux, D. H., Maudlin, I. and Welburn, S. (1990). Identification of midgut trypanolysin and trypano-agglutinins in *Glossina palpalis ssp* (Diptera:*Glossinidae*). *Parasitology*. **101**: 369-376.

Trivedi, S. M., Carmelita, G., Frondoza, G. and Humphrey, R. L. (1983). Modified technique for periodic acid Schiff staining of glycoproteins on agarose-film electrograms. *Clinical Chemistry*. **29** (5): 836–839.

Vickerman, K. (1965). Polymorphism and mitochondrial activity in sleeping sickness trypanosomes. *Nature*. **208**: 762-766.

Vickerman, K. (1985). Developmental cycles and biology of pathogenic trypanosomes. *British Medical Bulletin*. **4**: 105–114.

Vickerman, K., Tatley L., Hendley, A. and Turner, M. (1988). Biology of African trypanosomes in tsetse fly. *Biology of the Cell*. **64**: 109-119.

Vryesen, M. J. B., Saleh, K. M., Ali, M. Y., Abdulla, A. M., Zhu, Z., Juma, K. G., Dyck, A., Msangi, A. R., Mkonyi, P. A. and Feldmann, A. M., (2000). *Glossina austeni* (Diptera: Glossinidae) eradicated on the Island of Unguja, Zanzibar, using the sterile insect technique. *Journal of Economic Entomology*. **93**: 123–135.

Waiswa, C., Olaho-Mukani, W. and Katunguka-Rwakishaya, E. (2003). Domestic animals as reservoirs of sleeping sickness in three endemic foci in southern Uganda. *Annals of Tropical Medicine and Parasitology*. **97** (2): 149-155.

Welburn, S. C.and Maudlin, I. (1991). Ricketsia-like organisms, puparial temperature and susceptility to trypanosome infection in *Glosina morsitans*. *Parasitology*. **102**: 201-206.

Welburn, S. C., Amold K., Maudlin, I. and Gooday, G. W. (1993). Ricketsia-like organisms and chitinase production in relation to transmission of trypanosomes by tsetse flies. *Parasitology.* **107**: 141-145.

Welburn, S. C. and Maudlin, I. (1999). Tsetse-trypanosome interaction: rites of passage. *Parasitology Today.* **15**: 399-403.

Woolhouse, M. E. J., Bealby, K. A., Mc Namara, J. J. and Silutongwe, J. (1994). Trypanosome infections of tsetse fly *Glossina pallidipes* in Luangwa valley, Zambia. *International Journal of Parasitology.* **24**: 987-993.

World Health Organisation (1995). The world health report, www.who.int/whr/1995/media_centre/executive_summary2/en/index2.html, Date accessed 01/02/08.

World Health Organisation (1998). Expert Committee on Control and Surveillance of African trypanosomosis. *WHO Technical Report Series*, No.881.

World Health Organisation (2006). African trypanosomiasis (sleeping sickness) fact sheet No 259, http://www.who.int/mediacentre/factsheets/fs259/en/, Date accessed 13/02/2008.

Wray, W., Boulikas T., Wray, V. P. and Hancock R. (1981). Silver staining of proteins in polyacrylamide gels. *Annals of Biochemistry.* **188**: 197-203.

Yan J., Cheng', Q., Li C-B and Aksoy, S. (2002). Molecular characterization of three gut genes from *Glosssina morsitans morsitans*: cathepsin B, metalloprotease, Zinc- metalloprotease and Zinc carboxy peptidase. *Insect Molecular Biology.* **11** (1): 57–65.

Yeates, R. A. and Steiger, S. (1981). Ultrastructural damage of *in vitro* cultured ookinites of *Plasmodium gallinecium* (Brumpt) by purified proteases of susceptible *Aedes aegypti (L).* Z, *Parasitenkd.* **118**: 197-203.

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