

East African Medical Journal Vol. 80 No. 11 November 2003

PARTIAL CHARACTERISATION OF A TRYPANOSOME-LYSING FACTOR FROM THE MIDGUT OF THE DESERT LOCUST, *SCHISTOCERCA GREGARIA*

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ABSTRACT

Objective: Screening and biochemical characterisation of trypanosome-lysing factor (trypanolysin) from non-vector insect, *Schistocerca gregaria*.

Design: Laboratory based experiment.

Setting: Department of Biochemistry, University of Nairobi.

Results: Lysis of isolated trypanosomes was demonstrated with midgut homogenates of natural vector *Glossina morsitans centralis* as well in non-vector insects. The highest trypanolytic activity was observed in midgut homogenate of the desert locust, *Schistocerca gregaria* followed by the cockroach, *Periplaneta americana* (L). Further studies on the *S. gregaria* trypanolytic factor showed its proteinaceous nature due to its sensitivity to temperatures above 40°C and to proteases. Additionally, the factor showed lectin-like properties since the activity was blocked by D-glucosamine.

Conclusion: The trypanolytic factor has the potential of being used to modulate tsetse fly vectorial capacity.

INTRODUCTION

The life cycle of African trypanosomes (*Kinetoplastida, Trypanosomatidae*) is marked by several rounds of differentiation and proliferation within the invertebrate host, the tsetse fly (*Diptera: Glossinidae*). At each stage, the survival and successful replication of the parasite improve the chance of continuing with the life cycle. Of the various stages of development, the midgut stage is thought to be very crucial for successful development(1). Infection rates of the tsetse vectors under natural conditions are generally very low for all the three major trypanosome groupings. Moreover, infection rates have been found to vary considerably among the various tsetse-trypanosome interactions(2,3). Infection rates are determined by a complex interaction of factors originating from the tsetse fly, host bloodmeal as well as the species of trypanosome involved(4-7).

Croft *et al* (8) were the first to demonstrate that tsetse haemolymph contain factors that reduce the motility of procyclic *Trypanosoma brucei brucei* Plimmer and Bradford, *in vitro*, but has no effect on *Crithidia* or *Leishmania* parasites. Ibrahim *et al.*(9) further showed that the haemolymph factor was a lectin and that the midgut also contained another lectin with different specificity. In *Rhodnius prolixus* Stal, the vector for *Trypanosoma cruzi* Chagas, lectins from the haemolymph and the gut agglutinated epimastigotes *in vitro*(10). Further studies have shown that tsetse refractoriness in most fly population is correlated to

their ability to remove invading trypanosomes from the guts by secreting specific lectin(s) that binds to trypanosomes resulting in lysis and cell death(4,11). Variation in susceptibility between tsetse populations and species is attributed to inherent differences in the levels and specificity of midgut lectin. This hypothesis is supported by the demonstration that tsetse fed infective bloodmeal containing D-glucosamine, which binds to tsetse midgut lectin, shows very high infection rates compared to controls(12,13). Additional evidence comes from the observation that high levels of rickettsia-like organisms (RLO) are found in the midgut of susceptible flies(4,6). RLOs produce chitinase that degrade chitin leading to high levels of chitin-derived D-glucosamine that blocks lectin-mediated trypanocidal activity in the midgut. Levels of bloodmeal-induced trypsin have also been shown to be correlated to refractoriness in a similar manner(14).

Through co-evolution, parasites have managed to exploit vector-derived molecules for their survival. For example, midgut lectins are implicated in the differentiation of trypanosomes *in vivo* although high levels kill them(6,12). In *Triatoma infestans* the vector of *T. cruzi*, gut homogenates were shown to stimulate differentiation of epimastigotes, *in vitro* (15). Similarly, midgut trypsin is required for successful establishment of trypanosomes in the tsetse vector but higher levels kill them(14,16).

A lot of effort has so far been directed towards understanding the molecular basis of vector-parasite

interactions. This has been done with the sole aim of finding weak points that could be exploited in blocking transmission cycle(17). Little, however, is known about the factors responsible for total exclusion of parasite development in non-vector insects. However, earlier *in vitro* studies revealed the presence of parasite agglutinins against the trypanosomatid flagellates *Trypanosoma brucei* and *Leishmania hertigi* in the haemolymph of *Schistocerca gregaria* and *Periplaneta Americana* (18). Our own preliminary investigations showed that *S. gregaria* midgut homogenate lyse trypanosomes *in vitro*. This study was therefore initiated to elucidate the properties of a trypanosome-lysing factor(s) hereafter referred to as 'trypanolysin' from the midgut of a non-vector insect, *S. gregaria*.

MATERIALS AND METHODS

Experimental animals and insects: Adult albino rats were obtained from the Department of Zoology, University of Nairobi and maintained on rat pellet. Adult desert locusts, *Schistocerca gregaria*, cockroach, *Periplaneta Americana* (L), mosquito, *Aedes aegypti* (L) and the body louse (*Phthiraptera: Pediculidae*), *Pediculus humanus humanus* (L), (from a colony from Hebrew University) were all obtained from the Insectary at the Department of Zoology, University of Nairobi. The insects were maintained at 28°C -34°C, 40-50% relative humidity and photoperiod of 12h:12h (light:dark). The tsetse flies, *Glossina morsitans centralis* Machado, used as controls, were obtained from the International Livestock Research Institute (ILRI) at Nairobi. The flies were maintained at a 12h:12h light:dark photoperiod at 75-80% relative humidity and 25°C.

Parasite maintenance and isolation: The strain of *Trypanosoma brucei brucei* (ILTAT 1.4) used in all the experiments was originally obtained from the International Livestock Research Institute (ILRI) and passaged severally within the Department of Biochemistry, University of Nairobi. Rats were subcutaneously injected with approximately 10^3 trypanosomes suspended in 2 ml phosphate buffered saline (PBS) (0.1M phosphate, 0.15M NaCl), pH 8.0 containing 1% glucose (PBSG). Parasitaemia was monitored every two days by piercing the tip of the tail with a sterile lancet to obtain a drop of blood. In order to isolate parasites, infected rat blood was drawn by cardiac puncture with a 21G needle using a 10ml syringe. Sodium citrate (14% in PBSG) was used as the anticoagulant. The blood was centrifuged (1000 xg for 10 min) and the buffy layer containing mainly trypanosomes resuspended in PBSG. The trypanosomes were then separated from contaminating cells by anion exchange chromatography on DEAE-cellulose(19). Trypanosomes were counted using an improved Neubauer ruled haemocytometer(20) and the final concentration adjusted to 5×10^6 trypanosomes per ml in PBSG.

Midgut preparation and haemolymph collection: Insects starved overnight were briefly chilled and midguts dissected in phosphate buffered saline pH 7.2 (PBS). Pooled samples were homogenized in PBS using glass homogenizer, centrifuged (10,000 xg, 4°C, 30 min) and the supernatant stored at -20°C for subsequent use(21). Haemolymph was collected from the insects through an incision below the coxa using microcapillary tubes and immediately mixed with PBS containing 5 mM EDTA (22). The haemolymph was then

centrifuged (5,000 x g, 10 min, 4°C) and the supernatant kept at -20°C. Protein estimation was carried out using the bicinchoninic acid (BCA) protein assay method (Pierce, Rockford, IL, USA) according to instructions supplied by the manufacturer. Bovine serum albumin was used as the protein standard.

Trypanolysin assay: Trypanolysin assay was carried out with a starting protein concentration of 1mg/ml for the test samples. Midgut homogenate and haemolymph samples were serially diluted on a microtitre plates using 20µl of PBSG. An aliquot of parasite suspension (20µl) was then added to each well, mixed, then incubated at room temperature. Lysis of the trypanosomes was checked at different time points using a phase-contrast microscope at a magnification of x40 objective lens. Control experiments were also set up in the wells using PBSG and parasites. Percentage lysis was estimated by comparison to the value obtained from the controls.

Effect of various factors on trypanolysin activity

Temperature: Midgut homogenate samples (1mg/ml) were separately incubated at temperatures of 30°C, 40°C, 50°C, 60°C and 70°C for 1 hour. The samples were then cooled to room temperature and 20µl of each sample used for trypanolysin assay.

Proteases and anti-proteases: The effect of the proteolytic enzymes, trypsin, papain and leucine aminopeptidase and the protease inhibitors, antipain, pepstatin and leupeptin on trypanolysin activity were tested. Preliminary assays at enzyme/inhibitor: sample weight ratios of 1:1, 1:3, 1:5 and 1:25 were carried out. The enzymes and the protease inhibitors were then used at optimized enzyme/inhibitors:sample weight ratio of 1:3. The *S. gregaria* midgut extract samples containing these proteases or anti-proteases were incubated for 1 hour at 30°C then cooled to room temperature before carrying out the trypanolysin assay. Controls were set up by incubating the enzyme or anti-proteases with parasites in the absence of midgut homogenate.

Divalent cations: The effect of divalent cations was evaluated as previously described(18). Divalent cations present in the *S. gregaria* midgut homogenates were removed by incubating the samples overnight with 16mM EDTA at 4°C. The samples were then dialyzed against PBS to remove the EDTA-ion complexes and trypanolysin activity assessed. The salts, magnesium chloride and or calcium chloride (10 mg/ml) were separately added to the EDTA-treated samples and used in trypanolysin assays.

Sugars: Various sugars namely, N-acetylglucosamine, D-glucosamine, D-galactose and D-glucose at concentration of 500 mM were incubated with the *S. gregaria* midgut homogenate at 30°C for 30 minutes before assays. Control experiments were set up with each of the sugars but without the trypanolysin test samples. Trypanolysin assay was then carried out as outlined above.

RESULTS

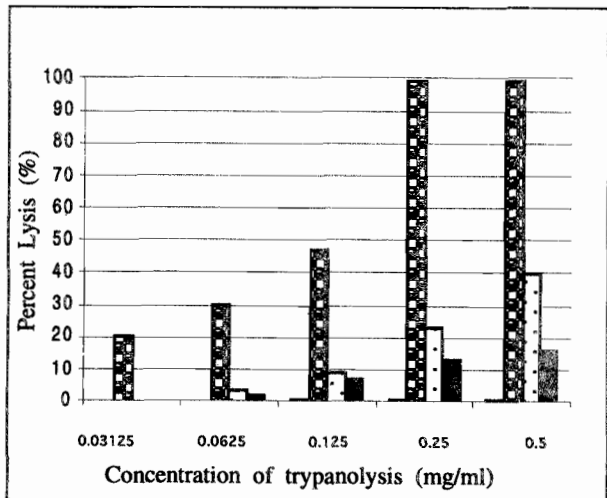
Screening for trypanolysin activity: Trypanolysin activity was detected in all the midgut homogenates with the locust, *S. gregaria*, having the highest followed by the cockroach. For the other insects the order of trypanolysin activity starting from the highest was as follows. *Pediculus humanus humanus*, *Glossina morsitans centralis* and *Aedes aegypti*. In midgut

homogenate and haemolymph samples, trypanolysin increased with the protein concentration with locust midgut showing highest activity at all concentrations tested (Figure 1). Complete lysis was observed with 0.25 mg/ml of protein of locust midgut homogenate. In contrast the same concentration resulted in only 17% and 26% lysis with locust haemolymph and tsetse midgut samples, respectively.

The time course profile of trypanolysin activity was assessed over a three hour period (Figure 2). Initially, lysis increased with incubation time with the locust midgut showing the highest activity followed by tsetse midgut. After two hours of incubation, a 100% lysis was realised in locust midgut sample compared to only 26% and 14% in *G. m. centralis* midgut and locust haemolymph, respectively. Incubation for another 1 hour increased lysis to 30% and 25% in the *G. m. centralis* midgut and locust haemolymph respectively. Since the locust midgut homogenate showed the highest trypanolysin activity, further analysis was carried out using this sample. Trypanolysin assays were optimized for protein concentration of 1 mg/ml, parasite titre at 5×10^6 trypanosomes/ml and incubation time of 2 hours and unless otherwise stated, assays were carried out under these conditions.

Figure 1

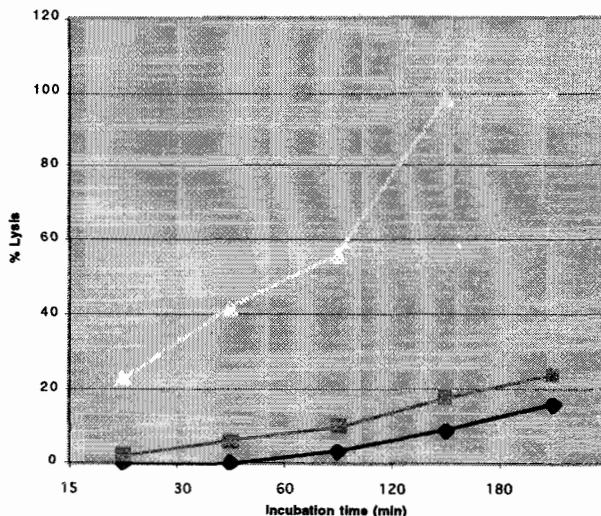
Trypanolysin activity at different concentrations of midgut homogenate and haemolymph



Serial dilutions of samples with a starting protein concentration of 1mg/ml were made then incubated with parasite for two hours. ▣ *S. gregaria* midgut, ▤ *G. morsitans* midgut and ▥ *S. gregaria* haemolymph.

Figure 2

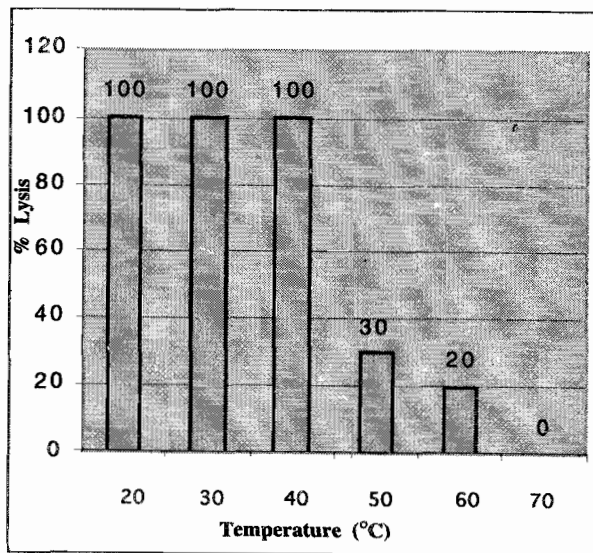
Trypanolysin activity at various incubation times



Test samples (1 mg/ml) were incubated with parasites at various times. Δ *S. gregaria* midgut. \square *G. M. centralis* and \blacklozenge *S. gregaria* haemolymph.

Figure 3

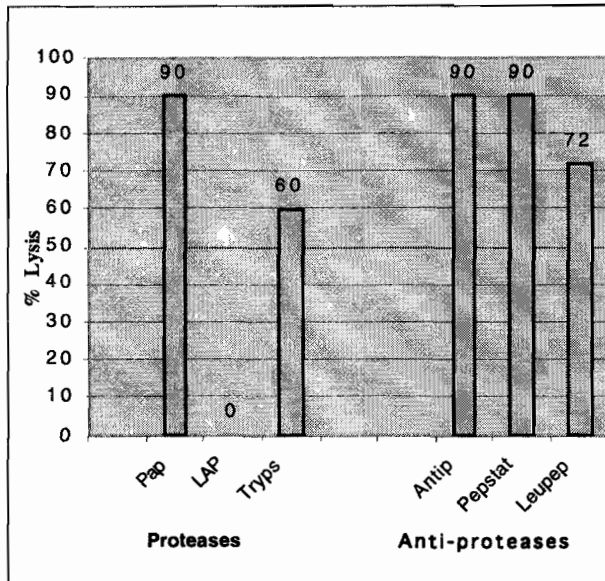
Thermosensitivity of *S. gregaria* midgut trypanolysin activity



Midgut homogenate samples (1mg/ml) were separately incubated at the specified temperatures for 1 hour, cooled then trypanolysin assay carried out.

Figure 4

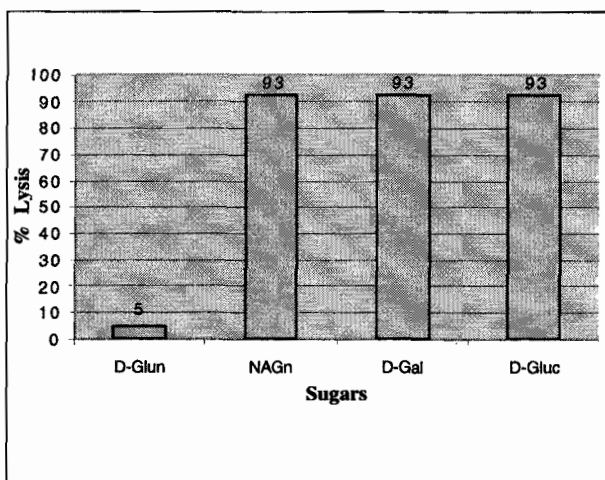
Effect of proteases and protease inhibitory of trypanolysin activity



Midgut extract samples containing these proteases or anti-proteases were incubated for 1 hour at 30°C then cooled to room temperature for trypanolysin assay, Pap (papain), Lap (Leucine amino peptidase), Tryps (trypsin), Anti P (antipain), pepstat (pepstatin), Leupep (leupeptin).

Figure 5

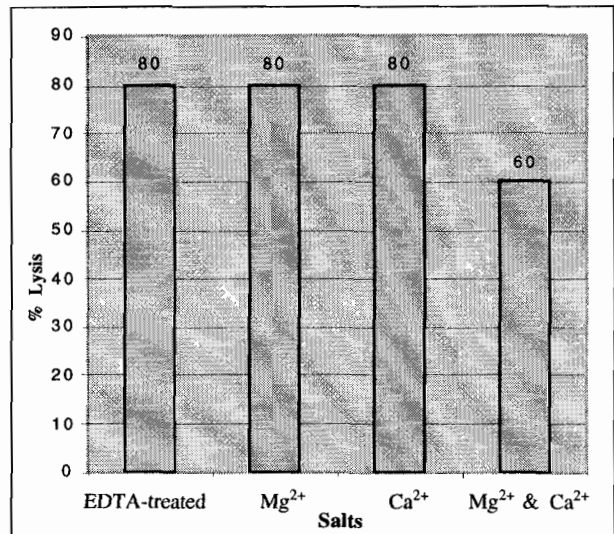
Effect of sugars on S. gregaria midgut trypanolysin activity



Various sugars at 500mM were incubated with the *S. gregaria* midgut homogenate at 30°C for 30 minutes before assay. D-Gluc= D-glucosamine, NAGn= N-Acetyl-glucosamine, D-Gal= D-galactose and D-Gluc= D-glucose were used.

Figure 6

Effect of divalent cations on trypanolysin activity



Midgut homogenates were incubated overnight with EDTA at 4°C. The samples were then dialyzed then salts, magnesium chloride and/or calcium chloride (10 mg/ml) were separately added to the EDTA-treated samples and used in trypanolysin assays.

Factors affecting trypanolysin activity:

Thermosensitivity of trypanolysin was tested by incubation of locust midgut samples at various temperatures for 1 hour prior to the bioassays (Figure 3). Activity of trypanolysin was unaffected by temperatures below 40°C. However, at 50°C and 60°C, 70% and 80% of the activity was destroyed, respectively. At 70°C, trypanolytic activity in the midgut extract was completely eliminated.

The effect of proteases and protease inhibitors on trypanolysin activity was studied by treating the samples as detailed in Materials and Methods before bioassays. Leucine aminopeptidase and trypsin resulted in 100% and 40% loss of activity, respectively, while papain had no effect (Figure 4). Among the protease inhibitors tested, only leupeptin had an effect, causing a reduction in activity of 28% (Figure 4).

The effect of the sugars (N-acetylglucosamine, D-glucosamin, D-galactose and D-glucose) and divalent cation on the trypanolysin activity was also assessed. The sugar, D-glucosamine at a concentration of 500mM showed the highest inhibition of 95% while other sugars had only limited inhibition of 7% (Figure 5). Following the removal of divalent cations through dialysis against EDTA, trypanolysin activity decreased by 20% (Figure 6). The addition of CaCl₂ and MgCl₂ separately to the EDTA-treated samples, did not result in any change in activity. Surprisingly, addition of both cations resulted in a further 20% decrease in activity (Figure 6).

DISCUSSION

Vector insects are known to possess both cellular and humoral defense capabilities(23,24). Thus, successful development of a parasite within the vector requires circumvention of the immune system. The parasite's failure to establish and develop to maturation in non-vector insects represents the inability of the parasites to circumvent the various general and or specific barriers. It is also equally possible that the interaction of the parasite with non-vector lacks the necessary stimuli/signal for further development.

The results of this study demonstrates the presence of trypanolytic activity in the tsetse fly-vector, *G. morsitans centralis*, as well in the non-vector insects; *S. gregaria*, *P. Americana*, *P. humanus humanus* and *A. aegyti*. The highest trypanolytic activity was observed in the midgut homogenates of *S. gregaria* followed by cockroach. The occurrence of trypanolytic factor in both vector and non-vector insects albeit in different concentrations confirms that resistance to infection is mounted even in vector insects (4,11). The specific recognition of parasites by the vector through lectin-like molecules has also been observed in many insects including the mosquitoes, blackflies and sandflies (*Diptera: Psychodidae*) (23,25). Among tsetse species and populations, variations in susceptibility to trypanosomes has been attributed partly to inherent differences in titre and specificity of the midgut lectins(4,26). The *S. gregaria* trypanolytic factor showed lectin-like properties due to inhibition of activity by the sugar D-glucosamine. Earlier *in vitro* studies also revealed the presence of parasite agglutinins against the trypanosomatid flagellates, *Trypanosoma brucei* and *Leishmania hertigi* in the haemolymph of *S. gregaria* and *P. Americana*(18). The present study showed that the trypanosome-lysing factor was present in both haemolymph and the midgut of *S. gregaria* but activity was much higher in the latter.

The predominant molecules in the secretions of insect midgut are, notably trypsin and chymotrypsin-like proteases as well as lectin-like/agglutinin activities(27,28). These factors have been shown to be involved in elimination of parasites from the gut as well as a means for parasite establishment(24). The trypanosome adaptation to the effect of the midgut trypsin and lectins is such that optimal levels stimulate differentiation but higher levels kill them(14). The isolation of bifunctional molecule with both lectin and trypsin activity from the midgut of *Glossina longipennis* Corti that could induce *Trypanosoma brucei* differentiation *in vitro* supports the hypothesis and also show how intimate the two molecules function(29). The trypanolysin factor from *S. gregaria* was shown to be proteinaceous due to inactivation by temperatures above 40°C and inhibition by proteases (leucine aminopeptidase and trypsin). In addition the molecule showed some

proteolytic activity as evident from partial inhibition by antiprotease, leupeptin. Trypanolysin factor also had lectin like activity since it was inhibited by N-acetylglucosamine. The function of trypanolysin does not appear to be significantly affected by EDTA-treatment which suggests that divalent cations(Ca^{2+} Mg^{2+}) do not influence trypanolysin activity. Our results concur with those of Ingram *et al.* (18), with respect to agglutinin in locust haemolymph against *Trypanosoma brucei*.

The *S. gregaria*-derived trypanolytic molecule if well studied will be very important in understanding trypanosome-tsetse interaction and has the potential of being exploited in modulating arthropod vectorial capacity using modern approaches in biotechnology (17,30). The modulation of arthropod vector competence using genetically altered symbiotic bacteria has already been demonstrated by the constitutive expression of recombinant cecropin-A in the hindgut of *R. prolixus*(31). The expressed molecule was lytic to developing trypanosomes and the transformed bacteria maintained a stable symbiotic relationship with insect host. The results by Durvasula *et al.* (31), provides an impetus into studies which may lead to identification of trypanolytic factors that may be introduced into midgut of tsetse and probably other vectors by using midgut endosymbionts(17). Further studies on the *S. gregaria* trypanolysin factor are therefore required towards the isolation and characterisation of trypanolysin and the gene(s) that codes for it.

ACKNOWLEDGEMENTS

To the Departments of Biochemistry and Zoology for supplying the animals and insects used in the experiment respectively. The work was supported in part by re-entry grant to DOO from ICIPE.

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