Properties Of A Midgut Trypanolysin From The Tsetse Fly, *Glossina Morsitans Morsitans*

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Abstract: The properties of a bloodmeal-induced trypanolysin from the midgut of the tsetse, *G. m. morsitans* was studied *in vitro*. The semi-purified trypanolysin from twice-fed tsetse had the highest trypanolysin activity against bloodstream trypanosomes followed by those once-fed and the unfed flies. Serum found to display trypanolysin activity. The trypanolysin had no trypsin activity nor even affected by the enzyme. In addition, trypanolysin was not affected by protease inhibitors such as soy bean trypsin inhibitor (STI), *N*-a-*p*-Tosyl-*L*-lysine chromethyl ketone (TLCK), phenylmethyl sulphonyl fluoride (PMSF), diisopropyl fluoro-phosphate (DFP) and tosylamide-2-phenylethyl chloromethyl ketone (TPCK). However, the activity was completely inhibited by diethyl pyrocarbonate (DEPC) and partially by aprotinin. The induction of trypanolysin activity by bloodmeal increased gradually reaching a peak at 72-120 h after the bloodmeal, and then decreased rapidly, with only 25% of the peak activity remaining after 192 h. The trypanolysin was inactivated during storage at 27℃ and 4℃ after 15 and 32 days, respectively. Similarly, heating the midguts trypanolysin to 60 - 80℃ led to loss of activity. On the other hand, 50℃ was found to be the optimum temperature for trypanolysin activity. The activity was also unstable by freeze-thaw at 80℃, -70℃, -20℃ and 0℃ after 33, 41, 55 and 63 days, respectively. Trypanolysin caused lyses of bloodstream-form *T. b. brucei*, while the procyclic trypanosomes were unaffected. The highest trypanolysin activity in different tsetse species was found with *Glossina longipennis*, followed by *Glossina pallidipes*, *Glossina morsitans centralis, Glossina fuscipes fuscipes* and *G. m. morsitan*s. When the midgut homogenate was separated by anion-exchange chromatography, the trypanolysin activity was recovered in the bound fraction. These results suggest that the midgut trypanolysin plays an important role in the establishment of trypanosome infections in tsetse.

Index Terms: Midgut, property, trypanolysin, trypanosomes, tsetse.

1 Introduction

An important step in the establishment of gut-adapted trypanosome infections in tsetse involves their differentiation from bloodstream into procyclic (midgut) forms (Gibson and Bailey, 2003). This complex process is mediated by a wide variety of factors, all of which are intrinsic to both the tsetse vector and the host blood (Maudlin, 1982). Most of the ingested trypanosomes are thought to be lysed by a wide variety of factors including enzymes, agglutinins, lectins and other factors that are yet to be characterised. The small number of trypanosomes that survive lysis continue with the process of development and establish themselves into effective forms within the tsetse. Of the many factors that have been implicated in tsetse-trypanosome interaction, trypsin or trypsin-like enzymes (Cheeseman and Gooding, 1985; Imbuga *et al*., 1992 a, b) and lectin-like molecules (Maudlin, 1991; Osir *et al.*, 1995) have received the most attention. In addition, innate immune response products have been implicated to contribute to these factors (Hao *et al*., 2001).

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For example, at least six proteolytic enzymes including trypsin, trypsin-like enzyme, chymotrypsin and carboxypeptidases among others have been mentioned (Gooding and Rolseth, 1976). Also, proteolytic lectin serine protease (GpI) isolated from *G.f.fucipes* as have a role in trypanosome differentiation *in vitro* (Abubakar *et al*., 2006). Moreover, tsetse harbor different *Sodalis glossinidius* (Askoy, 2000). Similarly, the purification and characterization of a lectin-trypsin complex has been reported (Osir *et al*., 1995). Although it has been suggested that this molecule plays an important role in the differentiation of trypanosomes, the actual mechanisms still remain unknown. A part from the trypsins and lectin-like molecules, a molecule that is specifically involved in lysis of trypanosomes has been described in the midguts of *G. palpalis* (Stiles *et al*., 1990) and *G. fuscipes* (Osir *et al*., 1990). The trypanolysin destroys trypanosomes and may serve to remove trypanosomes from unsuitable sites in the midgut. It is however not known whether Glossina species other than these studied posses varying levels of this molecule. In order to gain insight into the mechanisms that are involved in innate refractoriness, we have studied the properties of a bloodmeal-induced trypanolysin from *G. m. morsitans*. The distribution of this molecule in other tsetse species has also been elucidated.

2 Materials And Methods

2.1 Preparation of insect midgut homogenates and isolation of trypanosomes

Male tsetse *G. m. morsitans, G. m. centralis, G. f. fuscipes* (Diptera: Glossinidae) and male rats 2-4 months old (Wistar Strains) were supplied by the Animal Breeding and Quarantine Unit (ABQU) of the International Center of Insect Physiology and Ecology (ICIPE). The tsetse were reared as previously described (Osir *et al*., 1993).) The *G. longipennis* Corti and *G. pallidipes* were provided by the Tsetse Vector Laboratory of the International Livestock Research Institute (ILRI). These tsetse were maintained on rabbit blood *in vivo* at 25 - 26□, 65 -75% relative humidity (RH) and a 12 h: 12 h light: dark photoperiod cycle. Three hundred teneral tsetse of each species (24 h after emergence) were fed on rats and then

maintained (72 h, 27□). The tsetse were immobilized by brief chilling (4° C) after which their midguts homogenates prepared as previously described (Abubakar *et al*., 1995). Rats were infected with a Pleomorphic *T. b. brucei* of a stock derived in 1969 from East African Trypanosomiasis Research Organization (EATRO) (Otieno *et a*l., 1983). The bloodstream form trypanosomes was isolated as previously described (Abubakar *et al*., 1995). Procyclic-form trypanosomes *(T. b. brucei*) were obtained by transferring the bloodstream-forms into a Semi-defined medium –70 (Brun and Jenni, 1977, Brun and Schonenberger, 1970) containing 10% foetal calf serum (FCS). The trypanosomes were cultivated (27° C) with twice weekly passages. No antibiotics were included in the medium. Prior to use, the trypanosomes were pelleted from the medium by centrifugation (3,000 g, 10 min, 4° C) and washed once in PBS in order to remove serum components.

2.2 Induction of trypanolysin activity by bloodmeal and estimations of protein

All trypanolysin assays were carried out in triplicates in Microtitre plates (Nunc, Denmark). Double serial dilutions of the filtered strored midgut homogenates from *G. m. morsitans, G. m. centralis, G. f. fuscipes, G. pallidipes, G. longipennis* and semi-purified trypanolysin from *G. m. morsitans* were prepared in 20 mM Tris-HCl, pH 8.0. An equal volume of freshly isolated bloodstream-form (≈ 5 X 10^6 trypanosomes/ml) was added to each dilution. In another set of experiment, the procyclic form trypanosome was adjusted to an equal volume of ≈ 5 X 10^5 trypanosomes /ml and mixed with double serial dilutions of semi purified trypanolysin. After mixing, the plates were incubated (2 h, 27° C) and lysis checked at 30 mins intervals using an inverted microscope (Leitz Dialux 600, Germany). Controls consisted of trypanosomes mixed with buffer only. Data is expressed as reciprocal of the least dilution that caused lysis of the trypanosomes. Protein estimations were carried out using the bicinchonic acid (BCA) method (Pierce, Rockforf, III, USA) with bovine serum albumin (BSA, Pierce) as the protein standard.

2.3 Effect and time course of number of feeds on typanolysin activity in *G. m. morsitans*

The trypanolysin activities of midgut homogenates obtained from *G. m. morsitans* of different feeding states were assessed as follows: the unfed tsetse had their midguts dissected 24 h after emergence. The once-fed were given a bloodmeal 24 h after emergence and the midguts dissected after 72 h. The twice-fed tsetse were fed 24 h after emergence, starved for 72h, fed again and midguts obtained after 72 h. The homogenates from these groups of tsetse were both assayed for their abilities to lyse bloodstream-form trypanosomes as described above. In another experiment, the time course of trypanolysin induction was assayed as follows: 200 teneral *G. m. morsitans* were given a bloodmeal 24 h after emergence. Of these, 20 were dissected immediately after feeding (0 h) and another 20 flies 5 h after feeding. The rest of the tsetse (160) were dissected at 24 h intervals and trypanolysin activities assayed as described above.

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

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

2.5 Effects of protease inhibitors and temperatures on trypanolysin activity

The effect of soy bean trypsin inhibitor (STI, Millipore Corp. Freehad, USA), *N*-α-*p*-tosyl-*L*-Lysine chloromethyl kethone (TLCK), phenyl methyl sulphonyl flouride (PMSF), diisopropyl flourophosphate (DFP), tosylamide - 2 - phenylethyl chloromethyl ketone (TPCK),diethyl pyrocarbonate (DEPC) and aprotinin (Sigma, st. louis USA) on trypanolysin activity was assessed as follows: increasing concentrations (0.0 - 1.0 mg/ml) of inhibitors were mixed with trypanolysin prior to addition of the bloodstream-form trypanosomes. Lysis of the trypanosomes was assessed as described above. In another study, midgut homogenates from tsetse 72 h after feeding were incubated (20 min) in a water bath maintained at 37, 40, 50, 60, 70 and 80℃. The samples were subsequently allowed to attain room temperature (27℃) and the trypanolysin assays carried out as described above. A sample maintained at 27℃ served as the control.

2.6 Effects of freezing-thawing and of storage on trypanolysin activity

The effect of freezing and thawing on the activity of isolated trypanolysin was assessed by frozen the samples at -20° C, -70℃ and -80° C and thawed at 27° C. The activity was assayed and estimated by the method described above. In a separate study, the trypanolysin assays were carried out under standard conditions except that the temperature of the incubations was 27, 4 and 0° C.

2.7 Partial purification of trypanolysin

Midguts from *G. m. morsitans* were dissected 72 h after feeding and homogenized. After homogenisation, the homogenate was centrifuged (12,000 x g, 15min, 4℃) in a Heraecus minifuge and the resulting supernatant solution dialysed overnight (three changes) against 20 mM Tris-HCL buffer, pH 8.0 and then filtered through a 0.22 μm Millipore filter (Nalge, Rochester, New York). The sample was separated by anion-exchange chromatography K 16 (Pharmacia, Uppsala, Sweeden; type K 16/29, diameter 1.6 cm, length 20 cm and bed volume 40 ml diethylaminoethyl-DEAE-Sephacel) column which had been pre-equilibrated in 20 mM Trish-HCL buffer, pH 8.0. The filtrated midguts homogenate (3.0 ml) was then loaded and the column washed with the same buffer at the rate of $2.0\,ml/min^{-12}$. The absorbance was continuously monitored at 280 nm. After washing, the bound proteins eluted using a linear gradient (0.0 - 500 mM NaCl). Fractions (2.0 ml) were collected and assayed for their abilities to lyse bloodstream-form trypanosomes.

3 RESULTS

3.1 Induction of trypanolysin activity by bloodmeal and estimations of protein

Trypanolysin was found in all tsetse species examined. Protein estimation of midgut homogenates from the different species of tsetse were estimated to be; *G. longipennis* (45 mg/ml), *G. pallidipes* (34 mg/ml), *G. f. fuscipes* (22.2 mg/ml), *G. m. centralis* (17. 5 mg/ml) and *G. m. morsitans* (14.34 mg/ml). The assays of trypanolysin activity titre showed that, *G. longipennis* has the highest trypanolysin activity (512) folowed by *G. pallidipes* (128), *G. f. fuscipes* (64) and *G. m. centralis* (32). However, *G. m. morsitans* gave the lowest activity (16) against blood stream form trypanosomes compared to the other species included in the bioassay (Table 1). It was observed that, while blood-stream form trypanosomes were lysed by trypanolysin neither midgut homogenate from examined tsetse species nor semi-purified trypanolysin was able to lyse procyclic-forms of *T. b. brucei.*

TABLE 1
INDUCTION OF TRYPANOLYSIN ACTIVITY BY BLOODMEAL AND ESTIMATIONS OF PROTEIN

Tsetse species	Protein estimations (mg/ml)	Trypanolysin titre
G. longipennis	45	512
G. pallidipes	34	128
G. f. fuscipes	22.2	64
G. m. centralis	17.5	32
G. m. morsitans	14.34	16

3.2. Effect and time course of number of feeds on trypanolysin activity in *G. m. morsitans*

Trypanolysin activity was induced by bloodmeal. In this case, the activity increased gradually with time reaching peak level 72-120 h post feeding. Thereafter, the activity decreased to only 25% of the peak activity after 192 h (Figure 1).

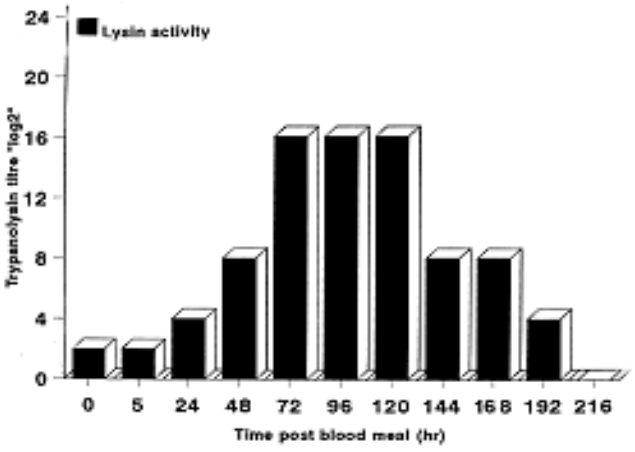

Fig. 1. *Time course of trypanolysin activity in G. m. morsitans.*

The activity also increased with the number of times that the tsetse were fed. For example, at 72 h, tsetse fed twice had the highest trypanolysin titre (32) with the highest protein estimation (16.25 mg/ml) followed by those fed only once with trypanolysin titre (16) and protein estimation as (14.34 mg/ml) and the unfed which gave the lowest trypanolysin activity (4) and the lowest protein estimation as (3.16 mg/ml) (Table 2).

TABLE 2
EFFECT OF NUMBER OF FEEDS ON TRYPANOLYSIN ACTIVITY

Number of feed	Protein estimation (mg/ml)	Trypanolysin titre
Unfed	3.16	4
Once-fed	14.34	16
Twice-fed	16.25	32
Isolated trypanolysin first step of purification	4.70	8

Protein estimation of isolated trypanolysin by an ion-exchange was found as 4.70 mg/ml and trypanolysin titre as 8.

3.3. Induction of trypanolysin activity by serum, red blood cells and whole blood

Serum membrane-fed tsetse displayed slighty higher trypanolysin activity (32) compared to those fed on red blood cells (16) and whole blood (16) (Table 3). However, tsetse fed on red blood cells gave the same titre as those fed on whole blood.

TABLE 3
INDUCTION OF TRYPANOLYSIN ACTIVITY BY SERUM, RED BLOOD CELLS AND WHOLE BLOOD

Bloodmeal	Trypanolysin titre
Whole blood meal (control)	16
Serum meal	32
Red blood cells meal	16

3.4. Effect of proteases inhibitors and temperatures on trypanolysin activity

Trypanolysin was not affected by diisopropryl fluorophosphate, Tosylamide-2-phenyl chloromethyl ketone, soybean trypsin inhibitor, *N*-α-*p*-Tosyl-*L*-lysine chloromethyl ketone or phenyl methyl sulphonyl fluoride. On the other hand, while aprotinin partially inhibited trypanolysin activity, diethyl pyrocarbonate completely inactivated the activity (Table 4). Temperatures ranging from 27 to 50° C have no effect on trypanolysin activity. Temperature of about 60° C reduced the trypanolysin activity by 50% and the activity declined further by 50% at 70 °C, whilst at 80° C, virtually all the activity was lost (0) (Table 4). From this experiment, 50° C was found to be the optimum temperature for trypanolysin activity.

TABLE 4

EFFECT OF PROTEASE INHBITORS AND TEMPERATURES ON TRYPANOLYSIN ACTIVITY

Protease inhibitors	Trypanolysin titre	Temperature in °C	Trypanolysin titre
Control	64	27	64
Diisopropyl fluorophosphate (DPF)	64	37	64
Tosylamide -2-phenyl chloromethyl ketone (TPCK)	64	40	64
Soybean Trypsin Inhibitor (STI)	64	50	64
N-a-*p*-Tosyl-l-lysine chloromethyl ketone (TLCK)	64	60	32
Phenyl methyl sulphonyl fluoride (PMSF)	64	70	16
Aprotinin	8	80	0
Diethylpyrocarbonate (DEPC)	0	37	64

3.5. Effects of freezing-thawing and of storage on trypanolysin activity

It has been observed that, after the 30th freeze-thaw cycle of typanolysin, the sample was found to be very stable with no appreciable loss of activity. The activity, however, progressively decreased after the 33rd, 41st and 55th cycle at -80, - 70, and - 20°C, respectively (Table 5).

TABLE 5

EFFECT OF FREEZING AND THAWING ON TRYPANOLYSIN ACTIVITY

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

The effect on trypanolysin activity exposed to different temperatures indicated that the activity was completely lost by storage both at 27° C (15 days) and 4° C (32 days). However, the activity of the molecule remained stable at 0° C up to the end of the experiment (60 days) (Table 6).

TABLE 6

EFFECT OF STORAGE ON TRYPANOLYSIN ACTIVITY

Days of storage	Trypanolysin titre Temperature ° C		
	27° C	4° C	0° C
2	64	64	64
5	64	64	64
7	32	64	64
10	16	64	64
15	0	64	64
20	0	64	64
25	0	32	64
30	0	8	64
32	0	0	64
60	0	0	64

3.6. Partial purification of trypanolysin

The separation of midgut homogenates was achieved by an ion exchange chromatography column using gradient procedure and elution of the bound proteins was obtained with increasing concentrations of NaCl (0.1 - 0.5 M) in 20 mM Tris - HCl, pH 8.0. The highest trypanosylin activity was only recovered in the bound fractions (95%, 0.5 M NaCl). The eluted peack fractions (35-50) were pooled, concentrated, dialysed, tested and used.

4 DISCUSSION

This study showed that teneral tsetse had very low titres of midgut trypanolysin, suggesting that trypanolysin is secreted in response to bloodmeal. This low level of trypanolysin release might explain the increased susceptibility of teneral tsetse to trypanosomes infection. Midgut homogenates from 72 h once-fed *G. m. morsitans* lysed bloodstream-form *T. b. brucei* while the procyclic forms were not affected. Possibly, the transformation from bloodstream-forms to procyclics might be one way in which the *T. b. brucei* evade the trypanolysin activity in the midgut. Trypanolysin as well as trypsin were involved in the lysis of the bloodstream-form trypanosomes but not procyclics. In contrast, trypanoagglutinin agglutinates procyclic-form trypanosomes. For the establishment of *T.b.brucei* in tsetse midgut, it is possible that, the trypanosome express green fluorescent protein by control of promoter of procyclin (Gibson and Bailey, 2003) hence differentiation of blood stream form to procyclis involves replacement of variant surface glycoproteins by procyclin (Vassella *et al*., 2001; Acosta, 2001; Gibson, 2003). Osir (1993) reported close relationship between lectins and trypsins. Imbuga (1992 a, b) provided evidence for the involvement of trypsin or trypsin-like enzymes in trypanosomes differentiation and lysis, and trypanolysin in thus, unlikely to be a lectin or act as one. The present study showed that trypanolysin was found in all Glossina species, with *G. longipennis* has the highest activity followed by *G. pallidipes, G. fuscipes,* and *G. m. centralis* however, *G. m. morsitans* gave the lowest activity compared to the other species included in the study suggesting that some component of bloodmeal is responsible for stimulating the release of trypanolysin. Stiles (1991) reported that in Glossina species, bloodmeal stimulates the release of different molecules including proteolytic enzymes, lectins and

trypanolysins. It was reported that *G. longipennis* has significantly low mature infections (Moolo, 1988) might probably be due to the high trypanolysin and trypanoagglutinin activities observed in these species (Osir, 1995; Stiles, 1990). Studies showed that the midgut killing by trypanolysin in *G. m. morsitans, G. m. centralis* and *G. fuscipes* was lower than in *G. pallidipes* and *G. longipennis.* It is possible that the highest concentration in midgut trypanolysin output of tsetse studied such as *G. longipennis* and *G. pallidipes* appeared to be more resistant while *G. m. morsitans, G. m. centralis* and *G. fuscipes* susceptible. It was reported that the morsitans group of tsetse are better of all trypanosome species (Maudlin, 1991)compared to *G. fuscipes* (Duke, 1936). The trypanolysin activity increased with number of times that the tsetse were fed, since the twice-fed flies showed a higher trypanolysin activity compared to the once-fed flies. Stiles *et a*l. (1990) reported that regular feeding helps to reduce the probability of infection. This might probably be due to the increased trypanolysin activity in non-teneral tsetse which lyses the trypanosomes. It was noted that, trypanolysin activity in *G. m. morsitans* started immediately 5 h after the bloodmeal, and increased gradually reaching a peak from 48-72 h later. Thereafter, the activity decreased with 50% of the peak activity remaining after 192 h. The bloodmeal induced trypanolysin activity in *G. m. morsitans* followed a trend quite similar to that of *G. fuscipes* trypanolysin (Osir, 1999) and also to that of trypsins (Van den Abbelle and Declair, 1992) and agglutinins (Stiles, 1990; Abubakar, 1995). It is possible that trypanolysin, trypsins and agglutinins secreted in tsetse midgut, in response to the serum bloodmeal, are normally responsible for lysing trypanosomes that enter the guts of refractory tsetse, susceptible tsetse simply do not secret enough trypanolysin to remove the invading trypanosomes. Heating the trypanolysin to 60-80□ led to loss of activity. It is possible that midgut trypanolysin activity was consistent with the fact that, trypanolysin, being proteinaceous are denatured by heat, just as was the case with lectins (Abubakar, 1995). It was observed that incubation (27℃) and storage (4℃) reduced trypanolysin inactive after 15 and 32 days, respectively. Also, after the 30^{th} freez-thaw cycle of trypanolysin, the molecule was found to be stable with no appreciable loss of activity. The activity however, progressively decreased after the 33rd, 41st and 55th and *63*□□ cycle at -80□, -70□, -20□, and 0□ respectively, suggesting that several freeze-thaw from the lowest temperature to 27□ denatured the molecule. The activity was however, partially inhibited by aprotinin and completely abrogated by diethylpyrocarbonate (DEPC). DEPC is a known inhibitor of histidine and tyrosine residues. This suggests that unlike trypsins which act through serine -195, trypanolysin lysis trypanosomes by binding through it is histidyl and tyrosyl residues. Ochieng Nyambega (Unpublished Master, 2002) reported that trypanolysin may derive its potency from tyrosine and histidine residues and it is highly likely that the variant surface of the glycoprotein (VSG) contains or the trypanolysin binding protein on the surface of the bloodstream-form *T. b. brucei*. However, is it not clear whether these residues are on the protein itself or on the trypanosome membrane. The observation that there is substrate inhibition of trypanolysin by DEPC very intriguing and it would be interesting to establish the nature of the inhibition and how it related to the *in vivo* levels of the substrate. When the midgut homogenate was separated by anion exchange chromatography, the trypanolysin activity was recovered in the bound fraction. Hence, further biochemistry work will be needed.

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