Purification And Characterization Of G. M. Morsitans Midgut Trypanolysin

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Abstract: In the present study, a trypanolysin induced by component of blood meal in the midgut of tsetse G. m. morsitans was isolated and purified in three steps. The first step was achieved by separation of the midgut homogenate on a conventional anion-exchange chromatography column. The highest trypanolysin activity was recovered in the bound fractions (95%, 05 M NaCl). In the second step, isolation was achieved on a Mono Q anion-exchange column by elution at 70-80% 1 M NaCl. The third step of isolation-purification was achieved by using epoxy-activated Sepharose 6-B-affinity chromatography column. In this case, the trypanolysin was eluted using 20 mM Tris-HCl. The purified native G. m. morsitans trypanolysin was of Mw ≈ 669 kDa, while ≈ 14 kDa trypanolysin was shown in denaturing trypanolysin SDS-PAGE gel for the same tsetse species. It was noticed that the purified trypanolysin was lipidated and also found to be glycosylated.

Index Terms: Characterization, G.m. morsitans, midgut, purification, trypanolysin.

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1 INTRODUCTION

Tb. brucei infects a wide range of mammals. However, human blood, unlike the blood of other mammals, has efficient trypanolytic activity that lyse T. b. brucei. This trypanolytic activity is known as apolipoprotein L-1 which is associated with high density-lipoprotein particles in human serum. Trypanoagglutinins, Glossina proteolytic lectin serine protease (Gpl) (Abubakar et al., 2006), trypsin or trypsin-like enzymes, tsetse cathepsin B, zink carboxypeptidase and zinc metallo-protease (Yan et al., 2002), and trypanolysins among others have attracted considerable interest among parasitologists due to the realization that they may be involved in host-parasite-vector interactions, in such important disease vectors as Glossina (Maudlin and Welburn, 1987) and Rhodnius prolixus (Pereira et al., 1981). For example, the establishment of trypanosome infections in tsetse vector is influenced by several factors, including trypanoagglutinins (Maudlin and Welburn, 1998a, b), trypsin or trypsin-like enzymes (Imbuga et al., 1992a), trypanolysin (Stiles et al., 1990), Wigglesworthia glossinidus (Pais et al., 2008) and Sodalis glossinidus (Attardo et al., 2008), fly species (Welburn et al., 1999) as well as the type of host blood involved (Mihok et al., 1993; Nguu, 1996). Tsetse flies exhibit a considerable level of refractoriness to trypanosome infection (Kubi et al., 2006).   Most of the trypanosomes entering the tsetse are lysed and those that escape continue with development (Gibson and Bailey 2003;

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Peacock et al., 2006). Indeed, it appears that tsetse are poor vectors of trypanosomes since infection in field caught tsetse rareyl exceeds 1-5% (Lehane et al., 2000; Msangi et al., 1998). A key factor in this refractoriness is the fly immune system (Hao et al., 2001). In order to gain insight into mechanisms involved in innate refractoriness, we have studied a molecule, trypanolysin, which plays an important role in lysis of trypanosomes in tsetse midgut.

2 MATERIALS AND METHODS

2.1 Isolation and purification of trypanolysin

Isolation of the molecule was carried out using a combination of ion-exchange and gel permeation chromatography. Midguts from teneral G m. morsitans were dissected 72 h after feeding and homogenized. The homogenate was centrifuged twice (12,000x g, 15 min, 4° C) in a Heraecus Minifuge and the resulting supernatant solution was dialysed overnight with three changes against 20 mM Tris-HCl buffer; pH 8.0 and then filtred through a 0.2 µm Millipore filter (Nalge, Rochester, New York). In the first step, separation was carried out on anion-exchange chromatography column K 16 (Pharmacia, Uppsala, Sweeden) type K 16/20, diameter 1.6 cm, length 20 cm and bed volume 40 ml diethyl aminoethyl (DEAE) Sephacel. This was based on reversible ionic interactions between the molecules of the crude midgut preparation and those of immobilized anion exchanger. The column was pre-equilibrated using 20 mM Tris-HCl buffer, pH 8.0. Crude midgut homogenate (3.0 ml) was then loaded and then the column washed with the same buffer at the rate of 2.0 ml/min^{-12}. The absorbance was continuously monitored at 280 nm. Elution of the bound proteins was carried out using a salt gradient (0.1-0.5 M NaCl) and eluted fractions collected. The eluted fractions were then assayed for trypanolysin activity and the active fractions pooled, concentrated to 0.5 ml using polyethylene glycol (PEG-20,000, Serva, Westbury, NY, USA). After overnight dialysis with three changes against 20 mM Tris-HCl, pH 8.0, the sample was frozen until required. The second step of isolation was carried out using a fast protein liquid chromatography (FPLC) system. The sample (0.5 ml) from the ion-exchange chromatography was loaded via a 500 µl loop into a Mono Q HR 5/5 an ion-exchange column (Pharmacia, Uppsala, Sweeden) attached to a FPLC system equipped with a model gradient programmer (GP-250). The flow rate was maintained at 1.0 ml/min^{-1} and the absorbances continuously monitored at 280 nm. The column

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was first washed with buffer A (20 mM Tris-HCl, pH 8.0) and the bound fractions eluted using a linear gradient of buffer B (20 mM Tris-HCl in 0.0-500 mM NaCl). Eluted Fractions were collected and each tested for trypanolysin activity. The active fractions were pooled, concentrated to 0.5 ml and dialysed against buffer A as described above. The third step of isolation, was carried out using epoxy-activated Sepharose 6-B. The basic procedure for using epoxy was to weigh 2.0 freeze-dried material (about 6.0 ml final gel volume). The gel was washed and reswolled on a sintered glass filter (G-3) using distilled water (100 ml/g). For coupling, the ligand (1.5 g glucosamine) was dissolved in 50 ml distilled water. The ligand solution was mixed with the gel suspension and incubated (16 h, 35° C) in a shaking water bath. The excess ligands was washed away using the coupling solution followed with distilled water, bicarbonate buffer (0.1 M, pH 8.0), acetate buffer (0.1 M, pH 4.0) and the remaining excess groups blocked with 1.0 M ethanolamine for 4 h. Finally, the treated FPLC sample was loaded onto the epoxy column. The flow rate was maintained at 0.5 ml/min^{-1} and the absorbance was continuously maintained at 280 nm. The column was first washed with 20 mM Tris-HCl, pH 8.0 and the fractions were collected, assayed for trypanolysin activity. The active fractions were pooled, concentrated 0.5 ml, dialysed against 20 mM Tris-HCl, pH8.0 as above and frozen until required.

2.2 Polyacrylamide gel electrophoresis (PAGE)
Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to Laemmli (1970). Gradients (4-15%) were cast using a gradient maker (BRL, Gaithersburg, USA). Samples were mixed in an equal volume of sample buffer (130 mM Tris-HCl, 20% glycerol, 0.002% bromophenol blue, 4% SDS, 1% ß-mercaptoethanol, pH 6.8) and boiled for 5 min in a water bath, prior to application on to the gel. Running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3) was used and electrophoresis was performed (27°C) with constant current of (30 milliampere). Electrophoresis under non-denaturing conditions was carried out (27°C) with a constant voltage (V) 70 as described for SDS-PAGE except that the buffers did not contain SDS and ß-mercaptoethanol and the samples were also not heated. After electrophoresis, the gels were stained overnight for proteins with Coomassie Brilliant Blue (0.6%) (Weber and Osborn, 1969) in a solution of acetic acid, methanol and distilled water in ratios of 9.2: 50: 40.8, respectively, overnight. The gels were then soaked (12-20 h, 27°C) with several changes of destaining solution that contained acetic acid, methanol, distilled water in ratios of 7.5: 5: 87.5. The gel was also silver stained.

2.3 Silver staining
The gel was fixed in 50% methanol and 10% acetic acid (2 min), rinsed twice with 50% methanol (10 min) each and then washed with distilled water (5 min). The gel was rehydrated twice with 50% methanol (10 min) each, and 10% of 25% aqueoss glutaraldehyde was added (30 min) then washed with distilled water (30 min). The gel was then stained in Silver stain (containing two solutions, A: 0.8 AgNO3 in 2.5 distilled water; B: 1.0 ml of 2 M NaOH in 20 ml distilled water) and while shaking, solution A was added drop-wise, slowly and carefully not to form a precipitate and then topped with distilled water up to 100 ml (15 min) and the stain removed by washing in distilled water (5 min). For colour development, the gel was

covered by the developer [2.5 ml of citric acid (1% w/v) in to 250 ml cylinder, plus 125 µl of 37-40% formaldehyde solution and then topped up to 250 ml with distilled water] and swirled around in the hands as satisfactory colour stain observed. The colour development was stopped with 5% acetic acid and the gel stored in 7% acetic acid in a plastic bag (Wray et al., 1981).

2.4 Characterization of trypanolysin

2.4.1 Estimation of molecular weight
Both Native and SDS-PAGE were used to determine the molecular weight of the isolated trypanolysin. For estimation of Native molecular weight, Pharmacia protein standards were used: α-lactal albumin ($M_r \approx$ 14,400), trypsin inhibitor ($M_r \approx$ 20,100), carbonic anhydrase ($M_r \approx$ 30,000), ovalbumin ($M_r \approx$ 43,000), albumin ($M_r \approx$ 67,000), phosphorylase b ($M_r \approx$ 94,000), lactase dehydrogenase ($M_r \approx$ 140.000), catalase ($M_r \approx$ 232,000), ferritin ($M_r \approx$ 440,000) and thyroglobulin ($M_r \approx$ 669,000). For SDS-PAGE, Bio-Rad protein standards were used: lysozyme ($M_r \approx$ 14,400), trypsin inhibitor ($M_r \approx$ 21,500), bovine carbonic anhydrase ($M_r \approx$ 31,100), ovalbumin ($M_r \approx$ 45,000), BSA ($M_r \approx$ 66,200) and phosphorylase b ($M_r \approx$ 97,400). The molecular weight of the trypanolysin was estimated from the plots of $\log_{10}$ molecular weights versus the relative migration of the standards.

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

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

3 RESULTS

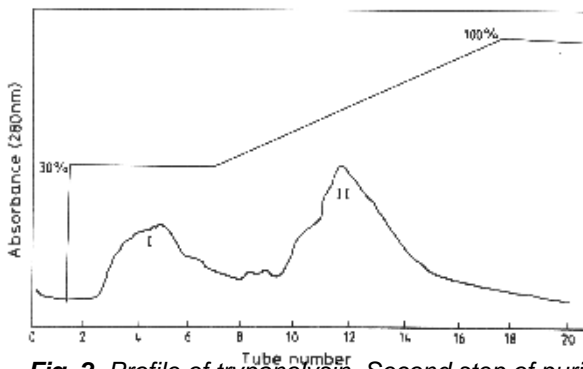### 3.1 Isolation and purification of trypanolysin
Trypanolysin was isolated in three steps. The first step was achieved by separation of the midgut homogenate on a conventional anion-exchange chromatography column using gradient procedure and elution of the bound proteins with increasing concentrations of NaCl (0.1-0.5 M) in 20 mM Tris-HCl, pH 8.0 i.e. changing ionic strength. The highest trypanolysin activity was recovered in the bound fractions (95%, 0.5 M NaCl) (Fig. 1). In the second step of isolation, fraction with trypanolysin activity was isolated on a Mono Q anion-exchange column. Elution of the bound proteins was carried out using a NaCl gradient (0.1-0.5 M) in 20 mM Tris-

HCl, pH 8.0. The first peak was eluted at 45% while the second peak eluted at 70-80% 1.0 M NaCl (Fig. 2). The third step of isolation was achieved by using epoxy-activated Sepharose 6-B-affinity chromatography column. In this case, the trypanolysin was eluted using 20 mM Tris-HCl, pH 8.0 (Fig. 3).
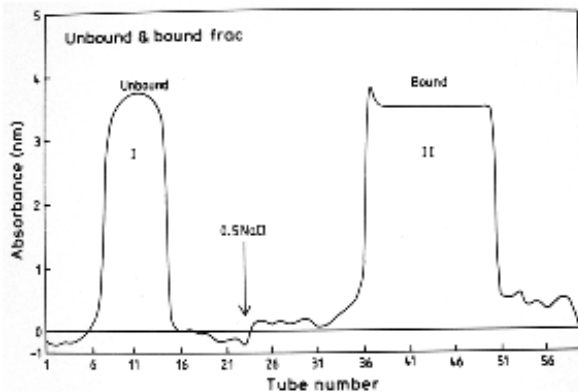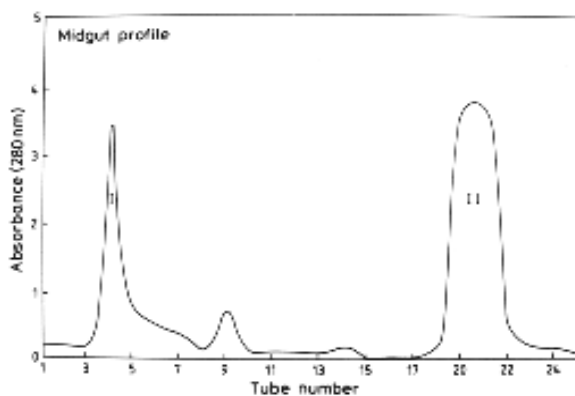

Fig. 1. Profile of trypanolysin. First step of purification.

Separation of G. m. morsitans crude midgut homogenate on anion exchange shows: peak I, the unbound fractions with 20mM Tris-HCl, pH 8.0. and peak II, the bound fraction eluted with increasing concentration of NaCl (0.1-0.5M) in 20 mM Tris-HCl, pH 8.0.

Fig. 2. Profile of trypanolysin. Second step of purification.

Purification of trypanolysin active fraction by Fast Protein Liquid Chromatography (FPLC) was resolved by NaCl gradient solution into two fractions (I and II).

Fig. 3. Profile of trypanolysin. Third step of purification.

The purification of trypanolysin was achieved on Epoxy-activated Sepharose 6-B affinity column. The trypanolysin active fractions was resolved into two peaks using 20mM Tris-HCl, pH 8.0 at a flow rate of 0.5 ml/min.

## 3.2. Characterization of trypanolysin

3.2.1 Molecular weight estimation
The corresponding fractions of semi-purified trypanolysin from ion-exchange and fast protein liquid chromatography columns are showed in Figs. 4 and 5, respectively. The purity of the isolated trypanolysin was ascertained by non-denaturing PAGE. A single band with a molecular weight of Mr≈669 kDa was observed (Fig. 6). Analysis of trypanolysin by SDS-PAGE revealed a single band of Mr ≈ 14 kDa (Fig. 7).

3.2.2 Staining for carbohydrates
The molecule was stained for carbohydrate with Periodic Acid Schiffs stain (PAS). The results showed that the high molecular weight protein was glycosylated (Fig. 8).

3.2.3 Staining for lipids
The presence of lipids in the trypanolysin was confirmed by staining with Sudan Black B. The high molecule weight protein was found to contain lipids (Fig. 9).
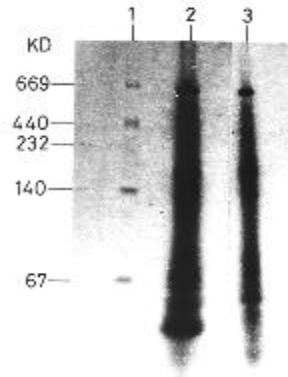
Fig. 4. Molecular weight estimation on non-denaturing –PAGE (14-15%)

(First step purified midgut homogenate fractions)
1. High molecular weight markers (6 µl) (Pharmacia)
2. Crude midgut homogenate (60 µg)
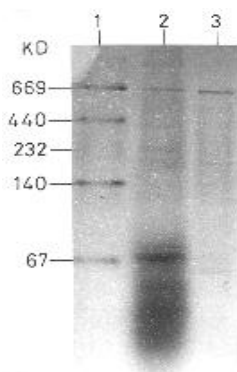3. Semi-purified trypanolysin (40 µg)
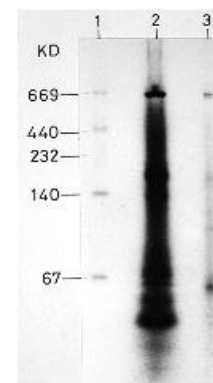
Fig. 5. Molecular weight estimation on non-denaturing-PAGE (4-15%)

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(Second step of purification of the trypanolysin active anion exchange-purified fraction-peak II).
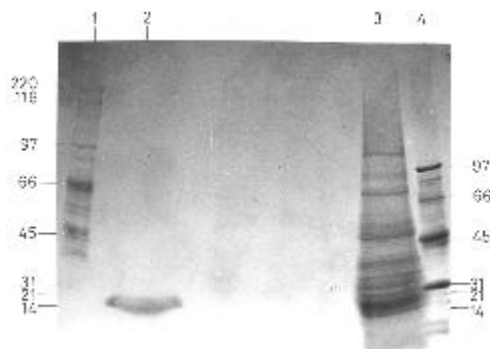1. High molecular weight markers (6 µl) (Pharmacia)
2. Crude midgut homogenate (60 µg)
3. **Semi-purified trypanolysin (40 µg)**

Fig. 6. *Molecular weight estimation on non-denaturing-PAGE (4-15%) (PURIFIED TRYPANOLYSIN)*

Crude midgut homogenates and purified typanolysin were subjected to non-denaturing-PAGE (4-15% Lane:
1. High molecular weight markers (6 µl) (Pharmacia)
2. Crude G. m. morsitans midgut homogenates (30 µg)
3. Purified trypanolysin (60 µg)

Fig. 7. *Molecular weight estimation on SDS-PAGE (4-15%) (Purified trypanolysin)*
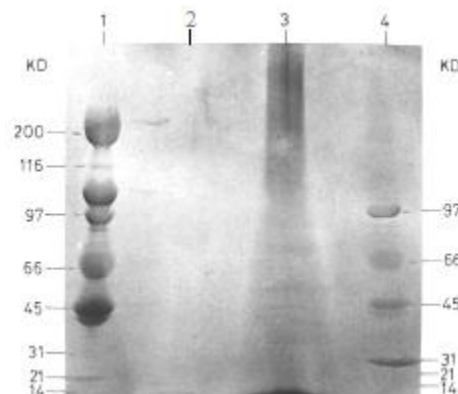
The protein is approximately 14 kDa.
1. High molecular weight markers (6 µl) (Pharmacia)
2. Purified trypanolysin (60 µg)
3. Crude G. m. morsitans midgut homogenates (30 µg)
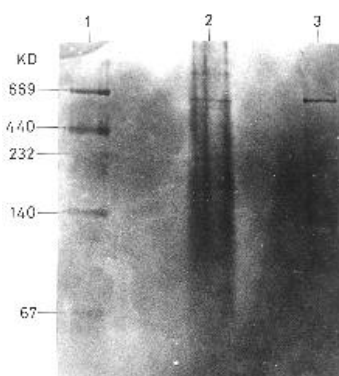
Fig. 8. *Staining for carbohydrates*

(Non-denaturing-PAGE (4-15%) stained with periodic acid Schiff stain) The carbohydrate moiety is at approximately 669 kDa similar to coomassie stained purified native trypanolysin.
1. High molecular weight markers (10 µl) (Pharmacia)
2. G. m. morsitans crude midgut homogenate (60 µg)
3. Purified trypanolysin (30 µg)

Fig. 9. *Staining for lipids*

(SDS-PAGE (4-15%) stained with Sudan Black-B stain) The lipid moiety is at approximately 14 kDa similar to silver stained purified SDS trypanolysin
1. High molecular weigh markers (10 µl) (Pharmacia)
2. Purified trypanolysin (30 µg)
3. G. m. morsitans crude midgut homogenate (60 µg)
4. Low molecular weight markers (10 µl) (Pharmacia)

4 DISCUSSION

In this study, a combination of anion-exchange and affinity chromatography was used to purify trypanolysin. Trypanolysin activity was noted only in the bound fractions on the anion-exchange column indicating a negatively charged active protein. Similar findings using fast protein liquid chromatography were reported for G. p. palpalis and G. p. gambiensis by Stiles et al. (1990). Other studies on G. m. morsitans and G. longipennis also reported that trypanoagglutinin activities were recovered in the bound fractions only (Abubakar et al., 1995). The purified native G. m. morsitans trypanolysin was of Mw ≈ 669 kDa similar to that of G. austeni as noted (Benson et al., 2002). While ≈ 14 kDa protein was shown in denaturing trypanolysin SDS-PAGE gel for G. m. morsitans, Benson et al., (2000) noted two subunits, ≈ 55 kDa and ≈ 29 kDa. The number and exact sizes of G. m. morsitans trypanolysin subunits was not investigated. The high molecular weight of ≈ 669 kDa is because the protein has a carbohydrate and lipid moieties. These core-migrated with the protein as shown in Figs. 8 and 9. The trypanolysin also compares to high molecular weight of Tricon protease (TRI) ≈ 720 kDa isolated from Thermoplasma acidophilum that formed a multi subunit proteolytic complex which composed of a single peptide of 120 kDa (Tamura et al., 1996). Other than the near similarity of TRI and trypanolysin in their Native molecular weight, Osir et al., (1999) eliminated the active involvement of cystine, serine, thiol and metal ions during trypanosome lysis by trypanolysin, whereas the TRI was inhibited by Tosyl-Lysine chloromethyl Ketone (TLCK) and Tosyl-phenyl chloromethyl Ketone (TPCK). This suggests that the TRI is a serine protease unlike trypanolysin. While the recombinant high molecular weight (rHMW) protease from

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Glossina austeni cleaved trypsin-like substrate, the 20 S proteosome from Thermoplasma acidophilum cleaved chymotrypsin-like substrates. There may be some other similarities not yet detected between the TRI and trypanolysin (Osir et al., 1999). Furthermore, the trypanolysin was found to be lipidated and glycosylated. It was reported that a carbohydrate moeity to the side-chain of a residue in a protein chain influences the physico-chemical properties of the protein. Glycosylation is known to alter proteolytic resistance, protein solubility, stability, local structure, life time in circulation and immunogenecity (Lis et al., 1998 and Hounsell et al., 1996). Some lipophorins so far studied consist of two apoproteins, apolipophorin-1 (apolp-1; Mr $\approx$ 210 -250 kDa) and apolipophorin-11 (apolp-11; Mr $\approx$ 70 kDa-85 kDa). Both apoproteins are glycosylated with mannose rich oligosaccharide chains (Ryan et al., 1984; Shapiro et al., 1988). The lipid moiety is predominantly composed of phospholipids and diacyglycerides. Ryan et al. (1984) also reported that a third protein, apolipophorin-111 (apolop-111, Mr$\approx$ 18-20 kDa) associates reversibly with lipophorin of certain insects especially those that utilize lipids for flight. Other workers also have since identified two or three isoform of apolp-111 in Locusta migratoria which are not indistinguishible by SDS-PAGE but separable by either non-denaturing PAGE or by ion-exchange chromatography (Chino and Yazawa, 1986; Van der Host et al., 1991). Chino and Yazawa (1986) have proposed that the isoforms may be due to variations in the phosphorylation of the oligosaccharide chains. Beenakers et al. (1988) also, reported that most of the haemolymph proteins have lipids moiety. Tytler et al. (1995) suggested that the trypanolysin lytic factor lipids do not have a direct role in lysis of trypanosomes but are necessary for the correct assembly of the lytic high density particle. Apolipoprotein A-1 (Apo A-1), apolipoprotein L-111 (apol-111) and apolipoprotein L-1 (apol-1) contributes to lysis in reconstituted particles but individually they are not cytotoxic. Similarly, Hajduk et al. (1995) fractionated trypanolytic activity from normal and Tangier of high-density lipoprotein by gel filtration chromatography and both normal and Tangier sera displayed two peaks of trypanosome lytic activity: one at Mr $\approx$ 1,500 to 600 kDa and the other at > 100 kDa. In addition, Fuanto et al. (1993) purified trypanolytic factor of human serum which was estimated to be of high molecular weight (Mr $\approx$ 3 x 1,000 kDa). Moreover, Mullan et al., (1985) isolated lytic enzyme by means of ion-exchange chromatography and further purified by gel filtration and ultrafiltration. The case of the lipidated trypanolysin in this study might be similar to that of most haemolymph proteins which had been found to have a lipid. However, no investigation has been done on the lipid trypanolysin.

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