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Properties of a blood-meal-induced midgut lectin from the tsetse fly *Glossina morsitans*

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Abstract The properties of a blood-meal-induced lectin (agglutinin) from the midgut of *Glossina morsitans* capable of agglutinating *Trypanosoma brucei* were studied in vitro. The midgut homogenate from flies that had been fed twice had the highest agglutination activity, followed by that from the once-fed flies and that from the unfed insects. As compared with the bloodstream-form trypanosomes, a much lower concentration of the midgut homogenate was required for agglutination of the procyclic parasites. Furthermore, the agglutination process was specifically inhibited by D-glucosamine. Soybean trypsin inhibitor abrogated agglutination of the bloodstream-form parasites, whereas the procyclics were unaffected. The agglutination process was temperature-sensitive, with little activity being evident between 4° and 15° C. Similarly, heating the midguts to 60°–100° C led to loss of activity. When the midgut homogenate was separated by anion-exchange chromatography, the agglutination activity co-eluted with trypsin activity at approximately 50% NaCl. These results suggest a very close relationship between midgut trypsin-like enzyme and the agglutinin. Since successful agglutination of bloodstream-form trypanosomes requires protease activity, it may be that the enzyme cleaves off some surface molecules on the parasite surface, thus exposing the lectin-binding sites.

Introduction

Agglutinins (lectins) are a group of carbohydrate-binding proteins with a ubiquitous distribution in nature (Barondes 1981). In insects and other invertebrates, agglutinins are believed to be involved in defence mechanisms that contribute to both cell-mediated and humoral immunity (Boman and Hultmark 1987; Natori 1990). More recently, however, these molecules have attracted considerable interest among parasitologists due to the realization that they may be involved in host-parasite vector interactions, especially in such important disease vectors as *Glossina* (Maudlin and Welburn 1987) and *Rhodnius prolixus* (Pereira et al. 1981). For example, in *Glossina* species the midgut lectins have been implicated in the establishment of trypanosome infection within the flies (Maudlin and Welburn 1988a, b).

According to a model proposed to explain this process, the lectins mediate both lysis and differentiation of the parasites (reviewed in Maudlin 1991). In susceptible tsetse flies the action of an endochitinase produced by *Rickettsia*-like organisms (RLOs) leads to accumulation of glucosamine in the fly midgut, which in turn blocks the lectin-mediated trypanocidal activity (reviewed in Molyneux and Stiles 1991). In contrast, refractory flies with few RLOs produce relatively less glucosamine and the parasites entering these flies are consequently more vulnerable to lysis by the lectins.

Although the importance of lectins in parasite-vector interactions has become widely recognized, there is insufficient information on the properties of these molecules from different tsetse fly species. In this paper we report on the properties of the midgut lectin (agglutinin) from *G. morsitans morsitans*.

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Materials and methods

Preparation of midgut homogenates and parasites

Male tsetse flies (*Glossina morsitans morsitans* Westwood) used in these studies were reared as previously described (Osir et al. 1993). A total of 100 teneral flies (24 h after emergence) were fed on rat blood and then starved for 72 h. The flies were immobilized by brief chilling at 4° C, after which their midguts were dissected, dipped several times in phosphate-buffered saline (PBS; 0.1 *M* sodium phosphate (pH 8.0) containing 0.15 *M* NaCl) to remove any haemolymph and then resuspended in 1.0 ml ice-cold PBS. The midguts were then homogenized briefly using a Virtis homogenizer (Gardiner, USA), centrifuged (12,000 *g*, 1 h, 4° C) in a Heraeus

2 Minifuge (Osterode, Germany) and the supernatant solution was stored at −20° C.

Male Wistar rats were infected with a *Trypanosoma brucei brucei* stock derived from EATRO 1969 (Otieno et al. 1983). Parasitized blood was obtained from the rats by cardiac puncture just before the peak of parasitaemia and the parasites were isolated as previously described (Lanham and Godfrey1970). Procyclic culture forms of the parasites were obtained by transferring the bloodstream forms into an SDM-79 cultivation medium (Brun and Jenni 1977) that contained 10% foetal calf serum. Antibiotics were excluded from the medium. The trypanosomes were cultivated at 27° C with twice-weekly passages. Before use, the parasites were pelleted from the medium by centrifugation (1,000 *g*, 10 min, 4° C) and washed once in PBS to remove the serum components. The parasites were counted using a haemocytometer equipped with improved Neubauer ruling. The procedures for trypsin assay and estimation of protein have been described elsewhere (Imbuga et al. 1992).

Agglutination assays

A sample of the crude midgut homogenate (~300 µg protein) was used to prepare doubling serial dilutions in PBS containing $MgCl_2$ (0.1 mg/ml) and $CaCl_2$ (0.13 mg/ml). The total volume was maintained at 0.02 ml. The samples were placed into the wells of a microtitre plate (Nunc, Denmark), and an equal volume of the trypanosomes (bloodstream or procyclic forms) containing ~5.0×10^6 parasites/ml was added to each dilution. After mixing, the plate was incubated (27° C, 2 h) and the agglutination was scored using an inverted microscope (Leitz Dialux, Germany). All tests were carried out in duplicate, with the controls consisting of only the parasites and PBS.

Induction of agglutination by blood meal

In this study, midguts were dissected from unfed flies at 24 h after emergence. The once-fed flies were given a blood meal at 24 h after emergence and the midguts were dissected after 72 h. The twice-fed flies were fed at 24 h after emergence, starved for 72 h and refed, and the midguts were obtained after 72 h. The homogenates prepared from these groups of flies were tested for their abilities to agglutinate bloodstream-form parasites as described above. In another experiment, 140 teneral flies were given a blood meal and 20 flies were killed at 24-h intervals. The midgut homogenates prepared from these flies were then assayed for agglutination activities.

Agglutination of bloodstream- and procyclic-form trypanosomes

In this study, midgut homogenate was prepared from once-fed flies as described above. Aliquots of the homogenate were separately incubated with either bloodstream- or procyclic-form trypanosomes and agglutination was scored as described above.

Effect of temperature on agglutination

The midgut homogenate prepared from once-fed flies was incubated for 20 min in a water bath maintained at different temperatures (37°–70° C). The samples were subsequently allowed to cool to room temperature (27° C), and the agglutination assays were carried out using procyclic-form parasites. The samples were maintained at 27° C in the control experiment.

Effect of sugars and a protease inhibitor on agglutination

Double serial dilutions of once-fed midgut homogenate were mixed with varying concentrations (50–700 m*M*) of different sugars (listed in Table 4). After incubation (27° C, 30 min), procyclic parasites were added to each mixture and agglutination was scored as described above. In a separate study, increasing concentrations (0–1 mg/ml) of soybean trypsin inhibitor (SBTI; Millipore Corp., Freehad, Wash., USA) were mixed with samples of midgut homogenate prior to addition of the bloodstream- or procyclic-form parasites. In both cases, agglutination of the parasites was assessed.

Partial purification of the agglutinin

Midgut homogenate prepared from 200 twice-fed flies (72 h after the last feed) was centrifuged (12,000 *g*, 1 h, 4° C) in a Heraeus 2 Minifuge. The supernatant fraction was dialysed (24 h, two changes) against 20 m*M* TRIS-HCl buffer (pH 8.0) and then filtered through a 0.2-µm Millipore filter (Nalge, Rochester, N.Y., USA). Separation of the sample was carried out on a Mono Q HR 5/5 anion-exchange column (Pharmacia, Uppsala, Sweden) attached to a fast protein liquid chromatography (FPLC) system equipped with a model G-250 gradient programmer.

Results

Induction of agglutination activity by blood meals

The induction of agglutination activity by blood meals is shown in Fig. 1. The activity increased, gradually reaching a peak at 48–72 h after the blood meal, and then decreased rapidly, with only 36% of the peak activity remaining after 144 h. The agglutination activity also increased with the number of times that the flies were fed. For example, at 72 h the twice-fed flies had the highest titre (4,096), followed by the once-fed flies (2,048) and then the unfed insects (2).

Agglutination of bloodstream- and procyclic-form trypanosomes

A sample of the midgut homogenate was tested for its ability to agglutinate bloodstream and procyclic culture forms of *Trypanosoma brucei brucei*. The bloodstream and procyclic forms gave agglutination titres of 64 and

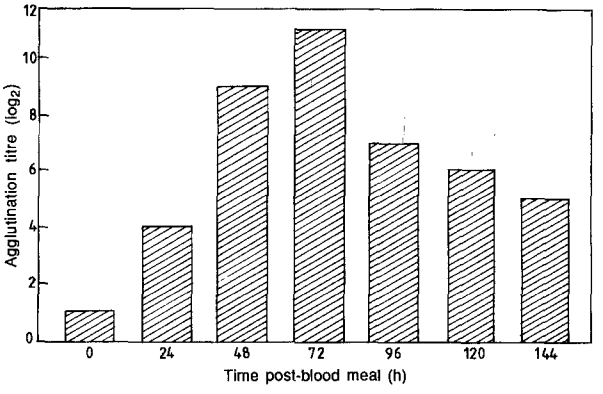

Fig. 1 Time course of agglutination activity in *Glossina morsitans*. Teneral flies (24 h post-emergence) were fed on uninfected rat blood. At 24-h intervals, five flies were killed and their midguts were dissected. The midgut extracts were used in agglutination assays with procyclic trypanosomes as described in Materials and methods. The agglutination titres are expressed as $\log_2$ values

2,048, respectively. Thus, as compared with the bloodstream-form parasites, a much lower concentration of the same midgut homogenate was required in the case of procyclic culture forms.

Effect of temperature and a protease inhibitor on agglutination

Exposing the midgut homogenate to various temperatures led to loss of the agglutination activity. At temperatures above 50° C the agglutination titres were reduced by 90% (Table 1). In a separate study, increasing concentrations of SBTI caused a marked inhibition of the agglutination of bloodstream-form parasites (Table 2). As compared with controls, SBTI at 1.0 mg/ml decreased agglutination by 94%. The same concentration of SBTI inhibited trypsin activity by 88%. In contrast, agglutination of the procyclic-form parasites was unaffected by SBTI even at 1.0 mg/ml. The highest concentration of SBTI tested also failed to affect the trypanosomes.

Table 1 Heat stability of the trypanoagglutinin[a]

Temperature (° C)	Agglutination titre
27	2,048
37	2,048
42	512
50	64
60	2
70	2

[a] Once-fed midgut homogenates were pre-incubated for 20 min at the respective temperatures and then brought to room temperature. Agglutination activity was determined as described in Materials and methods. Agglutination titres are expressed as reciprocals of end-point dilution

Table 2 Effect of a protease inhibitor on agglutination[a]

Inhibitor (mg/ml)	Agglutination titre		Trypsin activity (μmol ml^{-1} min^{-1})
	Bloodstream	Procyclics	
0.0	64	2,048	17.7
0.2	64	2,048	12.4
0.4	64	2,048	8.9
0.6	16	2,048	5.3
0.8	8	2,048	3.6
1.0	4	2,048	2.2

[a] Doubling serial dilutions of once-fed midgut homogenate were pre-incubated with SBTI (0–1 mg/ml) for 30 min. Agglutination and trypsin activities were determined as described in Materials and methods

Inhibition of agglutination by sugars

Of the sugars tested, D-glucosamine showed the strongest inhibition of agglutination activity (Table 3). At a sugar concentration of 200 m*M* the agglutination activity

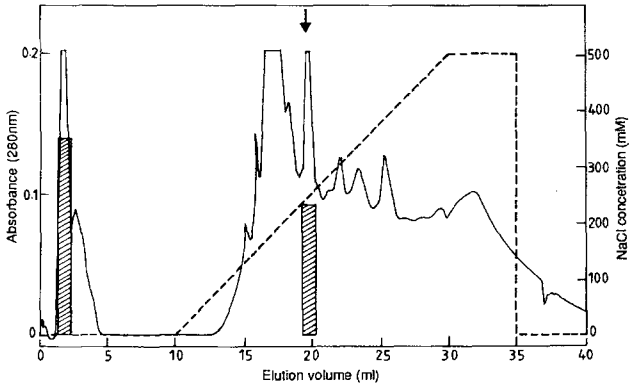

Fig. 2 Separation of crude midgut extracts by anion-exchange chromatography. Midguts were dissected from twice-fed flies at 72 h after the last feed. After homogenization, the extract (~15 mg protein) was loaded onto an FPLC Mono Q column via a 500-μl loop. The column was equilibrated with 20 m*M* TRIS-HCl (pH 8.0). The flow rate was maintained at 0.5 ml m^{-1} and the absorbance was continuously monitored at 280 nm. Elution was accomplished with a linear salt gradient ranging from 0 to 500 m*M* (----). Fractions (1.0 ml) were collected and assayed for agglutination (*arrow*) and trypsin (▨) activities. The *solid line* shows the absorbance at 280 nm

Table 3 Effect of carbohydrates on parasite agglutination[a]

Type of sugar	Concentration (m*M*)							
	0	100	200	300	400	500	600	700
p-Nitrophenyl glucopyranoside	2,048	2,048	1,024	1,024	1,024	1,024	1,024	1,024
m-Nitrophenyl glucopyranoside	2,048	2,048	1,024	1,024	1,024	1,024	1,024	1,024
Sucrose	2,048	2,048	1,024	1,024	1,024	512	512	256
D-Glucose	2,048	2,048	1,024	1,024	1,024	512	128	128
D-Galactose	2,048	1,024	1,024	1,024	256	128	128	128
N-Acetyl-D-glucosamine	2,048	512	256	256	256	256	256	256
D-Glucosamine	2,048	8	4	2	2	–	–	–

[a] Doubling serial dilutions of once-fed midgut homogenate were pre-incubated with the various sugars for 30 min and agglutination activities were determined using procyclic trypanosomes. The control consisted of reaction mixture in the absence of sugar. Agglutination titres are expressed as reciprocals of end-point dilutions

was reduced by 90%. On the other hand, the same concentration of *N*-acetyl glucosamine reduced the agglutination titre by only 30%. In the case of galactose, glucose, sucrose, m- and p-nitrophenyl glucopyranoside, a concentration of 200 m*M* reduced the agglutination activities by 10%. By contrast, N-acetyl-D-galactosamine, maltose, lactose and mannose had no inhibitory effect even at a concentration of 700 m*M*.

Partial purification of the agglutinin

Separation of crude midgut homogenates by anion-exchange chromatography gave trypsin activities in both the unbound and the bound fractions (Fig. 2). These fractions had elution volumes (V_e) of 2 and 20 ml, respectively. Interestingly, the agglutination activity co-eluted with the trypsin activity in the bound fraction. The agglutination activity was not detected in any other fraction.

Discussion

In *Glossina* species, a blood meal stimulates the release of different molecules, including proteolytic enzymes, lectins, lysins and probably other yet-uncharacterised molecules (Molyneux and Stiles 1991). In the case of a trypanosome-infected blood meal, the lectins (agglutinins) are proposed to stimulate parasite differentiation (Maudlin and Welburn 1988a, b), although the underlying mechanisms are poorly understood. Recent work in our laboratory has also shown the importance of the midgut trypsins in the differentiation process (Imbuga et al. 1992). This was based on the realization that the so-called tsetse midgut lectins and trypsins share several similarities, suggesting that these two molecules may be very closely related (Osir et al. 1993). The present study was therefore undertaken to examine this proposition further by studying the properties of the lectins in crude tsetse midgut homogenates.

The blood-meal-induced agglutination activity in *G. morsitans morsitans* followed a pattern quite similar to that of the trypsins (Van den Abbelle and Decleir 1992). Starting immediately after the blood meal, the activity increased and reached a peak 48–72 h later. Similar findings have been reported in *G. palpalis*, where peak agglutination activity occurs at 48 h post-blood meal (Stiles et al. 1990). An interesting observation was that as compared with the bloodstream-form parasites, a much lower concentration of the lectin was required to agglutinate the procyclics. A possible explanation for this observation may be that the lectin-binding sites on the surface of procyclics are more accessible such that both the interaction with the lectin and agglutination are facilitated.

The tsetse midgut lectin activity was heat-labile, an observation consistent with the observation that lectins, being proteins, are denaturable by heat. In insects, similar findings have been reported for *G. palpalis* midgut lectin (Stiles et al. 1990) and for haemolymph lectins from *G. fuscipes fuscipes* (Ingram and Molyneux 1990) and *Calliphora vomitoria* (McKenzie and Preston 1992). Interestingly, only the agglutination of bloodstream-form parasites was abrogated by a specific trypsin inhibitor, suggesting that the enzyme was not required for agglutination of the procyclics. It is likely that the enzyme may be required to cleave off some surface molecules on the bloodstream forms, a process that may expose the lectin-binding sites and thus facilitate agglutination. Other studies have also revealed that agglutination titres against red blood cells can be enhanced by proteolytic enzymes (Ingram and Molyneux 1990).

A major property of lectins is their specific saccharide-binding sites (Barondes 1981). In the case of *G. m. morsitans* midgut lectin, specificity was directed to D-glucosamine. The importance of a free amine group was evident since acetylation of this group as in *N*-acetyl-D-glucosamine led to poor inhibition. These results are in conformity with those of both Ibrahim et al. (1984) and Maudlin and Welburn (1988a, b). In contrast, the haemolymph lectin from *G. m. morsitans* is specific to galactose and its derivatives (Ingram and Molyneux 1990).

Work by Gooding and Rolseth (1976); Cheeseman and Gooding (1985) resulted in the identification of at least six proteolytic enzymes in the midgut of *G. m. morsitans*. Of these, two enzymes (trypsin and a trypsin-like enzyme) were described on the basis of differences in their molecular weights, Michaelis constants (K_m values) and affinities to diethylaminoethanol (DEAE)-cellulose. In this study, separation of the midgut homogenate by anion-exchange chromatography gave two enzyme activity peaks in both the unbound (trypsin) and the bound (a trypsin-like enzyme) fractions. Interestingly, the trypsin-like enzyme co-eluted with the lectin activity. We have to ascertain whether there are two separate molecules or one molecule with both lectin and enzyme activities.

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