Metalloproteases in *Trypanosoma rangeli*-infected *Rhodnius prolixus*

D Feder, SAO Gomes, ES Garcia, P Azambuja+

Departamento de Bioquímica e Biologia Molecular, Instituto Oswaldo Cruz, Av. Brasil 4365, 21045-900 Rio de Janeiro, RJ, Brasil

Protease activities in the haemolymph and fat body in a bloodsucking insect, Rhodnius prolixus, *infected with* Trypanosoma rangeli, *were investigated. After SDS-polyacrylamide gel electrophoresis containing gelatin as substrate, analysis of zymograms performed on samples of different tissues of controls and insects inoculated or orally infected with short or long epimastigotes of* T. rangeli, *demonstrated distinct patterns of protease activities: (i) proteases were detected in the haemolymph of insects which were fed on, or inoculated with, short epimastigotes of* T. rangeli *(39 kDa and 33 kDa, respectively), but they were not observed in the fat body taken from these insects; (ii) protease was also presented in the fat bodies derived from naive insects or controls inoculated with sterile phosphate-saline buffer (49 kDa), but it was not detected in the haemolymph of these insects; (iii) no protease activity was observed in both haemolymph and fat bodies taken from insects inoculated with, or fed on, long epimastigotes of* T. rangeli. *Furthermore, in short epimastigotes of* T. rangeli *extracts, three bands of the protease activities with apparent molecular weights of 297, 198 and 95 kDa were detected while long epimastigotes preparation presented only two bands of protease activities with molecular weights of 297 and 198 kDa. The proteases from the insect infected with* T. rangeli *and controls belong to the class of either metalloproteases or metal-activated enzymes since they are inhibited by 1,10-phenanthroline. The significance of these proteases in the insects infected with short epimastigotes of* T. rangeli *is discussed in relation to the success of the establishment of infection of these parasites in its vector,* R. prolixus.

Key words: fat body - haemolymph - *Rhodnius prolixus* - metalloprotease - *Trypanosoma rangeli*

Trypanosomes are digenetic parasites that usually have insects as vectors and infect humans beings and other animals as hosts (Brener 1973, D'Alessandro 1976, Garcia & Azambuja 1991). *Trypanosoma rangeli*, a South American trypanosome, is a harmless parasite of humans and various wild and domestic animals (Ellis et al. 1980). *T. rangeli* after being ingested as trypomastigotes by its vector *Rhodnius prolixus*, multiplies as epimastigotes in the midgut and invade the haemocoel. In a normal infection course, a few days after infection, short epimastigotes appear in the haemocoel of the insect. Soon, they disappear to be replaced by a massive colonisation by long epimastigotes (Mello et al. 1995). The epimastigotes survive in the haemolymph and/or inside the haemocytes, migrate to and complete their development in the salivary glands (Takle 1988). The parasites are transmitted when the insects are feeding blood from the vertebrate host (Grewal 1957, Tobie 1968, 1970, Cuba 1975, Cuba Cuba, 1998).

The interaction of protozoan parasites with their vectors may be modulated by a complex defensive response (Molyneux et al. 1986, Kaaya 1989, Ingram & Molyneux 1991). Gregorio and Ratcliffe (1991), using *in vitro* activation of the prophenoloxidase (proPO) system, demonstrated that the susceptibility of *R. prolixus* to *T. rangeli* haemolymph infection, at least in part, may have been associated with the suppression of the activation of proPO in the presence of this flagellate. On the other hand, several authors suggest that agglutinins and/or lectins of different insects are important for both the establishment of infection and the development of the parasites in the gut and/or haemolymph of the vectors (Ibrahim et al. 1984, Wallbanks et al. 1986, Barraco & Loch 1988, Ingram & Molyneux 1990, Welburn & Maudlin 1990, Mello et al. 1999).

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+Corresponding author. Fax: +55-21-590.3495. E-mail: azambuja@gene.dbbm.fiocruz.br
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In the present work, we extend our investigation on the interaction *T. rangeli* and its vector, *R. prolixus*, and demonstrate that short epimastigotes of *T. rangeli* (but not long ones) given orally or inoculated into the insects induce distinct patterns of protease activities in the insect's haemolymph. Both proteolytic activities have the same apparent molecular weights and are inhibited by 1,10-phenanthroline, suggesting that they might be either metalloproteases or metal-activated enzymes. In addition, epimastigotes of *T. rangeli* present protease activities with molecular weights distinct from the vector enzymes.

MATERIALS AND METHODS

Insect and feeding - Adults of *R. prolixus* originated from a colony raised and maintained in the laboratory at a RH of 60%-70% and 28±1°C (Garcia et al. 1984) were used. The insects were starved for 15 days after the imaginal ecdysis and then fed on human erythrocytes suspended in citrated human heat-inactivated plasma through a membrane feeder.

Parasites - *T. rangeli* strain H14 epimastigotes (donated by Maria Auxiliadora de Sousa, Fiocruz, Brazil) were maintained at 28°C in NNN and LIT media (Jaffe et al. 1984, Chiari & Camargo 1984) supplemented with 20% heat-inactivated foetal bovine serum. Long epimastigotes of *T. rangeli* (95-100% purity) were obtained from the late stationary growth phase (12 days of culture) of short epimastigotes (99-100% purity) were originated from the log growth phase (7 days of culture) of the parasites (Gomes et al. 1997).

T. rangeli infection of R. prolixus - The infections of *R. prolixus* adults were performed by inoculation of short and/or long epimastigotes of *T. rangeli* or by feeding on blood meals containing different forms of the parasites.

Blood meals were prepared by centrifugation of the citrated human blood just before feeding the insects. The erythrocytes were added, after heat-inactivation of the plasma, to complete the original volume of blood. Short or long epimastigotes of *T. rangeli* were suspended in the heat-inactivated plasma at a concentration of $1x10^6$ flagellates/ml of blood meal for oral infection. Control insects were fed on a blood meal. *T. rangeli* used for inoculation into insects were obtained from cultures of short or long epimastigotes as already described. Before inoculations the culture medium containing the parasites was centrifuged at 2,000 g for 10 min, and the pellets washed five times in 0.14M NaCl in 0.01 M phosphate buffer (PBS), pH 7.2. The flagellates were injected laterally into the thorax, five days after feeding, with 1 µl of trypanosome suspension ($1x10^6$ cells/ml) in PBS using a 10-µl Hamilton syringe connected to a fine needle. Control insects were naive or insects inoculated with sterile PBS five days after feeding. The percentage of mortality during the entire experiment was minimal (<5%).

Collection of haemolymph and abdominal fat bodies - Haemolymph was collected with micropipettes by cutting the metathoracic legs of 20 insects one day after inoculation. The haemolymph was pooled in Eppendorf tubes on ice, then centrifuged at 5,000 g for 5 min, and the supernatants were frozen at -20°C until use. Fat body extracts were prepared in 50 mM phosphate buffer, pH 8.0 (PB) from the bled insects by dorsal excision of the abdomen. Digestive tract, Malpighian tubules and reproductive organs were removed and the abdominal fat bodies were easily collected, immediately washed in *Rhodnius saline* (0.1M NaCl, 25 mM KCl and 10 mM $CaCl_2$), transferred to 100 µl of PB and kept at -20°C until use.

Trypanosome suspension - *T. rangeli* used for zymogram techniques were obtained from cultures of short or long epimastigotes, described above. Before assay, the culture medium containing the parasites was centrifuged and the pellets washed three times in 0.14M NaCl in 0.01 M phosphate buffer (PBS), pH 7.2 and then the samples were frozen at -20°C until use.

Zymogram technique for detection and partial characterisation of proteases - Proteases were assayed by 14% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) with 0.1% gelatin incorporated into the gel as substrate (Heussen & Dowdle 1980). Briefly, one whole insect abdominal fat body was homogenised in 100 µl of PB, centrifuged at 10,000 g for 15 min, and the supernatant collected. Proteins in the haemolymph and fat body samples were quantified with protein dosage kit (Sigma Chem. Co., USA) using bovine serum albumin (BSA) as standard (Lowry et al. 1951). Ten µl of fat body homogenates or 10 µl of haemolymph were added to 5 µl of sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 2% bromophenol blue) in a concentration approximately of 200 µg of protein per sample. From this material, without boiling, the gels were loaded with this aliquot and electrophoresis was carried out at a constant current of 20 mA at 4ºC. Then, gels were incubated for 48 hr at 37ºC in PB, pH 8.0, using slow rotation shaker, in a vial containing 10 volumes of 2.5% Triton X-100 in distilled water, and then stained for 30 min with Coomassie blue R-250 in methanol-acetic acid-water (10:10:80) and destained in the solvent. The molecular mobility of proteins was determined by interpolation from the mobilities of commercial prestained standards (Sigma Chem. Co., USA) by computer analysis. In another electrophoresis gel, to characterise the proteases, the following com-

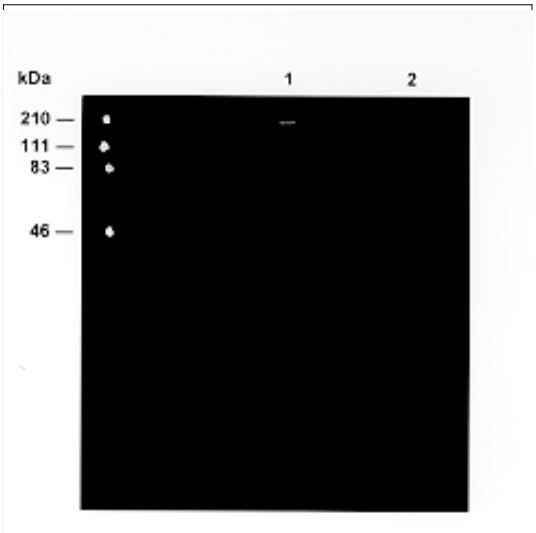

Fig. 1: analysis of the proteolytic activity of short or long epimastigotes of *Trypanosoma rangeli* using SDS-polyacrilamide gels containing gelatin as substrate. Lane 1: short epimastigotes; lane 2: long epimastigotes. The numbers refers to the position of the molecular weight markers.

pounds were added to PB: phenylmethanesulphonyl fluoride (PMSF- 0.1 and 1 mM), Elastatinal (10-100Mm), N-α-tosyl-L-lysylchlotomethyl ketone (TLCK-10-100 μM), L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64- 1-10 μM) in a 2 mM dithiothreitol (DTT), leupeptin (10 and 100 μM), pepstatin (1 μM) or 1,10-phenanthroline (5 and 10 mM). All these reagents were from Sigma Chemical Co., USA.

RESULTS

Protease activity of short and long epimastigotes of T. rangeli - SDS-PAGE containing gelatin demonstrated that in the short epimastigotes sample three bands of the protease activities with apparently molecular weights of 297, 198 and 95kDa (Fig. 1, lane 1). Long epimastigotes preparation presents only two bands of the protease activities with apparently molecular weight of 297 and 198 kDa (Fig. 1, lane 2).

Protease activity in the haemolymph and fat body of insects fed on blood meal containing short or long epimastigotes of T. rangeli - Haemolymph from control insects did not show any apparent protease activity (Fig. 2A, lane 3). However, one protease band could be detected in haemolymph from insects fed on blood containing short epimastigotes of *T. rangeli* (Fig. 2A, lane 1). This protease activity had an apparent molecular weights of 39 kDa. In contrast, fat bodies taken from insects fed on blood containing short epimastigotes of *T. rangeli* did not present any protease activity (Fig. 2B, lane1) whereas fat bodies from control insects had one protease band with an apparent molecular weight of 49 kDa (Fig. 2B, lane 3). However, in insects fed on blood containing long forms of epimastigotes of *T. rangeli* we did not observe any protease activity in the haemolymph (Fig. 2A, lane 2) or fat body (Fig. 2B lane 2). All these patterns were similar in five independent experiments.

Protease activity in the haemolymph and fat body of insects inoculated with short and long epimastigotes of T. rangeli - One day after inoculation of short epimastigotes the haemolymph

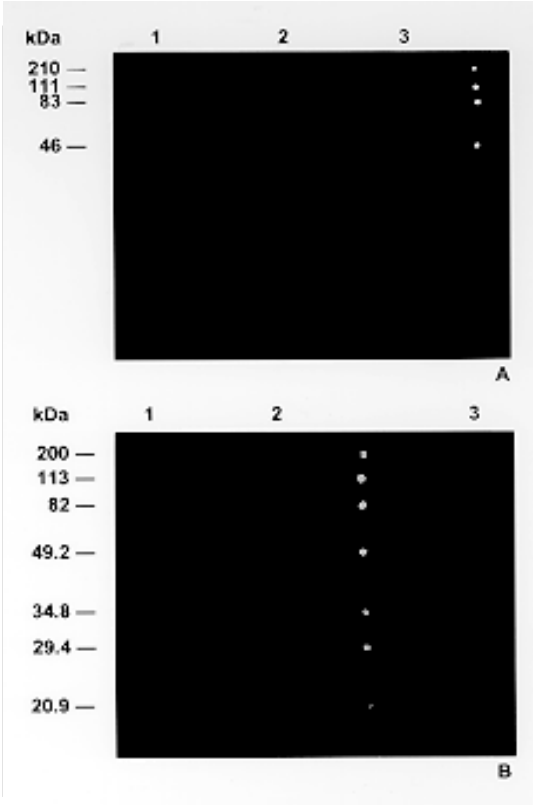

Fig. 2-A: analysis of the proteolytic activity of *Rhodnius prolixus* haemolymph from insects fed on blood meal containing short or long epimastigotes of *Trypanosoma rangeli* using SDS-polyacrilamide gels containing gelatin as substrate. Lane 1: haemolymph from insects infected with short epimastigotes; lane 2: haemolymph from insects infected orally with long epimastigotes; lane 3: haemolymph from control insects. The numbers refers to the position of the molecular weight markers. B: analysis of the proteolytic activity of *R. prolixus* fat body from insects fed on blood meal containing short or long epimastigotes of *T. rangeli* using SDS-polyacrilamide gels containing gelatin. Lane 1: fat body from insects infected with short epimastigotes; lane 2: fat body from insects infected with long epimastigotes; lane 3: fat body from control insects. The numbers refers to the position of the molecular weight markers.

sample showed two bands of protease activities with apparently molecular weights of 33 and 39 kDa (Fig. 3A, lane 3). Control insects inoculated with PBS or naive insects had no protease activity detected in the haemolymph (Fig. 3A, lane1). SDS-PAGE containing gelatin of fat bodies removed from insects inoculated with short forms of T. rangeli did not present any protease activity (Fig. 3B , lane 1) whereas fat bodies from control insects had one protease band with an apparent molecular weight of 49 kDa (Fig. 3B, lane 3). However, we did not observe any protease activity in the haemolymph (Fig. 3A, lane 2) and fat body preparation of insects inoculated with long epimastigotes of *T. rangeli* (Fig. 3B, lane 2). Also, these patterns were similar in five independent experiments.

Partial characterization of the proteases - The results displayed in Table show that the proteolytic activities detected in the haemolymph and fat body preparations were not altered in the presence of the cysteine- and serine-protease inhibitor, leupeptin, with the specific cysteine inhibitor, E64, or with the serine-protease inhibitors, elastatine, PMSF and TLCK nor with the aspartic protease inhibitor, pepstatin. Inspection of SDS-PAGE gelatin-containing gels run with the haemolymph of insects infected with short epimastigotes of *T. rangeli* and those observations of gels with the fat body preparation of control insects showed no proteolytic activity in the presence of 1,10-phenanthroline. These data indicate that the proteases from the haemolymph of *T. rangeli* infected insects and fat body preparations from controls are similar and belong to the class of either metalloproteases or metal-activated enzymes (Table).

DISCUSSION

The results described above demonstrate that protease activity is detected in the fat bodies of insects either naive or inoculated with sterile PBS, with molecular weights in the range of 49 kDa. In these insects the assays of proteolytic activity in gelatin-containing gels show that in the haemolymph no enzymes are observed. However, the oral infection with short epimastigotes of *T. rangeli* was able to induce protease activities in the haemolymph with a molecular weight of 39 kDa but in the fat bodies of these insects no proteolytic activity could be detected. Similarly, the inoculation of short epimastigotes of *T. rangeli* into the haemocoel expressed two proteases with molecular weights of 33 and 39 kDa in the haemolymph and no protease in the fat body preparation was detected. However, these findings are in contrast to the situation observed when feeding infected *R. prolixus* or by inoculation of long epimastigotes of *T. rangeli*. Inspection of the SDS-PAGE gelatin-containing gels demonstrates that no proteolytic activity was detected in either the haemolymph or fat body preparation of *R. prolixus* infected with long epimastigotes. Based on the molecular weights the proteases in the haemolymph and fat bodies clearly are not the same as those observed in the short and long epimastigotes of *T. rangeli* (compare Fig. 1 with the others figures) due to the differ-

TABLE

Effects of inhibitors on the protease activities in SDS-PAGE containing gelatin as substrate

Inhibitor class	Compounds[a]	Tissue	Concentration
No inhibition			
Serine protease	PMSF	FB	0.1 and 1 mM
		H	0.1 and 1 mM
	Elastatinal	FB	10 and 100 μM
		H	10 and 100 μM
	TLCK	FB	10 and 100 μM
		H	10 and 100 μM
Cysteine protease	E 64 [b]	FB	1 and 10 μM
		H	1 and 10 μM
Aspartic protease	Pepstatin	FB	1μM
		H	1μM
Serine/cysteine protease	Leupeptin	FB	10 and 100 μM
		H	10 and 100 μM
Inhibition metalloprotease	1,10-phenanthroline	FB	5 and 10 mM
		H	5 and 10 mM

a: see Materials and Methods; *b*: in a 2 mM DTT solution; H: haemolymph taken from insects infected with short epimastigotes of *Trypanosoma rangeli*; FB: fat body from insect controls.

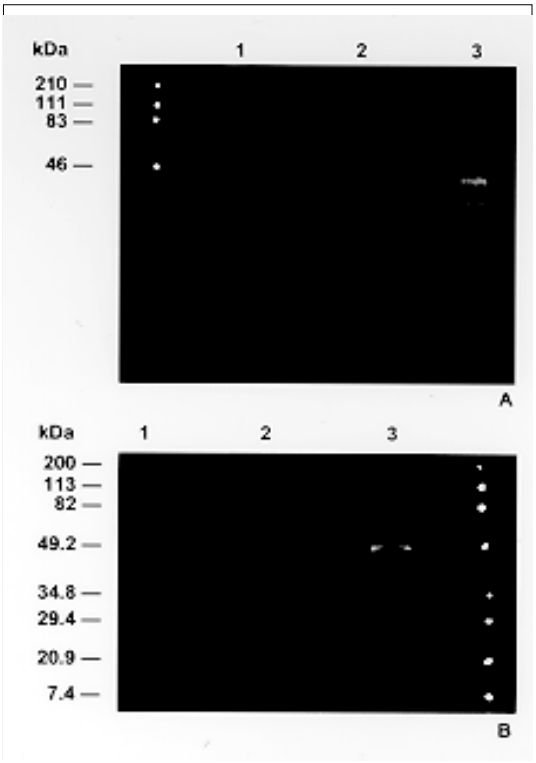

Fig. 3-A: analysis of the proteolytic activity of *Rhodnius prolixus* haemolymph from insects inoculated with short or long epimastigotes of *Trypanosoma rangeli* using SDS-polyacrilamide gels containing gelatin as substrate. Lane 1: haemolymph from control insects; lane 2: haemolymph from insects inoculated with long epimastigotes; lane 3: haemolymph from insects inoculated with short epimastigotes. The numbers refers to the position of the molecular weight markers. B: analysis of the proteolytic activity of *R. prolixus* fat body from inoculated insects with short or long epimastigotes of *T. rangeli* using SDS-polyacrilamide gels containing gelatin. Lane 1: fat body from insects inoculated with short epimastigotes; lane 2: fat body from insects inoculated with long epimastigotes; lane 3: fat body from control insects. The numbers refers to the position of the molecular weight markers.

ent molecular weights. It seems therefore that the infection with short epimastigotes of *T. rangeli* is able to mobilise for the haemolymph the proteases stored in the fat body while the long epimastigotes in some way inhibit the protease activities in the fat body or, if these proteases are mobilised, in the haemolymph.

No attempts were made to purify and further characterisation the enzymes, but the assay of protease activities in SDS-PAGE gelatin-containing gels in the presence of specific inhibitors indicates that the proteolytic activities were not related to cysteine, serine, and aspartic-proteases since these compounds were unable to block the formation of clear zones on the gelatin substrate. There is clearly evidence that these proteolytic activities in the haemolymph of insects infected with short epimastigotes and from the fat body of controls belong to metalloprotease or metal-activated protease classes since the metalloprotease specific inhibitor 1,10-phenanthroline in the SDS-PAGE containing 0.1% gelatin inhibited the formation of the proteolytic bands.

The occurrence of metalloprotease or metal-activated enzymes associated with *T. rangeli* infection in *R. prolixus* deserves attention. A complex of proteases and associated factors found mainly in the haemolymph are involved in the proPO system (Ashida & Yamazaki 1990, Ratcliffe 1991). Inactive proPO is converted to PO by a series of serine proteases which are themselves triggered by microbial cell wall components, such as β-1,3-glucans, lipopolysaccharides and peptidoglycans (Ashida & Yamazaki 1990). Recently, an interaction of *T. rangeli* with the *R. prolixus* proPO-activating system *in vivo* was described (Mello et al. 1995). Gomes et al. (1999) also demonstrated that short epimastigotes of *T. rangeli*, but nor long epimastigotes, activated, in *in vivo* and *in vitro* experiments, the proPO system leading to the deposition of melanotic substances. Thus, it is possible that some factor(s) from the parasites may be related to the activation of the proPO system. Preliminary experiments demonstrated that the protease inhibitor 1,10-phenanthroline blocked the activation of the proPO system *in vitro* while serine protease inhibitors such as PMSF and TLCK resulted in 70% of melanin formation induced by bacteria and *T. rangeli* in the haemolymph of *R. prolixus*. These findings indicate the possibility that serine proteases may not be involved in the proPO-activating system in this insect (Gomes & Azambuja, unpublished results). Alternately, Ham (1992) observed that besides an active serine protease, a cysteine protease was strongly induced following trauma or infection with Onchocerca microfilariae in the haemolymph of *Simulium ornatum*. Since serine proteases are involved in the activation of proPO system, it was postulated that proteases present in *S. ornatum* haemolymph may not be restricted to triggering proPO activation, but may be responsible for the direct killing of microfilariae (Ham 1992). However, this hypothesis is not adequate for *T. rangeli* infection due to the fact that protease does not kill the parasite, by contrary it survives and develops in *Rhodnius* haemolymph (Mello et al. 1995, Gomes et al. 1999).

It was described elsewhere that the inoculation of *Enterobacter* cloacae into *R. prolixus* haemocoel also induced two distinct patterns of protease ac-

tivities in the haemolymph with the apparent molecular weights of 46 and 56 kDa (Feder et al. 1998). Thus, the induction of these proteases in the haemolymph by the presence of parasite or bacterial infections is important. There is interest of our laboratory to investigate whether the metalloproteases or the metal-activated proteases are directly involved in the proPO system or in other defence reaction mechanisms of *R. prolixus* against parasite and bacterial infections.

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