Haemolymph and Fat Body Metallo-protease Associated with *Enterobacter cloacae* Infection in the Bloodsucking Insect, *Rhodnius prolixus*

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Analysis of zymograms with SDS-polyacrilamide gel electrophoresis containing gelatin as substrate, and performed on samples of haemolymph or fat body taken from Rhodnius prolixus *inoculated or not with* Enterobacter cloacae, *demonstrated distinct patterns of protease activities: (i) in the haemolymph two proteases were induced in insects inoculated with bacteria; (ii) two proteases were detected in the fat bodies derived from non-inoculated controls or insect inoculated with sterile culture medium; (iii) haemolymph and fat body had both the same apparent molecular weights proteases (46 and 56 kDa); and (iv) these enzymes were characterized as metallo-proteases. The association of these enzymes in* Rhodnius *infected with bacteria was discussed.*

Key words: fat body - haemolymph - *Rhodnius* - protease - prophenoloxidase - Triatomine

The interaction of bacteria with vectors is associated with several cellular and humoral factors (Hoffman 1995, Strand & Pech 1995, Nigam et al. 1996, Gillespie et al. 1997). For example, bacteria when invades the haemocel of insects triggers the cellular and humoral defence mechanisms to destroy the bacteria infection. Some well known humoral factors related to immune system of insects are the prophenoloxidase system (proPO), antibacterial induced peptides and lectins, while the cellular mechanisms involve phagocytoses, nodule formation and encapsulation of the invading organisms (Mello et al. 1995, Nigam et al. 1996, Gillespie et al. 1997, Feder et al. 1997, Azambuja et al. 1998).

In the present study, we describe *in vivo* experiments in the haematophagous bug, *Rhodnius prolixus*, which demonstrate the presence of two proteases in the haemolymph of insects inoculated with bacteria and in fat body from control insects. We also partially characterize both proteases and propose that the source of these proteases is the fat body, and that they are released from the fat body into the haemolymph after inoculation of bacteria. These studies may help the understanding of the *R. prolixus* defence reactions.

MATERIALS AND METHODS

Insect and feeding - All experiments were undertaken with adult of *R. prolixus*, reared and maintained as previously described (Garcia et al. 1984). The insects were starved for 15 days after the last ecdysis and then fed on citrated human blood through a membrane feeder (Garcia et al. 1984).

Culture medium and maintenance of microorganisms - The bacteria used for challenging the insects was *Enterobacter cloacae* b12 (obtained from Dr H Boman, Sweden). The bacteria was grown aerobically in liquid brain-heart culture medium (Biobras, Brazil) at 37ºC. Before inoculation, 4-hr-old brain-heart medium culture were centrifuged and the pellets washed three times in this medium. The insects were inoculated, five days after feeding, with 1 µl of sterile culture medium (controls) or with 1 µl of culture medium containing 1 X 10^6 live cells of *E. cloacae* b12 by using a 10 ml Hamilton microserynge. The percentage of mortality was minimal (< 5%).

Haemolymph and abdominal fat body collections - Haemolymph was collected carefully with micropipettes at different intervals after inoculation of bacteria, by cutting off the insect legs. Fat body preparation of *R. prolixus* was done by adults being opened by dorsal dissection in *Rhodnius* saline (0.1M NaCl, 25 mM KCl and 10 mM $CaCl_2$).

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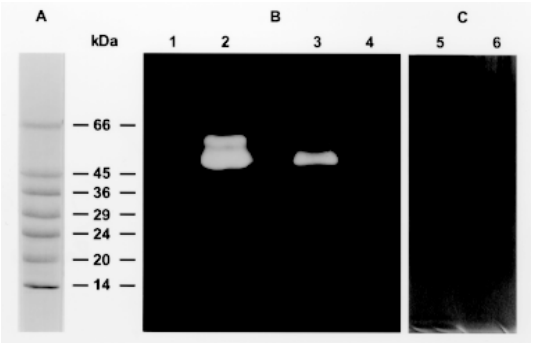
Midgut and reproductive organs were first removed to easier collect the abdominal fat body which was washed in *Rhodnius* saline and transferred to the 50mM phosphate buffer. After the homogenization the preparation was kept at -20°C until use.

Zymographic technique for detection and partial characterization of proteases - Proteases were assayed and partially characterized by 10% SDS-polyacrilamide gel electrophoresis (SDS-PAGE) with 0.1% gelatin incorporated into the gel as substrate (Heussen & Dowdle 1980). Briefly, 10 ml of pure haemolymph (approx. 200 mg of protein) was added to 5 ml of sample buffer (50 mM Tris-HCl, pH 6.8, 4% SDS, 20% glycerol, and 2% bromophenol blue, without boiling). Also, one abdominal fat body amount was homogenized in 100 ml of 50mM phosphate buffer, centrifuged, and kept at -20°C until use. Samples (approximately 200 mg protein) were prepared by adding half a volume of sample buffer, without boiling. Electrophoresis was performed at a constant current of 20 mA at 4°C. After the run, gels were incubated for 1 hr at room temperature in 10 volumes of 2.5% Triton X-100 with or without specific inhibitors of different classes of proteolytic enzymes (Sigma Chem. Co, USA). The inhibitors used were phenylmethanesulphonyl fluoride (PMSF), N-a-tosyl-L-lysylchlotomethyl ketone (TLCK), L-trans-epoxysuccinyl-leucylamide-(4-guanidino)-butane (E-64), leupeptin, pepstatin and 1,10-phenanthroline. Control activities (no inhibitors added) were tested in 50mM phosphate buffer at specific pH for 24-48 hr at 37°C and constant shaking. Then gels were stained for 30 min with Coomassie blue R-250 in methanol-acetic acid-water (10:10:80) and destained in the solvent. The molecular mobilities of proteins were determined by interpolation from mobilities of commercial pre-stained standards (Sigma Chem. Co, USA) by computer analysis.

Protein determination - Proteins of 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).

RESULTS AND DISCUSSION

Analysis for detection of haemolymph and fat body proteases - In order to investigate the proteases from homogenized fat body or haemolymph of *R. prolixus* a zymographic analysis was performed. In these assays haemolymph and fat body derived from control insects or from insects after 24 hr of inoculation with *E. cloacae* were used. After submitting the different samples to SDS-PAGE containing gelatin as a protease substrate, two gelatinase activities were observed both in the haemolymph from inoculated insects and in the fat bodies of control insects. The activities had apparent molecular weights of 46 and 56 kDa on both samples (Fig., lanes 2 and 3). Haemolymph from inoculated insects had a weak band of 56 kDa indicating a lower gelatinase activity. However, under our assay conditions, no activity could be detected on the haemolymph from control insects or on fat body taken from inoculated insects (Fig., lanes 1 and 4).



Analysis of the proteolytic activity of *Rhodnius prolixus* fat bodies homogenates and haemolymph from both untreated and *Enterobacter cloacae* b-12 inoculated insects through SDS-polyacrilamide gels containing gelatin. A = refers to the position of the molecular weight markers. B = zymogram performed (as described in the text) lane 1: fat body from inoculated insects and lane 2: fat body from control insects, lane 3: haemolymph from inoculated insects and lane 4: haemolymph from control insects. C = zymogam performed as in B plus 1,10 phenanthroline used as a protease inhibitor lane 5: haemolymph from *E. cloacae* b 12 treated insects and lane 6: fat body homogenates from non inoculated insects (control).

Partial characterization of the proteases - Aiming to further characterize these proteases, we tested their activity in SDS-PAGE gelatin-containing gels in the presence of specific protease inhibitors. The results presented in Table indicates that both enzymes are blocked in the presence of 1,10-phenanthroline, a specific metallo-protease inhibitor (Fig., lanes 5, 6). Conversely, the activities of these proteases were not altered in the presence of the cysteine- and serine-protease inhibitor (leupeptin), specific cysteine inhibitor (E64), or serine-proteases inhibitors (elastatine, PMSF and TLCK) or the aspartic protease inhibitor (pepstatin) (Table).

The principal results may be summarized as follows. Inoculation of *E. cloacae* into *R. prolixus* haemocoel induced two distinct patterns of protease activities in the haemolymph, two proteases were also detected in the fat bodies derived from

TABLE

Effects of protease inhibitors on the proteolytic activities of fat body homogenate controls (FB) and haemolymph taken from bacteria inoculated *Rhodnius prolixus* (H) in SDS-PAGE containing gelatin

Inhibitor class	Compounds[a]	Tissue	Concentration	Protease activity[b]
Serine protease	PMSF	FB	0.1 and 1 mM	+
		H	0.1 and 1 mM	+
	Elastatinal	FB	10 and 100 mM	+
		H	10 and 100 mM	+
	TLCK	FB	10 and 100 mM	+
		H	10 and 100 mM	+
Cysteine protease	E-64[c]	FB	1 and 10 mM	+
		H	1 and 10 mM	+
Aspartic protease	Pepstatin	FB	1 mM	+
		H	1 mM	+
Serine/cysteine protease	Leupeptin	FB	10 and 100 mM	+
		H	10 and100 mM	+
Metalloprotease	1,10-phenan throline	FB	5 and 10 mM	-
		H	5 and 10 mM	-

a: see Materials and Methods; *b*: results of three determinations by the SDS-PAGE gelatin method; *c*: in a 2 mM DTT solution.

non-inoculated controls or insect inoculated with sterile culture medium, proteases from haemolymph and fat body had the same apparent molecular weights (46 and 56 kDa) and both enzymes were characterized as metallo-proteases.

In this paper, we demonstrated for the first time, evidence of two proteases in both fat body of control insects and haemolymph of bacteria inoculated *R. prolixus* using SDS-PAGE with 0.1% gelatin as protease substrate (Fig.) The molecular weight determinations of these proteases gave values of 46 and 56 kDa for visible bands both in the haemolymph taken from inoculated insects and fat body derived from control ones. In contrast, these proteases were not detected in control haemolymph or in fat bodies taken from bacteria inoculated insects. Thus, it seems that fat body from inoculated insects, in some way, released these proteases into the haemolymph. Besides that, we showed that the metallo-protease specific inhibitor 1,10-phenanthroline in the SDS-PAGE containing 0.1% gelatin inhibited the formation of the both proteolytic bands, i.e., no protease activity was found either in the haemolymph from inoculated insects or in fat body control. Several serine-, cysteine- and aspartic-protease inhibitors were also tested but none of them was able to block the formation of clear zones on the gelatin substrate. Thus, *Rhodnius* has metallo-proteases or metal-activated proteases involved in its defence reaction mechanism.

It is possible that the bacteria infection by altering the fat body cell membrane, can in some way influence the liberation into the haemolymph of these two metallo-proteases. The occurrence of metallo-proteases associated with bacteria infection in *R. prolixus* deserves attention. According to Ashida and Yamazaki (1990) the enzymes involved in the immune response are serine-proteases which activate the proPo system in *Diptera* and *Lepidoptera*. This could be a consequence of the fact that the majority of the investigated insect species have serine-proteases as digestive enzymes (Applebaum 1985). However, the presence of functional digestive serine-proteases could not be demonstrated in *Rhodnius* or in any other hemipteran species (Terra 1988, Terra & Ferreira 1994). In some way, the detection of non-serine-proteases involved in the defence reactions could be expected. To assess the general validity of this hypothesis, it would be interesting to study the presence, distribution and function of metallo-proteases in other hemipteran bugs, with special care to their relation to bacteria infection.

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