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Prey digestion in the midgut of the predatory bug *Podisus nigrispinus* (Hemiptera: Pentatomidae)

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ABSTRACT

Pre-oral digestion is described as the liquefaction of the solid tissues of the prey by secretions of the predator. It is uncertain if pre-oral digestion means pre-oral dispersion of food or true digestion in the sense of the stepwise bond breaking of food polymers to release monomers to be absorbed. Collagenase is the only salivary proteinase, which activity is significant (10%) in relation to *Podisus nigrispinus* midgut activities. This suggests that pre-oral digestion in *P. nigrispinus* consists in prey tissue dispersion. This was confirmed by the finding of prey muscles fibers inside *P. nigrispinus* midguts. Soluble midgut hydrolases from *P. nigrispinus* were partially purified by ion-exchange chromatography, followed by gel filtration. Two cathepsin L-like proteinases (CAL1 and CAL2) were isolated with the properties: CAL1 (14.7 kDa, pH optimum (pHo) 5.5, km with carbobenzoxy-Phe-Arg-methylcoumarin, Z-FR-MCA, 32 μM); CAL2 (17 kDa, pHo 5.5, km 11 μM Z-FR-MCA). Only a single molecular species was found for the other enzymes with the following properties are: amylase (43 kDa, pHo 5.5, km 0.1% starch), aminopeptidase (125 kDa, pHo 5.5, km 0.11 mM L-Leucine-p-nitroanilide), α-glucosidase (90 kDa, pHo 5.0, km 5 mM with p-nitrophenyl α-D-glucoside). CAL molecular masses are probably underestimated due to interaction with the column. Taking into account the distribution of hydrolases along *P. nigrispinus* midguts, carbohydrate digestion takes place mainly at the anterior midgut, whereas protein digestion occurs mostly in middle and posterior midgut, as previously described in seed- sucker and blood-feeder hemipterans.

1. Introduction

Pre-oral digestion is described as the liquefaction of the solid tissues of the prey caused by secretions of the predator. This is highly prevalent on predaceous ground-dwelling arthropods and among heteropterans it is known in at least 38 of 62 families (Cohen, 1993, 1995). Liquefaction of an animal tissue can be caused by the hydrolytic cleavage of the extracellular matrix that is responsible for maintaining together the cells in tissues. The major components of the extracellular matrix are collagen and hyaluronic acid (Alberts et al., 2008), which means that collagenase or hyaluronidase may suffice to disrupt the tissue. In the case of plants, the cement among cells is mostly pectin that may be hydrolyzed by pectinase (Alberts et al., 2008). Both animal and plant tissues may also be disrupted by a phospholipase A. This enzyme removes a fatty acid moiety from the cell membrane phospholipids, allowing lysophospholipids that leave the membrane to form micelles. As a consequence, the cell membranes are solubilized and their contents are freed. Finally, tissue disruption may also be attained by the mechanical action of the mouthparts and saliva fluxes, as observed in the seed-sucker *Dysdercus peruvianus* (Heteroptera: Pyrrhocoridae) (Silva and Terra, 1994).

Digestion is the process by which food molecules are broken down into smaller molecules that are able to be absorbed by the gut tissue. Most food molecules requiring digestion are polymers, such as proteins and starch (or glycogen), and are subsequently digested through three phases. Primary digestion is the dispersion and reduction in molecular size of the polymers and results in oligomers. During intermediate digestion, these undergo a further reduction in molecular size to dimers, which in final digestion form monomers that are absorbed (Terra and Ferreira, 1994, 2012). The different phases of digestion occur at different compartments inside the midgut. In the case of insects having a peritrophic membrane (PM), initial digestion occurs inside PM, the intermediate digestion outside PM and final digestion at the surface midgut cells carried out by membrane-bound enzymes (Terra and Ferreira, 1994, 2012). Compartmentalization of digestion increases the

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efficiency of the digestive process (Terra, 2001; Bolognesi et al., 2008). In the case of insects lacking a PM, as exemplified by hemipterans, the midgut microvillar membranes are ensheathed by an unusual extra-cellular lipoprotein membrane. This membrane was named perimicrovillar membrane (PMv) (Terra, 1988) and is widespread among paraneopterans insects (Ferreira et al., 1988; Silva et al., 1995, 2004). PMv limits a closed space, the perimicrovillar space and in hemipterans, digestion occurs into the lumen, perimicrovillar space and at microvillar membranes surface (Ferreira et al., 1988; Silva et al., 1995).

Controversies regarding pre-oral digestion include its extent, that is, the evaluation of whether it is only a pre-oral disorganization of prey tissues or if it includes one of the phases of digestion (initial, intermediate or final), the enzymes involved and they are released from salivary glands or midgut. Differences are expected among insect representatives of different orders. For example, among coleopterans pre-oral digestion is carried out by enzymes from the midgut (Cheeseman and Gillott, 1987; Colepicolo-Neto et al., 1986) and at least in the case of the elaterid *Pyrearinus termitilluminans* (Coleoptera: Elateridae) (Colepicolo-Neto et al., 1986), pre-oral digestion includes initial and intermediate digestion. Pre-oral digestion among hemipterans is reported to occur under the action of salivary enzymes and trypsin in *Zellus renardii* (Heteroptera: Reduviidae) (Cohen, 1993) is frequently cited as the main enzyme. Accordingly, a trypsin gene was found to be active in the salivary gland of *Lygus lineolaris* (Heteroptera: Miridae) (Zeng et al., 2002). In spite of this, there is evidence of the presence of a cysteine proteinase (probably a cathepsin L-like proteinase) in salivary glands of *L. lineolaris* (Zeng et al., 2002; Zhu et al., 2003) and *Podisus maculiventris* (Heteroptera: Pentatomidae) (Bell et al., 2005). Although both works concluded that serine is more important than cysteine proteinase, their assay conditions do not favor cysteine proteinase action (no activators like cysteine were added). Furthermore, the finding that a part of the proteolytic activity in salivary glands of *P. maculiventris* is inhibited by EDTA (Bell et al., 2005) deserves further investigation. The inhibition was misinterpreted as due to carboxypeptidases which are not significantly active on intact protein molecules. It is, therefore, more probable that the enzyme inhibited was the metallopeptidase collagenase.

This paper was undertaken to evaluate the digestive enzymes in the salivary glands and midgut, as well as the role of a collagenase in pre-oral digestion in a predaceous hemipteran, *Podisus nigrispinus* (Heteroptera: Pentatomidae), and to provide evidence that pre-oral digestion in this case is actually a pre-oral dispersion of food and that digestion is carried out in midgut, essentially as described before for other non-predaceous hemipterans. *P. nigrispinus* was chosen in this study because it is an important predator of agricultural pests worldwide (De Clercq, 2000), including in Brazil (Zanuncio et al., 1994), and because the first evidence of the occurrence of a possible salivary metalloproteinase was described in an insect of the same genus (Bell et al., 2005). The results described in this paper suggest that a salivary collagenase (a metalloproteinase) injected into prey disrupts its tissues resulting in some cell clusters still seen inside in the midgut of predator and that protein digestion is accomplished mainly in its middle and posterior midgut and carbohydrate digestion mostly in anterior midgut.

2. Material and methods

2.1. Insects and preparation of samples from the midgut and salivary glands

Adult males of *P. nigrispinus* were obtained from the colony of the Laboratory of Biological Control of Insects of the Federal University of Viçosa, where they are kept at 25 ± 2 °C, 70% ± 5 relative humidity and 12 h photophase, this insects were fed on *Tenebrio molitor* (Coleoptera: Tenebrionidae) and *Eucalyptus cloeziana* leaves. We use only adult males, since preliminary studies using both male and females, resulted in large variation in enzymes activities, probably due to physiological reproductive variations in females. The insects were starved for 48 h and then fed *ad libitum* for 24 h with pupae of *T. molitor* L. Adults of *P. nigrispinus* were immobilized in cold and dissected in saline solution (0.1 M NaCl, 0.1 M KH_2PO_4, 0.1 M Na_2HPO_4, pH 7.2). Salivary glands and midguts were removed and stored at −80 °C until use. In some insects, the midgut was divided into three regions (anterior, middle and posterior). Samples of the salivary glands, whole midguts and midgut sections were homogenized in cold MilliQ water with the aid of a Potter–Elvehjem homogenizer. The homogenates were centrifuged at 16,000*g* for 30 min at 4 °C. The pellets and supernatants were stored at −20° C until use. For the enzymes assays pools of ten midguts were homogeneized in 500 μL of MiliQ water and 20 salivary glands in 100 μL in MiliQ water, whereas for enzymes purification a pool of 40 midguts were homogeneized in 1 mL of MiliQ water. No enzyme inactivation was detected on storage.

2.2. pH determination of the salivary glands and midgut contents

The contents of the salivary glands and the midgut sections were dispersed in 5 μL of MilliQ water and added to 5 μL of a 5-fold dilution of a universal pH indicator (E. Merck, Darmstadt, pH 4–10). The resulting colored solutions were compared with suitable standard solutions diluted in 5 μL of MilliQ water.

2.3. Protein determination, enzyme assays, optimum pH and kinetic parameters

Protein content in extracts was determined according to Smith et al. (1985) as modified by Morton and Evans (1992), using bovine serum albumin (BSA) as a standard.

Unless otherwise specified, hydrolase assays were performed as follows. α-Amylase activity was measured by determining the appearance of reducing groups (Noelting and Bernfeld, 1948) in 50 mM citrate–phosphate buffer at pH 6.0 using 0.5% (w/v) starch as substrate. Absorbance was measured at 550 nm. Aminopeptidase assays were accomplished using 1 mM L-leucine *p*-nitroanilide (LpNA) as substrate in 50 mM citrate–phosphate buffer pH 6.0, according to Erlanger et al. (1961) and absorbance measured at 550 nm. α-Glucosidase activity was determined by following the release of *p*-nitrophenolate from 5 mM p-nitrophenyl-α-D-glucoside (pNPαGlu) in 50 mM citrate–phosphate buffer pH 6.0 and absorbance measured at 420 nm, as described in Terra et al. (1979). Serine protease (trypsin and chymotrypsin) assays were performed in 0.1 M Tris–HCl, pH 7.5 as follows. Activities were quantified by determining the methyl-coumarin fluorescence (excitation 360 nm and emission 460 nm) released from 1 mM carbobenzoxy-Phe-Arg-7-amino-4-methylcoumarin (Z-FR-MCA) in the case of trypsin and 1 mM succinyl-Ala-Ala-Pro-Phe-7-amino-4-methyl-coumarin (Suc-AAPF-MCA) in the case of chymotrypsin. Cathepsin was assayed using 1 mM Z-FR-MCA or carbobenzoxy-Arg-Arg-7-amino-4-methylcoumarin (Z-RR-MCA) in 100 mM sodium acetate buffer at pH 6.0, containing 3 mM cysteine plus 3 mM sodium ethylenediaminetetraacetic acid (EDTA). The substrate Z-FR-MCA was used for determining cathepsin L and Z-RR-MCA was used for determining cathepsin B. Collagenase activity was determined by following the release of the amino acids from bovine Achilles tendon collagen (Sigma) as substrate in 50 mM Tris–HCl buffer pH 7.8. For this, 1 mg of collagen was added to 50 μL of the Tris buffer containing 1 mM $CaCl_2$ and 1 μL of the enzyme source. After different times at 30 °C, the assay tubes were removed and placed on ice and EDTA was added at a concentration

of 5 mM. The tubes were then processed according to Rosen (1957) to determine free amino acids. Thus, 200 μL of the cyanide-acetate buffer were pipetted to each tube, followed by the addition of 100 μL of the ninhydrin solution. After boiling the tubes for 10 min, 1 mL of isopropanol-water (1:1) solution was added to each tube that, after centrifuging at 16,100*g* for 10 min at 4 °C, had their absorbance read at 570 nm.

Proteinase inhibitors were tested and the concentrations used were: 10 mM L-trans-epoxysuccinyl-L-leucinamido-(4-guanidino) butane (E-64) and 5 mM EDTA. These compounds incubated for 15 min at 30 °C with the supernatants of salivary gland and midgut before adding the substrate. E-64 and EDTA are inhibitors of cysteine proteinases and metalloproteinases like collagenase, respectively.

To determine K_m, the effect of substrate concentration in the activity of semi-purified enzymes was determined using 10 different concentrations of the following substrates (range of concentrations used): LpNA (0.017–0.2 mM), Z-FR-MCA (1–300 μM), pNPαGlu (0.5–15 mM) and starch (0.025–0.35%). Data analysis was carried out with the software Enzfitter (Elsevier Biosoft, Cambridge, UK).

The buffers used in determination of pH optima were: 50 mM citrate–phosphate (pH 2.5–7.0) and 50 mM Tris–HCl (pH 7.0–9.5), both containing 0.2 M NaCl.

Incubations were carried out at 30 °C for at least four different time periods (up till 12 min for cathepsin and trypsin, 40 min amylase, 120 min α-glucosidase and 450 min Collagenase), and initial rates of hydrolysis were calculated. All assays were performed so that the measured activity was proportional to protein and to time. Controls without enzyme or without substrate were included. One unit of enzyme (*U*) is defined as the amount that hydrolyzes 1 μmol of substrate per minute.

2.4. Purification of digestive enzymes of P. nigrispinus midgut

The soluble fraction of midgut homogenates of *P. nigrispinus* corresponding to 40 individuals was loaded onto a HiTrap Q XL column (Amersham Biosciences), equilibrated and eluted with buffers that differed for each enzyme. Elution was accomplished with a gradient of NaCl from 0 to 1 M in the same buffer. The flow was 2.0 mL/min and fractions of 1.5 mL were collected. Elution buffers used were: for aminopeptidase, 0.1 M Tris–HCl at pH 7.0; for cathepsin-L, 20 mM Tris–HCl buffer pH 7.0, containing 1 mM methylmethanesulfonate (MMTS) at pH 7.0; and for α-glucosidase, 10 mM imidazole buffer pH 6.0. The use of MMTS in the case of cathepsin L was to prevent the oxidation of the sulfhydryl group of the enzyme during the purification steps. MMTS reacts with the sulfhydryl group, from which it is removed on cysteine addition during the assays Tyagi (1991).

Amylase was pre-purified before been applied to the column. One mL of the supernatant from midgut homogenates were added to 50 μL of 400 mM TAPS buffer pH 8.0, 60 μL of glycogen (17 mg/mL) solution and 80 μL of 96% ethanol. After 5 min in ice, the suspension was centrifuged at 9300*g* for 5 min at 4 °C. The supernatant (1.7 mL) was discarded and the pellet resuspended in 1.7 mL of 40% ethanol in TAPS buffer and centrifuged again after 5 min in ice. The new pellet was submitted to the same procedure as before. The resulting pellet was solubilized in 20 mM CAPS buffer pH 10.5, containing 100 mM benzamidine. After dialysis against 20 mM Tris–HCl buffer at pH 7.0, the dialysate was loaded onto the HiTrap column as described above.

The fractions corresponding to the single activity peak of each enzyme obtained at this step were pooled and submitted to chromatography in a Superdex 200 10/30 column (Pharmacia) to resolve aminopeptidase and Superdex 75 HR 10/30 (Pharmacia) to isolate amylase, cathepsin L and α-glucosidase. The column was equilibrated with two volumes (50 mL) of the different buffers and the flow was 0.5 mL/min and fractions of 0.4 ml were collected. Gel filtration was performed in the same conditions as described for HiTrap Q XL chromatography. Molecular masses were calculated according to Andrews (1964) with the following proteins as standards: β-amylase (200 kDa), BSA (66 kDa), ovalbumin (45 kDa), carbonic anhydrase (29 kDa) and cytochrome C (12.4 kDa). The column was calibrated with "blue dextran" (2000 kDa).

2.5. Transmission electron microscopy

For ultrastructural analyses of the midgut and its content, six males of *P. nigrispinus* from the rearing colony were starved for 48 h and then fed *ad libitum* for 24 h with *Anticarsia gemmatalis* (Lepidoptera: Noctuidae) larvae. Then the predators were dissected in 0.1 M sodium cacodylate buffer pH 7.4 containing 0.2 M sucrose. The midgut was divided into anterior, middle and posterior and the sections were fixed in 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4) and picric acid for two hours. The samples were post-fixed in 1% osmium tetroxide, then dehydrated in an ethanol series and embedded in LR White acrylic resin (Electron Microscopy Sciences, Ft Washington, USA), cut into ultrathin sections, stained with uranyl acetate and lead citrate (Reynolds, 1963) and, finally, examined in a Zeiss EM 109 electron microscopy.

3. Results

3.1. General morphology and luminal pH of salivary glands and midgut

As all Hemiptera, *P. nigrispinus* has piercing-sucking mouth parts with which it attacks its prey. The salivary complex is composed of two salivary glands (MG in Fig. 1A) having an anterior (AL) and a posterior (PL) lobes and two cylindrical accessory glands (AG) (Fig. 1A). A more detailed description is found in Oliveira et al. (2006). The midgut of *P. nigrispinus*, like other Heteroptera Pentatomorpha (see for example *D. peruvianus*, Silva et al., 1995), is divided into three major chambers, from which the first (AM, anterior midgut) and the last (PM, posterior midgut) are dilated and the middle chamber (MM, middle midgut) is cylindrical (Fig. 1B).

Salivary and midgut luminal contents are mildly acidic. The mean and standard errors of pH values ($n = 10$) are 6.0 ± 0.1 in salivary gland and 5.6 ± 0.1 in AM, 5.7 ± 0.1 in MM, and 5.8 ± 0.1 in PM.

3.2. Fine structure of midgut cells

The fine structure of *P. nigrispinus* midgut cells do not differ much from other Heteroptera Pentatomomorpha, like *D. peruvianus* (Silva et al., 1995) and *Brontocoris tabidus* (Heteroptera: Pentatomidae) (Guedes et al., 2007; Fialho et al., 2009). Hence, only the apical features that are interesting in the context of this work will be described.

The apex of midgut cells display microvilli that are ensheathed with glove-like finger membranes, the perimicrovillar membranes (Fig. 2A and B). What are remarkable are the muscles fibers visible in the midgut lumen (Fig. 2C and D). These ingested muscles fibers are discernible only in the anterior and middle midgut, suggesting that they are digested as the food moves toward the hindgut.

3.3. Digestive enzymes in salivary glands and midgut

Activities measured in extracts of midguts and salivary glands are described in table 1. The substrate Suc-AAPF-MCA was not hydrolyzed by homogenates of salivary glands and midguts, thus ruling out the occurrence of chymotrypsin as a digestive enzyme.

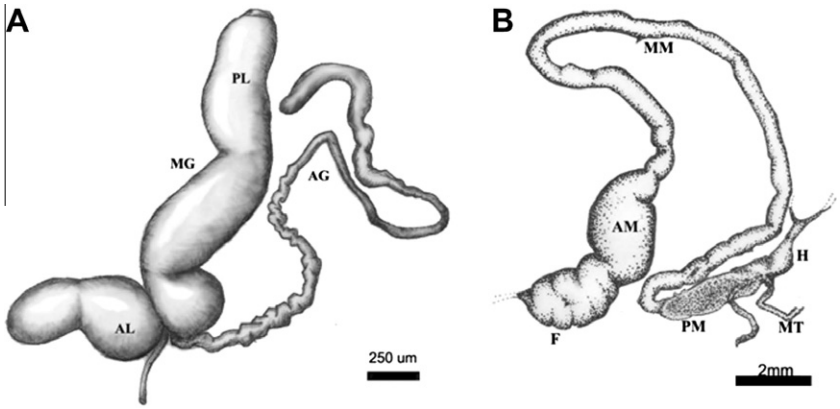

Fig. 1. Diagrammatic representation of the salivary complex (A) and digestive tract (B) of *P. nigrispinus*. The salivary complex is formed by the main gland (MG), with an anterior (AL) and a posterior (PL) lobe and the accessory gland (AG). The digestive tract include the foregut (F), anterior (AM), middle (MM), and posterior (PM) midgut and the hindgut (H). MT, Malpighian tubule.

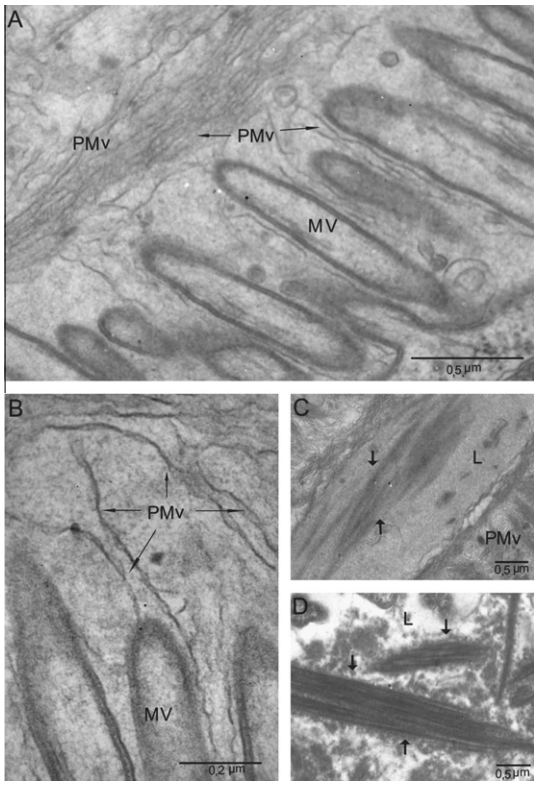

Fig. 2. Transmission electron micrographs of *P. nigrispinus* (Heteroptera: Pentatomidae) midgut cells. (A) Apex of an anterior midgut cell. Note perimicrovillar membranes (PMv). (B) Detail of the previous micrograph. (C) Anterior midgut luminal contents (L) showing ingested prey muscle fibers (arrows). (D) Detail of the ingested prey muscle fibers (arrows). MV, microvilli.

Z-FR-MCA (like benzoyl-Arg-p-nitroanilide, BApNA) may be hydrolyzed by both cathepsin L-like enzymes, which are cysteine proteinases, and trypsin, which is a serine proteinase. They may be distinguished, however, by their pH optima and their response in the presence of sulfhydryl reagents (usually Cys) and E-64 (Terra and Ferreira, 1994). By these criteria, the major proteinase in both salivary glands and midgut is cathepsin L, although a small amount of trypsin activity is present in salivary glands. These conclusions are based in the following observations: (1) assays with Z-FR-MCA at pH 6.0 in the presence of Cys were strongly inhibited by E-64 (100 ± 10% in midguts and 92 ± 7% in salivary glands), revealing the presence of a cathepsin L activity; (2) assays with Z-FR-MCA at pH 7.5 with salivary gland homogenates were inhibited by only 31 ± 8% in the presence of E-64, indicating the occurrence of a trypsin activity. No activity was found at those conditions in midgut samples, discounting the presence of significant trypsin activity in midguts. Activity on Z-RR-MCA is only 2% of the activity determined with Z-FR-MCA, confirming that the enzyme is really a cathepsin L, discounting the possibility of being a cathepsin B (also a cysteine proteinase), which would be more activity on Z-RR-MCA (Barrett et al., 2004).

Enzyme activities determined with native collagen (thus presenting a triple helix) correspond to the metalloproteinase collagenase. This was confirmed by the abolishment of its activity in the presence of 5 mM EDTA, as expected for true collagenases (Barrett et al., 2004).

Table 1 show that only collagenase and aminopeptidase have significant activities in salivary glands in comparison with midgut activities, as they amount to 8–10% of the latter.

Amylase and membrane-bound α-glucosidase predominate in the anterior midgut, whereas cathepsin L and collagenase are observed only in middle and posterior midguts and soluble α-glucosidase occurs along the whole midgut (Fig. 3).

3.4. Purification and properties of the major midgut hydrolases of *P. nigrispinus*

The supernatant obtained by centrifuging midgut homogenates of *P. nigrispinus* was adjusted to become 20 mM Tris–HCl buffer pH 7.0 with 1 mM MMTS and loaded onto a HiTrap Q XL column and eluted with the same buffer. Two cathepsin L-like proteinase activity peaks were observed (Fig. 4A): CAL1, the minor peak amounting to about 15% of midgut cathepsin L activity and CAL2, summing up 85% of cathepsin L activity. They were separately pooled and subsequently loaded on gel filtration columns (Fig. 4B and C).

The effect of pH (Fig. 4D) and substrate concentration (Fig. 4F) on the activity of semi-purified CAL1 were studied and the results displayed in Table 2. The same was done with CAL 2 (Fig. 4E and G, Table 2).

Amylase, aminopeptidase, and soluble α-glucosidase resulted in a single activity peak after ion-exchange chromatography. Pooled fractions corresponding to each enzyme were thereafter submitted to gel filtration, resulting again in single activity peaks (not showed). The pH optima, molecular masses and km values of the semi-purified enzymes are displayed in Table 2.

Table 1
Hydrolase activities in the salivary glands and midgut of the *P. nigrispinus* (Heteroptera: Pentatomidae).

Enzyme	Substrate	Salivary Glands		Midgut	
		Total Activity	Specific Activity	Total activity	Specific Activity
Amylase	Starch	0.23 ± 0.03 mU	0.01 ± 0.001 mU/mg	360 ± 70 mU	20 ± 4 mU/mg
Aminopeptidase	LpNA	5 ± 1 mU	0.19 ± 0.08 mU/mg	50 ± 4 mU	3.0 ± 0.5 mU/mg
Cathepsin L	Z-FR-MCA	370 ± 40 U	16 ± 2 U/mg	121,000 ± 20000 U	3800 ± 300 U/mg
α-Glucosidase	pNPαglu	6 ± 2 mU	0.3 ± 0.1 mU/mg	2300 ± 200 mU	150 ± 20 mU/mg
Collagenase	Collagen type I	490 ± 60 mU	42 ± 8 mU/mg	6000 ± 2000 mU	250 ± 80 mU/mg

Activities are means and SEM calculated from four assays performed in the soluble fraction of six different preparations obtained from 10 animals each. No chymotrypsin activity was found in salivary glands and midguts. Collagenase activity was abolished with 5 mM EDTA. Midgut is devoid of trypsin, but in salivary glands (24 μg protein/animal) it has a specific activity of 7 U/mg. Assays with Z-RR-MCA result in activities only 2% of those determined with Z-FR-MCA, confirming that the enzyme is actually a cathepsin L, discounting the occurrence of a cathepsin B.

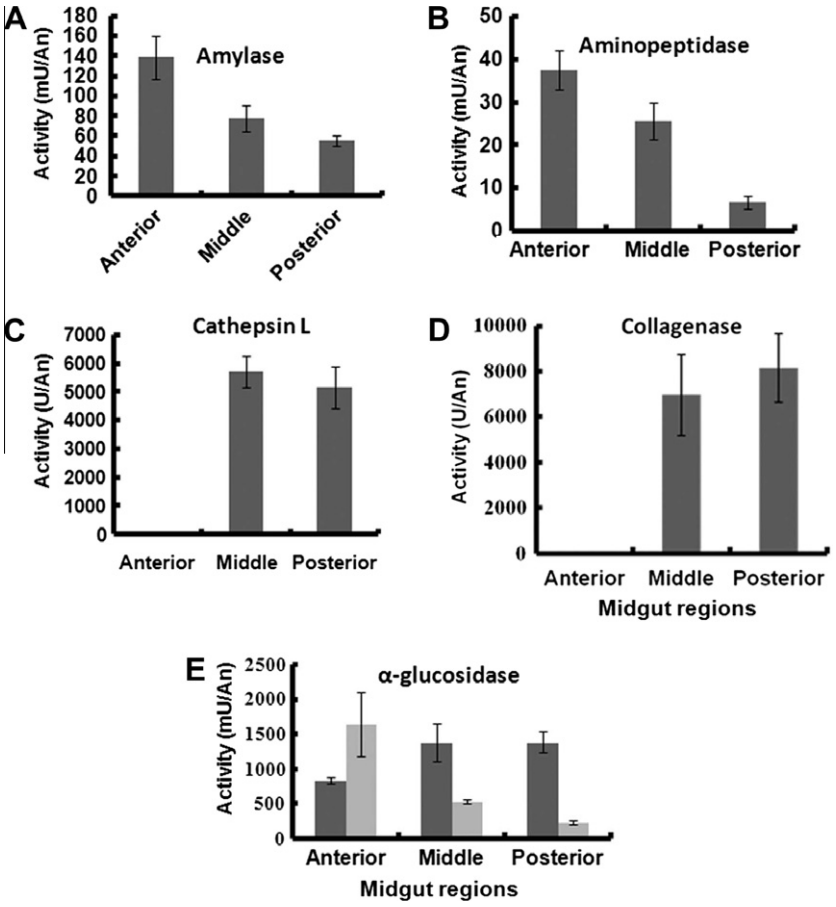

Fig. 3. Distribution of the major hydrolases along the midgut of *P. nigrispinus* (Heteroptera: Pentatomidae). Black bars, soluble fraction of tissues; gray bars, membrane fraction of the tissues. Determinations were carried out in three different preparations obtained from 10 animals each. Amounts of proteins in midgut sections (per animal): anterior, 78 μg; middle, 43 μg; posterior, 24 μg. An, animal.

4. Discussion

4.1. Properties of P. nigrispinus digestive enzymes

Two α-glucosidases were found in *P. nigrispinus* midguts: one soluble and another membrane bound. The latter should correspond to the enzyme marker of the perimicrovillar membranes found in hemipterans and insects pertaining to some other paraneopteran orders (Terra and Ferreira, 1994, 2012; Silva et al., 2004). There is a single molecular species of the soluble α-glucosidase, amylase, and aminopeptidase, which have properties similar to those described from other insects, including hemipterans (Terra and Ferreira, 1994, 2012). In *D. peruvianus*, a Hemiptera Pentatomomorpha like *P. nigrispinus*, the aminopeptidase is found in the space between the microvillar and perimicrovillar membranes, where it carries out the intermediate digestion of proteins (Silva et al., 1996).

Cathepsin Ls are major digestive proteinases in Cucujiformia beetles and in hemipterans. The digestive enzymes were derived from an ancestral gene that codes for a lysosomal cathepsin L. Digestive beetle cathepsin L seem to be more derived (farther from the lysosomal enzyme) than those from hemipterans (Terra and Ferreira, 2012). *P. nigrispinus* is not an exception among hemipterans, as no serine proteinases (chymotrypsin and trypsin) were found in their midguts. Nevertheless, it has two cathepsin Ls, identified by the fact they are inhibited by E-64 and are active on Z-FR-MCA, but not on Z-RR-MCA. The properties of these CALs,

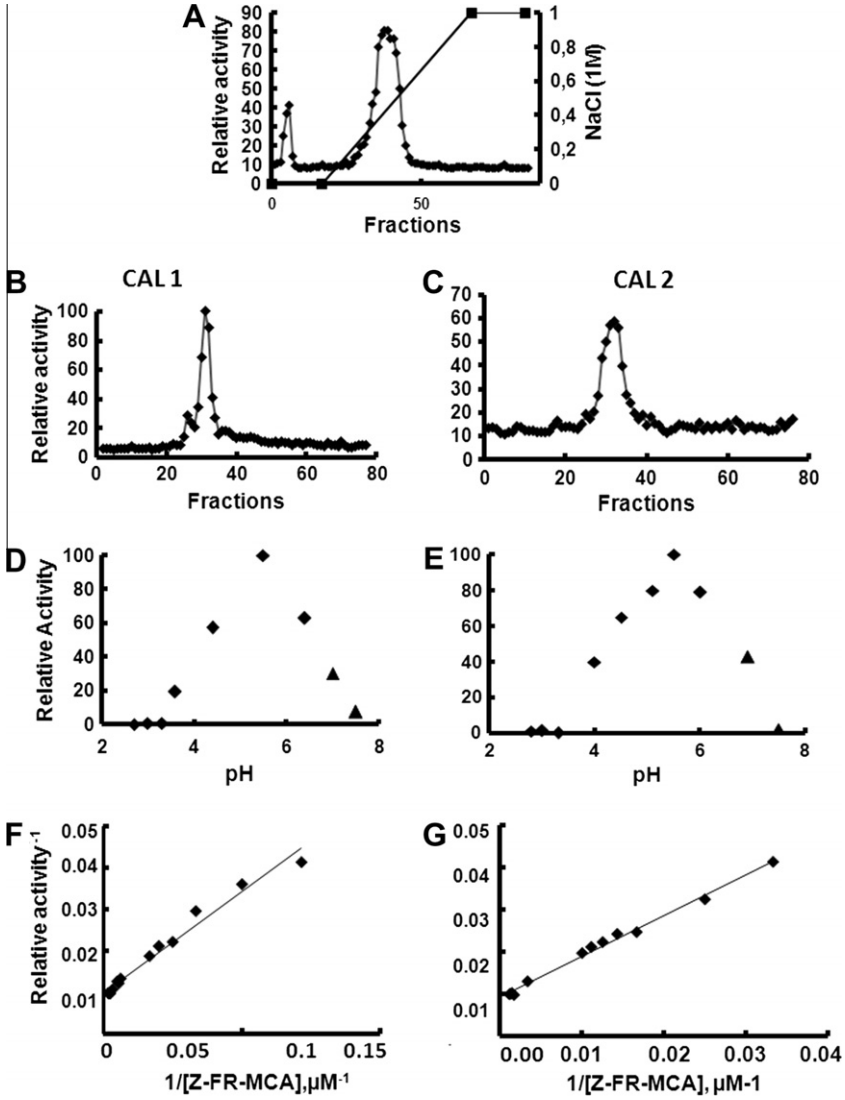

Fig. 4. Physical and kinetic properties of the major hydrolases from the midgut of *P. nigrispinus* (Heteroptera: Pentatomidae). (A) Ion-exchange chromatography on HiTrap Q equilibrated with 20 mM Tris–HCl (pH 7.0), containing 1 mM MMTS. Elution with a gradient of 0–1 M NaCl in the same buffer. Samples corresponding to peak 1 was labeled cathepsin L-1 (CAL1) and to peak 2, CAL2. (B) Gel filtration of CAL1 on Superdex 75 HR 10/30. The column was equilibrated and eluted with 20 mM Tris–HCl buffer pH 7.0, containing 1 mM MMTS. (C) Same as (B) with CAL2. (D) Effect of pH in the activity of CAL1. (E) Same as (D) with CAL2. (F) Effect of substrate (Z-FR-MCA) concentration in the activity of CAL1. (G) Same as (F) with CAL2.

Table 2
Physical and kinetic properties of the major midgut hydrolases from *P. nigrispinus* (Heteroptera: Pentatomidae).

Enzymes	pH optimum	MW (KDa)	km
Amylase	5.5	43	0.100 ± 0.008%
Aminopeptidase (soluble)	5.5	125	0.11 ± 0.02 mM
CAL1	5.5	14.7	32 ± 2 µM
CAL2	5.5	17	11.0 ± 0.7 µM
α-Glucosidase (soluble)	5.0	90	5.0 ± 0.5 mM

The enzymes were purified by ion-exchange chromatography followed by gel filtration, as detailed in Fig. 4 and in Section 2.

except for their molecular masses that apparently are underestimated due to interactions on the chromatographic column, are similar to those described before (Terra and Ferreira, 2012).

4.2. Prey digestion in P. nigrispinus

Predatory hemipterans are usually thought to rely on pre-oral digestion carried out by salivary enzymes. Although trypsin is usually described in salivary glands and considered to be responsible for prey tissue digestion, there is a lack of comparative work dealing with actual salivary hydrolase activities *vis a vis* midgut ones. Thus, unless this is done, one can not discount the possibility that prey tissues are pre-orally disrupted, but true digestion occurs only inside the midgut. For example, previous work on *P. nigrispinus* (Oliveira et al., 2006) and *B. tabidus* (Azevedo et al., 2007) implied a salivary trypsin on pre-oral digestion. Nevetheless, they did not rule out the possibility that they were assaying a cathepsin L instead of trypsin, nor evaluated the activity of this enzyme *vis a vis* the other proteinases to estimate its significance.

Prey digestive enzymes are sometimes considered to play a role in digestion by predators, although there is no experimental support for this. For instance, Pascual-Ruiz et al. (2009) suggested that *P. maculiventris* may well take advantage of prey proteolytic enzymes for digestion. Their conclusion is based on the increase of trypsin and chymotrypsin activity observed in *P. maculiventris* feeding on lepidopteran larvae in comparison to those feeding on beetles or dipteran pupae. As their proteolytic assays were done at pH 10, which favor lepidopteran enzymes (Terra and Ferreira,

1994, 2012) and maintain inactive hemipteran proteinases (this paper), their conclusions need to be re-evaluated.

Our findings showed that prey muscle fibers are observed inside *P. nigrispinus* midguts and that they are no longer visible at the posterior midgut. This suggests that pre-oral digestion is restricted to tissue disruption. Midgut proteinases are found only in middle and posterior midgut, what discount the possibility that these enzymes are injected into prey. The only salivary proteinase with significant activity in comparison with midgut enzymes is collagenase. Thus, it is probable that collagenase-containing saliva is injected into the prey. This enzyme acting on the extracellular matrix disrupts tissues. Isolated cells or cell aggregates, like the observed muscle fibers, are then ingested by the bugs. True protein digestion then occurs inside the midgut under the action of cathepsin L-like enzymes and aminopeptidase. Although carboxypeptidases and dipeptidases have not been assayed, it is highly probable that they are also involved in protein digestion (Terra and Ferreira, 1994, 2012). Prey glycogen should be initially digested by a midgut amylase and terminated under the action of a membrane-bound and a soluble α-glucosidase. The organization of the digestion here described is the same as found for other hemipterans such as the seed sucker, *D. peruvianus* (Silva and Terra, 1994) and a blood feeder, *Rhodnius prolixus* (Hemiptera: Reduviidae) (Ferreira et al., 1988; Terra and Ferreira, 2012).

Quantitative comparisons between salivary and midgut enzymes that include collagenase assays should be carried out in other predatory bugs. This will permit the evaluation as to whether true pre-oral digestion is actually as common as it is supposed to be or if it is usually only a pre-oral dispersion of prey tissues, as described here.

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