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Digestive physiology and characterization of digestive cathepsin L-like proteinase from the sugarcane weevil *Sphenophorus levis*

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ABSTRACT

Sugarcane is an important crop that has recently become subject to attacks from the weevil *Sphenophorus levis*, which is not efficiently controlled with chemical insecticides. This demands the development of new control devices for which digestive physiology data are needed. In the present study, ion-exchange chromatography of *S. levis* whole midgut homogenates, together with enzyme assays with natural and synthetic substrates and specific inhibitors, demonstrated that a cysteine proteinase is a major proteinase, trypsin is a minor one and chymotrypsin is probably negligible. Amylase, maltase and the cysteine proteinase occur in the gut contents and decrease throughout the midgut; trypsin is constant in the entire midgut, whereas a membrane-bound aminopeptidase predominates in the posterior midgut. The cysteine proteinase was purified to homogeneity through ion-exchange chromatography. The purified enzyme had a mass of 37 kDa and was able to hydrolyze Z-Phe-Arg-MCA and Z-Leu-Arg-MCA with k_{cat}/K_m values of $20.0 \pm 1.1\ \mu M^{-1}\ s^{-1}$ and $30.0 \pm 0.5\ \mu M^{-1}\ s^{-1}$, respectively, but not Z-Arg-Arg-MCA. The combined results suggest that protein digestion starts in the anterior midgut under the action of a cathepsin L-like proteinase and ends on the surface of posterior midgut cells. All starch digestion takes place in anterior midgut. These data will be instrumental to developing *S. levis*-resistant sugarcane.

1. Introduction

The weevil *Sphenophorus levis* (Coleoptera: Curculionidae) was identified in 1978 and has since become an increasingly important pest of sugarcane in Brazil, especially in the state of São Paulo (Vanin, 1990). This pest has larva length of about 15 mm and nocturnal habits. It lays its eggs in the soil, more specifically in the rhizomes of the sugarcane plants. The larvae penetrate the rhizome and build irregular galleries, where they remain until reaching adulthood. The larvae block the basal part of the plant and rhizomes, leading to plant death (Cerda et al., 1999). The behavior of *S. levis* larvae does not allow the use of chemical insecticides due to their location within the stem of the sugarcane. Some insecticides have been tried against this pest, but without success. For this reason, new strategies for controlling *S. levis* are desirable.

The use of transgenic plants expressing proteins that impair pest development is an important strategy that has been increasingly adopted in recent years (Haq et al., 2004). Such proteins may, for example, affect protein digestion by reducing the availability of amino acids and thereby hindering the synthesis of proteins necessary to the growth, development and reproduction of the pest (Broadway and Duffey, 1986). However, advances in this field depend on digestive physiology data, particularly protein digesting enzymes.

Coleoptera is divided into the major suborders Adephaga and Polyphaga. Polyphaga includes the major series Scarabaeiformia, Elateriformia, Bostrichiformia and Cucujiformia (Liebherr and McHugh, 2003). All coleopterans were once thought to rely mainly on digestive cysteine proteinases for protein digestion, based on a variety of whole midgut homogenate assays in the presence and absence of specific activators and inhibitors (Murdock et al., 1987; Wolfson and Murdock, 1990). However, Terra and Cristofoletti (1996) demonstrated that digestive cysteine proteinases are restricted to Cucujiformia beetles, which led the authors to propose that the Cucujiformia ancestor was a beetle adapted to

Abbreviations: DTT, dithiotreitol; SBTI, soybean trypsin inhibitor; Z-FR48 MCA, benzyloxycarbonyl-Phe-Arg-7-amino-4-methylcoumarin; Z-RR-MCA, benzyloxycarbonyl-Arg-Arg-7-amino-4-methylcoumarin; Z-LR-MCA, benzyloxycarbonyl-Leu-Arg-7-amino-4 methylcoumarin; E-64, L-trans-epoxysuccinyl-leucylamido (4 guanidine) butane (E-64).

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ingest seeds rich in naturally occurring serine proteinase inhibitors by using a cysteine proteinase for protein digestion instead of or in addition to serine proteinases.

Tenebrio molitor (Tenebrionidae) is the Cucujiformia beetle for which protein digestion is known in greater detail. Two trypsins (Vinokurov et al., 2006) and chymotrypsins (Elpidina et al., 2005; Lopes et al., 2009) are active in posterior midgut. The subsites of the active sites of these enzymes have been characterized in the search for determining insect–plant relationships (Lopes et al., 2004, 2006; Sato et al., 2008). Cysteine proteinases, actually cathepsin L-like proteinases (CALs), are active in the anterior midgut *of T. molitor*. There are two isoforms of a lysosomal CAL and two digestive CALs: CAL2 (the major CAL) and CAL 3 (Cristofoletti et al., 2005). Curiously, CAL2 is not expressed in the Russian strain of *T. molitor* (Prabhakar et al., 2007).

Research on *Diabrotica virgifera* (Chrysomelidae) has not progressed as far as with *T. molitor*, but the data clearly demonstrate that its major digestive proteinase is a cathepsin L-like proteinase, which has been purified to homogeneity (Koiwa et al., 2000) and the cDNA clones expressing it and other CALs have been described (Bown et al., 2004).

There are no comprehensive data on the digestive physiology of any Curculionidae (weevil) species. Protein digestion, however, has been the object of several studies suggesting that weevils rely on cysteine proteinases for protein digestion (Murdock et al., 1987; Wolfson and Murdock 1990). Purcell et al. (1992) were unable to detect cysteine proteinases in the midgut of the weevil *Anthonomus grandis*. Other authors interpreted their contrasting results to the fact that they used midgut contents, whereas other investigators used total midguts, which may include intracellular enzymes. Therefore, a cDNA coding cysteine proteinase was prepared from *A. grandis* midgut tissues (Oliveira-Neto et al., 2004) and insect performance was shown to be affected by trypsin inhibitors and synthetic epoxide peptide E-64.

Taking into account that Curculionidae is one of the largest families of organisms (about 40,000 species), including a great number of pests (Grimaldi and Engle, 2005), better knowledge on its digestive physiology is of fundamental importance. In this paper, the spatial organization of digestion in *S. levis* is described and the major digestive proteinase in this insect is shown to be a cathepsin L-like proteinase. These data will be instrumental to developing *S. levis*-resistant sugarcane.

2. Materials and methods

2.1. Animals, and preparation of samples from whole midgut, midgut tissue, and midgut contents

S. levis larvae were immobilized on crushed ice and dissected in cold 342 mM NaCl. The rinsed guts were transferred to a glass slide. The midgut was isolated and divided into four sections of similar length (V1, V2, V3, and V4) (Fig. 1). Midgut sections were separated into tissue and contents and were homogenized in cold double-distilled water using a Potter-Elvehjem homogenizer. The homogenates were centrifuged for 30 min at 20,000 × *g* at 4 °C. The supernatants were recovered and the pellets (except those of midgut contents) were resuspended in double-distilled water. The pellets are regarded as cell membrane fractions. The samples were stored at −20 °C until use. No enzyme inactivation was detected during storage.

2.2. pH of midgut contents

Midgut section contents (V1, V2 + V3, and V4), isolated as described above, were dispersed in 5 μl of the dissecting saline 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 appropriate standards.

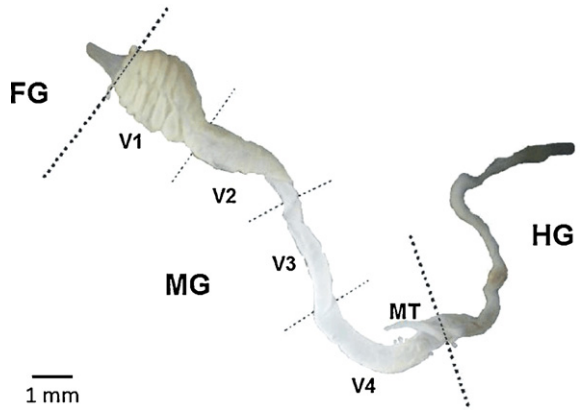

Fig. 1. A, Anatomical view of the midgut of *S. levis*; FG, foregut; HG, hindgut; MT, Malpighian tubules; V1–V4 are equal-length sections of the midgut; bar = 1 mm.

2.3. Protein determination and enzyme assays of midgut samples of whole midgut, midgut tissue and midgut contents

Protein was determined based on the method described by Bradford (1976), using ovalbumin as a standard. General proteolytic activity was determined with two different substrates: 0.5% (w/v) fluorescein isothiocyanate-labeled (FITC) casein (casein-FITC) (fluorescent substrate, useful at pH values above 5) or 0.5% hemoglobin-FITC (fluorescent substrate, useful at pH values below 4.5). The preparation of the substrates and the assays was based on the method described by Twining (1994) in 50 mM sodium citrate-phosphate buffer at pH 5.5 with casein-FITC or in the same buffer at pH 3.5 with hemoglobin-FITC as substrate.

Unless otherwise specified, other proteinase assays were carried out in 50 mM sodium citrate-phosphate buffer, pH 5.5, with the following fluorescent substrates: 10 μM carbobenzoxy-Phe-Arg-7-amino-4-methyl coumarin (Z-FR-MCA) (substrate for trypsin); 10 μM succinyl-Ala-Ala-Phe-MCA (S-AAF-MCA) (selective substrate for chymotrypsin); and 1 μM ε-amino-caproyl-leucyl-(S-benzyl) cysteinyl-MCA (selective substrate for cysteine proteinase). With these substrates, proteinase activity was measured by methylcoumarin fluorescence (excitation 380 nm and emission 460 nm). Inhibitors/activators were used at the following final concentrations: trans-epoxysuccinyl-L-leucyl-amido (4-guanidino butane) (E-64), 10 μM; benzamidine, 0.25 mM; EDTA, 5 mM; pepstatin A, 1 μM; chymostatin, 25 μM; EDTA/DTT, 3/1.5 mM; and soybean trypsin inhibitor (SBTI), 17 μM. These substances were pre-incubated with the supernadant of whole midgut homogenates at room temperature for 15 min before adding the substrate.

Unless otherwise specified, aminopeptidase, amylase and maltase were determined as follows: aminopeptidase was assayed in 50 mM Tris–HCl buffer (pH 7.0) using 1 mM L-leucyl-*p*-nitroanilide (LpNA), based on the method described by Erlanger et al. (1961); amylase was measured by determining the appearance of reducing groups (Noelting and Bernfeld, 1948) in 50 mM sodium citrate-phosphate buffer at pH 6.0 with 0.5% (w/v) starch as substrate in the presence of 10 mM NaCl; and maltase was assayed based on the method described by Dahlqvist (1968), using 7 mM maltose in 50 mM sodium citrate-phosphate buffer at pH 6.0.

Incubations were carried out at 30 °C in at least four different time periods and initial hydrolysis rates were calculated. All assays were performed under conditions in which the product was proportional to enzyme concentration and incubation time.

Controls without enzyme and others without substrate were included. One general proteinase unit is the amount of enzyme that causes an increase in the emission of 1000 units/60 min. For the other enzymes, one enzyme unit is the amount that hydrolyzes 1 μmol of substrate (or bond) per min. Enzyme activity is expressed in milli units (mU).

2.4. Purification of S. levis major whole midgut proteinase

Ten *S. levis* larvae were maintained at 4 °C for 5 min, dissected and the whole midgut were homogenized in buffer containing Tris–HCl 10 mM, NaCl 150 mM and 2% Triton X-100, pH 7.4 (2 ml). The mixture was centrifuged at 6000 × *g* for 30 min. The soluble fraction was applied to a DEAE-Sephadex column (25 cm × 1 cm) equilibrated with 0.1 M Tris–HCl, pH 8.0. The proteins were eluted with 1.0 M NaCl in the same buffer. The protein elution profile was followed by UV absorbance (280 nm). After protein elution, dialysis was performed in a buffer containing 10 mM Tris–HCl and 50 mM NaCl, pH 8.0.

2.5. Enzyme assays and kinetic parameters of purified S. levis peptidase

The hydrolysis of the fluorogenic peptides Z-FR-MCA, Z-LR-MCA and Z-RR-MCA (Calbiochem, La Jolla, CA, USA) by purified *S. levis* peptidase was continuously monitored in a Hitachi F-2500 spectrofluorimeter by measuring fluorescence at λ_{ex} = 380 nm and λ_{em} = 460 nm. Approximately 20 μM of purified enzyme were added to 0.1 M sodium acetate, pH 5.5, containing 2.5 mM DTT (1.0 ml final volume) and incubated for 3 min at 37 °C. The substrates were then added at different concentrations and the catalytic activity was monitored. The apparent second-order rate constant K_{cat}/K_m was determined under pseudo first-order conditions, in which $[S] \ll K_m$. Determinations were performed with different substrate concentrations and calculated using nonlinear regression data analysis with the aid of the GraFit program (Leatherbarrow, 2001). The molar concentration of the *S. levis* cysteine proteinase was determined by active site titration with E-64 inhibitor (Anastasi et al., 1983).

The pH dependence on Z-FR-MCA hydrolysis by *S. levis* proteinase was studied over a range of 4.0–9.0. Determinations were carried out at 37 °C using the following buffers: 0.1 M sodium acetate ($4.0 < pH < 5.5$); 0.1 M sodium phosphate ($6.0 < pH < 7.0$); 0.1 M Tris–HCl ($7.0 < pH < 8.5$) and 0.1 M sodium borate ($9.0 < pH < 10.0$). The enzyme was pre-activated with 2.5 mM DTT for 5 min at 37 °C before the addition of the substrate. Enzyme activity was monitored using the fluorimetric assay described above. For each pH value, enzyme activity was calculated using the Grafit program (Leatherbarrow, 2001). All experiments were carried out in triplicate and the values were converted to percentage of relative activity.

3. Results

3.1. General gut morphology, gut pH and presence of peritrophic membrane in S. levis larvae

The gut of the larvae is composed of a very short foregut, a large midgut that is anteriorly dilated and a medium-size hindgut (Fig. 1). The midgut is made up of a simple linear tube – ventriculus. The ventriculus is 1.58 ± 0.08 cm long and has a decreasing diameter from its anterior region (0.20) to the posterior

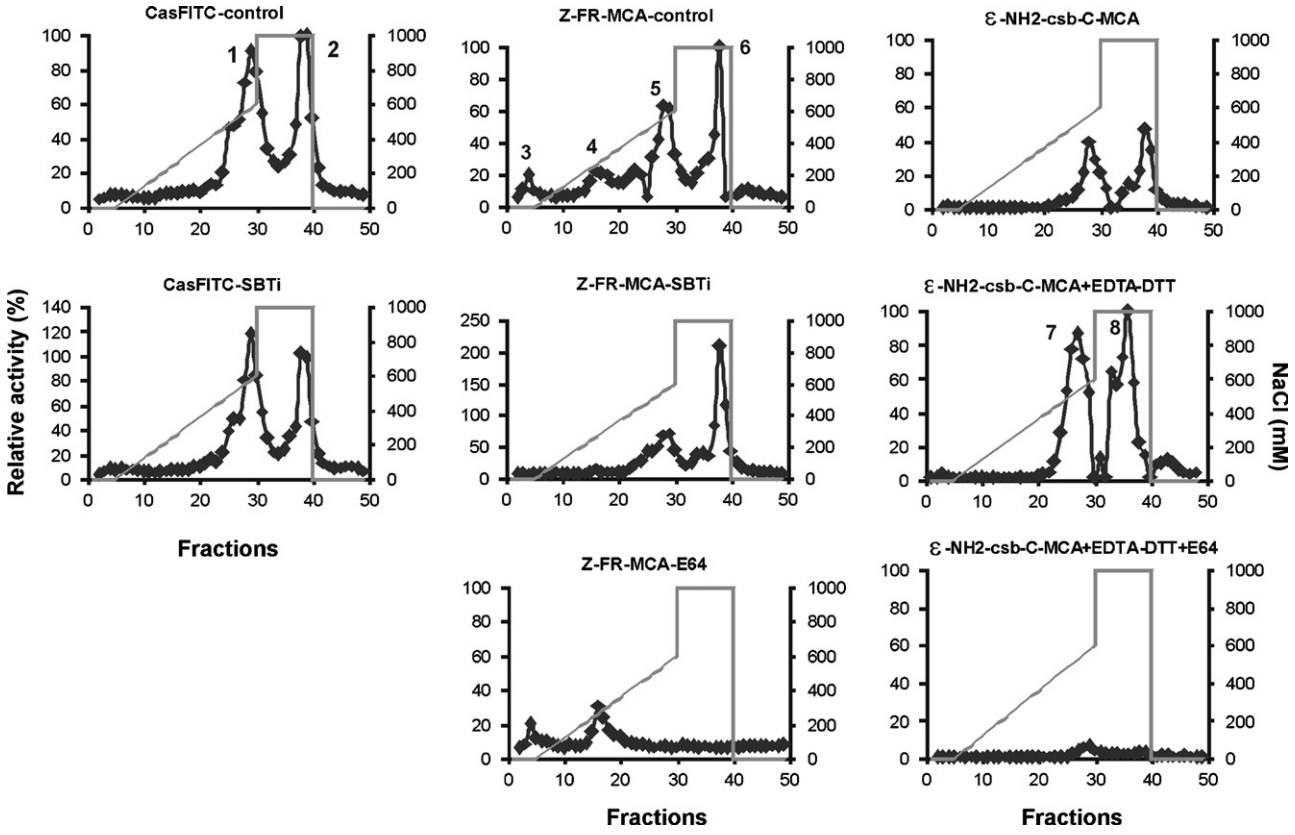

Fig. 2. Ion-exchange chromatography of proteins of midgut homogenates from *S. levis* larvae; same fractions assayed in the presence of different fluorescent substrates, EDTA + DTT were used as activators of cysteine proteinases, SBTI and E-64, a specific cysteine proteinase inhibitor, were used as inhibitors; straight lines correspond to NaCl gradient (0–600 mM); CasFITC, casein-FITC, fluorescent general substrate for proteinases; SBTI, soybean trypsin inhibitor; Z-FR-MCA, carbobenzoxy-Phe-Arg-MCA, substrate for trypsin; ε-NH_2-csb-C-MCA, ε-amino-caproyl-leucyl-(S-benzyl) cysteinyl-MCA (specific substrate for cysteine proteinase); all assays performed in 50 mM sodium citrate-phosphate buffer at pH 5.5; results displayed typical of three independent experiments.

region, with an average diameter of 0.06 cm. The hindgut is 0.78 ± 0.09 cm long and 0.050 ± 0.005 wide. The pH values (n = 7) vary throughout the contents of the midgut: 5.5 ± 0.2 in the anterior midgut (V1, see Fig. 1), 6.5 ± 0.1 in the middle portion of the midgut (V2 + V3) and 7.6 ± 0.2 in posterior midgut (V4).

The presence of the peritrophic membrane (PM) in the midgut was detected by dissection. In the anterior region, there is a viscous material surrounding food, whereas a PM may be picked up with a fine forceps in the posterior midgut, especially in V3 and V4. These results signify that the contents are surrounded by a peritrophic gel (PG) in anterior midgut (Terra, 2001) and a PM in posterior midgut.

3.2. Digestive proteinases

There are two peaks (1 and 2) in activity with casein (general substrate for proteinase) assayed at pH 5.5 that are resolved by ion-exchange chromatography (Fig. 2). These peaks are unaffected by SBTI (Fig. 2, left column) and benzamidine (not shown), increase with the addition of EDTA plus DTT and are almost abolished in the presence of E-64 (not shown). This suggests the presence of two active midgut cysteine proteinases. Z-FR-MCA (substrate used for trypsin, but is also a substrate for cysteine proteinases) is hydrolyzed by activities corresponding to four peaks (peaks 3, 4, 5, and, 6, Fig. 2, middle column). Activities in peaks 3 and 4 are inhibited by SBTI and those in peaks 5 and 6 are inhibited by E-64 (Fig. 2, middle column). The occurrence of cysteine proteinase activity was further confirmed with the use of 1 μM ε-amino-caproyl-leucyl-(S-benzyl) cysteinyl-MCA, a substrate specific for cysteine proteinases (Alves et al., 1996), for which hydrolysis was increased by EDTA + DTT (peaks 7 and 8) and completely abolished by E-64.

As the contents in the posterior midgut of *S. levis* are alkaline, the experiments were replicated at pH 8. As observed at pH 5.5, the major activities (peaks 11 and 12, Fig. 3, left column) correspond to cysteine proteinases, as judged by inhibition by E-64 (not shown) and the lack of effect from SBTI (Fig. 3, left column). Data obtained with Z-FR-MCA as substrate at pH 8 (Fig. 3, middle column, peaks 13 and 14), confirm that the minor peaks active on casein (peaks 9 and 10) are trypsin-like enzymes, whereas the major peaks (peaks 11 and 12) are cysteine proteinases. However, the major peaks on Z-FR-MCA at pH 8 (peaks 13 and 14) correspond to trypsin-like enzymes.

The presence of a minor chymotrypsin-like enzyme is suggested by the action on Suc-AAF-MCA, which is inhibited by chymostatin (Fig. 3, right column). Assays of the chromatographic fractions with hemoglobin-FITC as substrate at pH 3.5 (not shown) were negative. This discounts aspartic proteinases as significant digestive enzymes in *S. levis*. The combined results indicate that the major *S. levis* midgut activities on proteins are cysteine proteinases, with minor trypsins and negligible chymotrypsins.

3.3. Purification and characterization of S. levis midgut peptidase

The crude extract of whole midgut *S. levis* larvae was submitted to ion exchange chromatography in DEAE-Sepharose. A large peak of inactive protein was eluted with 0.3 M NaCl. Two other peaks were eluted in 1 M NaCl (Fig. 4A). These two peaks hydrolyze Z-FR-MCA, but most of the activity was associated with the second peak. SDS-PAGE of the purified proteins revealed a single band corresponding to each eluted peak, displaying the same molecular mass of approximately 37 kDa (Fig. 4B). As the enzyme present in the second peak has greater activity and was more stable than the

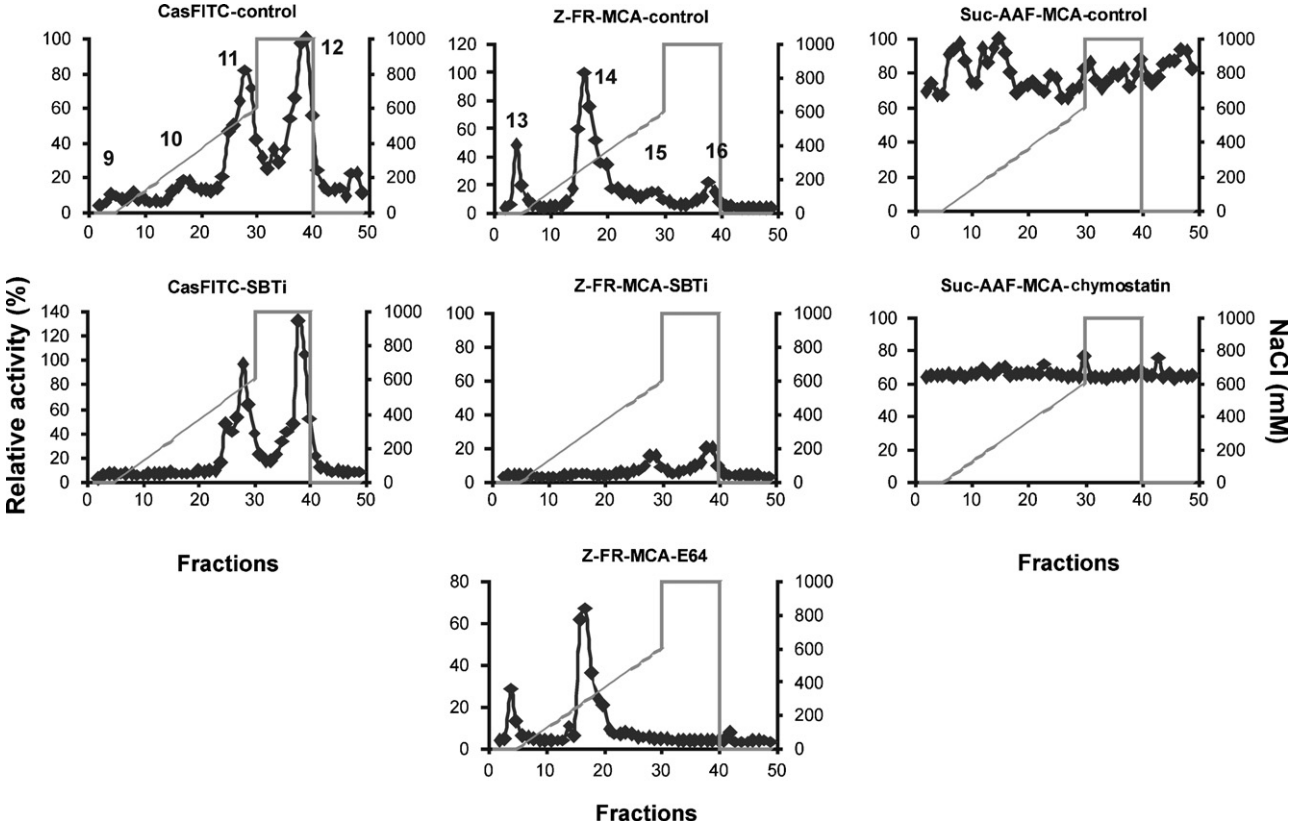

Fig. 3. Ion-exchange chromatography of proteins of midgut homogenates from *S. levis* larvae; same fractions assayed in the presence of different fluorescent substrates, EDTA + DTT were used as activators of cysteine proteinases, SBTI, Chymostatin (inhibitor of chymotrypsin) and E-64, a specific cysteine proteinase inhibitor, were used as inhibitors; straight lines correspond to NaCl gradient (0–600 mM); CasFITC, casein-FITC, fluorescent general substrate for proteinases; SBTI, soybean trypsin inhibitor; Z-FR-MCA, carbobenzoxy-Phe-Arg-MCA, substrate for trypsin; S-AAF-MCA, succinyl-Ala-Ala-Phe-MCA, specific substrate for chymotrypsin; all assays performed in 50 mM Tris–HCl buffer at pH 8; results displayed typical of 3 independent experiments.

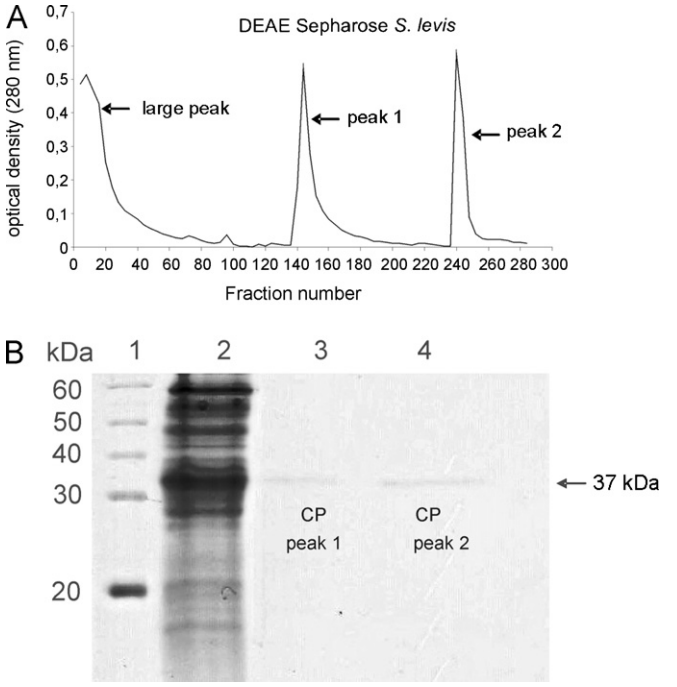

Fig. 4. Ion-exchange chromatography of soluble fraction of midgut from *S. levis* (A); note large peak of inactive protein eluted with 0.3 M NaCl and two peaks of cysteine proteinase activity eluted with 1 M NaCl; SDS-PAGE of fractions obtained in chromatography (B); Lane 1, molecular mass markers (Invitrogen); lane 2, crude extract of *S. levis* midgut; lane 3 (pool of fractions corresponding to peak 1); lane 4 (pool of fractions corresponding to peak 2); Note band of 37 kDa (arrow).

first, it was chosen for characterization. Thus, the data refer only to the major *S. levis* midgut cathepsin L.

The successfully purified enzyme is active on Z-FR-MCA, has an optimal pH of 6 (Fig. 5). The kinetic parameters for the hydrolysis of the fluorogenic peptides Z-FR-MCA, Z-RR-MCA and Z-LR-MCA by *S. levis* cysteine proteinase were determined. The greatest catalytic efficiency was obtained with Z-FR-MCA with k_{cat}/K_m value of $30.0 \pm 0.5\ \mu M^{-1}\ s^{-1}$. The substrate Z-LR-MCA was hydrolyzed with a k_{cat}/K_m value of $20.0 \pm 1.1\ \mu M^{-1}\ s^{-1}$ and Z-RR-MCA substrate was resistant to hydrolysis. The kinetic data and standard deviations were calculated from at least three separate determinations.

3.4. Distribution of enzyme activities throughout midgut

Amylase and maltase were assayed throughout the midgut to define the sites of initial (amylase) and final (maltase) starch digestion. Cysteine proteinase and trypsin were found to be the major and minor digestive proteinases, respectively, in *S. levis* (see previous item). Hence, both proteinase activities were selected to identify the site of initial protein digestion and that of final digestion of aminopeptidase. Optimal pH for the selected enzymes are (Fig. 5) 6–7 for amylase, 5–6 for maltase, 8–10 for trypsin, 7–8 for aminopeptidase and 6.0 for cysteine proteinase. The selected enzymes were analyzed in the midgut contents and in the soluble and membrane-bound fraction of the midgut tissue at different sites along the midgut (Fig. 6). Based on the data, amylase, maltase, cysteine proteinase and trypsin predominate in the luminal contents of the anterior (V1 and V2) midgut. However, trypsin also occurs in significant amounts in the tissue both as a soluble and as a membrane-bound enzyme (Fig. 6). An aminopeptidase is

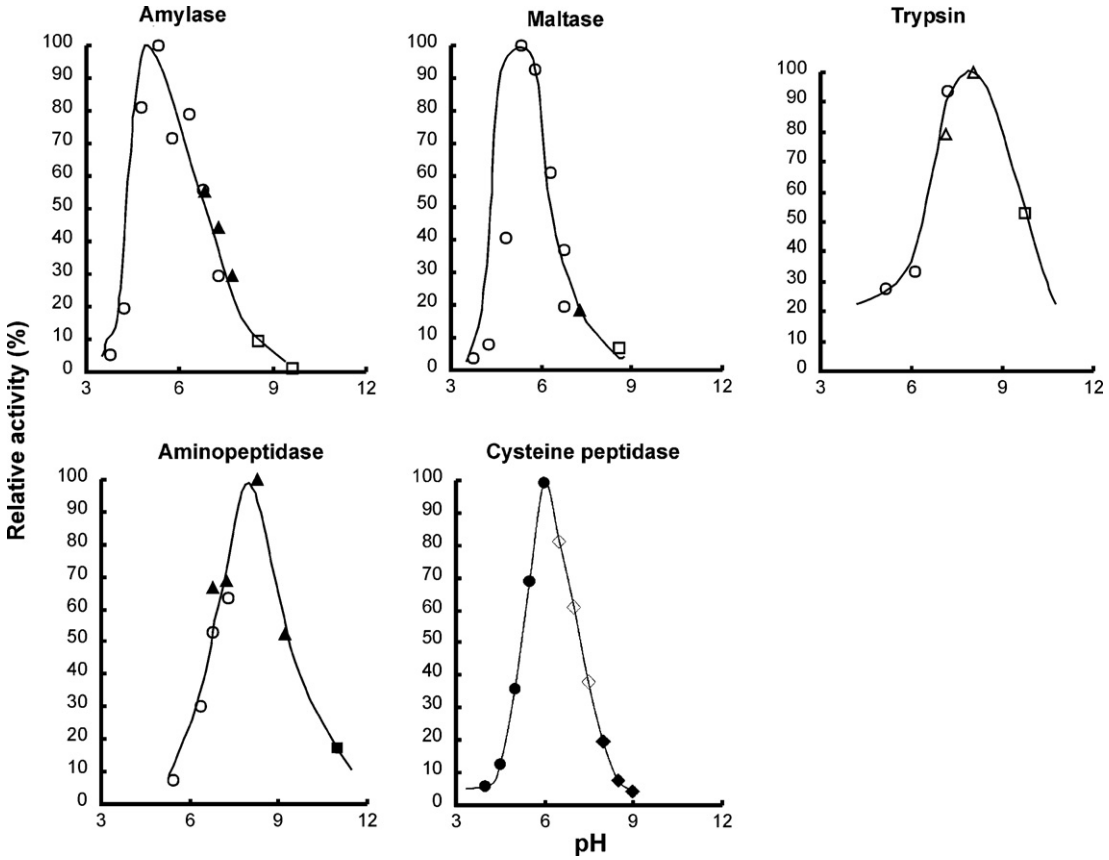

Fig. 5. Effect of pH on five hydrolase activities of midgut homogenates of *S. levis* larvae; buffers (200 mM) used for all enzymes, except for cysteine peptidase: sodium citrate-phosphate (○), triethanolamine (▲), sodium phosphate (△), glycine-NaOH (□, ■); for purified cysteine peptidase, buffers used: sodium acetate 100 mM (●), HEPES 100 mM (◇) and sodium borate 50 mM (◆).

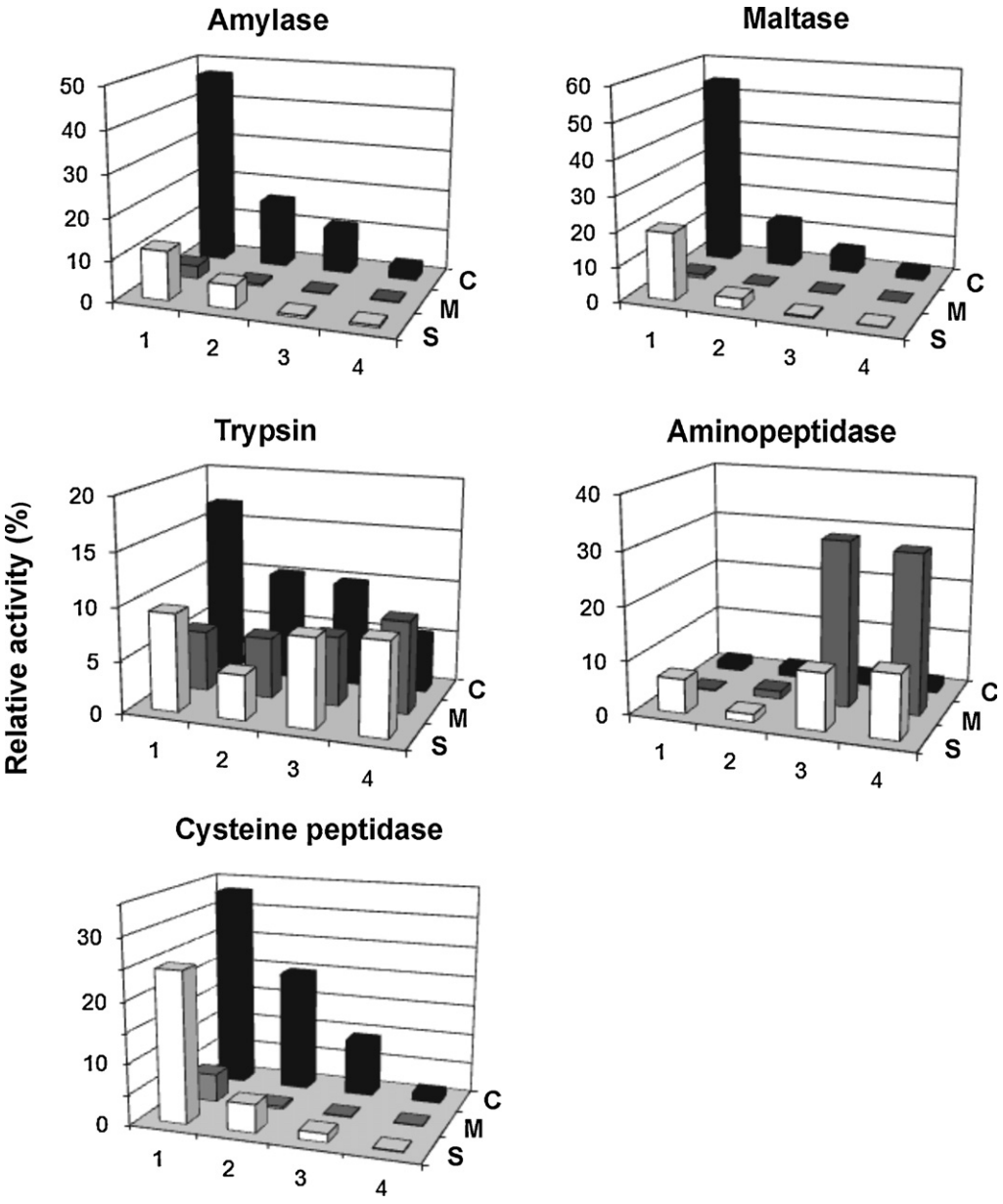

Fig. 6. Distribution of major hydrolases throughout gut of *S. levis*; C, content of the midgut; M, membrane fraction of tissues; S, soluble fraction of tissues; V, ventriculus (tubular section of the midgut) divided into four sections, as shown in Fig. 1: 1, V1; 2, V2; 3, V3; 4, V4; determinations carried out in three different preparations obtained from 10 insects each; SEM found to be 10–25% of means; activities (mU/animal) in whole midgut homogenates: amylase, 9500; maltase, 1700; trypsin, 1.19; aminopeptidase, 22.1; cysteine proteinase, 170,000; amount of proteins in gut sections (per animal): 1, 1.283 mg; 2, 0.226 mg; 3, 0.206 mg; 4, 0.128 mg.

found mainly in the posterior (V3 + V4) midgut as a membrane-bound enzyme (Fig. 6).

4. Discussion

4.1. Digestive proteinases in S. levis

The midgut of *S. levis* has two cysteine proteinases, two trypsins and perhaps a negligible chymotrypsin. SDS-PAGE analysis showed purified bands of cysteine proteinases both with 37 kDa eluted at 1 M NaCl as two peaks. This elution profile suggests the presence of two isoforms of cysteine proteinase that most likely differ in their charge or isoeletric point. *S. levis* cathepsin L exhibits elution profile similar to human cathepsin L (EC 3.4.22.15) purified from human kidneys (Turk, 1993). The major *S. levis* cysteine proteinase was purified to homogeneity and hydrolyzes Z-FR-MCA and Z-LR-MCA, but not Z-RR-MCA. As cathepsin L prefers a hydrophobic residue at P2 and cathepsin B prefers an arginine at the same position (Barrett et al., 1998), *S. levis* cysteine proteinase is a cathepsin L-like proteinase. Its molecular mass, as determined by SDS-PAGE (37 kDa), is somewhat larger than and its optimal pH (6.0) is similar to known insect cathepsin L-like proteinases (see, for example, Cristofoletti et al., 2005).

4.2. Digestive physiology in S. levis

'The food ingested by insects generally passes through the foregut and is enclosed by the PM in the midgut, where it is digested first by enzymes that penetrate into the endoperitrophic space (inside the PM), then by enzymes acting on diffuse material in the ectoperitrophic space (between the PM and midgut epithelium) and finally on the midgut cell surface (Terra and Ferreira, 1994; Terra and Ferreira, 2005). The PM is a film that surrounds the food bolus in most insects and is formed by a network of chitin and proteins to which enzymes and other components associate. This structure shares with the ancestral

gastrointestinal mucus the functions of protection against food abrasion and microorganisms, but also has specific functions in digestion associated to the compartmentalization of luminal contents (Terra, 2001; Bolognesi et al., 2008). Occasionally, the film surrounding the food may have a gel consistency, forming a non-membranous structure known as peritrophic gel (PG) (Terra, 2001; Terra and Ferreira, 2005). The presence of a conspicuous PM in *S. levis* was confirmed by dissection only in the middle and posterior regions of the midgut, whereas the observations of the present study indicate the occurrence of a PG in the anterior midgut.

Enzyme assays in *S. levis* demonstrated that the initial digestion of starch must be carried out by amylase in the anterior and middle portions of the midgut, whereas final starch digestion must be performed by maltase. Protein digestion starts under the action of a cathepsin L-like proteinase in the anterior and middle midgut, continues with trypsin in middle and posterior midgut and finishes on the surface of cells in the middle and posterior midgut by a membrane-bound aminopeptidase. Soluble trypsin and capthepsin L-like enzymes found associated with midgut tissue probably correspond to enzyme molecule entrapped in the cell glycocalyx, as shown in other beetles (Ferreira et al., 1990). Membrane-bound trypsin has been described in other insect midguts and seems to be enzyme molecules en route to be secreted (Terra and Ferreira, 1994, 2005).

The activities of amylase, cysteine proteinase and trypsin decrease throughout the contents of the midgut. This is what one would expect when there is a flux of fluid from the posterior midgut to the anterior midgut in the ectoperitrophic space, as described for most insects (for reviews, see Terra and Ferreira, 1994, 2005). This flux is thought to be caused by the secretion of fluid by posterior midgut cells and its absorption by anterior midgut cells. As the flux moves, it displaces forward enzymes and digestion products diffusing from the PM into the ectoperitrophic space. This counterflux prevents digestive enzymes from being lost to the feces and causes enzyme recycling.

Taking *S. levis* as a model, curculionid digestion differs from that of putative Coleoptera ancestors (Terra and Ferreira, 2005) in that most terminal digestion of proteins takes place on the surface of midgut cells.

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