Article

DIGESTIVE PROTEOLYSIS ORGANIZATION IN TWO CLOSELY RELATED TENEBRIONID BEETLES: RED FLOUR BEETLE (*Tribolium castaneum*) AND CONFUSED FLOUR BEETLE (*Tribolium confusum*)

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Abbreviations: AM, anterior midgut; BzRpNA, N_α-benzoyl-DL-arginine *p*-nitroanilide; DTT, dithiothreitol; DMF, dimethylformamide; E-64, L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane; EDTA, ethylene diamine tetraacetate; GlpFApNA, pyroglutamyl-phenylalanyl-alanine *p*-nitroanilide; PI, peptidase inhibitor; PM, posterior midgut; PB_{AM}, physiological buffer of anterior midgut (pH 5.6, 1 mM DTT); PB_{PM}, physiological buffer of posterior midgut (pH 7.9 without DTT); PMSF, phenylmethylsulphonyl fluoride; SucAAPFpNA, N_α-succinyl-alanyl-alanyl-prolyl-phenylalanine *p*-nitroanilide; SucAAPLpNA, N_α-succinyl-alanyl-alanyl-prolyl-leucine *p*-nitroanilide; STI, soybean Kunitz trypsin inhibitor; TCA, trichloroacetic acid; TPCK, tosyl-L-phenylalanine chloromethyl ketone; UB, universal buffer; ZFRpNA, benzyloxycarbonyl-phenylalanyl-arginine *p*-nitroanilide

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The spectra of Tribolium castaneum and T. confusum larval digestive peptidases were characterized with respect to the spatial organization of protein digestion in the midgut. The pH of midgut contents in both species increased from 5.6–6.0 in the anterior to 7.0–7.5 in the posterior midgut. However, the pH optimum of the total proteolytic activity of the gut extract from either insect was pH 4.1. Approximately 80% of the total proteolytic activity was in the anterior and 20% in the posterior midgut of either insect when evaluated in buffers simulating the pH and reducing conditions characteristic for each midgut section. The general peptidase activity of gut extracts from either insect in pH 5.6 buffer was mostly due to cysteine peptidases. In the weakly alkaline conditions of the posterior midgut, the serine peptidase contribution was 31 and 41% in T. castaneum and T. confusum, respectively. A postelectrophoretic peptidase activity assay with gelatin also revealed the important contribution of cysteine peptidases in protein digestion in both Tribolium species. The use of a postelectrophoretic activity assay with p-nitroanilide substrates and specific inhibitors revealed a set of cysteine and serine endopeptidases, 8 and 10 for T. castaneum, and 7 and 9 for T. confusum, respectively. Serine peptidases included trypsin-, chymotrypsin-, and elastase-like enzymes, the latter being for the first time reported in Tenebrionid insects. These data support a complex system of protein digestion in the Tribolium midgut with the fundamental role of cysteine peptidases.

Keywords: Coleopteran insects; insect digestive peptidases; organization of digestion; *Tribolium castaneum*; *Tribolium confusum*

INTRODUCTION

The red flour beetle (*Tribolium castaneum*) and confused flour beetle (*Tribolium confusum*), belonging to the family Tenebrionidae, are the most abundant and serious pests of stored grains and milled products in the world. Evidently, *T. castaneum* is of Indo-Australian origin and found more in temperate areas, whereas *T. confusum* originated in Africa and is more problematic in cooler climates (Smith and Whitman, 1992). Both beetles can coexist and compete for the same resources, but only *T. castaneum* can fly (Ryan et al., 1970). The developmental period from egg to adult is usually shorter for *T. castaneum* than for *T. confusum* (ARS Agriculture Handbook Number 500, 1986).

New effective protection methods that use transgenic plants and cereals expressing peptidase inhibitors (PIs) and Cry endotoxins of *Bacillus thuringiensis* are based on a thorough understanding of the organization of the midgut proteolytic complex of target insects. If protein digestion in an insect is spatially arranged, the knowledge of physico-chemical conditions (pH and rH), peptidase spectrum, and consequences of protein degradation in different midgut compartments is important with regard to the processing of PIs and Cry endotoxins in the gut.

The organization of digestive proteolysis in coleopterans in the Cucujiformia series including Tenebrionidae is quite complex, and includes cysteine (cathepsins L and B), serine (trypsin- and chymotrypsin-like), and in several families also aspartate (cathepsin D-like) peptidases (Terra and Cristofoletti 1996; Johnson and Rabosky, 2000; Prabhakar et al., 2007). Transcriptome analysis of the insect midgut suggests that many genes encoding digestive peptidases are up- or down-regulated in response to different diets (Zhu-Salzman et al., 2003; Bown et al., 2004; unpublished data). The complex pattern of digestive peptidases provides rapid adaptability of the digestive system to different agents. Indeed, resistance to PI's (Oppert et al., 1993, 2003; Mazumdar-Leighton and Broadway, 2001; Zhu-Salzman et al., 2003; Rivard et al., 2004) and Cry-endotoxins (Oppert et al., 1996, 1997a; Keller et al., 1996; Li et al., 2004; Karumbaiah et al., 2007) has been attributed to adaptive changes in the spectrum of insect digestive peptidases.

A few studies have examined the full spectra of different groups of digestive proteolytic enzymes secreted and simultaneously operating in the coleopteran midgut. Using diagnostic inhibitors and specific substrates, complex systems of digestive peptidases were described in the colorado potato beetle, *Leptinotarsa decemlineata* (F. Chrysomelidae) (Novillo et al., 1997), and rice water weevil, *Lissorhoptrus brevirostris* (F. Curculionidae) (Hernandez et al., 2003). Previously, we characterized in detail the digestive peptidase spectrum of the larvae of yellow mealworm, *Tenebrio molitor* (Vinokurov et al., 2006a,b; Elpidina and Goptar, 2007) with subsequent analysis of midgut cDNA transcripts encoding peptidases (Prabhakar et al., 2007). Larvae of *T. molitor* use a complex of digestive peptidases, including those from serine and cysteine classes, operating in a midgut with a sharp pH gradient (Terra et al., 1985; Vinokurov et al., 2006a). Cysteine peptidase activity is compartmentalized to the anterior region of the larval midgut (AM), whereas serine peptidase activity predominates in the posterior midgut (PM) (Thie and Houseman, 1990; Terra and Ferreira, 1994; Vinokurov et al., 2006a). A comprehensive biochemical analysis suggested that at least 15 different endopeptidase activities are expressed simultaneously in *T. molitor* larvae under normal dietary conditions, including six cysteine and nine serine peptidases (Vinokurov et al., 2006a,b).

The spectrum of midgut peptidases and the organization of midgut digestive proteolysis in *T. castaneum* are insufficiently characterized. Cysteine peptidases were reported to be the major digestive peptidases in *T. castaneum*, but the activity of serine (trypsin- and chymotrypsin-like) peptidases with alkaline pH-optima also was detected (Oppert et al., 2003). The annotation of cysteine cathepsins in the *T. castaneum* genome indicated that there were 25 cysteine cathepsin L and B peptidases (*Tribolium* Genome Sequencing Consortium, 2008), and subsequent transcriptome and proteome studies of this insect have confirmed the presence of many of these enzymes in the gut (Oppert and Elpidina, 2008). Seed cystatins significantly reduced azocaseinolytic activity of enzymes in the gut extract from this insect (Liang et al., 1991; Chen et al., 1992). However, only the combination of two class-specific inhibitors (cysteine and serine) in bioassays led to growth retardation and mortality in *T. castaneum* larvae (Oppert et al., 1993, 2003). Biochemical studies suggested that *T. castaneum* larvae shift from a cysteine- to serine-based protein digestion strategy when fed cysteine PIs (Oppert et al., 2005), a hypothesis that also has been supported by microarray data (unpublished data). A cathepsin D-like aspartic peptidase also was described in midgut extracts of *T. castaneum* (Blanco-Labra et al., 1996). There is no information regarding the midgut digestive peptidases of another *Tribolium* species, *T. confusum*.

To provide a more complete understanding of the complexity of digestion in *Tribolium* larvae reared under normal dietary conditions, this study examined the spectra of cysteine and serine digestive peptidases in *T. castaneum* and *T. confusum* larvae with respect to the pH of midgut contents and spatial organization of protein digestion in the midgut, using general proteinaceous and specific *p*-nitroanilide substrates in combination with inhibitor analyses. By applying a post-electrophoretic method of peptidase analysis (Vinokurov et al., 2005), each *T. castaneum* and *T. confusum* midgut digestive peptidase activity was assigned to a specific peptidase class.

MATERIALS AND METHODS

Chemicals

Azocasein, porcine hemoglobin, gelatin, bicinchoninic acid protein assay kit, N_α-benzoyl-D,L-arginine *p*-nitroanilide (BzRpNA), L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), tosyl-L-phenylalanine chloromethyl ketone (TPCK), soybean Kunitz trypsin inhibitor (STI), ethylenediamine tetraacetic acid (EDTA), and pepstatin A were purchased from Sigma-Aldrich (St. Louis, MO); phenylmethylsulphonyl fluoride (PMSF) and dithiothreitol (DTT) were from Fluka (Buchs, Switzerland). Fluorescently labeled casein (BODIPY-TR-X casein) was from Molecular Probes (Eugene, OR). N_α-succinyl-alanyl-alanyl-prolyl-leucine *p*-nitroanilide (SucAAPLpNA), N_α-succinyl-alanyl-alanyl-prolyl-phenylalanine *p*-nitroanilide (SucAAPFpNA), and benzyloxycarbonyl-phenylalanyl-arginine *p*-nitroanilide (ZFRpNA) were from Bachem AG (Bubendorf, Switzerland). A specific cysteine peptidase substrate pyroglutamyl-phenylalanyl-alanine *p*-nitroanilide (GlpFApNA) was synthesized at the Department of Chemistry of Natural Compounds, Chemical Faculty, Moscow State University (Moscow, Russia). Nitrocellulose membrane sheets with a 0.45-μm pore size were from Bio-Rad (Hercules, CA). For midgut pH evaluation, a set of indicator dyes from Merck (Darmstadt, Germany) was used.

Insects

Stock cultures of *T. castaneum* and *T. confusum* were maintained on a mixture (1:1) of milled oat flakes (Raisio, Finland) and wheat bran at 25°C. Approximately 1 wk prior to dissection, larvae were transferred to milled oat flakes that were processed at high temperature and were devoid of active peptidases and peptidase inhibitors (data not shown). At the time of dissection, larvae weighed on average 3.7 ± 0.11 mg (average of 10 groups of five larvae each, mean ± S.E.).

pH of Midgut Contents

A standard set of indicator dyes with overlapping regions of color change was used to determine pH in the midgut lumen. The set was composed of bromphenol blue, methyl red, bromcresol purple, bromthymol blue, phenol red, cresol red, and thymol blue. Before each experiment, larvae were starved for 5 d, and subsequently animals were fed 1 g of wheat flour soaked with 0.02–0.1% dye solution in 96% ethanol. The larvae were dissected after 2 h and the color of the AM and PM contents was evaluated in 10 replicates.

Preparation of Enzyme Extract

Larvae were chilled on ice, the anterior and posterior ends were removed, and the entire gut was removed from one end. After rinsing in precooled distilled water, either the entire guts or those divided into two equal parts (AM and PM) were pooled and homogenized in cold distilled water in a glass Downce homogenizer (approximately 50 AM or PM parts in 200 μl of water). The homogenate was centrifuged at 4°C for

10 min at 10,000*g*. To separate the midgut contents and tissue, 25 guts were excised, placed on a piece of parafilm, and slit longitudinally. The leaking content was diluted by 1 μl of 0.75% NaCl (or distilled water if the samples were analyzed electrophoretically) and transferred by micropipette to a tube with 80 μl of 0.75% NaCl or distilled water. The remaining tissue was carefully rinsed with 0.75% NaCl or water, dried by filter paper, and transferred to another tube containing the same volume of 0.75% NaCl or distilled water. Diluted gut content was vortexed gently, and the tissue was homogenized by sonication at 40–50 MHz (Sonoplus HD2070 Bandelin Electronics, Berlin, Germany) for 12 s in cold saline or water. The homogenate was centrifuged at 4°C for 15 min at 18,500*g*. The resulting supernatant was stored at −70°C until use.

Enzyme Assays and Protein Determination

The total proteolytic activity of extracts was assayed with azocasein (Charney and Tomarelli, 1947; Michaud et al., 1995). Enzyme extracts (0.2–0.5 gut equivalents) were diluted to 50 μl with 100 mM phosphate buffer, pH 5.6 or 7.5, and were incubated with 100 μl of 0.5% azocasein solution in the same buffer for 40 min at 30°C. Conditions for proteolytic measurements were determined experimentally as previously described (Vinokurov et al., 2006a). For both *Tribolium* spp., pH 5.6 buffer containing a reducing compound, 1 mM dithiothreitol (DTT), was used to approximate the AM physiological conditions (PB_{AM}), while the PM physiological conditions were approximated with a nonreducing buffer of pH 7.5 (PB_{PM}). The enzyme reaction was terminated by the addition of 150 μl of 12% trichloroacetic acid (TCA). The mixture was incubated for 15 min at 4°C and centrifuged for 10 min at 10,000*g* to remove precipitate. An equal volume of 1 M NaOH was added to a 100-μl aliquot of supernatant transferred to a 96-well plate, and the absorbance was measured at 450 nm with a Spectra Max 340 PC plate reader (Molecular Devices, Sunnyvale, CA).

At acidic pH (3.0), the proteolytic activity was measured with 1% porcine hemoglobin as a substrate in 100 mM universal acetate-phosphate-borate buffer (UB, Frugoni, 1957) according to the modified procedure of Houseman and Downe (1983). The formation of TCA-soluble hydrolysis products was evaluated with the bicinchoninic acid protein assay kit at 562 nm (Walker, 2002). All assays were adjusted so that the proteolytic activity was proportional to protein concentration and to time. One unit of total proteolytic activity with azocasein or hemoglobin was defined as the increase in absorbance by 0.1 unit per min per gut.

The pH-optimum of the total proteolytic activity was determined with fluorescently labeled casein, BODIPY-TR-X (Invitrogen, Carlsbad, CA; Oppert et al., 1997b), which, unlike azocasein, was soluble throughout the entire pH range. The substrate was diluted as per the manufacturer's recommendation, and 10 μl (0.1 μg) was added to each well containing the midgut extract (0.2 gut equivalents) diluted to 90 μl with 100 mM UB with pH from acidic to basic (2.0–11.0). After a 30-min incubation at 30°C, the fluorescence was measured (excitation 584 nm, emission 620 nm) using a Fluoroskan Ascent FL microplate reader (Labsystems, Thermo Electron Corp., Milford, MA).

Specific proteolytic activity was assessed with synthetic substrates, including GlpFApNA, specific for cysteine peptidases (Stepanov et al., 1985), ZFRpNA, specific for cysteine and trypsin-like peptidases (Tchoupé et al., 1991; Halfon and Craik, 1998; Volpicella et al., 2003), BzRpNA, specific for trypsin-like and some cysteine peptidases (Erlanger et al., 1961), and SucAAPFpNA and SucAAPLpNA, specific for chymotrypsin-like peptidases and pancreatic elastases type II (Del Mar et al., 1979, 1980).

Five microliters of 10 mM substrate diluted in dimethylformamide (DMF) were added to each well containing 0.3–0.4 equivalents of gut extract diluted to 195 μl with 100 mM phosphate buffer. Samples were incubated at 30°C, and absorbance of released *p*-nitroaniline was measured spectrophotometrically at 405 nm at 5-min intervals with a Spectra Max 340 PC plate reader. Enzyme activity was calculated in μmol/min per gut on the linear part of the time and protein concentration response curves. Determinations of enzyme activity were made in two biological replicates with 3–5 technical replicates. Data were expressed as mean ± S.E. and Student's *t*-test was used for statistical analysis of significance. Graph generation and statistical analysis were performed with GraphPad Prism 4 (GraphPad software, San Diego, CA). Assays for sulfhydryl (SH)-dependent activity with GlpFApNA, ZFRpNA and BzRpNA were performed with 1 mM DTT in the final reaction mixture. For pH-optimum determination of peptidase activity against *p*-nitroanilide substrates, aliquots of midgut extract were incubated in UB with pH values ranging from acidic to basic. Protein content in the extracts was measured with bicinchoninic acid reagent (Walker, 2002).

Inhibition Assays

For inhibition studies, aliquots of the total enzyme preparation were preincubated with different concentrations of inhibitors for 15 min at room temperature in 100 mM phosphate buffer (pH 5.6 and 7.5) or UB (pH 3.0), and residual activity against azocasein or hemoglobin was assayed as previously described. Diagnostic inhibitors of the active site included: PMSF (specific for serine peptidases) at 0.01, 0.1 and 1 mM, pepstatin A (specific for aspartic peptidases) at 0.001, 0.01, 0.1 mM, E-64 (specific for cysteine peptidases) and STI (specific for serine peptidases) at 0.0001, 0.001, 0.01 mM, EDTA (inhibitor of metallopeptidases) at 0.02, 0.2, and 2 mM final concentrations.

Native PAGE

Native PAGE was performed in 1-mm thick 12% polyacrylamide gels (Bio-Rad Mini Protean 3 system) with 35 mM HEPES and 43 mM imidazole buffer, pH 7.2, according to McLellan (1982) at 10 mA constant current and 4°C. The electrophoresis was performed in two directions: towards the anode for proteins with acidic pI ($<$7.2 pH units), and towards the cathode for proteins with basic pI ($>$7.2 pH units). Equal amounts (usually 0.75 gut equivalents) of either AM or PM preparations were loaded in each well.

Postelectrophoretic Activity Detection and Inhibition

Detection of peptidase activity in electrophoregrams was performed by two different methods. In the first approach, the total proteolytic activity was detected by means of hydrolysis of general peptidase substrate, gelatin (0.03%), incorporated into an 12% polyacrylamide indicator gel polymerized in 100 mM UB, pH 3.0, 5.6, or 7.5. The acid indicator gel was polymerized at higher concentrations of polymerization reagents (TEMED and ammonium persulfate) according to Díaz-Lópes et al. (1998). After polymerization, the indicator gel was placed into an appropriate UB solution for 40 min. When testing SH-dependent peptidase activity, the indicator gel was incubated in the same buffer containing 5 mM DTT. After electrophoresis, the resolving gel was washed in the reaction buffer of pH 3.0, 5.6, or 7.5 for 15 min and layered onto an appropriate indicator gel (with the same pH). The gels were incubated in a moist chamber for 1 h 30 min at 37°C. Proteolysis was terminated by transferring the gels into a staining solution of 0.15% (w/v) Coomassie Brilliant Blue R-250 in 30% ethanol, 10% acetic acid. The gels were destained in 15% ethanol and 5% acetic acid solution.

In the second approach, specific proteolytic activity was detected with *p*-nitroanilide substrates ZFRpNA, GlpFApNA, BzRpNA, SucAAPFpNA, and SucAAPLpNA using an overlay on the native polyacrylamide gel of a nitrocellulose membrane impregnated with the substrate (Vinokurov et al., 2005). After electrophoresis, the resolving gel was washed for 15 min in 100 mM UB, pH 5.6 or 7.5. The buffer was removed, and a nitrocellulose membrane, presoaked for 40 min in 0.25 mM solution of substrate in 100 mM UB, pH 5.6 or 7.5, was layered onto the surface of the gel. For SH-dependent proteolytic activity, the substrate solution contained 5 mM DTT. The membrane was incubated with the gel in a moist chamber at 37°C for 60 min until faint yellow bands became visible on the membrane. The gel was removed, and liberated *p*-nitroaniline was diazotized by subsequent incubations of 5 min each in 0.1% sodium nitrite in 1 M HCl, 0.5% ammonium sulfamate in 1 M HCl, and 0.05% N-(1-naphthyl)-ethylenediamine in 47.5% ethanol. Immediately after formation of the pink bands representing proteolytic activity, membranes were placed in heat-sealed plastic bags, scanned and stored at −20°C. A comparison of activity bands, obtained by both detection methods, was based on the R_f calculated for each fraction.

Inhibition studies of electrophoretically-separated peptidases were performed as follows. Individual lanes of the gel were excised, and each lane was incubated in 10 mM phosphate buffer, pH 6.8, containing diagnostic peptidase inhibitors: PMSF (2 mM), TPCK (0.3 mM), STI (0.02 mM), E-64 (0.05 mM) for 30 min at 25°C. Effect of pepstatin A (0.02 mM) was evaluated at acidic pH (3.0). After incubation, the gels were washed in 100 mM UB of appropriate pH (3.0, 5.6, or 7.5). Proteolytic activity was detected by one of the earlier described methods and compared to the control without inhibitor.

RESULTS

Midgut pH

The determination of pH in the midgut contents of *T. castaneum* and *T. confusum* revealed a pronounced gradient from slightly acidic pH in the anterior (5.6–6.0), increasing to neutral in the middle (6.0–7.0) and further increasing to weakly alkaline in the posterior third of the midgut (7.0–7.5). Interspecific differences in the midgut pH of the two *Tribolium* species were not detected.

Effect of pH on Proteolytic Activity of Entire Midgut Extracts

The total proteolytic activity in extracts of the entire midgut was approximated with fluorescently-labeled casein (Fig. 1). In extracts from either *T. castaneum* (Fig. 1A) or *T. confusum* (Fig. 1B), activity was maximal in acidic buffer at pH 4.1. Caseinolytic activity was much lower in buffers approximating the pH of the anterior and middle midgut contents (5.6–7.0), and activity decreased further in buffers with the slightly alkaline pH of the posterior midgut contents (7.0–7.5). When buffers contained 1 mM DTT, the activity only slightly increased in the acidic region, more so with *T. confusum* enzymes, and slightly decreased in the alkaline buffers. These profiles are similar to a previous profile (Oppert et al., 2003) and support the hypothesis that protein digestion in *Tribolium* spp. larvae is due primarily to cysteine peptidases with minor contributions from serine peptidases.

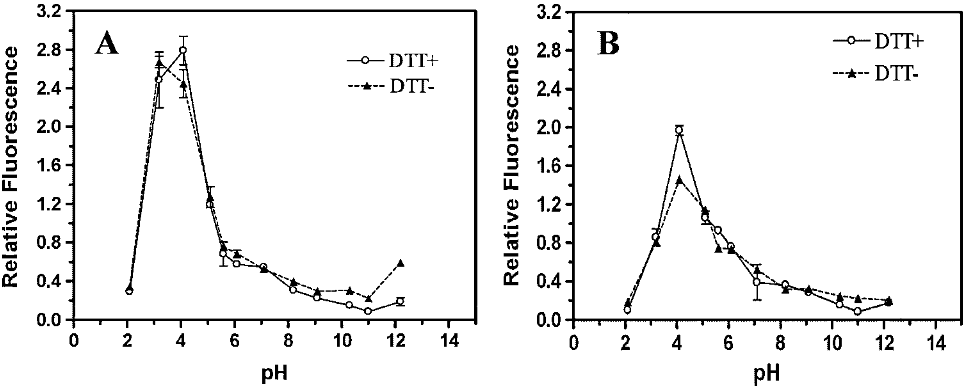

Figure 1. Effects of pH on the activity of extracts from *T. castaneum* (**A**) and *T. confusum* (**B**) entire larval midgut assayed with BODIPY-TR-X casein (mean ± S.E.) in the presence and absence of 1 mM DTT.

Analysis of the proteolytic activity in extracts from both species, measured with the specific cysteine peptidase substrate GlpFApNA, revealed a maximum activity zone from pH 4.0 to 8.0 (Fig. 2AB). Overall, the activity was significantly stimulated by DTT ($P < 0.05$), with the highest activation in the acidic part of the curve (close to the physiological conditions of anterior and middle midgut) and the maximal level at pH 4.1, which was more prominent with *T. castaneum* than *T. confusum* enzymes. In buffers without DTT, a noticeable peak in activity was observed at pH 7.0 for both extracts, and high levels of activity were retained in pH 7.5 buffer, characteristic of the posterior midgut (84% in *T. castaneum* and 88% in *T. confusum*). Throughout the entire pH interval, GlpFApNA-hydrolyzing activity was completely inhibited by 0.0001 mM E-64 but was not susceptible to 1 mM PMSF (results not shown). Therefore, cysteine peptidases may be highly active along the entire midgut of *Tribolium.*

The hydrolysis of chymotrypsin (SucAAPFpNA, Fig. 3A) and trypsin (BzRpNA, Fig. 3B) substrates by enzymes in larval midgut extracts was more efficient in alkaline buffers, with maximum activity at pH 8.0–10.5. Chymotrypsin-like activity in buffer approximating the pH of PM contents, 7.5, constituted 63 and 66% of the maximum activity (at pH 9.0) in *T. castaneum* and *T. confusum*, respectively. However, this activity was quite low in buffer with a pH approximating the AM contents, 5.6 (only 20% of the maximum). The trypsin-like activity in both species with BzRpNA at the pH of the PM constituted approximately 80% of the maximum activity and also was quite low at the pH of the AM (approximately 10%). Thus, the activities of serine peptidases in the two *Tribolium* species would be maximal in the pH conditions of the PM, and therefore these enzymes can participate mainly in the final stages of digestion.

Localization of Proteolytic Activities in the Larval Midguts

To study the localization of the total proteolytic activity in the midgut, activity was initially measured in the AM and PM using the general peptidase substrate azocasein in buffers that approximated the physiological conditions of each midgut section. Eighty percent of the total proteolytic activity of the entire *T. castaneum* midgut was located in the AM, and 20% was found in the PM (calculated as the sum of activity in the AM at PB_{AM}, 0.287, and in the PM at PB_{PM}, 0.072; Table 1). A similar activity distribution was found in the *T. confusum* midgut, with 70% of activity located in the AM

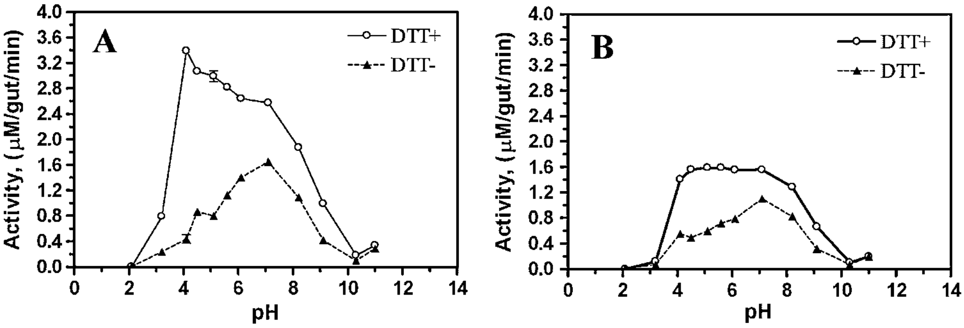

Figure 2. Effects of pH on the activity of extracts from *T. castaneum* (**A**) and *T. confusum* (**B**) entire larval midgut assayed with cysteine peptidase substrate GlpFApNA (mean ± S.E.) in the presence and absence of 1 mM DTT.

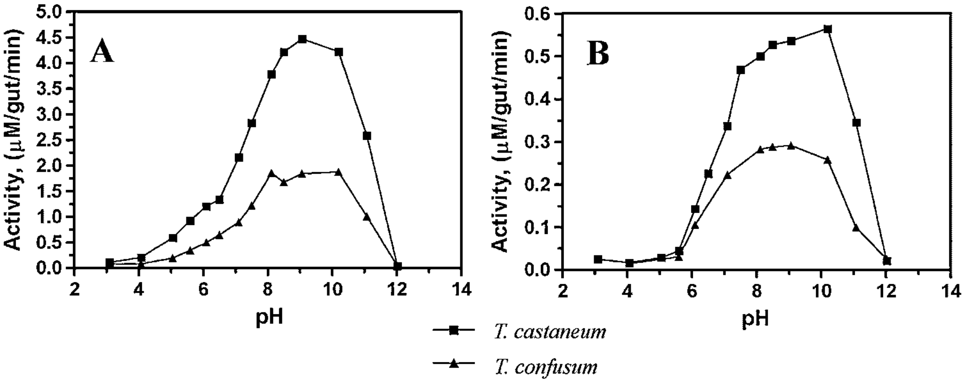

Figure 3. Effects of pH on the activity of extracts from *T. castaneum* and *T. confusum* entire larval midgut assayed with serine peptidase substrates. **A:** SucAAPFpNA. (chymotrypsin-like peptidases). **B:** BzRpNA (trypsin-like peptidases).

Table 1. Comparison of Proteolytic Activities With General Protein and Specific Synthetic Substrates in T. castaneum Larval AM and PM Extracts at pH 5.6 and 7.5 (Mean ± S.E.)

Substrate	Activity (U/min/gut)[a]			
	pH 5.6		pH 7.5	
	AM	PM	AM	PM
Azocasein	0.220 ± 0.050	0.108 ± 0.004	0.142 ± 0.002	0.072 ± 0.001
Azocasein[b]	0.287 ± 0.034	0.139 ± 0.005	0.094 ± 0.002	0.052 ± 0.001
GlpFApNA	0.093 ± 0.013	0.023 ± 0.002	0.220 ± 0.007	0.216 ± 0.005
GlpFApNA[b]	0.712 ± 0.010	0.317 ± 0.001	0.591 ± 0.007	0.336 ± 0.002
BzRpNA	0.082 ± 0.004	0.043 ± 0.001	0.487 ± 0.003	0.276 ± 0.003
BzRpNA[b]	0.140 ± 0.001	0.073 ± 0.001	0.525 ± 0.003	0.322 ± 0.002
SAAPFpNA	0.433 ± 0.008	0.436 ± 0.007	1.532 ± 0.034	1.523 ± 0.014
SAAPLpNA	0.106 ± 0.010	0.088 ± 0.001	0.320 ± 0.067	0.284 ± 0.004

[a] See Materials and Methods for activity units determination for total (azocaseinase) and specific peptidase activity.
[b] Activity was detected in the presence of 1 mM DTT.

Table 2. Comparison of Proteolytic Activities With General Protein and Specific Synthetic Substrates in T. confusum Larval AM and PM Extracts at pH 5.6 and 7.5 (Mean ± S.E.)

Substrate	Activity (U/min/gut/)[a]			
	pH 5.6		pH 7.5	
	AM	PM	AM	PM
Azocasein	0.129 ± 0.016	0.126 ± 0.005	0.103 ± 0.005	0.081 ± 0.001
Azocasein[b]	0.201 ± 0.002	0.145 ± 0.020	0.075 ± 0.012	0.056 ± 0.001
GlpFApNA	0.008 ± 0.002	0.006 ± 0.002	0.065 ± 0.012	0.089 ± 0.005
GlpFApNA[b]	0.405 ± 0.003	0.267 ± 0.001	0.401 ± 0.010	0.265 ± 0.020
BzRpNA	0.040 ± 0.001	0.026 ± 0.012	0.120 ± 0.001	0.089 ± 0.006
BzRpNA[b]	0.059 ± 0.001	0.040 ± 0.001	0.139 ± 0.002	0.107 ± 0.005
SAAPFpNA	0.118 ± 0.003	0.254 ± 0.002	0.603 ± 0.022	1.011 ± 0.006
SAAPLpNA	0.023 ± 0.001	0.037 ± 0.002	0.101 ± 0.001	0.143 ± 0.001

[a] See Materials and Methods for activity units determination for total (azocaseinase) and specific peptidase activity.
[b] Activity was detected in the presence of 1 mM DTT.

Table 3. Effect of Class-Specific Inhibitors on the Total Proteolytic Activity With Azocasein of T. castaneum Larval Midgut Extracts at pH 5.6 and 7.5 (Mean ± S.E.)

Inhibitor	Concentration (mM)	Residual activity (% of control)	
		pH 5.6	pH 7.5
E-64	0.0001	26.1 ± 0.40	66.7 ± 0.87
	0.001	3.30 ± 0.09	15.1 ± 0.40
	0.01	2.90 ± 0.047	13.9 ± 0.45
PMSF	0.01	111 ± 0.75	96.8 ± 0.05
	0.1	97.8 ± 1.31	90.8 ± 1.07
	1.0	88.4 ± 1.15	68.8 ± 1.32
EDTA	0.02	99.3 ± 0.22	100 ± 0.68
	0.2	95.8 ± 1.28	98.7 ± 0.54
	2.0	97.5 ± 0.35	105 ± 0.75
STI	0.0001	96.8 ± 0.25	97.8 ± 0.77
	0.001	96.1 ± 0.51	103 ± 5.04
	0.01	88.2 ± 0.85	96.9 ± 0.98
Pepstatin	0.001	100 ± 0.88	97.9 ± 1.32
	0.01	101 ± 0.97	96.4 ± 0.44
	0.1	97.9 ± 0.81	92.7 ± 1.34

(Table 2). The calculated total peptidase activity in the entire midgut was 27% higher in *T. castaneum* than in *T. confusum*.

The contribution of *Tribolium* spp. peptidases from different classes to the total midgut azocaseinolytic activity measured at the respective physiological pH was assessed by an inhibitor analysis (Tables 3 and 4). In both species at PB_{AM}, azocasein hydrolysis was greatly reduced (up to 97%) by an inhibitor of cysteine peptidases, E-64, at 0.001 mM concentration, while the effect of an inhibitor of serine peptidases, PMSF, was negligible, with approximately 11–12% inhibition at 1 mM concentration. However, at PB_{PM}, PMSF inhibited 31 and 41% of azocaseinase activity in *T. castaneum* and *T. confusum*, respectively. In this buffer, inhibition by E-64 also was high in both species (up to 85%), indicating that cysteine peptidases are likely responsible for the

Table 4. Effect of Class-Specific Inhibitors on the Total Proteolytic Activity With Azocasein of T. confusum Larval Midgut Extracts at pH 5.6 and 7.5 (Mean ± S.E.)

Inhibitor	Concentration (mM)	Residual activity (% of control)	
		pH 5.6	pH 7.5
E-64	0.0001	6.10 ± 0.13	44.8 ± 1.85
	0.001	4.90 ± 1.02	15.2 ± 0.53
	0.01	3.50 ± 0.44	15.3 ± 1.27
PMSF	0.01	109 ± 0.20	99.4 ± 0.46
	0.1	99.2 ± 0.52	91.3 ± 1.51
	1.0	89.2 ± 0.57	59.3 ± 1.45
EDTA	0.02	102 ± 0.46	102 ± 0.63
	0.2	101 ± 0.86	103 ± 0.83
	2.0	101 ± 0.90	107 ± 1.34
STI	0.0001	95.7 ± 0.87	93.4 ± 0.89
	0.001	95.9 ± 0.90	93.3 ± 0.95
	0.01	89.9 ± 1.03	91.9 ± 1,24
Pepstatin	0.001	99.1 ± 1.19	99.1 ± 1.36
	0.01	97.5 ± 0.47	97.4 ± 1.14
	0.1	92.3 ± 1.03	88.1 ± 5.22

majority of the total proteolytic activity of *Tribolium* larvae along the entire midgut. Kunitz trypsin inhibitor from soya beans (STI) displayed very low inhibitor activity, explained by the minor contribution of trypsin-like enzymes to the overall activity of serine peptidases against the proteinaceous substrates and/or the susceptibility of STI to hydrolysis by insensitive peptidases, mostly cysteine peptidases. An inhibitor of metallopeptidases (EDTA) was practically ineffective against azocaseinase activity. In the presence of 0.1 mM pepstatin, an inhibitor of aspartate peptidases, the activity was weakly decreased at both PB_{AM} and PB_{PM} as compared to the control, with more pronounced inhibition in *T. confusum* (about 10%).

To compare the location and relative activities of different types of peptidases along the midgut, activities in the AM and PM extracts of larvae were tested with specific *p*-nitroanilide substrates at two physiological pH values (Tables 1 and 2). The hydrolysis of the cysteine peptidase substrate GlpFApNA was maximal in pH 5.6 buffers, increased after the addition of DTT ($P < 0.05$), and was found primarily in AM extracts (ca. 80% in the AM at PB_{AM} and 20% in the PM at PB_{PM}) in both species. Hydrolysis of BzRpNA was maximal in pH 7.5 buffers, was slightly increased by the addition of DTT (significant, $P < 0.05$), and so at PB_{PM} was probably due both to trypsin-like and cysteine peptidases. However, inhibitor analysis (0.001 mM E-64) indicated that the cysteine peptidase contribution to BzRpNA-hydrolyzing activity at pH 7.5 was quite small and did not exceed 10% in either species. Chymotrypsin- and elastase-like peptidases substrates (SucAAPFpNA and SucAAPLpNA) were most actively hydrolyzed in PB_{PM} conditions, and the activity against SucAAPFpNA was significantly higher than that of SucAAPLpNA. In *T. castaneum*, the activity measured with SucAAPFpNA at PB_{PM} was almost equally distributed between AM and PM, but in *T. confusum* this activity was 1.6 times higher in PM. A comparison of specific activities per gut in both *Tribolium* species with *p*-nitroanilide substrates indicated that all are higher in *T. castaneum* than in *T. confusum*.

A more detailed study of the possible involvement of aspartate peptidases in *Tribolium* larvae digestion was performed at pH 3.0, an optimal pH for aspartate peptidase activity (Rawlings, 1998). The peptidase activity of *T. castaneum* entire midgut

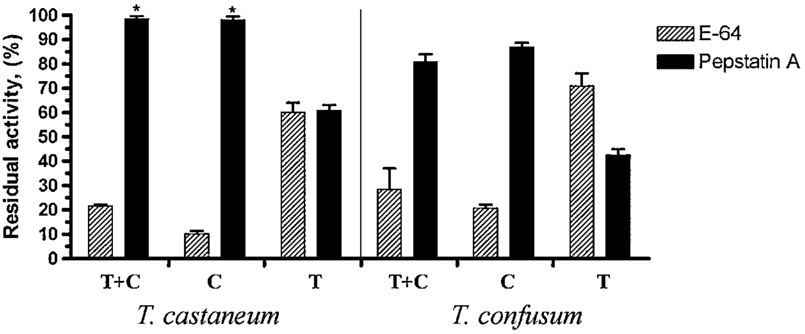

Figure 4. Inhibitor analysis of activity against hemoglobin at pH 3.0 in the entire midgut extracts (T+C), midgut contents (C), and midgut tissue (T) of *T. castaneum* and *T. confusum* with E-64 (0.001 mM) and pepstatin A (0.01 mM). *Enzyme activity is insensitive to the effect of pepstatin ($P > 0.05$).

extract (tissue+contents) and midgut contents only using hemoglobin as a substrate was measured in a pH 3.0 buffer with inhibition by E-64 (0.001 mM) and pepstatin (0.01 mM). This combined analysis revealed a substantial decrease in activity by E-64 (78 and 90%, respectively) and very low pepstatin inhibition (1.5–2%) (insignificant, $P > 0.05$) (Fig. 4). The higher level of inhibition of hemoglobinolytic activity by pepstatin (39%) was found only in the extract of midgut tissue. Thus, a highly active secreted midgut lumen digestive aspartate peptidase, previously described by Blanco-Labra et al. (1996) in *T. castaneum*, was not found. A similar determination was made for *T. confusum* enzymes, where the levels of pepstatin inhibition were also low, 19% for the entire midgut and only 13% for midgut contents. However, pepstatin inhibition here was slightly higher than for *T. castaneum*, coinciding with the data obtained for azocasein hydrolysis (Table 4). Significant inhibition by pepstatin (57%) was found only in the midgut tissue extract of *T. confusum* (Fig. 4). These data demonstrate that the major component of digestive peptidase activity in *T. confusum* at acidic pH belongs to cysteine, but not to aspartate, peptidases, in agreement with our previous data.

Postelectrophoretic Activity With Gelatin

Detailed characteristics of *T. castaneum* and *T. confusum* larval digestive peptidases were obtained by a combination of activity electrophoresis and inhibitor analysis in PB_{AM} and PB_{PM}. The total peptidase activity of midgut extracts was analyzed in gelatin-containing gel-replicas in contact with the strips of a resolving gel previously incubated with class-specific inhibitors (Figs. 5A,B, 6). In the preliminary experiments, gelatinase spectra of separate extracts from midgut contents and total midgut extracts (tissue + contents) were compared in *T. castaneum* and *T. confusum*. The gelatinase patterns for both sources of material assayed in both species at PB_{AM} and PB_{PM} were identical (results not shown), so for the subsequent peptidase spectrum characterizations, the extracts of the midgut with contents (usually divided into equal AM and PM parts) were used. All results on the characteristics of gelatinase fractions and enzymatic fractions hydrolyzing specific *p*-nitroanilide substrates are summarized in Tables 5 and 6.

In *T. castaneum*, gelatinolytic activity in the AM in PB_{AM} was due to at least 11 anionic fractions (fr1 through fr11; Fig. 5A). The highest activity was observed in fr8 and 9. Activity in these fractions was DTT sensitive, but not completely inhibited by E-64, slightly inhibited by serine peptidase inhibitors (PMSF and STI), and completely vanished if the gel was incubated in the mixture of E-64 and STI. Therefore, frs8-9

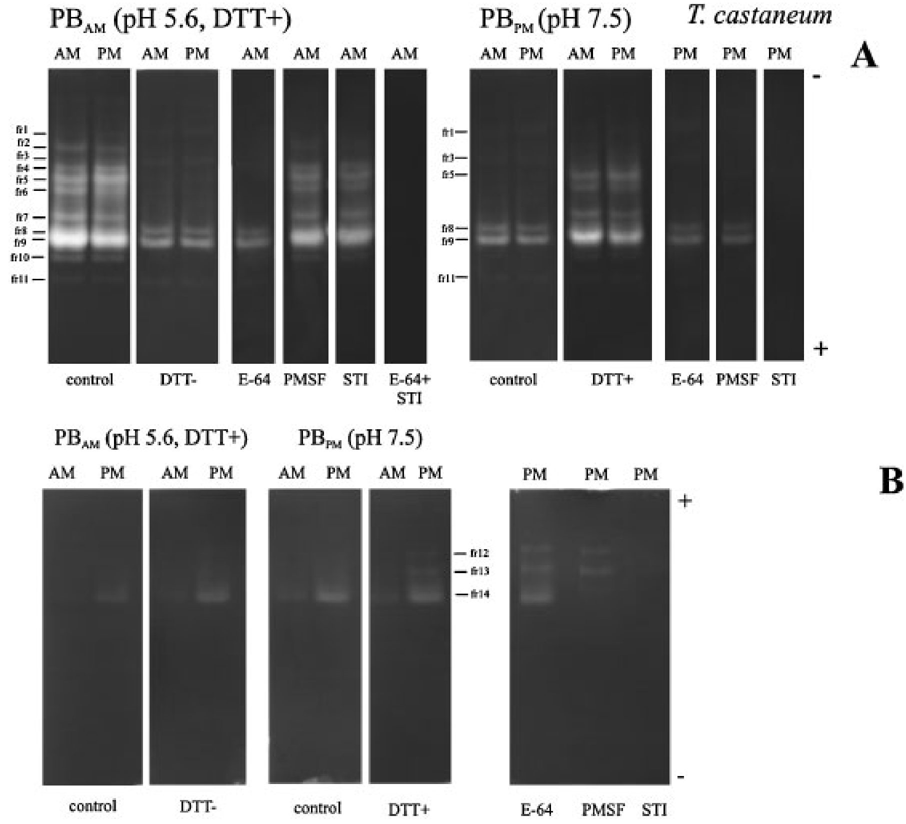

Figure 5. Postelectrophoretic anionic (**A**) and cationic (**B**) gelatinolytic activity of the AM and PM extracts of *T. castaneum* larvae. Activity was detected in PB$_{AM}$ (pH 5.6, DTT+) or PB$_{PM}$ (pH 7.5, without DTT) indicator gel. Each lane was incubated with or without inhibitors or DTT, as indicated.

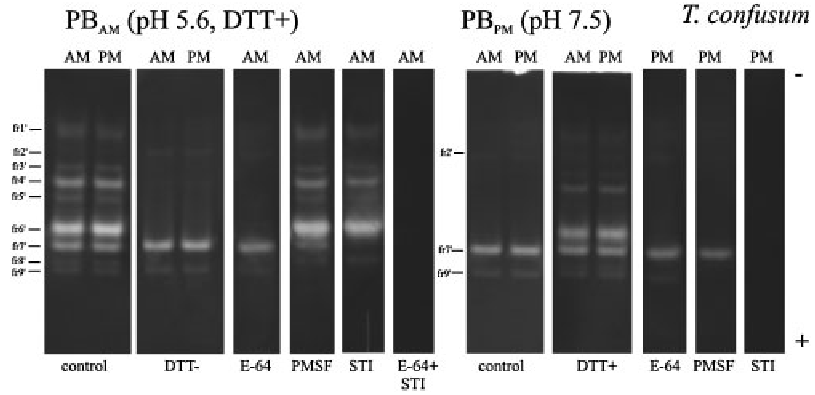

Figure 6. Postelectrophoretic gelatinolytic activity of the AM and PM extracts of *T. confusum* larvae. Activity was detected in PB$_{AM}$ (pH 5.6, DTT+) or PB$_{PM}$ (pH 7.5, without DTT) indicator gel. Each lane was incubated with or without inhibitors or DTT, as indicated.

likely contained comigrating cysteine and serine peptidases. Fraction 2, frs4-7, and fr10 were completely inhibited by E-64, were active only with DTT in the buffer, and were insensitive to PMSF and STI, and so they likely were cysteine peptidases. Minor

Table 5. Characteristics of Proteolytic Activities From the *T. castaneum* Larvae Midgut

Peptidase Type	Substrate activity						pI	Effective inhibitors	Ineffective inhibitors	Localization and relative activity			
	ZFRpNA	GlpFApNA	BzRpNA	SAAPLpNA	SAAPFpNA	Gelatin				AM in PB_{AM}	AM[a] in PB_{PM}	PM in PB_{PM}	PM[a] in PB_{AM}
Cysteine[b]						fr2	anionic	E-64	PMSF, STI	+			+
						fr4	anionic	E-64	PMSF, STI	+			+
	CYSa					fr5	anionic	E-64	PMSF, STI	+			+
	CYSb					fr6	anionic	E-64	PMSF, STI	+			+
	CYSc					fr7	anionic	E-64	PMSF, STI	+			+
	CYSd	CYSd		CYSd		fr8	anionic	E-64	PMSF, STI	++			+
	CYSe	CYSe		CYSe		fr9	anionic	E-64	PMSF, STI	+++			++
	CYSf	CYSf				fr10	anionic	E-64	PMSF, STI	++			+
Trypsin-like	TRYa		TRYa			fr8	anionic	STI	E-64		+	+	
	TRYb		TRYb			fr9	anionic	STI	E-64	+	+++	++	+
			TRYc[b]			fr12[b]	cationic	STI	E-64			+	
			TRYd[b]			fr13[b]	cationic	STI	E-64			+	
			TRYe[b]			fr14[b]	cationic	PMSF, STI	E-64		+	++	
Chymo-trypsin-like				CHYa	CHYa	fr1	anionic	PMSF, STI, TPCK	E-64	++	+++	++++	+++
				CHYb	CHYb	–	anionic	PMSF	E-64, TPCK	+++	++++	+++	++
				CHYc	CHYc	fr11	anionic	PMSF, STI	E-64, TPCK	++	+++	++	+
Elastase-like				ELA		–	anionic	PMSF, STI, TPCK	E-64		++	+	
Serine						fr3	anionic	PMSF, STI	E-64	+	+	+	+

[a] All activities were latent.
[b] All activities were increased by DTT.

Table 6. Characteristics of Proteolytic Activities From the* T. confusum *Larvae Midgut

Peptidase type	Substrate activity						pI	Effective inhibitors	Ineffective inhibitors	Localization and relative activity			
	ZFRpNA	GlpFApNA	BzRpNA	SAAPLpNA	SAAPFpNA	Gelatin				AM in PB$_{AM}$	AM^{a} in PB$_{PM}$	PM in PB$_{PM}$	PM^{a} in PB$_{AM}$
Cysteine[b]						fr1′	anionic	E-64	PMSF, STI	+			+
	CYSa′	CYSa′				fr3′	anionic	E-64	PMSF, STI	+			+
	CYSb′	CYSb′				fr4′	anionic	E-64	PMSF, STI	+			+
						fr5′	anionic	E-64	PMSF, STI	+			+
	CYSc′	CYSc′	CYSc′			fr6′	anionic	E-64	PMSF, STI	+++			++
	CYSd′	CYSd′				fr7′	anionic	E-64	PMSF, STI	++			++
						fr8′	anionic	E-64	PMSF, STI	+			+
Trypsin-like	TRY′		TRY′			fr7′	anionic	PMSF, STI	E-64	+	++	++	+
Chymo-trypsin-like				CHYa′	CHYa′	–	anionic	PMSF, STI, TPCK	E-64	+	++	+++	++
				CHYb′	CHYb′	–	anionic	PMSF, STI, TPCK	E-64	+	++	+++	++
				CHYc′	CHYc′	–	anionic	PMSF, STI, TPCK	E-64	+	+	+	+
					CHYd′	–	anionic	PMSF, STI	E-64, TPCK	+	+	+	+
					CHYe′	-	anionic	PMSF, STI	E-64, TPCK	+	+	+	+
				CHYf′	CHYf′	fr9′	anionic	PMSF, STI	E-64, TPCK	++	+++	++	++
Elastase-like				ELA′			anionic	PMSF, STI, TPCK	E-64	+	+	++	++
Serine						fr2′	anionic	PMSF, STI	E-64	+	+	+	+

[a] All activities were latent.

[b] All activities were increased by DTT.

fractions (fr1, fr3, and fr11) were inhibited by PMSF and STI and were not susceptible to the presence of DTT, characteristic of serine peptidases. Only one very weak cationic peptidase activity was detected in AM extract at PB_{AM}, fr14 (Fig. 5B).

The total proteolytic activity in PM extracts with gelatin at PB_{PM} was due to both anionic (Fig. 5A) and cationic peptidases (Fig. 5B) that were more pronounced than those in the AM at PB_{AM}. Anionic gelatinolytic activity of the PM extract was due to serine peptidases in fr1, 3, 8, 9, and 11 with the same relative mobility and inhibitor sensitivity as fractions from the AM. The minor fractions were completely inhibited by PMSF, whereas fr8 and 9 were inhibited only by STI. The activity of cysteine peptidases was negligible at PB_{PM}, but in the presence of DTT, fr5, 6, 7, and 10 became visible and the activity of fr8 and especially 9 increased. The cationic peptidase activity was higher in the PM at pH 7.5, consisting of 3 fractions (fr12–14) that were slightly activated by DTT. These activities were likely due to serine peptidases, because fr14 was sensitive to PMSF, and all cationic peptidases were completely inhibited by STI (Fig. 5B).

In *T. confusum*, gelatinolytic activity in the AM in PB_{AM} was due to 9 anionic fractions, fr1' through fr9' (Fig. 6). Cationic peptidases were not detected in this species at PB_{AM} and PB_{PM}. At both conditions (PB_{AM} and PB_{PM}), the total peptidase spectrum contained the same fractions in the AM and PM. In the AM at PB_{AM}, fractions 1′, 3′, 4′, 5′, 6′, and 8′ were DTT sensitive, completely inhibited by E-64, and not susceptible to PMSF and STI, characteristic of cysteine peptidases. Serine peptidases also were detected in the AM, since only the addition of inhibitors of both classes (E-64+STI) completely blocked all peptidase activity. These three serine peptidase fractions (fr2′, 7′, and 9′) constituted the total gelatinase activity in PM extracts in PB_{PM}. Their activity was partially susceptible to PMSF and completely inhibited by STI. The activity of cysteine peptidases was negligible at PB_{PM}. However, in the presence of DTT, activities became apparent that correspond to previously described cysteine fractions 4′, 6′, and 8′.

When PM extracts were analyzed in nonphysiological PB_{AM}, "latent" activities of cysteine peptidases were detected in *T. castaneum* (fr4, 5, 6, 7, 8, 9, and 10) and *T. confusum* (fr1′, 3′, 4′ 5′, 6′, and 8′). These activities were slightly lower than in AM and displayed the same inhibitor sensitivities as the cysteine peptidases from AM. In addition, extracts from the AM tested at pH 7.5 in the presence of DTT displayed a lower but detectable level of cysteine peptidase activity.

A postelectrophoresis assay for secreted aspartic (cathepsin D-like) activity in gut contents of either *Tribolium* species did not reveal pepstatin A–sensitive bands in the acidic (pH 3.0) gelatin-containing gel (data not shown).

Postelectrophoretic Activity with p-Nitroanilide Substrates

A more precise identification of specific proteolytic activities in electrophoretic fractions was achieved by the postelectrophoretic detection of activities with specific *p*-nitroanilide substrates combined with inhibitor analysis (Figs. 7A,B, 8A,B). These results were compared with the characteristics of gelatinolytic activities of equal mobility and are summarized in Tables 5 and 6.

In *T. castaneum* AM extract assayed in PB_{AM} (Fig. 7A), a high level of anionic activity was detected with the cysteine peptidase substrate GlpFApNA and a substrate of cysteine and trypsin-like peptidases, ZFRpNA. Activity against ZFRpNA was higher in the AM and was due to the major bands coinciding in mobility with gelatinolytic fr8 through fr10, and minor bands corresponding to fr5 through fr7. As was

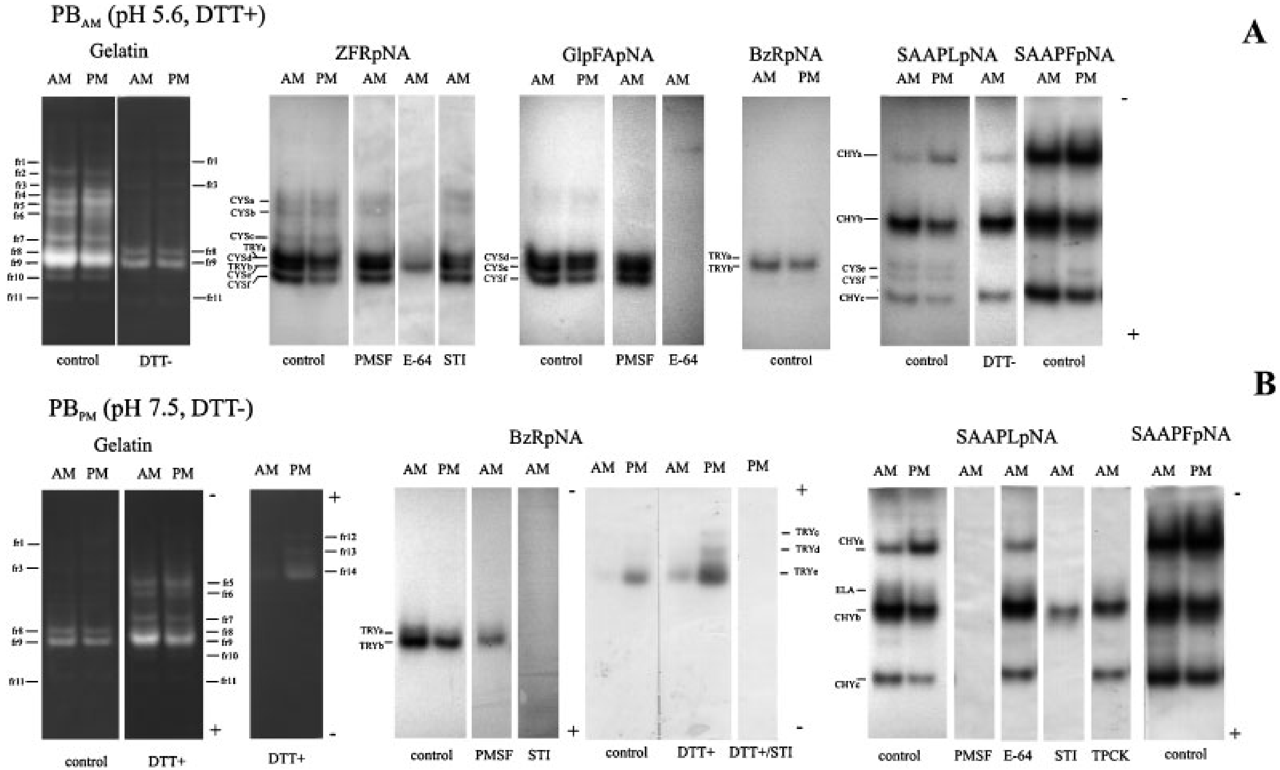

Figure 7. Postelectrophoretic activity with specific *p*-nitroanilide substrates of the AM and PM extracts of *T. castaneum* larvae. Activity was detected at PB$_{AM}$ (**A**) or PB$_{PM}$ (**B**) on nitrocellulose membrane. Each lane was incubated with different substrates and with or without inhibitors and DTT, as indicated.

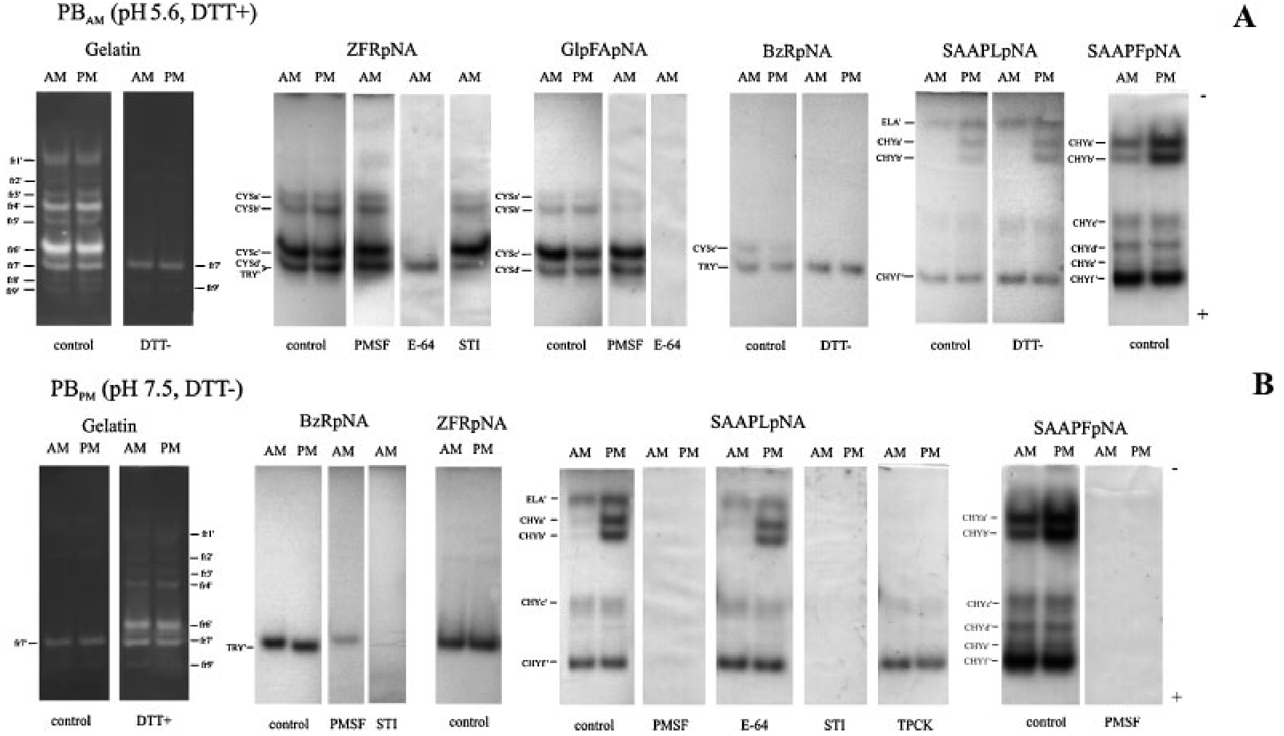

Figure 8. Postelectrophoretic activity with specific *p*-nitroanilide substrates of the AM and PM extracts of *T. confusum* larvae. Activity was detected at PB$_{AM}$ (**A**) or PB$_{PM}$ (**B**) on nitrocellulose membrane. Each lane was incubated with different substrates and with or without inhibitors and DTT, as indicated.

demonstrated previously, cysteine peptidase activity was identified with fr5 (CYSa), fr6 (CYSb), fr7 (CYSc), fr8 (CYSd), fr9 (CYSe), and fr10 (CYSf), which were active with ZFRpNA and were inhibited by E-64 completely or partially (frs8, 9). According to the results of inhibitor analysis (resistance to E-64), fr8 and 9 also contained trypsin-like serine peptidases TRYa and TRYb, respectively, also active with ZFRpNA.

The highly selective but less sensitive cysteine peptidase substrate, GlpFApNA, was hydrolyzed by three major fractions CYSd (fr8), CYSe (fr9), and CYSf (fr10); their activities were completely inhibited by E-64 and were insensitive to PMSF and thus undoubtedly were due to cysteine peptidases. Therefore, these data confirm that frs8 and 9 of the total gelatinase activity include a minimum of two equally migrating cysteine and trypsin-like peptidases (CYSd, TRYa for fr8 and CYSe, TRYb for fr9). The activity of minor low-mobility bands (CYSa, CYSb, and CYSc) was negligible with this substrate (Fig. 7A).

The more selective substrate of trypsin-like peptidases, BzRpNA, was hydrolyzed by two fractions of anionic peptidases in extracts from AM at PB_{AM}, more pronounced at PB_{PM} (Fig. 7A,B). These fractions coincided with TRYa and TRYb, previously identified with ZFRpNA. The activity of cationic enzymes with BzRpNA was negligible in AM extracts at PB_{AM} (data not shown).

The activity of anionic peptidases with a chymotrypsin substrate, SucAAPFpNA, in AM extracts in PB_{AM} was represented by three main fractions (CHYa, CHYb, CHYc) (Fig. 7A). The activities of CHYb and CHYc were higher in the AM while CHYa predominated in the PM. All fractions displayed high activity with SucAAPFpNA, but their activity against gelatin was relatively low. The band CHYa probably corresponded to serine gelatinolytic fr1 and CHYc to serine fr11. All three fractions of SucAAPFpNA-hydrolyzing activity were totally inhibited by PMSF (result not shown) and likely contained chymotrypsin-like peptidases. Activity against SucAAPLpNA, the major substrate for pancreatic elastase II but also hydrolyzed by chymotrypsin, was significantly lower and was mainly represented by the same set of fractions (CHYa, CHYb, CHYc). However, two additional DTT-sensitive bands coinciding in mobility with CYSe and CYSf, previously identified as cysteine peptidases (as there was no activity without DTT), were also detected (Fig. 7A). The activity of cationic peptidases against both substrates at PB_{AM} was not detected.

Further analysis of *T. castaneum* midgut peptidases was performed at physiological conditions of the PM (PB_{PM}). The activity of cysteine peptidases with GlpFApNA in PM extracts assayed at PB_{PM} was absent (data not shown). At PB_{PM}, BzRpNA was hydrolyzed also by two fractions of anionic peptidases, TRYa and TRYb, but the activity was higher in the AM than in the PM (Fig. 7B). All fractions were partially inhibited by PMSF and completely by STI and were identified as trypsin-like. Cationic peptidase activity with BzRpNA contained three DTT sensitive fractions (TRYc, TRYd, TRYe) co-migrating with serine gelatinolytic fr12, 13 and 14. Activities of these enzymes were predominant in the PM and completely inhibited by STI. Thus, trypsin-like activity with BzRpNA in *T. castaneum* larval midgut was due to two anionic fractions dominating in the AM (TRYa and TRYb) and three cationic (TRYc, TRYd, and TRYe) dominating in the PM.

Chymotrypsin-like activity against SucAAPFpNA at PB_{PM} was found in the same set of fractions (CHYa, CHYb, and CHYc) with a similar distribution between the AM and PM, but their activity was higher at PB_{PM} than at PB_{AM} (Fig. 7B). In the assay of activity with SucAAPLpNA, one new activity, presumably elastase-like (ELA), was detected in addition to three chymotrypsin-like fractions (CHYa, CHYb, and CHYc). All these activities were entirely inhibited by PMSF and not susceptible to E-64. CHYa, CHYc, and ELA were entirely inhibited by STI, and CHYa also by TPCK (Fig. 7B). The activity of cationic peptidases against both substrates at PB_{PM} was not detected. Enzymes in fr3, presumably serine peptidases, were not identified with *p*-nitroanilide substrates.

In *T. confusum*, the AM extract assayed in PB_{AM} against GlpFApNA contained a minimum of four fractions: CYSa′, CYSb′, CYSc′, and CYSd′, corresponding to fr3′, 4′, 6′, and 7′ of gelatinase activity, respectively (Fig. 8A). All bands were inhibited completely by E-64, and so gelatinase from fr7′, previously described as serine, also apparently contained cysteine peptidase. Activity against ZFRpNA was represented by the same set of fractions; however, CYSd′ was only partially inhibited by E-64 and also susceptible to STI. Thus, gelatinase from fr7′ contained comigrating cysteine (CYSd′) and trypsin-like (TRY′) peptidase activities. The activity of only one of the cysteine fractions, CYSc′, was higher in AM at PB_{AM} than the corresponding "latent" activity in PM.

Two different peptidases participated in BzRpNA hydrolysis at PB_{AM}. The substrate was weakly hydrolyzed by cysteine fraction CYSc′ (DTT sensitive band) as well as the trypsin-like fraction TRY′ that was slightly activated without DTT (Fig. 8A).

Chymotrypsin-like activity against SucAAPFpNA in AM at PB_{AM} was represented by six fractions CHYa′, CHYb′, CHYc′, CHYd′, CHYe′, and CHYf′. The activities of CHYa′ and CHYb′ were lower in the AM than in the PM, and the activity of CHYf′ was slightly higher in the AM (Fig. 8A). As it was described for *T. castaneum*, the high level of chymotrypsin-like activity was revealed only with *p*-nitroanilide substrates. Only the band CHYf′ corresponded to the minor serine gelatinolytic fr9′. All six fractions of SucAAPFpNA-hydrolyzing activity were totally inhibited by PMSF (result not shown) and likely were chymotrypsin-like peptidases. Activity against SucAAPLpNA was significantly lower than with SucAAPFpNA. Activity in the PM at PB_{AM} was represented by chymotrypsin-like fractions CHYa′, CHYb′, and CHYf′, and one additional slowly migrating presumably elastase-like (ELA′) band, not detected with SucAAPFpNA. Only weak SucAAPLpNA-hydrolyzing activity represented by ELA′ and CHYf′ was found in the AM at physiological PB_{AM}.

In PM extracts of *T. confusum* assayed at physiological PB_{PM}, the activity of cysteine peptidases with GlpFApNA was absent (data not shown). Hydrolysis of ZFRpNA at these conditions was identical to that detected with BzRpNA and was by a single trypsin-like peptidase (TRY′) susceptible to PMSF and STI (Fig. 8B). The same six SucAAPFpNA-hydrolyzing peptidases (CHYa′, CHYb′, CHYc′, CHYd′, CHYe′, and CHYf′) were found in the PM extracts at PB_{PM}, but their activities were higher than at PB_{AM}. The activity spectrum against SucAAPLpNA in the PM included chymotrypsin-like (CHYa′, CHYb′, CHYc′, and CHYf′) and elastase-like (ELA′) activities. Activities of CHYa′ and CHYb′ in the AM at PB_{PM} were negligible. All SucAAPLpNA activities were insensitive to E-64 and totally inhibited by PMSF and STI, while ELA′, CHYa′, CHYb′, and CHYc′ also were TPCK-sensitive.

DISCUSSION

Our investigation of the complex organization of midgut digestive proteolysis in Tenebrionid larvae began with *T. molitor* (Vinokurov et al., 2006a,b) and now is continued with two species from the genus *Tribolium*: the red flour beetle (*T. castaneum*) and confused flour beetle (*T. confusum*). These insects are among the most serious stored-products pests. However, because of the small size of *Tribolium* larvae, there are relatively few studies of their gut biochemistry, and little information is available on the organization of digestive proteolysis and the complement of the midgut digestive peptidases in these insects.

As it was described previously for *T. molitor* (Terra et al., 1985; Vinokurov et al., 2006a), the pH-gradient from the acidic (5.6) to the slightly alkaline (7.5) also was present along the midgut contents of both *Tribolium* larvae, although less pronounced than for *T. molitor*. These data provide a more detailed analysis of the larval midgut pH of *T. castaneum*, as previously the only information was an overall pH of 6.5 for larval gut contents (Oppert et al., 2003) or a range of 7.2–7.6 for that of adults (Sinha, 1959). The earlier data for *T. confusum* were for the midgut pH of adults: 6.8 in the AM and 5.2 in the PM (Sinha, 1959).

The combined data provide strong evidence that the primary digestive peptidases in larvae of both *Tribolium* species are cysteine peptidases. Most of the proteolytic activity was found in the AM where only about 10% of the total proteolytic activity was sensitive to the serine peptidase inhibitor PMSF, and in the PM PMSF-sensitive activity did not exceed 40%. These results differ from data for other species belonging to the family Tenebrionidae. In *T. molitor* larvae, digestive enzyme activity, assayed at the physiological conditions of each midgut section, was represented in the AM by enzymes from both peptidase classes: cysteine (64% E-64-sensitive) and serine (ca. 36% PMSF-sensitive), and in the PM the major proteolysis was due to serine peptidases, as only 20% of caseinolytic activity was E-64-sensitive (Vinokurov et al., 2006a). For another Tenebrionid stored-product pest, the larger black flour beetle (*Cyaneus angustus*), serine peptidases were the predominate peptidases of 9th instar larvae (Oppert et al., 2006). In that insect, total peptidase activity at physiological conditions of the midgut (pH 6.0) was highly susceptible only to serine peptidase inhibitors, PMSF and proteinaceous soybean Kunitz and Bowman-Birk inhibitors, whereas the effect of E-64 was negligible. Thus, it is possible that within the family Tenebrionidae, at least three different digestive proteolysis strategies in the midgut are operative.

Although there was a relatively low level of general proteolytic activity in the alkaline pH found in the PM in *Tribolium* species, the hydrolysis of specific *p*-nitroanilide substrates provided evidence of both trypsin- and chymotrypsin-like (SucAAPFpNA) activities in midgut extracts of *T. castaneum* and *T. confusum*. In the *Tribolium* larval gut, the activities of these enzymes displayed alkaline pH-optima, and were largely reduced by the acidic pH of the AM. Alternatively, in some coleopterans serine peptidases apparently are adapted to slightly acidic (5.5–6.0) pH and display an acidic pH-optimum (Novillo et al., 1997). This also is the first report of digestive elastase-like activity in *Tribolium*.

In this study, the electrophoretic fractionation of the total peptidase preparations of *Tribolium* beetles was performed in conditions with minimal denaturing influence (neutral pH, absence of SDS, low temperature). Previously, the use of a standard SDS-PAGE method combined with postelectrophoretic activity detection resulted in the loss of peptidase activity, especially that of cysteine peptidases (Liang et al., 1991; Oppert et al., 2005), probably due to enzyme inactivation in the presence of SDS and/or alkaline pH. A native electrophoresis system at neutral pH with bidirectional electrophoresis (toward the anode for anionic proteins with acidic pI, and toward the cathode for cationic proteins with basic pI) used in combination with a thorough inhibitor analysis provided the high-resolution screening capability of *T. castaneum* and *T. confusum* digestive peptidases found in this study.

The major cysteine digestive peptidases of *T. castaneum* were represented by eight anionic fractions, with three major activities among them, CYSd, CYSe, and CYSf. They were maximal in the physiological conditions approximating the AM and were located mainly in the AM. Four main types of serine peptidase activity were found in *T. castaneum*: trypsin-like, chymotrypsin-like, elastase-like, and an unidentified serine peptidase fraction. Trypsin-like peptidases were represented by two anionic fractions, TRYa and TRYb, located mainly in the AM when measured at alkaline pH, but at physiological conditions their activity was higher in the PM. The PM also contained three cationic trypsin-like fractions, TRYc, TRYd, and TRYe, the former two being minor. Chymotrypsin-like peptidases were represented by three anionic fractions. At slightly alkaline pH 7.5, the fraction with the highest activity, CHYb, and fraction CHYc were located mainly in AM, but displayed almost equal activity in AM and PM at their physiological conditions. CHYa displayed higher activity in the PM. The elastase-like activity was located mainly in the AM, and uncharacterized serine peptidase fr3 was equally distributed along the midgut. The preferential location of several serine peptidases in the AM was observed also for *T. molitor* (Vinokurov et al., 2006a) and might probably be due to their secretion in this part of the gut with subsequent transport with the food bolus to the PM, where their activity substantially increased.

The set of midgut digestive peptidases in *T. confusum* contained only anionic enzymes. Cysteine peptidases were represented by seven fractions with two major activities, CYSc′ and CYSd′. They were located mainly in the AM. Serine peptidases were presented by one trypsin-like fraction, six chymotrypsin-like fractions with the major CHYf′, one elastase-like fraction, and one unidentified serine peptidase fraction. The majority of serine peptidase activities was almost equally located in both midgut parts, while activities of two chymotrypsin-like fractions, CHYa′ and CHYb′, and the elastase-like fraction were mostly in the PM. Another chymotrypsin-like fraction, CHYf′, displayed higher activity in the AM only at nonphysiological pH 7.5.

Although the organization of protein digestion in *T. castaneum* and *T. confusum* was similar, the total azocaseinase activity and activities with specific substrates were greater with enzymes from the *T. castaneum* midgut than those from *T. confusum* when calculated per gut. This difference may facilitate the slightly faster developmental time for *T. castaneum* (ARS Agriculture Handbook Number 500, 1986). Therefore, when all conditions are equal, *T. castaneum* may probably have a slight advantage, although geographical distribution suggests that each is thermally adapted to its environment. Certainly, direct bioassays are needed to test this hypothesis.

Since previously a digestive cathepsin D-like aspartate peptidase was isolated from the *T. castaneum* larval midgut (Blanco-Labra et al., 1996), we investigated more precisely the location of an aspartate peptidase in the *T. castaneum* and *T. confusum* midgut. Although the hemoglobinolytic activity at pH 3.0 was severely reduced by E-64, significant inhibition by pepstatin was found only in midgut tissue extracts (up to 57% in *T. confusum*) and was due to an intracellular aspartate peptidase. These data are confirmed by our observations of the *T. castaneum* gut transcriptome and proteome, where cathepsin L and B, trypsin, and chymotrypsin are the predominant gene transcripts and proteins, respectively (Oppert and Elpidina, 2008). The participation of aspartate peptidases in *T. molitor* digestion also is unlikely (Terra and Christofoletti, 1996; Vinokurov et al., 2006a). In the previously cited work on *C. angustus* (Oppert et al., 2006), some pepstatin-sensitive proteolytic activity also was detected in the total midgut extracts, but the possibility that this activity was due to intracellular enzymes remains. Digestive cysteine (cathepsin L, B- and H-like) peptidases are widely distributed among most coleopteran families, belonging to the series Cucujiformia, excluding only wood-feeding larvae of Cerambycidae (Johnson and Rabosky, 2000). However, digestive cathepsin D-like peptidases were found only in Chrysomelidae, Bruchidae, and Curculionidae (Lemos et al., 1990; Silva and Xavier-Filho, 1991; Novillo et al., 1997; Wilhite et al., 2000; Hernández et al., 2003).

Thus, in both *Tribolium* species studied at normal dietary conditions, the midgut digestive proteolytic complex includes a set of digestive enzymes represented by cysteine and serine peptidases. According to the spatial location of peptidases and their different activity with the proteinaceous substrates casein and gelatin, we propose that cysteine peptidases perform the initial unspecific breakdown of food proteins, whereas serine peptidases act preferentially on the partially digested products in the more alkaline (pH 7.5) contents of the PM. Previously, when larvae of *T. castaneum* were fed a cysteine protease inhibitor, they were able to shift their proteolytic enzyme profile from cysteine to serine to compensate for the decrease of digestion efficiency (Oppert et al., 2005). From this point of view, the study of possible mechanisms of adaptation to the cysteine proteinase inhibitors in *T. castaneum* related to the complete shift of the enzyme profile from cysteine to serine peptidases is of great interest as a model in understanding of the adaptive transformations occurring in the insect gut. Such a dramatic change requires a significant remodeling of midgut digestive physiology, including the changes in peptidase spectra and related changes of midgut lumen physico-chemical conditions for proper functioning of newly secreted peptidases.

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