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Diversity of digestive proteinases in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae

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Abstract

The spectrum of *Tenebrio molitor* larval digestive proteinases was studied in the context of the spatial organization of protein digestion in the midgut. The pH of midgut contents increased from 5.2–5.6 to 7.8–8.2 from the anterior to the posterior. This pH gradient was reflected in the pH optima of the total proteolytic activity, 5.2 in the anterior and 9.0 in the posterior midgut. When measured at the pH and reducing conditions characteristic of each midgut section, 64% of the total proteolytic activity was in the anterior and 36% in the posterior midgut. In the anterior midgut, two-thirds of the total activity was due to cysteine proteinases, whereas the rest was from serine proteinases. In contrast, most (76%) of the proteolytic activity in the posterior midgut was from serine proteinases. Cysteine proteinases from the anterior were represented by a group of anionic fractions with similar electrophoretic mobility. Trypsin-like activity was predominant in the posterior midgut and was due to one cationic and three anionic proteinases. Chymotrypsin-like proteinases also were prominent in the posterior midgut and consisted of one cationic and four anionic proteinases, four with an extended binding site. Latent proteinase activity was detected in each midgut section. These data support a complex system of protein digestion, and the correlation of proteinase activity and pH indicates a physiological mechanism of enzyme regulation in the gut.
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Keywords: *Tenebrio molitor*; Yellow mealworm; Organization of digestion; Insect digestive proteinases

1. Introduction

Insects generally have a wide spectrum of digestive proteinases that are spatially and temporally expressed in the midgut (Terra and Ferreira, 1994; Terra et al., 1996; Reeck et al., 1999). Knowledge of the relative composition, arrangement and functioning of all component proteinases is essential for the effective design of insect control products based on biological agents, such as proteinase inhibitors (Jongsma et al., 1996; Hilder and Boulter, 1999) and *Bacillus thuringiensis* toxins (Oppert, 1999). The data from genomic studies indicate that multiple gene families of proteolytic enzymes are found in insects (Elvin et al., 1993, 1994; Müller et al., 1993; Casu et al., 1994; Bown et al., 1997), yet there are few biochemical studies devoted to the spectra of different groups of digestive proteolytic enzymes secreted and simultaneously operating in the insect midgut.

Studies of a stored product pest, the yellow mealworm, *Tenebrio molitor* L (Coleoptera: Tenebrionidae), are among the first on digestive proteinases in an insect (for a review see

Abbreviations: AM, anterior midgut; BApNA, N_α-benzoyl-DL-arginine *p*-nitroanilide; DTT, dithiothreitol; DMF, dimethyl formamide; E-64, L-*trans*-epoxysuccinyl-L-leucylamido(4-guanidino) butane; EDTA, ethylene diamine tetraacetate; GlpAALpNA, pyroglutamyl-alanyl-alanyl-leucine *p*-nitroanilide; GlpFpNA, pyroglutamyl-phenylalanine *p*-nitroanilide; GlpFApNA, pyroglutamyl-phenylalanyl-alanine *p*-nitroanilide; 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); PM, posterior midgut; PMSF, phenylmethylsulphonyl fluoride; STI, soybean Kunitz trypsin inhibitor; TCA, trichloroacetic acid; TLCK, N_α-tosyl-L-lysine chloromethyl ketone; TPCK, tosyl-L-phenylalanine chloromethyl ketone; U, unit of activity; UB, universal buffer.

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Applebaum, 1985). Nevertheless, these data are fragmentary and incomplete as to the complexity of the spectrum of midgut proteinases in this insect. Most of the studies were devoted to the characteristics and midgut localization of specific larval enzymes: trypsin (Applebaum et al., 1964; Levinsky et al., 1977; Terra et al., 1985; Ferreira et al., 1990; Thie and Houseman, 1990; Cristofoletti et al., 2001; Tsybina et al., 2005) and, to a smaller extent, chymotrypsin and cysteine proteinases (Applebaum, 1985; Terra and Cristofoletti, 1996; Cristofoletti et al., 2005; Elpidina et al., 2005). In addition to larval enzymes, digestive serine "α- and β-proteases" and trypsin and chymotrypsin from the imago have been described (Zwilling, 1968; Zwilling et al., 1972; Applebaum, 1985).

To provide a more complete understanding of the complexity of digestion in *T. molitor* larvae, this study examined the spectra of serine (trypsin and chymotrypsin) and cysteine digestive proteinases with respect to the spatial organization of midgut and pH by a combination of general proteinaceous and specific *p*-nitroanilide substrates with inhibitor analyses. A post-electrophoretic method of proteinase analysis (Vinokurov et al., 2005) was used to assign each *T. molitor* midgut digestive proteinase activity to a specific proteinase class. The data provide a more complete representation of the digestive proteinases of *T. molitor* larvae that are fed oat flakes as their primary diet.

2. Materials and methods

2.1. Chemicals

Azocasein, bovine hemoglobin, gelatin, phenylmethylsulfonyl fluoride (PMSF), L-*trans*-epoxysuccinyl-L-leucylamido (4-guanidino) butane (E-64) and pepstatin A were purchased from Sigma-Aldrich (St. Louis, MO, USA); soybean Kunitz trypsin inhibitor (STI) and ethylenediamine tetraacetic acid (EDTA) were from Reanal (Budapest, Hungary); N_{α}-benzoyl-D, L-arginine *p*-nitroanilide (BApNA), dithiothreitol (DTT), tosyl-L-phenylalanine chloromethyl ketone (TPCK) and N_{α}-tosyl-L-lysine chloromethyl ketone (TLCK) were from Fluka (Buchs, Switzerland). Leupeptin (Ac-LLR-CHO), cathepsin B inhibitor II (Ac-LVK-CHO), cathepsin L inhibitor I (Z-FF-CH_2F) and cathepsin L inhibitor II (Z-FY-CHO) were from Calbiochem (San Diego, CA, USA). Fluorescently labeled casein (BODIPY-TR-X casein) was from Molecular Probes (Eugene, OR, USA). Other *p*-nitroanilide substrates included pyroglutamyl-phenylalanyl-alanine *p*-nitroanilide (GlpFApNA), pyroglutamyl-alanyl-alanyl-leucine *p*-nitroanilide (GlpAALpNA), and pyroglutamyl-phenylalanine *p*-nitroanilide (GlpFpNA), synthesized at the Department of Chemistry of Natural Compounds, Chemical Faculty, Moscow State University (Moscow, Russia). Nitrocellulose membrane sheets with 0.45 μm pore size were from Amersham (Austria).

2.2. Insects

A stock culture of *T. molitor* was maintained on a mixture (1:1) of milled oat flakes (Raisio, Finland) and wheat bran at 25 °C. Actively feeding fourth instar *T. molitor* larvae were used in the experiments. Approximately 1–1.5 wk prior to dissection, larvae were transferred to milled oat flakes that were processed at high temperature and were devoid of active proteases and proteinase inhibitors (data not shown).

2.3. pH of midgut contents

For pH determination, midgut contents within the peritrophic matrix of 10 larvae were isolated and divided into three parts (anterior, middle, posterior), and the contents were extruded onto a paraffin plate. The pH in the drop of contents was measured immediately with a contact pH electrode (Pharmacia-LKB, Sweden) using a Radiometer pHM84 pH-meter (Denmark).

2.4. Preparation of enzyme extract

Larvae were immobilized on ice, the posterior and anterior tips of the larvae were removed in 0.15 M NaCl, and the gut was removed from one end. The gut was washed in precooled distilled water and was divided into two equal parts, anterior midgut (AM) and posterior midgut (PM), and each was homogenized in MilliQ (Millipore, France) water in a glass Downce homogenizer (50 AM or PM parts in 350 μl of water). The homogenate was centrifuged for 5 min at 10,000 ×*g*. The resulting supernatant was stored at −70 °C until use.

2.5. 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 (0.15 gut equivalents) were diluted to 100 μL with 100 mM acetate-phosphate-borate universal buffer (UB; Frugoni, 1957), pH 5.6 or 7.9, and were incubated with 200 μL of 1% azocasein solution in the same buffer for 30 min at 37 °C. The enzyme reaction was terminated by the addition of 300 μL of 12% trichloroacetic acid (TCA). The mixture was incubated for 30 min at 4 °C for precipitate formation and centrifuged for 5 min at 10,000 ×*g*. An equal volume of 500 mM NaOH was added to the supernatant, and the absorbance was measured at 440 nm with a Spectronic UV/VIS (Thermo Electron Corp., Rochester, NY) spectrophotometer. For estimation of the cysteine proteinase contribution to the total proteolytic activity, dithiothreitol (DTT) was added to the reaction mixture to 1 mM final concentration. At acidic pH (3.0), the proteolytic activity was measured with 1% bovine hemoglobin as a substrate in 100 mM UB, according to Houseman and Downe (1983).

The optimum pH of the total proteolytic activity was determined by a microplate assay with fluorescently labeled casein, BODIPY-TR-X (Invitrogen, Carlsbad, CA; USA, Oppert et al., 1997), which, unlike azocasein, was soluble throughout the entire pH range studied. The substrate was diluted per the manufacturer's recommendation, and 10 μl

(0.1 μg) was added to each well containing the extract of AM (0.18 gut equivalents) or PM (0.30 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 37 °C, the fluorescence was measured (excitation 584 nm, emission 620 nm) with a Fluoroskan Ascent FL microplate reader (Labsystems, Thermo Electron Corp., Milford, MA, USA).

Proteolytic activity was assessed with 0.5 mM synthetic substrates, including GlpFApNA, specific for cysteine proteinases (Stepanov et al., 1985), BApNA, specific for trypsin-like and some cysteine proteinases (Erlanger et al., 1961), and GlpFpNA and GlpAALpNA, specific for chymotrypsin-like proteinases (Lyublinskaya et al., 1987), measured spectrophotometrically at 410 nm by *p*-nitroaniline release. One hundred μL of diluted enzyme preparation was mixed with 680 μL of 100 mM UB of appropriate pH and incubated with 20 μL of 20 mM substrate solution in dimethylformamide (DMF) for 0.5–1 h at 37 °C. The enzyme concentration was chosen so that the absorbance at 410 nm was in the interval of 0.3–0.55 optical U. The reaction was stopped by the addition of 100 μL of a 30% solution of cold acetic acid. Assays for sulfhydryl (SH)-dependent activity with GlpFApNA and BApNA were performed with 1 mM DTT in the final reaction mixture. All assays were adjusted so that the proteolytic activity was proportional to protein concentration and to time. One unit (U) of proteolytic activity was defined as the amount of enzyme (mg) that caused an increase in optical density by 0.1 per min in 1 mL of the reaction mixture. Determinations of enzyme activity were made in 3 to 5 replicates.

Protein concentration was determined according to Lowry et al. (1951) and spectrophotometrically at 280 nm.

2.6. 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 UB, and residual activity with appropriate *p*-nitroanilide substrate was assayed as previously described. Diagnostic inhibitors and their specificity included: PMSF (serine proteinases) and pepstatin A (aspartic proteinases) at 0.01, 0.1, and 1 mM; E-64 (mostly cysteine proteinases) at 0.001, 0.01, 0.1, and 1 mM; leupeptin (cysteine and some serine proteinases), cathepsin B inhibitor II (cathepsin B), cathepsin L inhibitor I and II (cathepsin L) at 0.001, 0.01, and 0.1 mM; and EDTA (metalloproteases) at 0.1, 1 and 10 mM final concentrations.

2.7. Native PAGE

Native PAGE was performed in 1-mm thick 12% polyacrylamide gels with 35 mM HEPES and 43 mM imidazole buffer, pH 7.2, according to McLellan (1982) at 75 V constant voltage and 4 °C for 2 h. The electrophoresis was performed in two directions: toward the anode for proteins with acidic p*I* (<7.2 pH units), and toward the cathode for proteins with basic p*I* (>7.2 pH units). Each 4 mm^2 well was loaded with 0.28 gut equivalents of either AM or PM preparations.

2.8. Postelectrophoretic activity detection and inhibition

Detection of proteinase activity in electropherograms was performed by two different methods. In the first approach, the total proteolytic activity was detected by means of hydrolysis of general proteinase substrate, gelatin (0.025%), incorporated into an 8% polyacrylamide indicator gel polymerized in 100 mM UB, pH 5.6 or 7.9. The acid indicator gel was polymerized with increased concentrations of polymerization reagents (TEMED and ammonium persulfate) according to Díaz-López et al. (1998). After polymerization, the indicator gel was placed into an appropriate UB solution for 40 min. When testing SH-dependent proteinase 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 with pH 5.6 or 7.9 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 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 and 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 BApNA, GlpFApNA, GlpAALpNA, and GlpFpNA by an overlay on the native polyacrylamide gel of a nitrocellulose membrane impregnated with substrate (Vinokurov et al., 2005). After electrophoresis, the resolving gel was washed for 15 min in 100 mM UB, pH 5.6 or 7.9. The buffer was removed, and a nitrocellulose membrane, presoaked for 40 min in 1 mM solution of substrate in 100 mM UB, pH 5.6 or 7.9, 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 30 to 60 min, until faint yellow bands became visible on the membrane. The gel was removed, and liberated *p*-nitroanilide 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 Rf calculated for each fraction.

Inhibition studies of electrophoretically-separated proteinases 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 proteinase inhibitors: PMSF (5 mM), TLCK (0.3 mM), TPCK (0.3 mM), STI (0.02 mM), E-64 (0.05 mM), pepstatin A (0.025 mM), and EDTA (10 mM) for 20 min at 25 °C. After incubation, the gels were washed in 100 mM UB of appropriate pH (5.6 or 7.9). Proteolytic activity was detected by one of the earlier

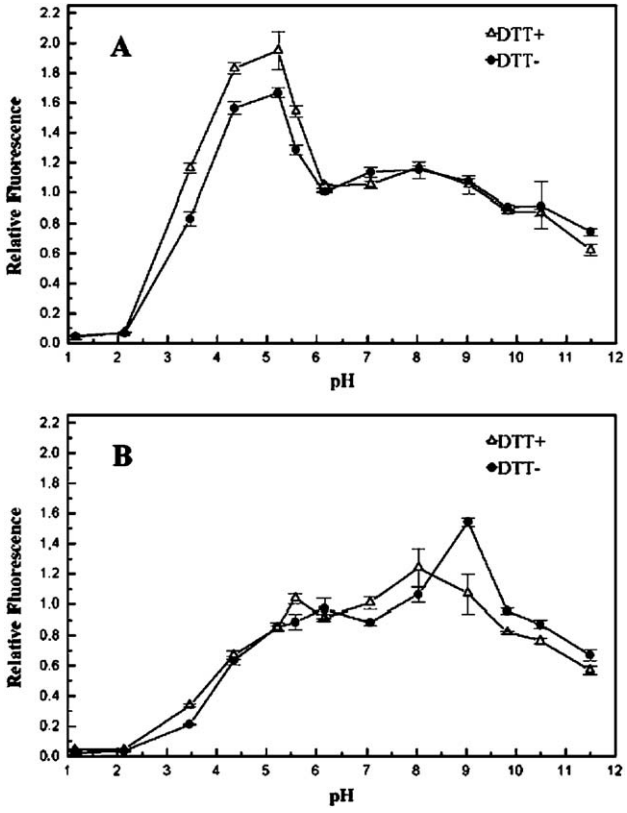

Fig. 1. Effects of pH on the activity of extracts from *T. molitor* larval AM (A) and PM (B) assayed with BODIPY-TR-X casein (mean±S. E.) in the presence and absence of 1 mM DTT.

described methods and compared with the control without inhibitor.

3. Results

3.1. Midgut pH

To obtain a more detailed analysis of physiological gut pH, the midgut of *T. molitor* larvae was divided into three equal parts. The average pH of the anterior third of midgut contents varied in the range 5.2–5.6. In the middle midgut, the pH was slightly increased, ranging from 5.9–6.5. The contents of the posterior third of the midgut were alkaline and displayed average pH from 7.8 to 8.2.

3.2. Effect of pH on the total proteolytic activity

The effect of pH on the total proteolytic activity of AM and PM extracts from *T. molitor* larvae was measured with a general proteinase substrate (Fig. 1). Proteolytic activity of the AM was maximal in the acid region, with a peak at pH 5.2, and a flat shoulder in the neutral and alkaline regions, with a slight increase at pH 8.0 (Fig. 1A). Activity in the PM was maximal in the alkaline region, with a peak at pH 9.0, and a flat shoulder in the neutral and acidic regions, with a small increase at pH 5.5–6.0 (Fig. 1B). In the presence of 1 mM DTT, activity increased only in the acidic region (3.5–5.5). Addition of a reducing reagent to buffers induced a shift of maximal activity in PM extracts from 9.0 to 8.0 and decreased the maximum activity by 25%.

3.3. Localization of proteolytic activities in T. molitor larvae midgut

The distributions of the total proteolytic activity (with azocasein as a substrate) in extracts from the AM and PM were compared in buffers at approximate physiological pH, 5.6 for the AM and 7.9 for the PM (Table 1). Substantial activation of proteolysis in the presence of 1 mM DTT, a characteristic of cysteine proteinases, was observed only in AM extracts in pH 5.6 buffer. If the AM extract was dialyzed in 10 mM phosphate buffer, pH 7.0, the total activity at pH 5.6 sharply decreased due to the removal of reducing agents, but the activity increased 2-fold after the addition of 1 mM DTT. In the dialyzed PM extract assayed at pH 7.9, addition of DTT resulted in a 20% decrease of activity. Therefore, we used pH 5.6 buffer containing a reducing compound (DTT) to approximate the AM physiological conditions (PB_{AM}), whereas the PM physiological conditions were approximated with a nonreducing buffer of pH 7.9 (PB_{PM}). Of the total proteolytic activity measured in each midgut extract in their respective physiological buffers, 64% was located in the AM, and 36% was found in the PM.

Table 1
Comparison of proteolytic activities with general protein and specific synthetic substrates in *T. molitor* larval AM and PM extracts at pH 5.6 and 7.9 (mean±S.E)

Substrate	Activity (U/gut)			
	pH 5.6		pH 7.9	
	AM	PM	AM	PM
Azocasein	0.316±0.033	0.191±0.024	0.15±0.011	0.213±0.031
Azocasein[a]	0.370±0.021	0.210±0.019	0.15±0.025[b]	0.209±0.04[b]
GlpFApNA	0.488±0.01	0.207±0.003	0.213±0.011	0.221±0.004
GlpFApNA[a]	1.30±0.04	0.584±0.023	0.474±0.005	0.314±0.005
BApNA	0.0323±0.0045	0.0617±0.0076	0.183±0.045	0.667±0.021
BApNA[a]	0.0519±0.007	0.07±0.001	0.186±0.005[b]	0.669±0.011[b]
GlpFpNA	0.0142±0.002	0.102±0.002	0.16±0.005	0.53±0.065
GlpAALpNA	0.245±0.007	0.235±0.0042	0.827±0.019	0.977±0.022

[a] Activity was detected in the presence of 1 mM DTT.
[b] Enzyme activity was insensitive to DTT (according to the Student's *t* test $P<0.05$).

Table 2
Effect of class-specific inhibitors on the total proteolytic activity with azocasein of *T. molitor* larval AM and PM extracts at physiological pH

Inhibitor	Inhibitor concentration (mM)	Residual activity (% of control)	
		AM (pH 5.6)	PM (pH 7.9)
E-64	0.01	37.6	80.1
	0.1	34.3	80.7
	1	35.8	72.3
PMSF	0.01	85.1	62.5
	0.1	77.3	42.2
	1	64.2	23.6
EDTA	0.1	100.3	86.3
	1	100.3	83.7
	10	90.4	77.5
Pepstatin A	0.01	104.5	93.8
	0.1	101.9	94.1
	1	100.0	91.2

The contribution of proteinases from different classes in the AM and PM to the total azocaseinolytic activity measured at the respective physiological pH was assessed by an inhibitor analysis (Table 2). In the AM extracts at pH 5.6, the addition of an inhibitor of cysteine proteinases, E-64, resulted in 64% reduction in activity, whereas an inhibitor of serine proteinases, PMSF, suppressed 36% of activity. PMSF inhibited 76% of caseinolytic activity in PM extracts at pH 7.9, whereas E-64 and EDTA only inhibited 20 to 27% of the activity, indicating that serine proteinases are likely responsible for the majority of the activity in the PM. An inhibitor of aspartic proteinases, pepstatin, had no effect on activity from either AM or PM extracts. To further confirm the absence of aspartic proteinases sensitive to pepstatin at acidic pH, the inhibitor analysis was performed at pH 3.0 with hemoglobin as a substrate. Pepstatin at 0.1 mM concentration was ineffective against hemoglobinolytic activity from extracts of the isolated AM and PM contents.

To compare the location and relative activities of different types of enzymes in the AM and PM, extracts were tested with specific *p*-nitroanilide substrates at two different pH values (Table 1). The hydrolysis of GlpFApNA, a specific substrate for cysteine proteinases, was maximal in pH 5.6 buffers, increased after the addition of DTT, and was found primarily in AM extracts. Several general and specific inhibitors of cysteine proteinases were tested against this activity at different concentrations (Fig. 2). At the lowest concentration (1 μM), GlpFApNA hydrolysis was completely inhibited by E-64. Leupeptin, an inhibitor of cysteine and serine proteinases, was not as effective, resulting in 67% inhibition. The most potent among specific cysteine proteinase inhibitors was cathepsin B inhibitor II, resulting in 71% inhibition. Cathepsin L inhibitors I and II were less effective at the lowest concentration, only reducing the activity by 12 and 33%, respectively. At the highest concentration (100 μM), all inhibitors reduced the activity more than 95%, with the exception of cathepsin L inhibitor I.

Hydrolysis of BApNA was maximal in pH 7.9 buffers, was not increased by the addition of DTT, and so at alkaline pH was due primarily to trypsin-like enzymes (Table 1). The majority of this activity was located in the PM extracts of the *T. molitor* larvae midgut. In pH 5.6 buffer, BApNA hydrolysis was decreased and was due to trypsin and also cysteine proteinases because of an increased activity in reducing buffers (see Section 3.5). Hydrolyses of chymotrypsin-like proteinases with GlpFpNA and GlpAALpNA were greater at pH 7.9 and mainly were located in PM extracts, 97% and 80%, respectively. The activity of PM extracts with GlpAALpNA was about 2-fold more than that of GlpFpNA.

3.4. Postelectrophoretic activity of the total enzyme preparations from AM and PM with gelatin

Detailed characteristics of *T. molitor* larvae digestive proteinases were obtained by a combination of activity electrophoresis and inhibitor analysis in PB_{AM} and PB_{PM}. To evaluate the effect of inhibitors on the total proteolytic activity, AM and PM extracts were subjected to electrophoresis in gelatin-containing gels and incubated with class-specific inhibitors (Fig. 3). In the AM in PB_{AM}, gelatinolytic activity was due to at least 9 anionic fractions (fr1 through fr9; Fig. 3A). The highest activity was observed in fractions with close relative mobilities in the middle of the gel, fr4, 5, and 6. These fractions, together with minor activities from fr2, 3 and 7, were completely inhibited by E-64, were only present with DTT in the buffer, and were insensitive to PMSF, STI, EDTA, and pepstatin. Therefore, proteinases in fr2 through fr7 likely were cysteine proteinases. Other proteinase activities, fr1 and fr8 and 9, were inhibited to various degrees only by inhibitors of serine proteinases. The relatively strong gelatinase in fr1 was inhibited only by STI. Minor fractions (fr8 and 9) were inhibited by PMSF and slightly by STI, and were more active in the absence of DTT, characteristic of serine proteinases. Therefore, activities from fr1 and fr8 and 9 were classified as serine proteinases. Cationic proteinases in AM extracts were not detected in PB_{AM} (data not shown).

The total proteolytic activity in PM extracts with gelatin in PB_{PM} was due to both anionic and cationic proteinases (Fig. 3B and C). Anionic gelatinolytic activity of the PM extract,

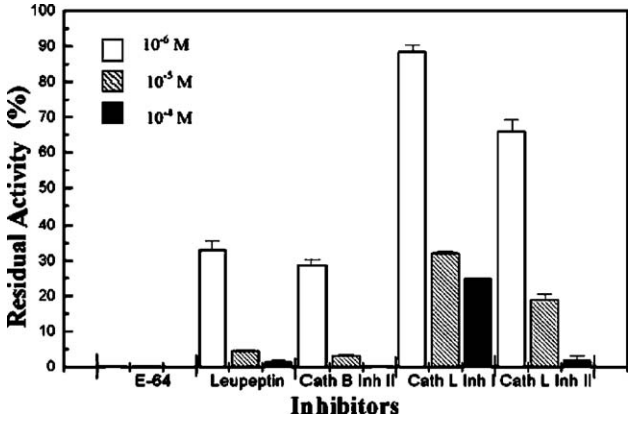

Fig. 2. Effects of different cysteine proteinase inhibitors on the proteolytic activity of the AM extract from *T. molitor* larvae with 0.5 mM GlpFApNA, assayed in 100 mM UB, pH 5.6, and 1 mM DTT (PB_{AM}).

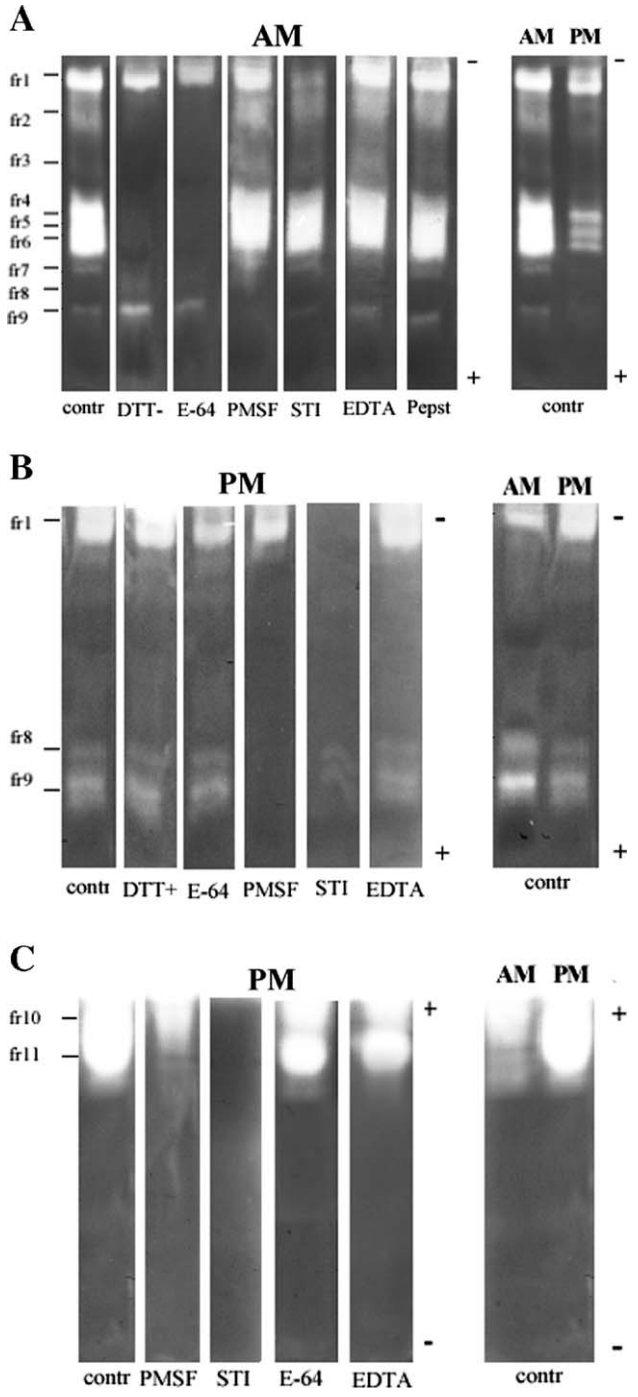

Fig. 3. Postelectrophoretic gelatinolytic activity of the AM and PM extracts of *T. molitor* larvae. Anionic activity was detected in PB_{AM} (A) or PB_{PM} (B) indicator gel, and cationic activity was detected in a PB_{PM} indicator gel (C). Each lane was incubated with or without inhibitors or DTT, as indicated.

equivalent to that of the AM extract, was due to serine proteinases in fr1, 8, and 9 with the same relative mobility and inhibitor sensitivity as fractions from the AM (Fig. 3B). The activity of cysteine proteinases, represented by fr2 through fr7 in the AM extracts, was negligible in the PM. The cationic gelatinolytic activity of PM extracts was represented by two slowly migrating fractions, fr10 and 11 (Fig. 3C). The cationic PM activity likely was due to serine proteinases, in as much as it was highly susceptible to PMSF, completely inhibited by STI, insensitive to E-64, and slightly inhibited by EDTA. The activities of cationic fr10 and 11 and anionic fr1 were predominant in the entire spectrum of proteinases from the PM. Metalloproteinases, highly susceptible to chelating agents, were not detected in either midgut extract.

When the PM extract was analyzed in PB_{AM} (Fig. 3A), "latent" activity (i.e., the activity that was evident under nonphysiological conditions) of cysteine proteinases in fr2 and fr4 through fr6 was detected. The activity was much less than in the AM and displayed the same inhibitor sensitivity as cysteine proteinases from the AM (data not shown). Examination of proteolytic activity from the AM in PB_{PM} also revealed latent cationic fractions of serine proteinases (Fig. 3C). The activity of anionic serine proteinases in fr8 and 9 was greater in PB_{PM} (Fig. 3B) than in PB_{AM} (Fig. 3A).

3.5. Postelectrophoretic activity testing with specific 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 (Fig. 4). These results were compared with the characteristics of gelatinolytic activities of equal mobility, as summarized in Table 3. AM extracts assayed in PB_{AM} displayed the highest level of anionic activity with the cysteine proteinase substrate GlpFApNA (Fig. 4A). The activity was due to a large zone, CYSc, coinciding in mobility with cysteine gelatinolytic fr4 through fr6, and two minor activities, CYSb and CYSd, corresponding to gelatinolytic fr3 and fr7, respectively (Fig. 3A). All were previously identified as cysteine proteinases, and this identification was corroborated by the data in this experiment, in that they hydrolyzed GlpFApNA, were inhibited completely by E-64 and insensitive to PMSF, and were active only in DTT buffers (data not shown). Activity with GlpFApNA, previously identified as cysteine proteinase gelatinolytic fr2, was negligible in the extracts, but was clearly visible after gel filtration (fraction CYSa, Vinokurov et al., 2006-this volume). The activity of cationic enzymes with this substrate was not observed (data not shown).

BApNA was hydrolyzed by three fractions of anionic proteinases in extracts from the AM in PB_{AM} (Fig. 4A). Low- and high-mobility bands, TRYa and TRYc with BApNA-hydrolyzing activity, had the same mobilities as activities in fr1 and 9, previously identified as serine proteinases (Fig. 3A). These zones of activity were inhibited by STI and likely were trypsin-like proteinases. The middle band contained activities that were similar in mobility to one of the previously identified cysteine proteinases from fr4 through fr6 and CYSc. These proteinases were completely inhibited by E-64, significantly reduced without DTT, more intense in the AM than in the PM in PB_{AM}; therefore, these enzymes were likely cysteine proteinases (CYSc′). The activity of cationic enzymes with BApNA was negligible in AM extracts (data not shown).

The activity of anionic proteinases with a chymotrypsin substrate, GlpAALpNA, in AM extracts in PB_{AM} was

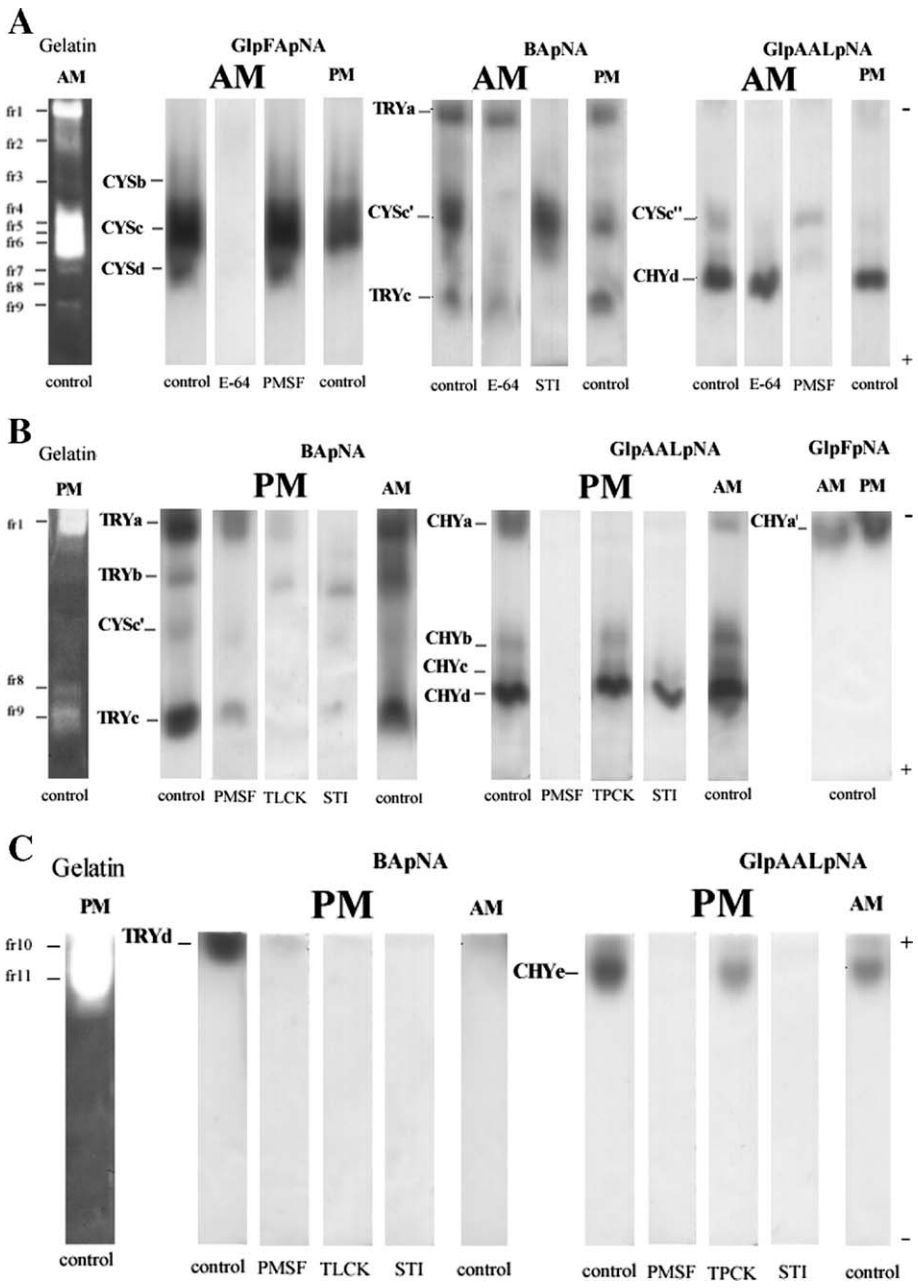

Fig. 4. Postelectrophoretic activity with specific *p*-nitroanilide substrates of the AM and PM extracts of *T. molitor* larvae. Anionic activity was detected in PB_{AM} (A) or PB_{PM} (B), and cationic activity was detected in a PB_{PM} (C). Each lane was incubated with different substrates and with or without inhibitors, as indicated.

represented by two fractions (Fig. 4A). CHYd displayed high activity with GlpAALpNA, but relatively low activity with gelatin (serine gelatinolytic fr8, Fig. 3A), was inhibited by PMSF, and likely was a chymotrypsin-like proteinase. The other fraction coincided in mobility with the major cysteine proteinase activity CYSc, was selectively inhibited by E-64, and was identified as a cysteine proteinase (CYSc″). The activity of cationic proteinases with GlpAALpNA in PC_{AM} was very limited (data not shown).

The activity of cysteine proteinases with GlpFApNA was absent in PM extracts assayed in PB_{PM} (data not shown). The activities of fractions CYSb and CYSc were visible in nonphysiological buffer PB_{AM} (Fig. 4A) and, thus, represented latent forms of proteinases in the PM.

BApNA was hydrolyzed by four fractions of anionic proteinases from the PM in PB_{PM} (Fig. 4B). Two fractions were identical in mobility to AM fractions TRYa and TRYc. Minor activity TRYb migrated between fr2 and fr3 of gelatinolytic activities. All fractions were inhibited to various degrees by PMSF, STI, and TLCK and were identified as trypsin-like. A relatively weak fourth activity represented previously identified cysteine proteinases from CYSc′. The cationic BApNA-hydrolyzing activity in the PM was represented by the fraction TRYd, co-migrating with serine gelatinolytic fr10, and was completely inhibited by all tested inhibitors of trypsin (PMSF, TLCK, and STI) (Fig. 4C).

A comparison of trypsin-like activities in the AM (Fig. 4A) and PM (Fig. 4B and C) revealed that the major enzyme, TRYd,

Table 3
Characteristics of proteolytic activities from the *T. molitor* larvae midgut

Proteinase	Substrate activity					*pI*; Mm	Effective inhibitors	Ineffective inhibitors	Localization and relative activity			
	GlpFApNA	BApNA	GlpAALpNA	GlpFpNA	Gelatin				AM in PB_{AM}	AM [a] in PB_{PM}	PM in PB_{PM}	PM [a] in PB_{AM}
Cysteine [b]	CYSa [c]				fr2	Anionic; unknown	E-64	EDTA pepstatin PMSF STI	+			+
	CYSb				fr3	Anionic; unknown	E-64	EDTA pepstatin PMSF STI	+			+
	CYSc	CYSc′	CYSc″		fr4-6	Anionic; unknown	E-64	EDTA pepstatin PMSF STI	+++			++
	CYSd				fr7	Anionic; unknown	E-64	EDTA pepstatin PMSF STI	++			
Trypsin		TRYa			fr1	Anionic; unknown	STI TLCK	E-64 EDTA pepstatin PMSF	++	++	+++	++
		TRYb			–	Anionic; unknown	PMSF TLCK	E-64		++	+	
		TRYc			fr9	Anionic; unknown	DTT [d] PMSF STI TLCK	E-64 EDTA pepstatin	+	++	+++	++
		TRYd [e]			fr10	7.4; 25.5 kDa	DTT [d] EDTA [d] PMSF STI TLCK	E-64			+++	
Chymotrypsin			CHYa	CHYa′	fr1	Anionic; unknown	PMSF TPCK STI	E-64 EDTA pepstatin		+	+++	
			CHYb		fr4-6	Anionic; unknown	PMSF STI	E-64 TPCK		++	+	
			CHYc		fr4-6	Anionic; unknown	PMSF STI	E-64 TPCK		++	+	
			CHYd		fr8	Anionic; unknown	DTT [d] PMSF STI [d]	E-64 EDTA pepstatin TPCK	+++	+++	+++	+++
			CHYe [f]		fr11	8.4; 23.2 kDa	DTT [d] EDTA [d] PMSF STI TPCK [d]	E-64		+	+++	

[a] All activities were latent.
[b] All activities in this class were activated by DTT.
[c] Identified with GlpFApNA in Vinokurov et al., 2006-this volume.
[d] Slight inhibition.
[e] Previously identified as "TmT1" (Tsybina et al., 2005).
[f] Previously identified as "TmC1" (Elpidina et al., 2005).

previously described in detail (Tsybina et al., 2005), was located entirely in the PM. Activities of TRYa and TRYc were predominant in the PM. The activity of TRYb from the AM in PB_{AM} was negligible, but in PB_{PM} the activity was greater than in the PM (Fig. 3B). Thus, trypsin-like activity with BApNA in the *T. molitor* larvae midgut was due to four fractions: three anionic (TRYa, TRYb, and TRYc) and one cationic (TRYd).

There were four anionic activities in PM extracts with GlpAALpNA in PB_{PM}: CHYa, CHYb, CHYc, and CHYd (Fig. 4B), and only one of them, CHYd, was previously identified in the AM. Fraction CHYa, coinciding with serine gelatinolytic fr1, was completely inhibited by PMSF, STI, and TPCK, typical of chymotrypsin-like proteinases. The other fractions (CHYb, CHYc, and CHYd) were entirely inhibited by PMSF and partly inhibited by STI, but were insensitive to TPCK, and presumably were TPCK-insensitive chymotrypsin-like proteinases. Minor fractions (CHYb and CHYc) co-migrated with fractions of cysteine proteinase activity (CYSc and CYSd).

Cationic enzymes detected in the PM with GlpAALpNA were due to CHYe (Fig. 4C), co-migrating with serine gelatinolytic fr11. The activity of CHYe was completely inhibited by PMSF and STI and was partly inhibited by TPCK, and was a chymotrypsin proteinase described previously (Elpidina et al., 2005).

The major anionic chymotrypsin-like fraction, CHYd, was active in both midgut sections; cationic CHYe and anionic fractions CHYa through CHYc were active only in the PM. If chymotrypsin-like activity in the AM was measured in PB_{PM}, however, latent chymotrypsin-like activity of all fractions was evident, and the activity of fractions CHYb through CHYd was more than the activity from PM extracts.

Hydrolysis of a short-chain chymotrypsin substrate, GlpFpNA, was detected only among anionic proteins (Fig. 4B). This activity, CHYa', coincided in mobility with the previously described CHYa, displayed the same inhibitor sensitivity (data not shown), was predominantly localized in the PM, and was a latent proteinase in the AM. This proteinase was presumably the same chymotrypsin-like enzyme as CHYa, hydrolyzing short- and long-peptide substrates, as has been observed with mammalian chymotrypsin.

4. Discussion

In our study, we observed an integral relationship between pH and the location of enzymes in the gut of *T. molitor* larvae. The average pH of the contents from the anterior, middle, and

posterior of the midgut demonstrated a sharp pH gradient (2.2 to 3.0 pH units) from the beginning to the end. These data are inclusive of the average pH of the AM (5.6) and PM (7.9) contents previously reported for *T. molitor* (Terra et al., 1985). Most insects lack such a sharp gradient in the midgut, with a transition from acid to alkaline pH (Berenbaum, 1980; Terra and Ferreira, 1994; Nation, 2002). In the family Tenebrionidae, two stored-product pests from the genus *Tribolium*, *T. confusum* and *T. castaneum*, had midgut pH values in the ranges 6.8–5.2 and 7.2–7.6, respectively (Sinha, 1959). In another tenebrionid, *Zophobas rugipes*, the pH of midgut contents of larvae was from 5.7 in the AM to 6.5 in the PM (Vinokurov, unpublished results). Six beetle species from the families Scarabaeidae, Passalidae, Chrysomelidae and Cerambycidae also lacked a pH gradient along the midgut (Grayson, 1958). A sharp gradient of acid to alkaline midgut pH only was reported for representatives of the most ancient and unspecialized, mainly detritophagous, insect groups, including the order Collembola, *Sinella coeca*, (Humbert, 1974), and two families of Blattoptera–Blaberidae, *Byrsotria fumigata* (Fisk and Rao, 1964) and *Nauphoeta cinerea* (Elpidina et al., 2001), and Blattellidae, *Blatella germanica* (Day and Powning, 1949). Regions with strong acidic pH (3.2–3.4) in the middle midgut, however, were found between the neutral AM and PM compartments in dipteran families Muscidae, Calliphoridae, and Tephritidae (Terra et al., 1996). These Cyclorrhaphous larvae have adapted to feeding on decaying substrates abundantly contaminated by bacteria, and use such extremely acidified gut compartments for killing and digesting bacterial cells (Espinoza-Fuentes and Terra, 1987; Lemos and Terra, 1991).

Cysteine and serine digestive proteinases are found together in a limited number of insect groups, including orders Thysanura, Hemiptera, and series Cucujiformia (including the family Tenebrionidae) of the order Coleoptera (Murdock et al., 1987; Terra and Ferreira, 1994; Johnson and Rabosky, 2000). In insects lacking a pH gradient, enzymes may be evenly distributed in the midgut, such as was found in the Colorado potato beetle *Leptinotarsa decemlineata* (Novillo et al., 1997). In this insect, the pH of the midgut was from 5.9 to 6.6 (Grayson, 1958), and a chymotrypsin-like digestive proteinase with unusually acidic pH optimum in the range of 5.5–6.5 was identified (Novillo et al., 1997). In the midgut of *T. molitor* larvae, serine proteinases had higher activity at alkaline pH, while cysteine proteinases were active more at acidic pH, and the gradient from acidic to alkaline pH and enzyme compartmentalization negates the need for structural adaptations in digestive enzymes. The high level of diversity of digestive proteinases related to the sharp pH gradient may be characteristic of the nonspecialized type of digestion of this cosmopolitan stored product pest, serving as a mechanism for the efficient incorporation of a wide variety of diets with differences in storage proteins and inhibitors. Indeed, insect larvae reared on diets with quantitatively and qualitatively different complements of proteinase inhibitors have different sets of digestive proteinases due to adaptive responses (Jongsma et al., 1996; Mazumdar-Leighton and Broadway, 2001; Oppert et al., 2005).

Differences in midgut pH are related to different complements of digestive proteases within each midgut compartment. The pH optima for the total proteolytic activity in *T. molitor* larvae differed significantly in the AM and PM and were similar to the pH of the midgut environment. The activity in AM extracts was slightly increased in reducing buffers, suggesting the presence of cysteine proteinases in this compartment. Such an increase was most prominent in dialyzed extracts, when intrinsic reducing compounds of low molecular mass were eliminated, as was observed for extracts from the AM but not from the PM. This information guided the standardization of buffers for experiments with AM and PM extracts. Although the entire midgut sections were taken as a starting material, all data were attributed to secreted digestive proteinases. The activities of intracellular epithelial proteinases were below the level of detection, as measured by gelatinolytic activity in extracts from isolated midgut epithelium loaded in standard midgut equivalents (data not shown).

The majority of the total proteolytic activity was in the AM, where food is introduced to the midgut digestive compartment. Inhibitor analysis of the total proteolytic activity revealed two classes of proteinases: cysteine and serine. In the AM, cysteine proteinases were predominant (64%), while in the PM, serine proteinases were predominant (76%), correlating with previous data in this insect (Terra et al., 1985; Thie and Houseman, 1990). The activity of aspartic proteinases was absent both in the AM and PM, in accordance with the data of Terra and Cristofoletti (1996). A slight inhibition of activity by the metalloproteinase inhibitor chelating agent EDTA, especially noted in PM extracts, was probably due to the sensitivity of trypsin- and chymotrypsin-like serine proteinases of *T. molitor* to chelating agents (Tsybina et al., 2005; Elpidina et al., 2005). The sensitivity of trypsin-like activity to chelating agents similarly was reported in another coleopteran, the European cockchafer *Melolontha melolontha* (Wagner et al., 2002).

The activity of cysteine proteinases, as determined by the hydrolysis of a specific cysteine proteinase substrate, GlpFApNA, was localized predominantly in the AM, in agreement with the data from the inhibitor analysis. This substrate was highly selective for cysteine proteinases of *T. molitor* larvae, as the corresponding activity was completely inhibited by a wide range of cysteine proteinase inhibitors. The activities of trypsin-like proteinases with BApNA and chymotrypsin-like proteinases with GlpAALpNA and GlpFpNA were primarily found in enzymes from the PM extracts.

Electrophoretic fractionation of the total proteinase preparation was performed in conditions with minimal denaturing influence (neutral pH, absence of SDS, low temperature). Our use of a popular SDS-PAGE method combined with postelectrophoretic activity detection (Michaud et al., 1993) resulted in the loss of cysteine proteinase activities, presumably due to inactivation in the presence of SDS and/or alkaline pH (data not shown). Therefore, the activity detection method was adapted to a native system at neutral pH, and electrophoresis was conducted in two directions: toward the anode for anionic proteins with acidic p*I*, and toward the cathode for cationic proteins with basic p*I*. With this technique, the electrophoretic

spectra of the total gelatinase activity differed in the AM and PM. In the AM in PB_{AM}, only anionic proteinases were observed, represented by at least 9 fractions (fr1 through fr9), with the highest activity due to proteins with intermediate mobility (fr4 through fr6). An evaluation of the residual activity of enzymes from fr2 through fr7 in the presence of different inhibitors led to their identification as cysteine proteinases. By a similar analysis, slowly migrating fr1 and fast migrating fr8 and 9 contained serine proteinases. Anionic proteinases from the PM in PB_{PM} were represented by fr1 and fr8 and 9 and were classified as serine proteinases. The activity of cationic proteinases in the PM also was due to serine proteinases (fr10 and 11). The activity of cysteine proteinases was negligible in PB_{PM}.

Further identification of electrophoretic activity was performed with specific *p*-nitroanilide substrates combined with inhibitor analysis. Proteinases from the AM in PB_{AM} were most active with GlpFApNA, and inhibitor analysis confirmed the identification of cysteine proteinases in these fractions. The predominant zone of this activity coincided with gelatinases fr4, 5, and 6 and were most active with gelatin and GlpFApNA, but also were considerably less active with BApNA (CYSc′) and only slightly active with GlpAALpNA (CYSc″). All activities were completely inhibited by E-64. Two fractions of weak trypsin-like activity (TRYa and TRYc) and one faction of strong chymotrypsin-like activity (CHYd) were found in AM extracts when assayed in PB_{AM}.

PM extracts in PB_{PM} contained trypsin-like proteinases, represented by three anionic (TRYa, TRYb, and TRYc) fractions and one cationic (TRYd) fraction. The detailed characteristics of cationic TRYd (TmT1) were presented elsewhere (Tsybina et al., 2005). In the physiological buffers of the midgut, fractions TRYb and TRYd were active only in the PM. Fractions TRYa and TRYc were active in both sections of the midgut, but their activity in the PM was greater.

Chymotrypsin-like proteinases from four anionic (CHYa, CHYb, CHYc and CHYd) and one cationic (CHYe) fractions from the PM hydrolyzed a long-chain substrate, GlpAALpNA, in PB_{PM}. The major anionic fraction (CHYd) was highly active in both sections of the midgut. The other anionic (CHYa, CHYb, and CHYc) fractions and cationic (CHYe) fraction were active only in the PM. Anionic CHYa also hydrolyzed the short-chain chymotrypsin substrate GlpFpNA, indicating that *T. molitor* larvae have at least two different types of digestive chymotrypsins. Four chymotrypsin-like fractions, including the most active, CHYd, and cationic CHYe, hydrolyzed only the long-chain peptide substrate GlpAALpNA and were completely or partly TPCK-insensitive, characteristic of insect chymotrypsins with an extended binding site (Johnston et al., 1995; Lee and Anstee, 1995; Gatehouse et al., 1999; Mazumdar-Leighton and Broadway, 2001). A detailed study of the cationic chymotrypsin CHYe with an extended binding site (TmC1) was made previously (Elpidina et al., 2005). A fifth chymotrypsin-like proteinase fraction was anionic and had the lowest mobility (CHYa), hydrolyzed both synthetic chymotrypsin substrates, was completely inhibited by TPCK, and resembled typical chymotrypsins of mammals. Such enzymes were described as the first insect chymotrypsins in a wide range of species (Terra and Ferreira, 1994).

The spectra of *T. molitor* larval digestive proteinases described herein were characterized in buffers representing physiological pH. When enzymes from each midgut section were analyzed in the physiological buffers of their counterpart, latent activities were observed. In the AM extracts assayed in PB_{PM}, one trypsin-like (TRYb) and four chymotrypsin-like (CHYa, CHYb, CHYc and CHYe) activities were observed, and most anionic activities (except CHYa) were greater than the corresponding activities isolated from the PM under the same conditions. These enzymes may be synthesized in the AM but are activated only when they are translocated with the food bolus to the alkaline pH of the PM. Latent forms of cysteine proteinases also were found in the PM as assayed in PB_{AM}, but the activity of these enzymes was less than those in the AM. Translocation of cysteine proteinases to the PM may represent a regulatory mechanism for enzyme inactivation in the gut.

The results of the analysis of the digestive proteinase spectra of *T. molitor* larvae indicate that complex enzyme diversity can be revealed only by general proteinaceous substrates and a set of specific substrates, combined with inhibitor analyses, all within the framework of physiological pH. The gelatinolytic fractions often contained several different proteinases with the same mobility, but their spectrum represented all major proteolytic activities. The use of specific substrates permitted the differentiation of these activities, but care must be taken to ensure that specific substrates can distinguish all gelatinolytic fractions. Thus, the wide level of diversity of digestive proteinases in *T. molitor* larvae combined with a sharp pH gradient in the midgut may represent a nonspecialized type of digestion of this cosmopolitan stored-product pest, and serves as a mechanism to overcome the negative effects of dietary proteinase inhibitors found in stored grains and meals.

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