



Cysteine digestive peptidases function as post-glutamine cleaving enzymes in tenebrionid stored-product pests

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ABSTRACT

The major storage proteins in cereals, prolamins, have an abundance of the amino acids glutamine and proline. Storage pests need specific digestive enzymes to efficiently hydrolyze these storage proteins. Therefore, post-glutamine cleaving peptidases (PGP) were isolated from the midgut of the stored-product pest, *Tenebrio molitor* (yellow mealworm). Three distinct PGP activities were found in the anterior and posterior midgut using the highly-specific chromogenic peptide substrate *N*-benzyloxycarbonyl-L-Ala-L-Ala-L-Gln *p*-nitroanilide. PGP peptidases were characterized according to gel elution times, activity profiles in buffers of different pH, electrophoretic mobility under native conditions, and inhibitor sensitivity. The results indicate that PGP activity is due to cysteine and not serine chymotrypsin-like peptidases from the *T. molitor* larvae midgut. We propose that the evolutionary conservation of cysteine peptidases in the complement of digestive peptidases of tenebrionid stored-product beetles is due not only to the adaptation of insects to plants rich in serine peptidase inhibitors, but also to accommodate the need to efficiently cleave major dietary proteins rich in glutamine.

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1. Introduction

The yellow mealworm, *Tenebrio molitor*, (Coleoptera: Tenebrionidae) belongs to a group of stored-product pests that use grains and their products as their primary food source. Digestive peptidases in *T. molitor* larvae include cysteine peptidases that are represented by at least four to six distinct enzymes (Vinokurov et al., 2006a,b; Prabhakar et al., 2007) with the major peptidase activity attributed to cathepsin L (Cristofolletti et al., 2005). In addition, *T. molitor* larvae have at least four trypsin-like and five chymotrypsin-like serine peptidases for protein digestion (Elpidina et al., 2005; Tsybina et al., 2005; Vinokurov et al., 2006a), as well as a membrane-bound aminopeptidase (Cristofolletti and Terra, 1999; Cristofolletti and Terra, 2000) and soluble carboxypeptidase (Ferreira et al., 1990; Prabhakar et al., 2007). A sharp pH gradient, from 5.6 in the anterior midgut (AM) to 7.9 in posterior midgut (PM), results in restricted activity of digestive enzymes in different areas of the larval midgut (Terra et al., 1985; Vinokurov et al., 2006a). Cysteine peptidases and carbohydrases are located mainly in the AM due to their acidic pH optima, and serine

peptidases are found mainly in the PM due to neutral or alkaline pH optima (Terra et al., 1985; Terra and Cristofolletti, 1996; Vinokurov et al., 2006a; Elpidina and Goptar, 2007; Prabhakar et al., 2007). The complement of digestive peptidases in related tenebrionids, *Tribolium castaneum* and *Tribolium confusum*, are similar, with a pH gradient from 5.6 to 7.5 in the midgut (Vinokurov et al., 2009). A bioinformatic study of peptidase genes in *T. castaneum* found 25 cysteine peptidases in the genome (*Tribolium Genome Sequencing Consortium*, 2008), and approximately half of them, including cathepsin B and L, were expressed in the gut (Morris et al., 2009).

Our research has focused on the collection of digestive peptidases in stored-product tenebrionid beetles as determined by the structure of their dietary proteins. The main dietary proteins of *T. molitor* and related cereal-feeding insects are storage proteins of cereal grains. The major fraction of storage proteins, prolamins, are as much as 50% of the total seed protein, and prolamins contain 30–50% glutamine and 10–30% proline residues (Shewry and Tatham, 1990; Shewry and Halford, 2002). Therefore, we predicted that *T. molitor* has digestive enzymes that can specifically and efficiently cleave peptide bonds following either glutamine or proline residues. Indeed, in an earlier study, we described the first proline-specific digestive peptidase in the midgut of *T. molitor* larvae, which is a serine peptidase that specifically cleaves after

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proline, has an acidic pH optimum (5.3), and is found mainly in the AM (Goptar et al., 2008a,b).

The identification of glutamine-specific peptidases represents an important area of research for insects such as stored-product pests that need to digest dietary protein high in glutamine content. Furthermore, these enzymes may initiate the liberation of glutamine, a key amino acid critical to insect immunity (Zou et al., 2007), plant-herbivore interactions (Pare et al., 1998), and cellular regulation and gene expression control in mammalian cells (Brasse-Lagnel et al., 2009; Rhoads and Wu, 2009). In the present study, we partially purified and characterized endopeptidases from the *T. molitor* larval midgut that hydrolyze the peptide bonds formed by the carboxyl group of glutamine residues, providing a crucial role in the digestion of the major dietary proteins of this insect.

2. Materials and methods

2.1. Preparation of enzyme extracts

Actively feeding fourth instar *T. molitor* larvae were reared on a mixture of milled oat flakes and bran. Larvae were transferred to milled oat flakes (Raisio, Finland) without bran 1–1.5 weeks prior to dissection. Midguts were isolated in 0.9% NaCl and divided into two equal parts, AM and PM. For fractionation by gel filtration chromatography, the AM and PM preparations with contents were homogenized in double-distilled water in a glass Downce homogenizer (approximately 50 AM or PM parts in 350 μ L of water) and centrifuged for 10 min at 15,000 g. To separate the midgut contents and tissue for further studies, AM and PM sections were sectioned longitudinally, the contents extruded, and the tissue was rinsed with 0.9% NaCl. The lumen contents or the washed gut tissues were homogenized separately and centrifuged as described above. All supernatants were stored at -70°C .

2.2. Gel chromatography of AM and PM extracts

The extract from 200 pooled AM or PM gut sections (2–2.5 mL) was applied to a Sephadex G-100 column (2.5 \times 120 cm) equilibrated with 500 mM NaCl in 10 mM phosphate buffer, pH 5.6, containing 0.02% NaN_3 . Fractionation was performed at 4°C . Fractions of 9.0 mL were collected and analyzed for protein content and hydrolytic activity with *p*-nitroanilides of benzyloxycarbonyl-L-alanyl-L-alanyl-L-glutamine (ZAAQpNA), pyroglutamyl-L-phenylalanyl-L-alanine (GlpFapNA), and pyroglutamyl-L-alanyl-L-alanyl-L-leucine (GlpAALpNA) in equal aliquots as described in the following section. Fractions with post-glutamine proteolytic activity were pooled and labeled PGP1_{PM} and PGP2_{PM} from the PM, and PGP2_{AM} from the AM. Pooled fractions were concentrated and desalted on Amicon YM3 membranes (Amicon, the Netherlands) at 4°C , and were either used immediately for further analysis, or were stored at -70°C . To assess the approximate molecular mass of eluted enzymes in each fraction, the column was calibrated using horse cytochrome c (13 kDa), soybean Kunitz trypsin inhibitor (STI) (22 kDa), ovalbumin (45 kDa) and BSA (67 kDa).

2.3. Enzyme assays and protein determination

Post-glutamine cleaving activity was assayed by the hydrolysis of the chromogenic peptide substrate ZAAQpNA, synthesized according to standard procedures (Pennington and Dunn, 1994). Activities of cysteine and chymotrypsin-like proteinases were assessed with specific substrates GlpFapNA (Stepanov et al., 1985; Vinokurov et al., 2006b) and GlpAALpNA (Lyublinskaya et al., 1987; Tsybina et al., 2005), respectively, and compared to those previously characterized (Vinokurov et al., 2006a; Vinokurov et al., 2006b). *p*-Nitroaniline release was measured spectrophotometrically at 405 nm (Erlanger et al., 1961) in 96 well plates with a StatFax 2100 microplate reader

(Awareness Technology Inc., FL, USA) using a differential filter of 492 nm. The activity of cysteine peptidases was assayed at pH 5.6, and of chymotrypsin-like peptidases at pH 7.9 (Vinokurov et al., 2006a). The standard pH of 5.6 was used for PGP activity measurement unless noted. Enzymatic activity was calculated by the formula:

$$a = k \frac{(A_t - A_0) \cdot V_{pr}}{t \cdot V_a}$$

where *a* is the activity of the preparation, nmol/min; *k* = 30.8 nmol, the *p*-nitroaniline amount at which optical absorbance of the solution is equal to 1 optical unit (*k* was determined from standard curve by plotting absorbance versus *p*-nitroaniline amount); *A_t*, absorbance of the reaction mixture at a specific time *t*, optical units; *A₀*, absorbance when reaction initiated at time 0, optical units; *V_{pr}*, total reaction volume in mL; *t*, reaction time, min; and *V_a*, the volume of the enzyme aliquot added to reaction mixture, mL. For comparative purposes enzymatic activity was calculated per 1 gut as *a*/200.

For peptidase activity measurements, 10–50 μ L of enzyme solution at 0.1–8 mg/mL concentration were added to a microplate well and diluted with Frugoni's Universal 100 mM acetate-phosphate-borate buffer (UB) (Frugoni, 1957), pH 5.6 or 7.9, to a final volume of 197 μ L. Three μ L of 20 mM substrate solution in dimethyl formamide (DMF) was added (the final substrate concentration was 0.3 mM; the DMF concentration in the reaction mixture, 1.5% v/v) and initial optical absorption at time zero was measured. The mixture was incubated at 37°C , and absorbance was measured at specific time intervals. Enzyme activity was calculated in nmol/min per gut on the linear part of the time and protein concentration response curves. All assays were run in triplicate.

For assays of sulfhydryl (SH)-dependent activity with the substrates GlpFapNA and ZAAQpNA, 5 μ L of freshly prepared 120 mM dithiothreitol (DTT) solution in water (final concentration of 3 mM) was added to the enzyme solution before the addition of substrate, and the mixture was incubated at 23°C for 20 min.

The effect of pH on peptidase activity against *p*-nitroanilide substrates was measured using 100 mM UB at various pH ranging from 3.9 to 8.6 in the presence of 3 mM DTT.

Protein content in the eluted fractions was assessed spectrophotometrically at 280 nm.

2.4. Inhibition assays

For inhibition studies, 5–7 μ L of PGP preparations were incubated with different concentrations of inhibitor in 100 mM UB, pH 5.6, at 23°C for 20 min, and the residual activity was assayed as described in Section 2.3 in triplicate. Diagnostic inhibitors of the active site included: phenylmethylsulphonyl fluoride (PMSF, specific for serine peptidases) at 0.01, 0.1 and 1 mM, pepstatin A (specific for aspartic peptidases), *L*-trans-epoxysuccinyl-L-leucylamido(4-guanidino)butane (E-64, specific for cysteine peptidases) at 0.001, 0.01, 0.1 mM, EDTA (an inhibitor of metallopeptidases) at 0.1, 1.0 and 10 mM final concentrations.

2.5. Postelectrophoretic proteolytic activity detection

Electrophoresis was carried out under native conditions in 12% separating and 4% concentrating polyacrylamide gels containing 35 mM HEPES and 43 mM imidazole at pH 7.2 according to McLellan (1982). An aliquot of partially purified gut enzymes with predetermined activity of 1 nmol/min was added to each well. Electrophoresis was performed toward the anode at a constant current of 10 mA for 45 min at 4°C .

Postelectrophoretic detection of specific proteolytic activity was performed using a nitrocellulose overlay impregnated with *p*-nitroanilide substrate (Vinokurov et al., 2005). After electrophoresis,

the resolving gel was washed for 15 min in 100 mM UB, pH 5.6, 5 mM DTT and was overlaid with a 0.45 μ m nitrocellulose membrane that was presoaked for 40 min in 0.5 mM solution of substrate in 100 mM UB (diluted from 20 mM substrate solution in DMF), pH 5.6, 3 mM DTT, and slightly dried before applying to the surface of the gel. The membrane was incubated with the gel in a moist chamber at 37 °C for 50 min. 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 the formation of the pink bands representing proteolytic activity, membranes were placed in heat-sealed plastic bags, scanned and stored at –20 °C.

2.6. Proteolysis of gliadins

The proteolysis of gliadins was studied by two different methods. In the first approach, hydrolysis products were visualized using sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE). The reaction mixtures consisted of 0.5 mg of gliadins (MP Biomedicals, CA, USA) in 50 μ L of 100 mM UB, pH 5.6, 5 mM DTT, and 2–10 μ L of fraction PGP2_{AM} from the *T. molitor* larval AM, preincubated for 20 min at 23 °C with 1 mM PMSF (to inhibit serine-like proteinases) or 0.1 mM E-64 (to inhibit cysteine-like proteinases). The reaction mixtures were incubated at 23 °C for 15 min, 30 min, 1 h, or 2 h. The enzyme reaction was terminated by the addition of 60 μ L of 10% SDS, 60 μ L of 10 M urea, and 60 μ L of glycerol with subsequent 5–10 min boiling. Hydrolysis products were separated by SDS-PAGE according to the modified method of Laemmli (1970). Urea (2 M) was included in gels to enable the solubilization of gliadins. Prior to loading, all samples were mixed with 10 μ L of 2-mercaptoethanol and placed in boiling water bath for 5 min. Nine μ L of the resulting mixtures was applied to each well. After electrophoresis, the gels were stained in a solution of 0.1% Coomassie Brilliant Blue R-250 in a mixture ethanol/acetic acid/water (3:1:6, v/v) and destained in the same solution. The relative amount of protein in all of the gliadin bands from enzyme treatment (with subtraction of control enzyme preparation) and control gliadins sample was calculated by means of LabWorks 4.6 software (UVP Bioimaging Systems, CA, USA).

In the second approach, gliadin proteolysis was measured in solution using ninhydrin reagent to observe an increase in free amino groups (Meyer, 1957). To assay the effect of serine peptidase activity on gliadins, 750 μ g of gliadin was added to 220 μ L of 100 mM UB, pH 5.6, 60 μ L of 10 M urea, and 7.5 μ L of 120 mM DTT, then mixed with 20 μ L of PGP2_{AM} preparation containing 0.1 mM E-64. For cysteine peptidase activity assay, 20 μ L of the same PGP2_{AM} preparation contained 1 mM PMSF. Ten μ L of each reaction mixture was removed at time zero, and each 20 min thereafter over a 2 h incubation time at room temperature (23 °C). At the designated time-point, the reaction mixture aliquots were supplemented with 190 μ L of 0.1% solution of ninhydrin in ethanol and incubated at 80 °C for 1 h. The optical absorbance of the colored reaction product was measured at 545 nm with a microplate reader.

The quantitative ratio of partially purified gut enzyme preparations and gliadin in the reaction mixture was chosen based on the predicted amount of peptidase present in the AM and percentage of gliadin present in diet.

3. Results

3.1. Localization of PGP activity in the midgut of *T. molitor* larvae

To determine the localization of PGP in the *T. molitor* larval midgut, activity was assayed in the lumen contents of the AM and PM, where secreted digestive enzymes are found, and in the AM and PM tissues, containing membrane-localized digestive and tissue

enzymes. The measurements were performed with the PGP-specific substrate ZAAQpNA at the physiological pH of the *T. molitor* larval midgut contents in each gut section: 5.6 in the AM and 7.9 in the PM (Terra et al., 1985). The activity of PGP was 2 to 3-fold higher at pH 5.6 than at pH 7.9 in all lumen and tissue extracts (Table 1). Correspondingly, PGP activity was 2.3 to 2.7-fold higher in lumen extracts of the AM than that of the PM contents at both pH values. PGP activity in midgut tissues from both sections and at both pH values was considerably lower (up to 100-fold lower) compared with the activity in the lumen contents. These data indicated that PGP activity was soluble, more active at the physiological pH values found in the AM, and was located primarily in the AM.

3.2. Gel chromatography of AM and PM extracts

To obtain partially purified PGP for further study, extracts from the AM and PM of *T. molitor* larvae were fractionated by gel filtration chromatography. Fractions were tested for post-glutamine proteolytic activity with ZAAQpNA, as well as the activities of cysteine and chymotrypsin-like serine peptidases using the specific substrates GlpFapNA and GlpAALpNA, respectively (Fig. 1).

There was a single peak of PGP activity in the elution profile of AM proteins (PGP2_{AM}; Fig. 1A), eluting very close to that of cysteine peptidase activity, previously referred to as “Cys II from AM” (Vinokurov et al., 2006b). The PGP profile of the PM also closely eluted with cysteine peptidase activity (Fig. 1B), but with two partially resolved peaks of activity, PGP1_{PM} and PGP2_{PM}. The putative molecular masses of PGPs were similar to those of previously described cysteine peptidases: 21 kDa for PGP2_{AM}, 39 kDa for PGP1_{PM} and 23 kDa for PGP2_{PM} (Fig. 1C) (Vinokurov et al., 2006b). The data presented in Fig. 1 demonstrate that the ratio of activities specific for GlpFapNA and ZAAQpNA were the same both for AM and PM profiles. The PGP activity profile partially overlapped with the activity profile of chymotrypsin-like peptidases, as was measured by the hydrolysis of GlpAALpNA. However, in the AM, the level of chymotrypsin-like activity was similar to that of PGP, while in the PM it was 4.5-fold higher. Hence, the activities that were determined by the hydrolysis of substrates GlpFapNA and ZAAQpNA may be attributed to the same enzymes, while chymotrypsin-like activities with GlpAALpNA were distinct.

3.3. Effect of pH on the activity of PGP

Fractions from the gel filtration chromatography containing PGP activities were pooled for further analysis. The effect of pH on the activity of enzymes in PGP2_{AM}, PGP1_{PM}, and PGP2_{PM} fractions was studied using ZAAQpNA and GlpFapNA as substrates (Fig. 2). The activity patterns for all preparations with both substrates showed similar pH patterns. All preparations were active within a wide pH range, with activity exceeding 50% of the maximal level within the pH 4.5–7.9 interval. The activities of enzymes in PGP2_{AM} and PGP2_{PM} were maximal at pH 7.4, and those in PGP1_{PM} at pH 7.2. Activity values detected with both substrates for each pooled enzyme sample were comparable and varied only 20–30%.

Table 1

Localization of glutamine-specific proteolytic activity, as determined by the hydrolysis of ZAAQpNA, in the *T. molitor* larval midgut (mean \pm SE; n = 3, significant at P < 0.05).

Midgut localization		Activity (nmol/min/gut)	
		pH 5.6	pH 7.9
AM	Lumen contents	32.5 \pm 5.53	12.5 \pm 1.37
	Tissues	0.38 \pm 0.06	0.18 \pm 0.06
PM	Lumen contents	11.9 \pm 0.77	5.10 \pm 0.16
	Tissues	0.35 \pm 0.03	0.12 \pm 0.03

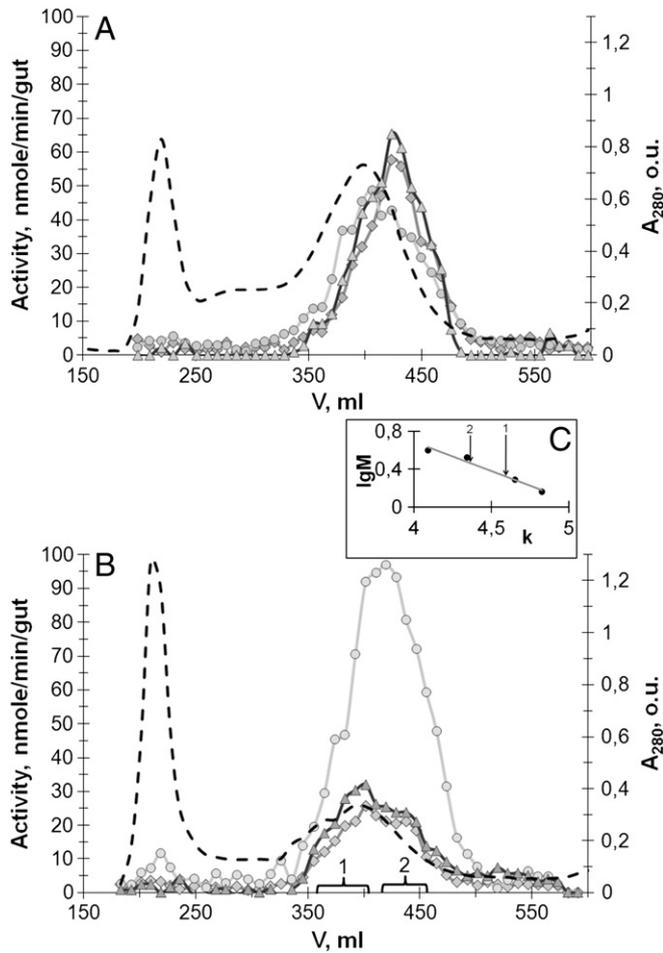


Fig. 1. Gel filtration of the AM (A) and PM (B) extracts from *T. molitor* larvae on Sephadex G-100. PGP activities were measured with ZAAQpNA (—◆—), cysteine peptidases with GlpFAPNA (—▲—), and chymotrypsin-like peptidases with GlpAALpNA (—●—). Protein concentration was assessed by absorbance at 280 nm (---). C. Determination of native molecular mass of PGP1_{PM} (1), and PGP2_{PM} (2) by gel exclusion chromatography on a Sephadex G-100 column. Standard markers were cytochrome c (13 kDa), soybean Kunitz trypsin inhibitor (22 kDa), ovalbumin (45 kDa) and BSA (67 kDa). $K = (V - V_0) / (V_{tot} - V_0)$, where V – elution volume of protein, V_0 – void volume, and V_{tot} – total column volume.

3.4. Inhibitory analysis

PGP2_{AM}, PGP1_{PM}, and PGP2_{PM} enzyme preparations were examined using class specific inhibitors of serine peptidases, PMSF; aspartic peptidases, pepstatin A; metallopeptidases, EDTA; cysteine peptidases, E-64; and an activator of SH-dependent peptidases, DTT (Table 2). All enzymes were active only in the presence of DTT, and the activity was completely inhibited by E-64 at all concentrations tested, indicating that cysteine-like peptidases are responsible for ZAAQpNA-hydrolysis. As the EDTA concentration increased up to 10 mM, the PGP activity increased approximately 25%, possibly due to the binding of bivalent cations that can inhibit cysteine peptidases. PMSF and pepstatin A had no effect on the activity of all preparations. These data indicated that PGP activity was due to cysteine peptidases that have properties similar to the digestive cysteine peptidases previously described in this insect (Vinokurov et al., 2006a,b).

3.5. Postelectrophoretic proteolytic activity

The three partially purified PGP fractions were tested for postelectrophoretic proteolytic activities with GlpFAPNA and ZAAQpNA at pH 5.6. A single band of similar electrophoretic mobility was observed for both GlpFAPNA and ZAAQpNA substrates for all three partially

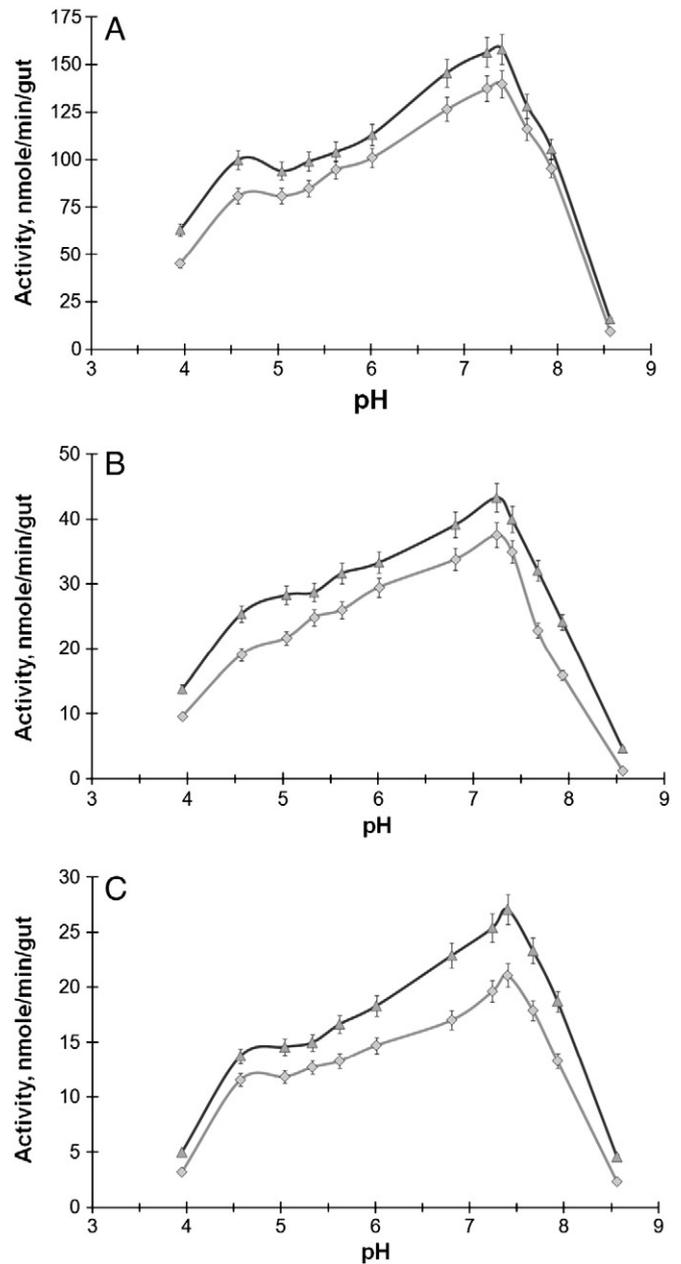


Fig. 2. Effect of pH on the activities of PGP2_{AM} (A), PGP1_{PM} (B), and PGP2_{PM} (C) preparations, assayed with ZAAQpNA (—◆—) and GlpFAPNA (—▲—).

purified enzyme fractions (Fig. 3). Therefore, we were unable to distinguish between PGP and general cysteine peptidase activities.

3.6. Gliadin hydrolysis by PGP2_{AM}

As the activity of PGP was more prominent in the AM fraction, we chose to examine the contribution of PGP activity against the glutamine-rich gliadin proteins *in vitro* using enzymes in the PGP2_{AM} fraction and conditions approximating the physiological pH of the AM of *T. molitor* larvae (Terra et al., 1985; Vinokurov et al., 2006a). The PGP2_{AM} preparation contained two types of proteolytic activity (cysteine- and serine-like) that were not resolved by size exclusion chromatography (Fig. 1A). The contribution of cysteine peptidases was assessed by the addition of the serine peptidase inhibitor PMSF (Vinokurov et al., 2006a), while the activity of serine peptidases was evaluated with the cysteine peptidase inhibitor E-64 (Fig. 4).

Table 2

Effect of inhibitors and activators on the activity of enzymes in PGP2_{AM}, PGP1_{PM}, and PGP2_{PM} with ZAAQpNA (mean ± SE; n = 3).

Reagent	Concentration (mM)	Residual activity ^a (%)		
		PGP2 _{AM}	PGP1 _{PM}	PGP2 _{PM}
DTT	0	0	0	0
	0.3	31 ± 3	33 ± 5	35 ± 4
	3.0	100 ± 11	100 ± 10	100 ± 10
PMSF ^b	1.0	110 ± 7	106 ± 8	91 ± 6
	0.1	107 ± 8	101 ± 8	90 ± 5
	0.01	95 ± 7	92 ± 8	100 ± 7
Pepstatin A ^b	0.1	98 ± 5	93 ± 7	85 ± 5
	0.01	103 ± 7	96 ± 5	87 ± 7
	0.001	96 ± 7	102 ± 6	96 ± 6
EDTA ^b	10.0	127 ± 11	121 ± 10	129 ± 10
	1.0	114 ± 10	111 ± 11	116 ± 11
	0.1	103 ± 9	102 ± 9	105 ± 10
E-64 ^b	0.1	0	0	0
	0.01	0	0	0
	0.001	0	0	0

^a Activity in the presence of 3 mM DTT was taken as 100%.

^b All measurements were performed in the presence of 3 mM DTT.

Using electrophoretic analysis, we found that the total gliadin preparation contained a mixture of proteins in major abundance ranging from 31 to 45 kDa, and proteins of minor abundance ranging from high molecular mass (72, 94 and 96 kDa) to low molecular mass (about 15 kDa) (Fig. 4A, lane 2). In the presence of both inhibitors E-64 and PMSF, gliadin proteolysis by PGP2_{AM} was weak even after 2 h (Fig. 4A, lane 3). Hydrolysis by PGP2_{AM} with E-64, to observe the effect of only serine peptidases, also was weak (Fig. 4A, lanes 9–12), and after a 2 h incubation, only half of the gliadin was hydrolyzed by presumably serine peptidases (Fig. 4B). However, the incubation of gliadin proteins with PGP2_{AM} and PMSF, to observe only cysteine peptidase activity, efficiently cleaved gliadin proteins (Fig. 4A, lanes 5–8), with 50% of gliadins hydrolyzed in 30 min (Fig. 4B), and nearly complete hydrolysis after 2 h (Fig. 4B).

The rates of gliadin hydrolysis by the enzymes from the PGP2_{AM} preparation were compared using ninhydrin to detect the formation of free NH₂-groups (Fig. 5). Cysteine peptidases (PGP2_{AM} with PMSF) hydrolyzed gliadins four times faster than serine peptidases (PGP2_{AM} with E-64). These results agree with our electrophoretic analysis (Fig. 4) and demonstrate that the PGP enzymes in *T. molitor* are represented by cysteine-like peptidases that efficiently hydrolyze glutamine-rich protein substrates.

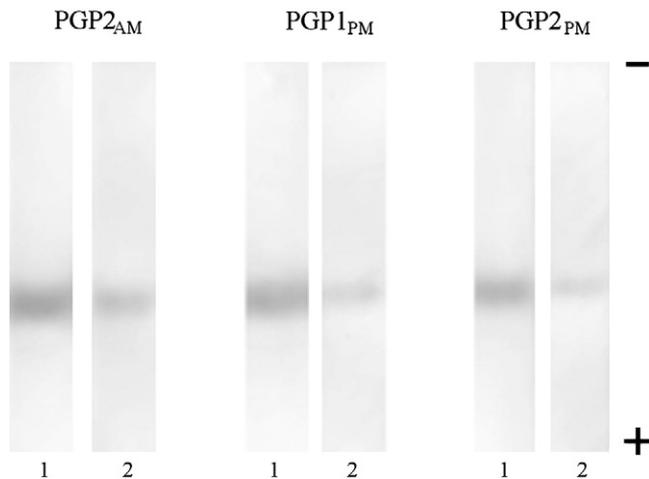


Fig. 3. Postelectrophoretic activity of PGP2_{AM}, PGP1_{PM}, and PGP2_{PM} assayed with GlpFapNA (1) and ZAAQpNA (2) at pH 5.6 in the presence of 3 mM DTT.

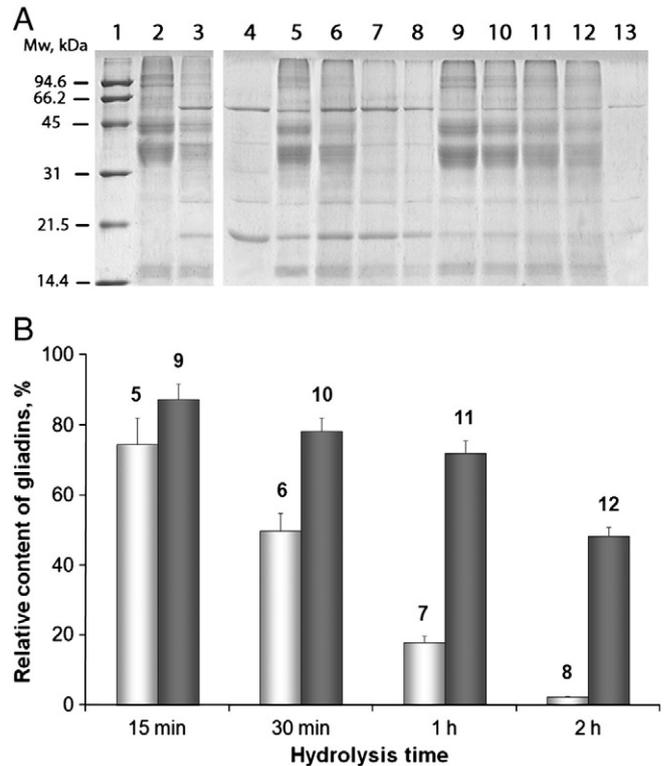


Fig. 4. Electrophoretic study of gliadin hydrolysis by enzymes in the PGP2_{AM} fraction. A. Electrophoregram of stained gel. 1 — molecular mass standards; 2 — gliadins; 3 — gliadins + PGP2_{AM} + E-64 + PMSF, 2 h incubation; 4 — PGP2_{AM} + PMSF control; 5, 6, 7, 8 — gliadins + PGP2_{AM} + PMSF after 15 min, 30 min, 1 h, and 2 h incubation; 9, 10, 11, 12 — gliadins + PGP2_{AM} + E-64 after 15 min, 30 min, 1 h, and 2 h incubation; 13 — PGP2_{AM} + E-64 control. B. Postelectrophoretic processing of the gel with Lab-Works 4.6 software (UVP Bioimaging Systems, USA). Light columns represent the activity (mean ± SE) of cysteine peptidases (lanes 5–8) and dark columns of serine peptidases (lanes 9–12); the figures above the columns correspond to the gel lanes.

4. Discussion

The primary natural food of *T. molitor*, as well as *T. castaneum* and *T. confusum*, is grain and grain products, which also serve as important food for people and some mammals. Cereal grains are characterized by a high content of storage proteins, and the major storage proteins are prolamins, which are comprised of up to 50% of glutamine residues (Shewry and Halford, 2002). In humans, the hydrolysis of food proteins is performed by the aspartic peptidase pepsin, secreted in the stomach under acidic conditions (Mosolov, 1971; Athauda

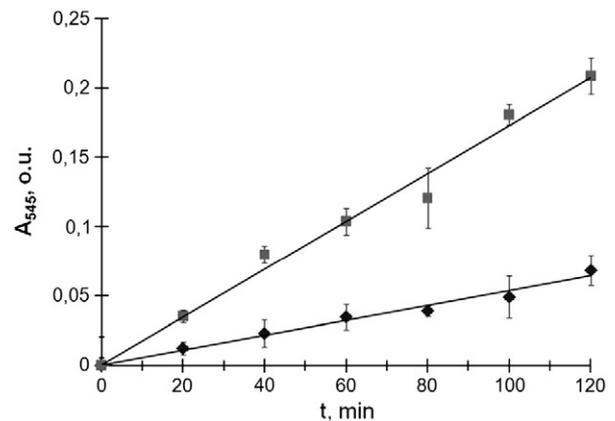


Fig. 5. Gliadin hydrolysis by enzymes in the PGP2_{AM} fraction in the presence of 1 mM PMSF (■) (cysteine peptidases) or 0.1 mM E-64 (◆) (serine peptidases), assessed with ninhydrin reagent.

and Takahashi, 2002), and chymotrypsin C, a serine peptidase from the pancreas (Folk and Cole, 1965). Aspartic peptidases have not been found among digestive peptidases of *T. molitor* (Vinokurov et al., 2006a; Elpidina and Goptar, 2007) or *T. castaneum* (Vinokurov et al., 2009) larvae, and our data indicate that the post-glutamine activity in *T. molitor* is due to cysteine, and not chymotrypsin-like, peptidases.

These data provide the first report of post-glutamine cleaving activity in an insect using a synthetic substrate ZAAQpNA. PGP activity was found in the contents of both AM and PM of *T. molitor* larvae, but most of the activity (70%) was located in the AM contents in the acidic part of the gut. Cysteine peptidase activity is reduced in alkaline pH (Vinokurov et al., 2006b), and therefore PGP enzymes in the PM are less active. Finding the enzyme activity in the midgut contents demonstrates that PGP are soluble enzymes. Gel chromatography profiles of activities with ZAAQpNA coincided with activity profiles with a specific cysteine peptidase substrate, GlpFapNA (Stepanov et al., 1985; Vinokurov et al., 2006a,b). Inhibitory analysis of PGP_{2AM}, PGP_{1PM}, and PGP_{2PM} revealed that PMSF, pepstatin A, and EDTA did not inhibit activity, while an inhibitor of cysteine peptidases, E-64, completely inhibited activity. Furthermore, without DTT, an activator of cysteine peptidases, PGP activity was not observed. These results support the identification of PGP activity, detected with the specific substrate ZAAQpNA, as cysteine peptidases. Similar effects of pH were observed on PGP_{2AM}, PGP_{1PM}, and PGP_{2PM} activities with ZAAQpNA and GlpFapNA substrates, as well as indistinguishable electrophoretic mobilities of activities for both substrates.

The major cysteine digestive peptidase activities in *T. molitor* and *T. castaneum* are cathepsin L (Cristofolletti et al., 2005; Morris et al., 2009; Oppert et al., 2010). In the present study, and in accordance with our previous results (Vinokurov et al., 2006a,b), the most active fractions with the specific cysteine peptidase substrate GlpFapNA corresponded to PGP_{2AM} and PGP_{2PM}, capable of cleaving the bonds formed by glutamine carboxyl, and the level of hydrolytic activity with ZAAQpNA was comparable to that with GlpFapNA. Therefore, we speculate that cathepsin L enzymes could be responsible for PGP activity in tenebrionid beetles. Our data correlate with a previous report demonstrating post-glutamine proteolytic activity in several mammalian cysteine cathepsins, including lysosomal cathepsin L, and plant cysteine peptidases (Choe et al., 2006).

The evaluation of the contribution of insect PGP to the hydrolysis of glutamine-rich gliadins revealed that these peptidases are located in the acidic AM of *T. molitor* larvae and likely contribute to early stages of protein digestion. The predominance of cysteine peptidases in the midgut of coleopteran larvae has been proposed as an adaptation to cereals rich in naturally occurring serine proteinases inhibitors (Terra and Cristofolletti, 1996). We propose that another reason for the retention of cysteine peptidases in storage pests is the need to efficiently digest seed storage proteins rich in glutamine, and to specifically cleave peptide bonds at the glutamine carboxyl. This hypothesis is consistent with an assumption that the shift to cysteine peptidases occurred in early seed-feeding beetles and contributed to their success (Johnson and Rabosky, 2000). The data also correlate with the exclusive role of cysteine peptidases in the digestion of phaseolin, the major seed protein of beans, by seed-feeding bruchid beetles (Wieman and Nielsen, 1988).

We conclude that the path of hydrolysis of cereal prolamins is different in insects and in humans, due at least in part to the involvement of peptidases from different subclasses in recognizing the predominant amino acid residue, glutamine. This difference provides a potential target for the development of novel transgenic cereal inhibitors to control damage to storage products by tenebrionid insects.

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