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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 (Cristofoletti 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 (Cristofoletti and Terra, 1999; Cristofoletti 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 Cristofoletti, 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 μL 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 × 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:

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

where $\boldsymbol{a}$ is the activity of the preparation, nmol/min; $\boldsymbol{k} = 30.8$ nmol, the *p*-nitroaniline amount at which optical absorbance of the solution is equal to 1 optical unit ($\boldsymbol{k}$ was determined from standard curve by plotting absorbance versus *p*-nitroaniline amount); $\boldsymbol{A_t}$, absorbance of the reaction mixture at a specific time $\boldsymbol{t}$, optical units; $\boldsymbol{A_0}$, absorbance when reaction initiated at time 0, optical units; $\boldsymbol{V_{pr}}$, total reaction volume in mL; $\boldsymbol{t}$, reaction time, min; and $\boldsymbol{V_a}$, the volume of the enzyme aliquot added to reaction mixture, mL. For comparative purposes enzymatic activity was calculated per 1 gut as $\boldsymbol{a}/200$.

For peptidase activity measurements, 10–50 μL 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 μL. Three μL 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 μL 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 μL 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,