

# Fractionation of digestive proteinases from *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae and role in protein digestion

K.S. Vinokurov<sup>a</sup>, E.N. Elpidina<sup>b</sup>, B. Oppert<sup>c,\*</sup>, S. Prabhakar<sup>d</sup>, D.P. Zhuzhikov<sup>a</sup>,  
Y.E. Dunaevsky<sup>b</sup>, M.A. Belozersky<sup>b</sup>

<sup>a</sup> Department of Entomology, Biological Faculty, Moscow State University, Moscow 119992, Russia

<sup>b</sup> A.N. Belozersky Institute of Physico-Chemical Biology, Moscow State University, Moscow 119992, Russia

<sup>c</sup> USDA ARS Grain Marketing and Production Research Center, 1515 College Ave., Manhattan, KS 66502, USA

<sup>d</sup> Department of Entomology, Kansas State University, Manhattan, KS 66506, USA

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## Abstract

*Tenebrio molitor* larval digestive proteinases were purified and characterized by gel filtration chromatography combined with activity electrophoresis. Cysteine proteinases, consisting of at least six distinct activities, were found in three chromatographic peaks in anterior and posterior midgut chromatographies. The major activity in the anterior midgut, peak cys II, consisted of cysteine proteinases with Mm of 23 kDa. The predominant peak in the posterior, cys I, was represented by 38 kDa proteinases. The activities of all cysteine proteinases were maximal in buffers from pH 5.0 to 7.0, with 80% stability at pH values from 4.0 to 7.0. In the conditions of the last third of the midgut, the activity and stability of cysteine proteinases was sharply decreased. Trypsin-like activity included a minor peak of “heavy” trypsins with Mm 59 kDa, located mainly in the anterior midgut. An in vitro study of the initial stages of digestion of the main dietary protein, oat 12S globulin, by anterior midgut proteinases revealed that hydrolysis occurred through the formation of intermediate high-Mm products, similar to those formed during oat seed germination. Cysteine proteinases from the cys III peak and heavy trypsins were capable of only limited proteolysis of the protein, whereas incubation with cys II proteinases resulted in substantial hydrolysis of the globulin.

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**Keywords:** *Tenebrio molitor*; Yellow mealworm; Insect digestive proteinases; Cysteine proteinases; Oat 12S globulin

## 1. Introduction

In a companion paper (Vinokurov et al., 2006), the enumeration of digestive proteinases of larvae of the stored product pest *Tenebrio molitor* was provided in connection with the spatial organization of protein digestion in the midgut. In that study, the

pH of the midgut contents increased from 5.2–5.6 in the anterior midgut (AM) to 7.8–8.2 in the posterior midgut (PM). Cysteine proteinases were located mainly in the AM and were represented by a group of anionic fractions with closely related electrophoretic mobility. The activity of cysteine proteinases after electrophoretic fractionation depended on the presence of a reducing agent (dithiothreitol, DTT), and so the predicted low redox potential of the acidic AM is conducive to the activity of cysteine proteinases. Trypsin-like activity, predominant in the alkaline PM, was due to one cationic and three anionic electrophoretic fractions. Chymotrypsin-like proteinases also were prevalent in the PM and consisted of one cationic and four anionic fractions; four fractions had an extended binding site. The activity of *T. molitor* serine proteinases was either unaffected or slightly inhibited by DTT (Elpidina et al., 2005). Both sections of the midgut contained “latent” proteinases, active only in conditions that mimicked the opposite part of the midgut.

**Abbreviations:** AM, anterior midgut; BApNA, *N*<sub>α</sub>-benzoyl-D,L-arginine *p*-nitroanilide; DTT, dithiothreitol; DMF, dimethyl formamide; E-64, L-trans-epoxysuccinyl-L-leucylamido(4-guanidino) butane; GlpAALpNA, pyroglutamyl-alanyl-alanyl-leucine *p*-nitroanilide; GlpFpNA, pyroglutamyl-phenylalanine *p*-nitroanilide; GlpFAPNA, pyroglutamyl-phenylalanyl-alanine *p*-nitroanilide; PB<sub>AM</sub>, physiological buffer of anterior midgut (pH 5.6, 1 mM DTT); PB<sub>PM</sub>, physiological buffer of posterior midgut (pH 7.9 without DTT); PM, posterior midgut; PMSF, phenylmethylsulfonyl fluoride; STI, soybean Kunitz trypsin inhibitor; U, unit of activity; UB, universal buffer.

\* Corresponding author. Tel.: +1 785 776 2780; fax: +1 785 537 5584.

E-mail address: [bsop@ksu.edu](mailto:bsop@ksu.edu) (B. Oppert).

Although we had a great deal of information on the number and types of proteinases in *T. molitor* larvae, the sequential process of protein digestion by these proteinases in vivo was unknown. Dietary protein hydrolysis is affected by physiological conditions in the insect gut, as well as by the relative complement of digestive enzymes and their compartmentalization in the gut. The primary dietary proteins of stored product insects are storage proteins, which are predominant among seed proteins (Popineau, 1991). Within the seed, storage proteins are resistant to proteolysis, and their degradation (mobilization) during germination is a complex regulated process, including the sequential action of several proteinases (Shutov and Vaintraub, 1987; Müntz et al., 2001). The details of seed storage protein hydrolysis in insects are unknown, but it can be assumed that it also is sequential, because a native seed storage protein, phaseolin, from the common bean was hydrolyzed by a purified digestive cysteine proteinase of a bruchid storage pest, *Acanthoscelides obtectus*, to large peptides between 20 and 29 kDa (Wieman and Nielsen, 1988). Therefore, the proteinase initiating the hydrolysis of storage proteins may be a key enzyme to target for the interruption of digestive processes in the insect. When a cysteine proteinase inhibitor was fed to *Callosobruchus maculatus* larvae, 50% mortality was reported (Macedo et al., 2004).

To gain insights into the digestive process in *T. molitor* larvae, the preparative AM and PM extracts were fractionated to isolate digestive proteinases in conditions that preserve enzyme activity. This partial enzyme purification enabled a more detailed characterization of the spectrum of digestive proteinases in this insect, with emphasis on cysteine proteinases. Proteinases from AM fractions were studied further to elucidate their role in the initial stages of hydrolysis of the main dietary protein, oat 12S globulin, by *T. molitor* larvae reared on oat flakes.

## 2. Materials and methods

### 2.1. Chemicals

*L-trans*-epoxysuccinyl-*L*-leucylamido(4-guanidino) butane (E-64) and phenylmethylsulfonyl fluoride (PMSF) were purchased from Sigma (St. Louis, MO); soybean Kunitz trypsin inhibitor (STI) was from Reanal (Budapest, Hungary); *N*<sub>α</sub>-benzoyl-*D,L*-arginine *p*-nitroanilide (BAPNA) and dithiothreitol (DTT) were from Fluka (Buchs, Switzerland). Molecular weight markers, except for STI, were from ICN (Aurora, OH). Other *p*-nitroanilide substrates: pyroglutamyl-phenylalanyl-alanine *p*-nitroanilide (GlpFAPNA), pyroglutamyl-alanyl-alanyl-leucine *p*-nitroanilide (GlpAALpNA), and pyroglutamyl-phenylalanine *p*-nitroanilide (GlpFpNA) were 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) and Sephadex G-100 were from Amersham (Austria). Ultrafiltration membranes YM3 were purchased from Amicon (Beverly, MA).

### 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 weeks before dissection, larvae were transferred to milled oat flakes that were processed at high temperature by the manufacturer and were devoid of active proteases and proteinase inhibitors (data not shown).

### 2.3. Preparation of enzyme extracts

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 divided into two equal parts: anterior midgut (AM) and posterior midgut (PM), and each were 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 supernatant was stored at –70 °C until use.

### 2.4. Enzyme assays and protein determination

Proteolytic activity with 0.5 mM 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), was measured spectrophotometrically at 410 nm by *p*-nitroaniline release. A 100 μl diluted enzyme preparation was mixed with 680 μl of 100 mM acetate-phosphate-borate universal buffer (UB; Frugoni, 1957) 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 absorbance units. 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 were performed with 1 mM DTT in the final reaction mixture. The blanks consisted of the enzyme added to the mixture after termination of the reaction by acetic acid. The proteolytic activity in the assays was proportional to protein concentration and to time. One U of activity was defined as the amount of partially purified enzyme preparation (mg) resulting in an increase of 0.1 absorbance unit per min in 1 mL of reaction mixture. Determinations of enzyme activity were made in 3–5 replicates.

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

### 2.5. pH stability determination

The pH stability of cysteine proteinases was determined after preincubation of enzyme fractions in 10 mM UB, pH 3.0–9.0 (total volume 100 μl) at 25 °C for 2 h. The enzyme solution

was adjusted to pH 5.6 by the addition of 700  $\mu$ L 100 mM UB (with or without 1 mM DTT) containing 0.5 mM GlpFapNA, and after 30 min incubation at 37 °C, cysteine proteinase activity was measured as described earlier. Maximal stability of the enzyme was assumed as 100%.

## 2.6. Gel filtration chromatography

Enzyme extract from preparations of 200 pooled AM or PM, containing 70–80 mg of protein, was applied to a Sephadex G-100 column (2.5 $\times$ 124 cm) equilibrated with 500 mM NaCl in 10 mM phosphate buffer, pH 5.6, containing 0.02% NaN<sub>3</sub>. Fractions of 9.0 mL were collected and analyzed for protein content and activity with different substrates. Active fractions were pooled, concentrated, and desalted on Amicon YM3 membranes and were used for further analysis. To assess the molecular mass (Mm) of enzymes, the column was calibrated with human insulin (6 kDa), horse cytochrome *c* (13 kDa), horse myoglobin (18 kDa), STI (22 kDa), porcine pepsin (36 kDa), ovalbumin (45 kDa), and BSA (67 kDa).

## 2.7. Native PAGE

Native PAGE was performed in a 1 mm 12% polyacrylamide gel in 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 pI (<7.2 pH units), and toward the cathode for proteins with basic pI (>7.2 pH units).

## 2.8. Postelectrophoretic activity detection

Proteolytic activity in the electrophoretic fractions was detected with *p*-nitroanilide substrates BApNA, GlpFapNA, GlpAALpNA, and GlpFpNA by using an overlay of a nitrocellulose membrane impregnated with substrate on the native polyacrylamide gel, as previously described (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, pre-soaked for 40 min in 1 mM solution of substrate in 100 mM UB, pH 5.6 or 7.9, with or without 5 mM DTT, was layered onto the surface of the gel. The membrane was incubated with the gel in a moist chamber at 37 °C for 30–60 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 the formation of pink bands of proteolytic activity, membranes were placed in heat-sealed bags, scanned, and stored at –20 °C. Identification of the electrophoretic activity fractions was according to the calculated  $R_f$  values, as previously described (Vinokurov et al., 2006).

## 2.9. Investigation of oat globulin hydrolysis

A total preparation of oat globulins, primarily the 12S globulin fraction, was isolated from milled oat seeds (var. “Horizon”) by sequential salt extraction, according to a previously described method (Mikola and Jones, 2000). For in vitro hydrolysis of oat globulins, pooled, concentrated, and desalted gel filtration fractions of different proteinases from the AM of *T. molitor* larvae were used. The aliquot of each enzyme preparation used in the study of the initial stages of globulin hydrolysis contained 0.2 U of azocaseinolytic activity, assayed as previously described (Vinokurov et al., 2006) in 0.1 M phosphate buffer, pH 5.6, with 1 mM DTT, incubated for 30 min at 25 °C. Two microliters of 0.025% globulin preparation (w/v) were mixed with 10  $\mu$ L of enzyme fraction containing 5–20  $\mu$ g of protein in 0.1 M phosphate buffer, pH 5.6, with 1 mM DTT and incubated for 30 min at 25 °C. The later stages of globulin hydrolysis were studied with a 4 h incubation interval. As indicated, the enzyme aliquot was preincubated with class-specific inhibitors (8 mM PMSF, specific for serine proteinases, 0.8 mM E-64, specific for cysteine proteinases, or 0.04 mM STI) for 15 min before the addition of substrate. Twelve microliters of nonreducing SDS-PAGE sample buffer were added, and the samples were analyzed by standard SDS-PAGE (Laemmli, 1970) in a 12% acrylamide gel. The separated proteins were stained with 0.15% Coomassie Brilliant Blue R-250, and destained in 15% ethanol and 5% acetic acid. The relative amount of protein in the major 12S globulin band was calculated by means of LabWorks 4.6 software (UVP Bioimaging Systems, CA, USA).

## 3. Results

### 3.1. Fractionation of AM and PM extracts from *T. molitor* larvae

Digestive proteinases in extracts from the AM and PM of *T. molitor* larvae were fractionated by gel filtration chromatography (Figs. 1 and 2, respectively). Each fraction was tested for cysteine, trypsin-like, and chymotrypsin-like proteinase activities with specific *p*-nitroanilide substrates. Fractions of different activity peaks were pooled as indicated in the chromatograms and were concentrated, desalted, and analyzed by activity electrophoresis, using the same substrates under the same conditions.

The highest activity in AM fractions was with the cysteine proteinase substrate GlpFapNA, as measured in the buffer simulating the physiological conditions of the AM (PB<sub>AM</sub>) (Vinokurov et al., 2006), with a pH of 5.6 and 1 mM DTT (Fig. 1A). The AM cysteine proteinase activity eluted in two peaks (cys II and cys III) and one shoulder (cys I). Proteins in the major peak, cys II, were 23 kDa, and the minor peak (cys III) proteins were about 7 kDa. The cys III peak was free of contamination by serine proteinases.

In an analysis of fractions from the PM chromatogram, activity with GlpFapNA was less than in fractions from the AM, and this activity was eluted in three distinct peaks (Fig.

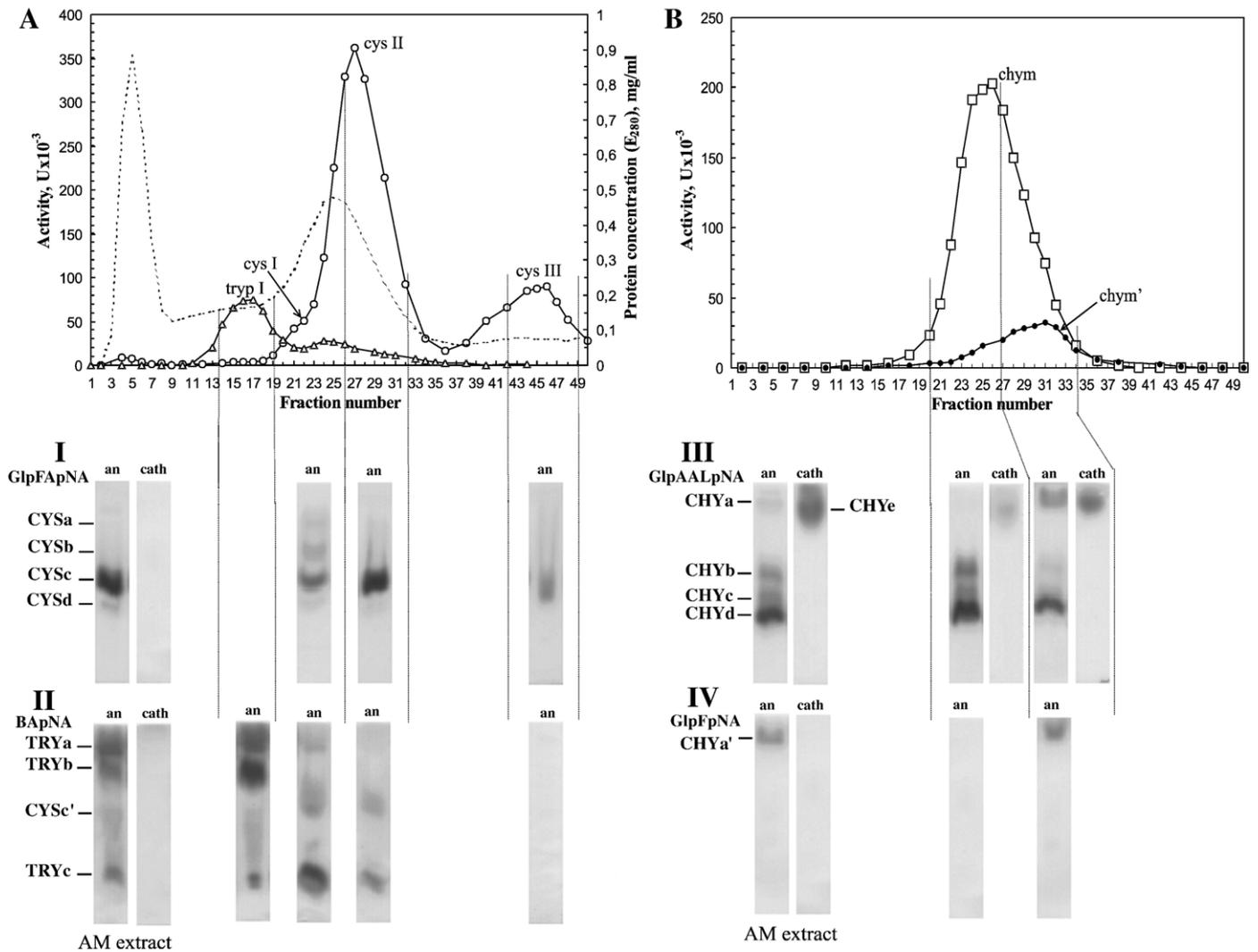


Fig. 1. Gel filtration chromatogram of the AM extract from *T. molitor* larvae. Protein was measured spectrophotometrically at 280 nm, and enzyme activity with specific *p*-nitroanilide substrates was measured at 410 nm. (A) (---) protein; (—○—) GlpFApNA, pH 5.6, 1 mM DTT; (—△—) BApNA, pH 7.9. (B) (—□—) GlpAALpNA, pH 7.9 (—●—); GlpFpNA, pH 7.9. Fractions (as marked by vertical dotted line) were pooled, concentrated, desalted, and subjected to native PAGE followed by postelectrophoretic activity analysis: I—GlpFApNA; II—BApNA; III—GlpAALpNA; IV—GlpFpNA; an—anionic proteins, cath—cationic proteins. Proteinases are denoted to the left (or right for cationic) of each substrate analysis from the total AM extract before chromatography.

2A). The highest activity was found in cys I, containing 38 kDa proteins, and corresponded to the shoulder (cys I) in the AM chromatogram (Fig. 1A). Proteinases in the other two peaks (cys II and cys III) from the PM also had the same Mm as those from the AM. The hydrolysis of GlpFApNA by all proteinases was stimulated in buffers containing DTT. The levels of cys III activities were similar in both midgut sections, whereas the ratio of cys I and cys II activities differed in the AM and PM. In proteinases from the PM, activity with GlpFApNA was latent and was detectable only in PB<sub>AM</sub>, as was previously observed (Vinokurov et al., 2006).

The postelectrophoretic detection of the hydrolysis of GlpFApNA by proteinases in the pooled fractions from either chromatography demonstrated that peaks cys II and cys III contained proteinases from the major activity zone, CYS<sub>c</sub>, observed in the electrophoregrams of unfractionated extracts from both AM and PM in PB<sub>AM</sub> (Fig. 1, I). The most prominent

activity peak in the PM, cys I, was not entirely resolved with cys II and contained, in addition to CYS<sub>c</sub> proteinases, three minor activities of CYS<sub>a</sub>, CYS<sub>b</sub>, and CYS<sub>d</sub> (Fig. 2, I), previously identified as cysteine proteinases by inhibitor analysis (Vinokurov et al., 2006). These same activities were observed as minor activities in AM extracts. Cationic activity with GlpFApNA was not found in the fractions, in agreement with previous data (Vinokurov et al., 2006). Therefore, at least six distinct cysteine proteinase activities were observed in midgut extracts from *T. molitor* larvae.

As previously described, activities with serine proteinase substrates were much greater in PM extracts in alkaline conditions (Vinokurov et al., 2006). Activities from both AM and PM fractions were compared in the buffer simulating the physiological conditions of the PM (PB<sub>PM</sub>) (Vinokurov et al., 2006), with a pH of 7.9 without DTT. In this buffer, the AM fractions contained a combination of native and latent activities

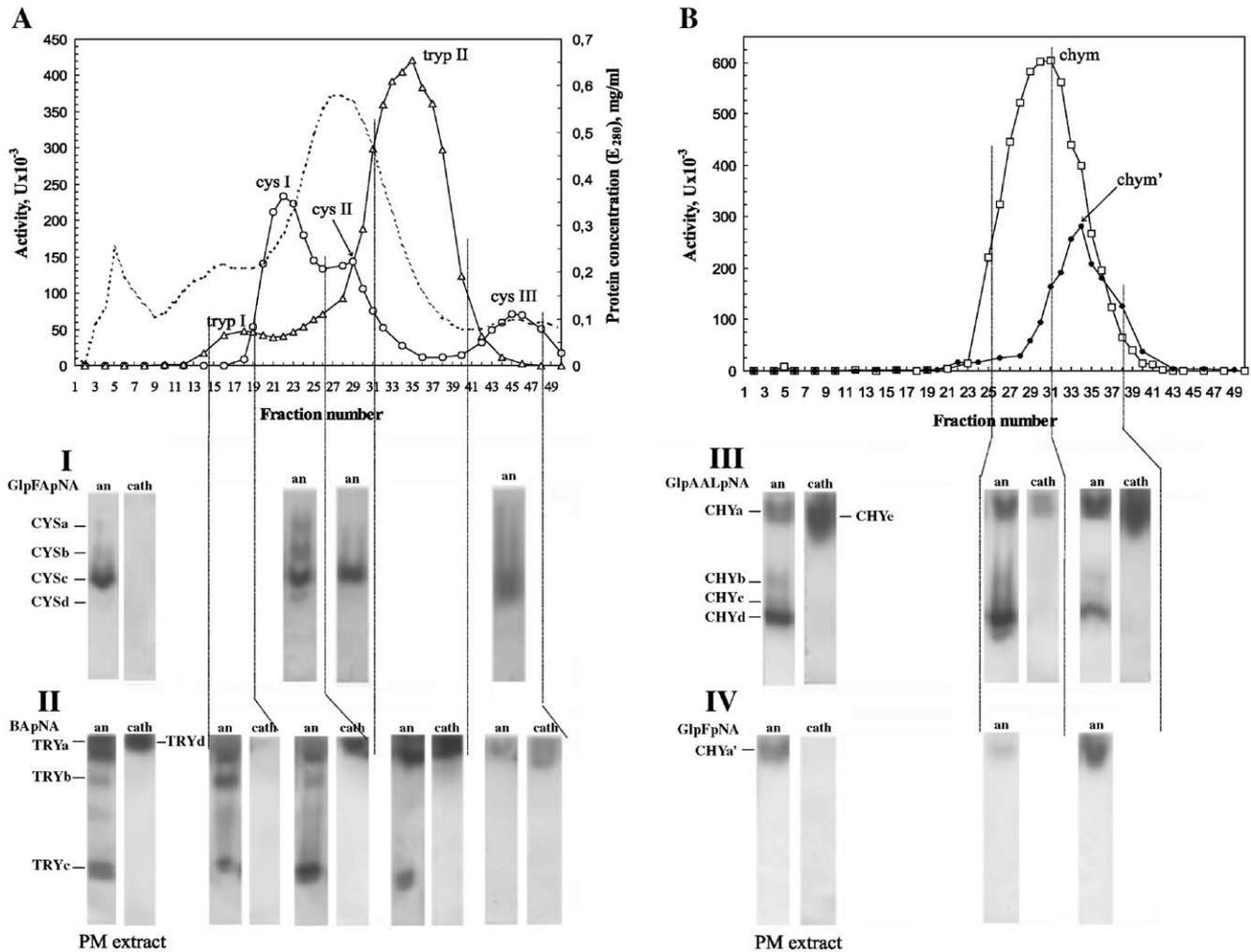


Fig. 2. Gel filtration chromatogram of the PM extract of *T. molitor* larvae. Protein was measured spectrophotometrically at 280 nm, and enzyme activity with specific *p*-nitroanilide substrates was measured at 410 nm. (A) (---) protein; (—○—) GlpFAPNA, pH 5.6, 1 mM DTT; (—△—) BApNA, pH 7.9. (B) (—□—) GlpAALpNA, pH 7.9 (—●—); GlpFpNA, pH 7.9. Fractions (as marked by vertical dotted line) were pooled, concentrated, desalted, and subjected to native PAGE followed by postelectrophoretic activity analysis: I—GlpFAPNA; II—BApNA; III—GlpAALpNA; IV—GlpFpNA; an—anionic proteins, cath—cationic proteins. Proteinases are denoted to the left (or right for cationic) of each substrate analysis from the total PM extract before chromatography.

(Vinokurov et al., 2006). Trypsin-like activity was measured with BApNA. In  $PB_{PM}$ , the major part of this activity was insensitive or slightly inhibited by DTT (data not shown) and, therefore, was not attributed to cysteine proteinases. In the PM chromatogram, BApNA activity eluted in a major peak of “light trypsins” (tryp II) with Mm of 17 kDa, a prominent left shoulder and a minor peak of “heavy trypsins” (tryp I) with approximate Mm of 59 kDa (Fig. 2A). In the AM chromatogram, the activity in the region of tryp II was low, but the peak tryp I of heavy trypsins was greater than in the PM chromatogram. In the post-electrophoretic detection, PM proteinases in tryp II consisted mainly of anionic TRYa, cationic TRYd, and minor amounts of anionic TRYc from prefractionated proteinases (Fig. 2, II). In tryp II proteinases from the AM, only slight activities of TRYc and CYSc' were visible (Fig. 1, II). CYSc' activity was previously characterized as cysteine proteinases (Vinokurov et al., 2006), whereas the activity of TRYc was residual in this region of both chromatograms (AM and PM), and eluted predominantly between peaks tryp I and tryp II. TRYb was the

most prominent activity in the tryp I peak from the AM and PM, but was greater in the AM. In both midgut sections, the tryp I peak also contained TRYa and residual amounts of TRYc. TRYd was the major proteinase activity in the PM, but was absent in the AM.

Chymotrypsin-like activity with GlpAALpNA in  $PB_{PM}$  also was much greater in fractions from the PM than in those from the AM. These proteinases were eluted as a wide single peak, chym, from both AM and PM extracts, with a Mm of 26 kDa in the AM and 22.5 kDa in the PM (Figs. 1 B and 2B, respectively). The postelectrophoretic detection of GlpAALpNA activity in  $PC_{PM}$  by proteinases in the left and right shoulders of each peak was performed separately. In chromatographies of both midgut extracts, the left shoulder contained the majority of activities CHYb–CHYd, and the right shoulder contained mostly CHYa and CHYe (Figs. 1, III and 2, III). The smaller Mm of the chymotrypsin-like activity peak from the PM may be due to the increased level of activity of fractions CHYa and CHYe (from the right shoulder) in this extract.

Activity with GlpFpNA, characteristic for chymotrypsin-like proteinases of mammals, eluted in a smaller peak with Mm of 18.5 kDa (chym'), and also was relatively greater in PM than in AM fractions (Figs. 1B and 2B). The activity coincided with the right shoulder of peak chym with GlpAALpNA and was represented by a single CHYa' electrophoretic activity fraction. The hydrolysis of GlpFpNA was observed only by anionic proteinases (Figs. 1, IV and 2, IV).

### 3.2. Characteristics of cysteine proteinases

Cysteine proteinases are responsible for the majority of proteolytic activity in the AM (Vinokurov et al., 2006), and presumably provide the initial stages of dietary protein hydrolysis. Therefore, detailed characteristics for these proteinases in different chromatographic peaks were obtained.

The effect of pH on the activity of proteinases in cys II and cys III from the AM (Fig. 3A) and cys I, cys II, and cys III from the PM (Fig. 3B) was similar. Activity slowly increased from pH 4.0 to a maximal value at pH 7.0, and sharply decreased at increased pH. At pH 7.9, the same pH as that of the PM, the activity of these proteinases was 31–65% of the activity at pH 5.6, the same as the AM pH, both measured in the presence of DTT. Therefore, the localization of cysteine proteinases in the

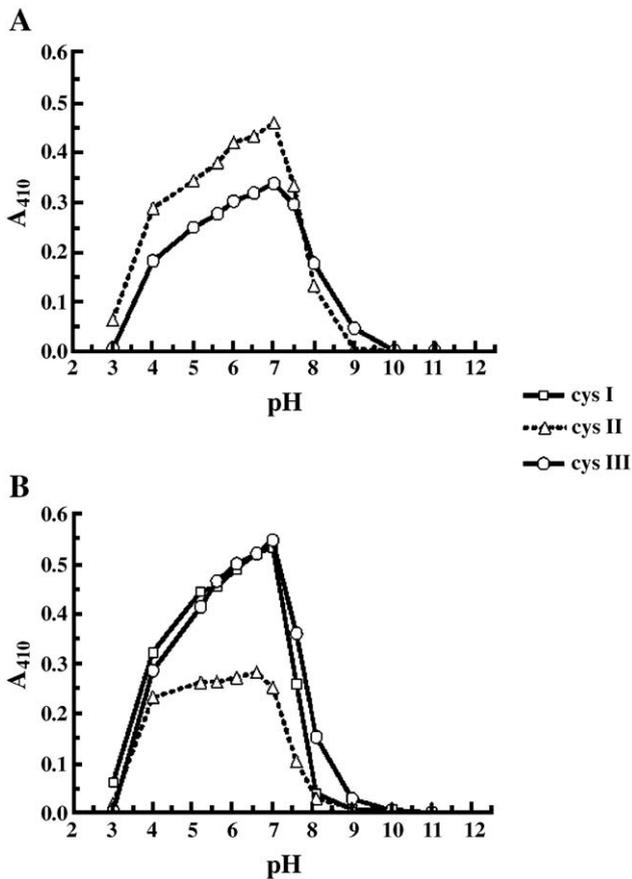


Fig. 3. Effect of pH on the activity of cysteine proteinases with 0.5 mM GlpFpNA from different peaks after gel filtration of AM and PM extracts from *T. molitor* larvae. (A) cys II and cys III from the AM; (B) cys I–cys III from the PM.

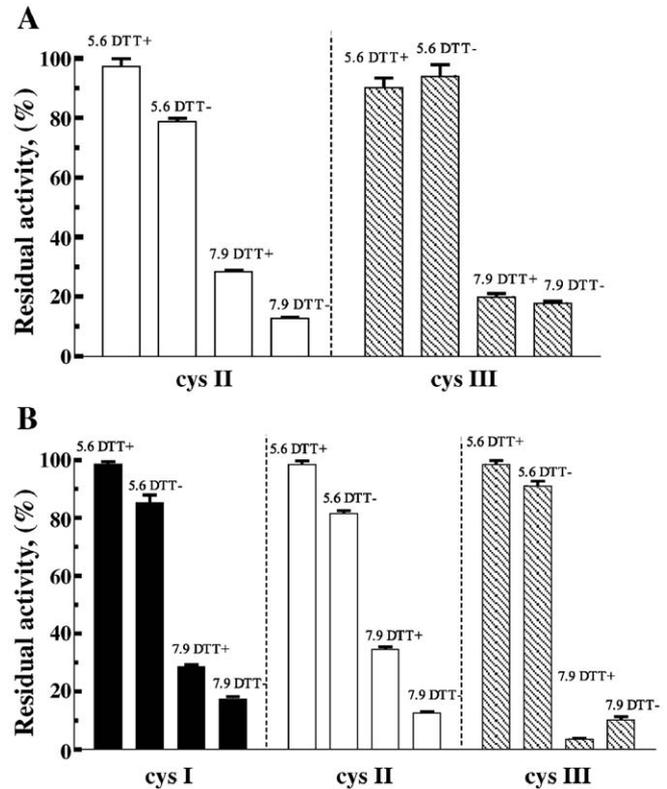


Fig. 4. Effect of DTT (1 mM) on the pH stability of cysteine proteinases from different peaks resulting from gel filtration of midgut extracts of *T. molitor* larvae, with 0.5 mM GlpFpNA as a substrate and measured at pH 5.6 and 7.9. (A) AM cys II and cys III, (B) PM cys I–cys III. Maximal activity of the enzyme was assumed as 100%.

midgut corresponded to the conditions for their optimal activity.

The pH stability of cysteine proteinases also was similar in AM (Fig. 4A) and PM (Fig. 4B) peaks. All proteinases were at least 80% stable in the 4.0–7.0 pH interval, but stability sharply declined outside of this range, as was demonstrated for PM proteinases (Fig. 5). Cysteine proteinases from peaks cys I and cys II were about 3-fold less stable at pH 7.9 than at pH 5.6, whereas for proteinases from cys III, the difference was up to 20-fold greater (Fig. 4). Inclusion of DTT in buffers increased the stability of cys I and cys II by about 20% at pH 5.6 and up to 3-fold at pH 7.9; there was no obvious DTT effect on cys III stability (Fig. 4). The loss of activity for cys II and cys III proteinases from either the AM or PM during a 2 h incubation in PB<sub>PM</sub> was not restored by a subsequent 2 h incubation in PB<sub>AM</sub>, but a slight restoration of activity (14%) was observed for cys I proteinases from the PM under the same conditions. Thus, cysteine proteinases irreversibly lose an essential portion of their activity in moving from the AM to the PM with the food bolus.

### 3.3. In vitro hydrolysis of oat seed globulin by proteinases from the AM of *T. molitor*

A study of the relative contribution of *T. molitor* larval proteinases to the initial stages of hydrolysis of the main storage

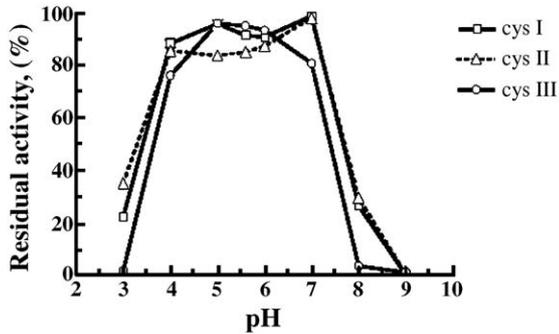


Fig. 5. pH-Stability of cysteine proteinases with 0.5 mM GlpFapNA from different gel filtration peaks (cys I–cys III) of the extracts from the PM of *T. molitor* larval midgut. Maximal activity of the enzyme was assumed as 100%.

protein of oat seeds, 12S globulin, was performed in vitro in  $PB_{AM}$  by using the partially purified proteinase fractions from the AM and class-specific inhibitors (Fig. 6). A non-digested oat globulin preparation was represented by a major protein band, with approximate Mm of 50 kDa, and three minor bands, one heavy (Mm > 100 kDa) and two light (38 and 34 kDa) (Fig. 6, lane 2). After a 30 min incubation of globulin with cysteine proteinase fraction cys II, which also contained chymotrypsin-like activity, only 2% of the initial 50 kDa globulin band remained, and a major intermediate product of 31 kDa was observed (Fig. 6, lane 3). The addition of PMSF, which entirely inhibited chymotrypsin-like activity (Vinokurov et al., 2006), and therefore provided the net impact of cysteine proteinases from cys II, resulted in 4% of the initial 50 kDa band and a 2.5 times increased intermediate product of 31 kDa (Fig. 6, lane 5). By adding E-64, cysteine proteinases in the peak were inhibited and the hydrolysis was due to chymotrypsin-like enzymes, resulting in 17% of the initial 50 kDa band and two intermediate products of 31 and 40 kDa (Fig. 6, lane 4). The combined addition of E-64 and PMSF totally disrupted oat globulin hydrolysis by proteinases in this fraction (Fig. 6, compare lanes 2 and 6). Therefore, cysteine proteinases from the cys II fraction primarily contribute to the initial stage of oat globulin hydrolysis. Highly purified cys III proteinases hydrolyzed

30% of the major globulin band, and approximately the same amount of stained material was found in a 31 kDa intermediate band (lane 8). The formation of 31 kDa band by cys III proteinases was blocked completely by the addition of E-64, but was not susceptible to PMSF (Fig. 6, lanes 9 and 10). The addition of heavy trypsins from tryp I proteinases resulted in a 40% hydrolysis of the initial 50 kDa globulin and formation of 31 and 40 kDa intermediate products with combined intensity equal to the hydrolyzed protein (Fig. 6, lane 12). This hydrolysis was completely prevented by serine proteinase inhibitors PMSF and STI (Fig. 6, lanes 13 and 14) and was not susceptible to E-64 (data not shown). Prolongation of the incubation to 4 h did not change significantly the products of globulin hydrolysis by proteinases from cys III and tryp I peaks, whereas cys II proteinases converted the 31 kDa intermediate polypeptide predominantly to a 24 kDa product (data not shown).

#### 4. Discussion

Digestive proteinases from the AM and PM of *T. molitor* larvae have been separated and further characterized to illustrate the spectrum of digestive proteinases in this insect. A graphic comparison of the levels of proteinase activities in the anterior and posterior *T. molitor* larval midgut provides more information about proteinases previously described in electrophoretic fractions (Vinokurov et al., 2006). Gel filtration is the mildest type of fractionation, and resulted in the separation of proteinases with retention of enzymatic activities (data not shown). In both midgut extracts, the most relevant results were obtained when proteolytic activities were measured in conditions optimal for their activity, and both active and latent proteinases contributed to the activity in each midgut section.

The highest level of activity in the AM eluate was assayed with the cysteine proteinase substrate GlpFapNA, and the identification of cysteine proteinases was supported by inhibitor analysis (Vinokurov et al., 2006). In the PM eluate, the activity of cysteine proteinases was latent and less than the corresponding activity in the AM. The activities of cysteine proteinases in

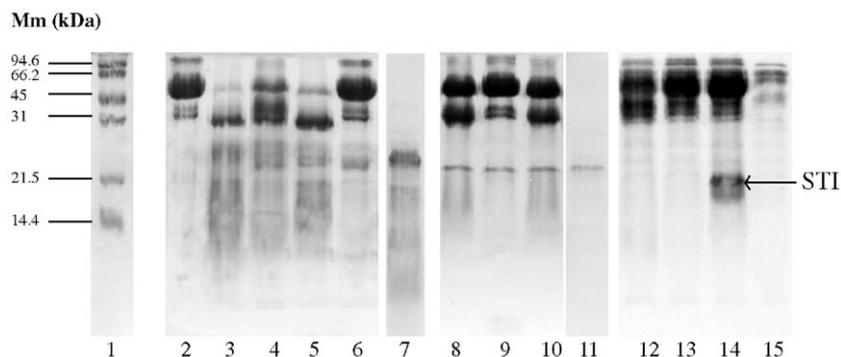


Fig. 6. Hydrolysis of oat globulins by proteinases of *T. molitor* larvae from the AM gel filtrations. Globulins extracted from resting seeds were incubated at pH 5.6 in the presence of 5 mM DTT with the indicated proteinase fraction for 30 min, and the products were analyzed by SDS-PAGE. Where indicated, specific inhibitors were added before the incubation. Lane 1, Molecular mass standards; lane 2, Control: globulin without enzyme; lane 3, Globulin+cys II; lane 4, Globulin+cys II+E-64 (0.4 mM); lane 5, Globulin+cys II+PMSF (5 mM); lane 6, Globulin+cys II+E-64 (0.4 mM)+PMSF (0.8 mM); lane 7, cys II without globulin; lane 8, Globulin+cys III; lane 9, Globulin+cys III+E-64; lane 10, Globulin+cys III+PMSF; lane 11, Cys III without globulin; lane 12, Globulin+tryp I; lane 13, Globulin+tryp I+PMSF; lane 14, Globulin+tryp I+STI; lane 15, Tryp I without globulin.

the AM were resolved into two main peaks (cys II and cys III), and three distinct peaks (cys I, cys II and cys III) were obtained from the PM. Further electrophoretic analysis of proteolytic activities in each peak demonstrated that there were at least six cysteine proteinase activities discernible in the *T. molitor* larval midgut. The major cysteine proteinase activity in the AM was found in the cys II peak, with Mm of 23 kDa. In contrast, the major cysteine proteinase activity in the PM was from a 38 kDa proteinases in cys I. Peak cys III, with similar levels of activity in the AM and PM, had a very low Mm by gel filtration, and was eluted late apparently due to Sephadex affinity. In contrast to our results, Terra and Cristofolletti (1996) resolved cysteine proteinases of *T. molitor* larvae in two fractions, with Mm of 31 and 51 kDa. The 31 kDa proteinases were found only in the PM. In the most recent research report, Cristofolletti et al. (2005) found that the major cathepsin L-like proteinase in the AM from *T. molitor* larvae was 30 kDa, according to SDS-PAGE.

Trypsin-like proteinases in the midgut of *T. molitor* larvae were separated by gel filtration in two main peaks: tryp II with a Mm of 17 kDa, and tryp I with an unusually high Mm of about 59 kDa. tryp II included the major activities in the PM, cationic TRYd and anionic TRYa, which were absent in the AM. These are presumably the trypsins that previously were shown to be synthesized and located in the PM (Ferreira et al., 1990; Cristofolletti et al., 2001). The smaller Mm obtained by gel filtration also was due to retardation on the column, because purified cationic trypsin TRYd (identical to the previously characterized TmT1) had an increased Mm of 25.5 kDa according to SDS-PAGE (Tsybina et al., 2005). Similar discrepancies in the mass estimation of these enzymes were reported earlier by Levinsky et al. (1977), in which the major cationic trypsin from the PM of *T. molitor* larvae was 24.3 kDa by SDS-PAGE and 18.3 kDa by ultracentrifugal analysis.

An electrophoretic fraction with high mobility, TRYc, found in both sections of the midgut, eluted primarily between tryp II and tryp I peaks. The major electrophoretic fraction in the heavy tryp I peak was TRYb, which was latent in the AM, but in PB<sub>PM</sub> was found mainly in the AM, and presumably was synthesized in this midgut section. Alternatively, tryp I proteinases may be oligomers of smaller molecular mass trypsins, presumably TRYc, that form in the acid conditions of the AM. Terra et al. (1985) reported that PM trypsin from *T. molitor* larvae had more Mm (46 kDa) than typical trypsins. The authors proposed that the enzyme dimerizes under their experimental conditions. Heavy trypsins with Mm of 56 and 63 kDa were found in the midgut of another coleopteran, *Melolontha melolontha* (Wagner et al., 2002). Two high Mm trypsins (70 and 67 kDa) also were reported in the midgut of the larvae of the lepidopteran *Heliothis virescens* when larvae were fed a natural diet containing proteinase inhibitors, whereas insects fed inhibitor-free artificial diet lacked high Mm trypsins (Brito et al., 2001). The authors provided some evidence that these heavy trypsins could be formed by the oligomerization of light trypsins.

The results of gel filtration confirmed our previous observations of two types of chymotrypsin-like proteinases in *T. molitor* larvae (Vinokurov et al., in press). The major portion of this type of activity was due to chymotrypsin-like proteinases

with an extended binding site, hydrolyzing only the long chain chymotrypsin peptide substrate GlpAALpNA. The results of the early papers on the characteristics of serine digestive proteinases from several insects (Terra and Ferreira, 1994), in which chymotrypsin-like activity was low, were probably due to the use of short chain chymotrypsin substrates, and the activity of chymotrypsin-like proteinases with an extended binding site was not detected.

To characterize the changes in the activity of cysteine proteinases that are translocated along the midgut with the food bolus, the effect of pH on the activity and stability of partially purified cysteine proteinases from the AM (cys II and cys III) and PM (cys I–cys III) was studied. The pH stability of cysteine proteinases was tested after an interval of two hours, which is slightly less than the passage time of food in the gut of *T. molitor* larvae (Terra et al., 1985). The characteristics of all fractions from the AM and PM were similar. Digestive cysteine proteinases of *T. molitor* larvae were active and stable in the pH of the first two thirds of the midgut; in the last third of the midgut, these enzymatic parameters were decreased significantly. The major proteolytic activities in the PM were from serine proteinases. These results agree with previous data (Terra and Cristofolletti, 1996; Cristofolletti et al., 2005).

Finally, the spectrum of digestive proteinases in *T. molitor* larvae was related to food digestion in the insect. The main dietary protein for larvae grown on oat flakes is 12S globulin, the main storage protein of oat seeds (Mikola and Jones, 2000). In the course of seed germination, this protein is sequentially hydrolyzed by different seed cysteine proteinases. An intermediate product with Mm about 35 kDa was formed under the limited action of a cysteine proteinase, followed by exhaustive hydrolysis by other cysteine proteinases (Mikola and Jones, 2000). Therefore, we evaluated whether the sequential digestion of 12S globulin by *T. molitor* also occurs in the larval midgut. To study the initial stages of 12S globulin hydrolysis, we evaluated the effect of partially purified enzyme preparations from *T. molitor* larval AM in buffers similar to the AM conditions at short periods of incubation, close to a quarter of food passage time through the larval midgut (Terra et al., 1985). The most effective hydrolysis of the major seed globulin was observed with cysteine proteinases from fraction cys II. Chymotrypsin-like proteinases from the same fraction were less effective. Cysteine proteinases from the fraction cys III, and “heavy” trypsin-like enzymes from fraction tryp I, produced only partial hydrolysis of the globulin. In all cases, high Mm intermediate products were formed. Incubation with both cysteine proteinase fractions led to the formation of a 31 kDa product, similar to the intermediate product formed by cysteine proteinases of germinated oat seeds. Serine proteinases produced 31 and 40 kDa products. When the time of incubation was increased to twice the time of food passage, the effects of proteinases from cys III and tryp I peaks did not change significantly. The effect of cys II proteinases was extended, and the main product of hydrolysis was a 24 kDa polypeptide.

Thus, the two main fractions of cysteine proteinases, cys II and cys III, from the AM of *T. molitor* larvae demonstrate similar pH optima and stability, but have different roles in the

dietary globulin hydrolysis. The hydrolysis of oat 12S globulin by the main larval digestive proteinases from the AM proceeds through the formation of high Mm intermediate products, similar to the products formed during oat seed germination. cys III and tryp I proteinases are capable of performing only limited proteolysis of the protein, whereas cys II proteinases perform more extensive hydrolysis of the globulin.

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