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ScienceDirect
Biochimie 90 (2008) 508–514
BIOCHIMIE
www.elsevier.com/locate/biochi

Research paper

Localization of post-proline cleaving peptidases in *Tenebrio molitor* larval midgut

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Received 16 August 2007; accepted 6 November 2007
Available online 17 November 2007

Abstract

Two soluble post-proline cleaving peptidase activities, PPCP1 and PPCP2, were demonstrated in *Tenebrio molitor* larval midgut with the substrate benzyloxycarbonyl-L-alanyl-L-proline *p*-nitroanilide. Both activities were serine peptidases. PPCP1 was active in acidic buffers, with maximum activity at pH 5.3, and was located mainly in the more acidic anterior midgut lumen. The dynamics of PPCP1 activity and the total activity of soluble digestive peptidases in the course of food digestion were similar, suggesting that the enzyme participates in protein digestion. PPCP2 is a nondigestive soluble tissue enzyme evenly distributed along the midgut. An increase in the activity of PPCP2 was observed in buffers of pH 5.6–8.6 and was maximal at pH 7.4. The sensitivity of PPCP2 to inhibitors and the effect of pH are similar to prolyl oligopeptidases with a cysteine residue near the substrate binding site.

Keywords: Digestive peptidase; Prolyl oligopeptidase; *Tenebrio molitor*; Serine peptidase; Yellow mealworm

1. Introduction

Proline is a unique genetically coded imino acid in proteins, and peptide bonds formed by proline are cleaved only by post-proline cleaving peptidases (PPCPs) [1], including prolyl oligopeptidases (POPs) (EC 3.4.21.26) [2,3]. POPs have been widely reported as cytosolic enzymes [2]. A membrane form of POP was isolated from the mammalian brain [4,5]. Extracellular POP activity is present in mammalian plasma [6,7] and also is secreted by the protozoan *Trypanosoma cruzi* [8,9].

Although POPs have been found in all major groups of living organisms, and several POPs were purified and characterized, their functions have been studied mainly in mammals. Many investigations have addressed the physiological role of POP, usually by the use of specific inhibitors, activity measurements in clinical samples, and (neuro) peptide degradation studies, but the exact functions of this enzyme remain obscure. POP is thought to be involved in neurodegenerative and affective disorders, and probably cell death, protein secretion and the phosphoinositide pathway [10]. A fungus, *Aspergillus niger*, secretes another type of post-proline cleaving endopeptidase, a member of the S28 family of clan SC of serine peptidases [11].

Abbreviations: AM, anterior midgut; BBMV, brush border membrane vesicles; DFP, diisopropylfluorophosphate; DTT, dithiothreitol; E-64, L-transepoxysuccinyl-L-leucylamido(4-guanidino) butane; LAP, leucine aminopeptidase; LpNA, L-leucine *p*-nitroanilide; PPCP, post-proline cleaving peptidase; pepst, pepstatin A; PM, posterior midgut; PMSF, phenylmethylsulphonyl fluoride; POP, prolyl oligopeptidase; UB, universal buffer; ZAPpNA, benzyloxycarbonyl-L-alanyl-L-proline *p*-nitroanilide; ZPPal, benzyloxycarbonyl-proline-prolinal.

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doi:10.1016/j.biochi.2007.11.002

In this study, we present the first data on digestive and gut tissue PPCPs in insects. Two different PPCPs were identified in the midgut of a stored product pest, the yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae).

2. Materials and methods

2.1. Animals

Actively feeding fourth instar *T. molitor* larvae were reared on a mixture of wheat flour, bran and brewer's yeast at 26 °C and were transferred to milled oat flakes (Raisio, Finland) 1–1.5 wk prior to dissection.

To study the dynamics of proteolytic activity in the course of food digestion, larvae were maintained without food on a wire mesh to exclude consumption of feces. After 3 d starvation, the larvae were transferred to milled oat flakes wetted with water (1:1), fed for 2 h, and transferred back to empty dishes. A control group consisted of unfed (starved) larvae. Four experimental groups were selected from actively feeding larvae, obtained after 2, 4, 6 and 8 h from the beginning of feeding ($n = 10$). The larvae from the control and experimental groups were immediately dissected, and enzyme extracts were prepared as described in Section 2.2.

2.2. Preparation of enzyme extracts

Preparation of enzyme extracts was performed at 4 °C. Larvae were dissected and the midgut was isolated and sectioned into halves representing the anterior midgut (AM) and posterior midgut (PM). The tube of the isolated AM and PM sections was split longitudinally, the contents were extruded, and the tissue was rinsed with 0.9% saline. The lumen contents or the remaining gut tissues from 50 AM or PM were homogenized separately in double-distilled water, and the homogenate was clarified by centrifugation as previously described [12]. The same procedure was used to study the dynamics of proteolytic activity. All supernatants were stored at −70 °C.

2.3. Isolation of brush border membrane vesicles (BBMV)

The isolation of BBMV was performed according to Wolfersberger et al. [13] at 4 °C. Three hundred frozen AM or PM sections containing lumen contents were thawed, suspended in 15 ml of 17 mM Tris–HCl buffer, pH 7.5, 300 mM mannitol, 5 mM EGTA (buffer A), and homogenized in a glass Downce homogenizer with subsequent passage through a gel loading pipette tip. An equal volume of 24 mM $MgCl_2$ was added to the homogenate, the mixture was thoroughly suspended, incubated on ice for 15 min, and centrifuged at 1500*g* for 20 min. The pellet contained cell debris, subcellular organelles, endoplasmic reticulum and basement–lateral membranes (p1). The supernatant (s1) was further centrifuged at 20,800*g* for 30 min, and the resulting supernatant (s2) contained ribosomes and soluble components of the cytoplasm and midgut contents. The pellet, containing crude BBMV (p2), was thoroughly resuspended in 3 ml of buffer A, mixed with an equal volume of 24 mM $MgCl_2$ and centrifugations at 1500*g* and 20,800*g* were repeated. The resulting supernatant (s3) was combined with s2 and was designated as S. The pellet resulting from the second 20,800*g* centrifugation and containing purified BBMV (P2) was suspended in buffer A. The pellets after 1500*g* centrifugations were combined, designated as P1, and also suspended in buffer A. The resulting fractions S, P1 and P2 were assayed for activity of PPCP and leucine aminopeptidase (LAP), a marker of the BBMV fraction [14].

2.4. Enzyme activity assays

The specific activity of peptidases was assayed with chromogenic *p*-nitroanilide substrates spectrophotometrically according to Erlanger et al. [15] at 405 nm using a StatFax 2100 microplate reader (Awareness Technology Inc., FL) using a differential filter, 492 nm. PPCP activity was assayed with 0.3 mM benzyloxycarbonyl-L-alanyl-L-proline *p*-nitroanilide (ZAPpNA). The substrate was synthesized from benzyloxycarbonyl-L-alanine and L-proline *p*-nitroanilide according to a previously described procedure [16] and was initially dissolved 20 mM in dimethylformamide. Three microliters of 20 mM ZAPpNA were added to each well containing 0.25 equivalents of AM or PM extracts from one larval equivalent, diluted to 197 µl with 0.1 M universal acetate–phosphate–borate buffer (UB) [17] of appropriate pH. The mixture was incubated at 37 °C and the absorbance was measured periodically. The initial (zero time) absorption was subtracted. Enzyme activity was calculated in nmoles/min per gut on the linear part of the time– and protein concentration–response curves. The effect of pH on PPCP activity was determined with 0.3 mM ZAPpNA in 0.1 M UB in the pH range 3.9–8.9.

The activity of LAP was assayed with 0.5 mM L-leucine *p*-nitroanilide (LpNA) (Sigma) at pH 7.9 [14] with the same procedure.

The total proteolytic activity of extracts was assayed with azocasein [18]. Enzyme extracts (0.15 AM or PM equivalents) were diluted to 100 µl with 0.1 M UB, pH 5.6 for those from the AM and pH 7.9 for the PM, and were incubated with 200 µl of 1% azocasein solution in the same buffer for 60 min at 37 °C. The enzyme reaction was terminated by the addition of 300 µl of 12% trichloroacetic acid. The mixture was incubated for 30 min at 4 °C for precipitate formation, and then was centrifuged for 5 min at 10,000*g*. An equal volume of 0.5 M NaOH was added to the supernatant, and the absorbance was measured at 450 nm using a 630 nm differential filter. Total proteolytic activity was calculated in units/min per gut, where 1 unit was defined as ΔOD equal to 1.

All determinations of enzyme activity were made in 3–5 replicates in two or three independent experiments.

2.5. Electrophoretic studies and post-electrophoretic activity detection

Native PAGE was performed in 12% separating and 4% stacking gels with 35 mM HEPES and 43 mM imidazole buffer,

pH 7.2, according to McLellan [19] at 10 mA and 4 °C for 45 min. Usually the electrophoresis was performed towards the anode, and 0.3 equivalents of either AM or PM extracts were loaded in each well.

The detection of the specific proteolytic activity of PPCP in electrophoregrams was performed with the *p*-nitroanilide substrate ZAPpNA, using an overlay on the native polyacrylamide slab of a nitrocellulose membrane impregnated with substrate [20]. After electrophoresis, individual lanes of the gel were excised, and each lane was incubated for 20 min at 25 °C in 0.1 M UB, pH 5.6 or 7.9, with or without specific inhibitors: 1 mM $HgCl_2$, 1 μM L-transepoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), 1 μM pepstatin A (pepst), 10 mM EDTA, 1 mM phenylmethylsulphonyl fluoride (PMSF), 1 mM diisopropylfluorophosphate (DFP), 1 μM Z-Pro-prolinal (ZPPal), or an activator of SH-dependent enzymes, 3 mM dithiothreitol (DTT). A nitrocellulose membrane, pre-soaked for 60 min in 0.3 mM solution of substrate in 0.1 M UB, pH 5.6 or 7.9, was layered onto the surface of the gel. The membrane was incubated with the gel in a moist chamber at 37 °C for 75 min. Liberated *p*-nitroaniline was diazotized, and PPCP activity was visualized as pink bands on a white membrane. Relative enzymatic activity in each band was calculated by means of LabWorks 4.6 software measuring relative band intensity values (UVP Bioimaging Systems, USA).

3. Results

3.1. Detection and localization of PPCP activity in the midgut of T. molitor

The activity of PPCP with the specific substrate ZAPpNA was assayed at the physiological pH of midgut contents: 5.6 in the AM and 7.9 in the PM [12,21]. The measurements were performed with the extracts of (i) the lumen contents of the AM and PM, where secreted digestive enzymes are located and (ii) the AM and PM tissue, containing membrane digestive and tissue enzymes (Table 1). The activity of PPCP in the extracts of AM and PM tissue was demonstrated at both pH values with comparable activity. In the extracts of AM and PM lumen contents, detectable activity was evident only at pH 5.6. This activity was determined to be due to insect enzyme(s) secreted into the contents, because the extract of larvae food, oat flakes, lacked PPCP activity at pH 5.6.

Table 1
PPCP activity with 0.3 mM ZAPpNA in the extracts from different parts of *T. molitor* midgut (mean ± SE)

Midgut part		Activity, nmole/min per gut		Activity in the presence of 1 mM $HgCl_2$, nmole/min per gut	
		pH 5.6	pH 7.9	pH 5.6	pH 7.9
AM	Contents	1.10 ± 0.17	0.08 ± 0.05	1.05 ± 0.13	0
	Wall	1.86 ± 0.17	2.25 ± 0.19	0	0
PM	Contents	0.44 ± 0.13	0.09 ± 0.06	0.39 ± 0.14	0
	Wall	1.97 ± 0.18	2.36 ± 0.19	0	0

To distinguish between PPCP proteinases, we used an inhibitor of SH-dependent enzymes, 1 mM $HgCl_2$. Incubation with this reagent resulted in the complete inhibition of PPCP activity in the AM and PM tissue. However, the activity of PPCP in the AM and PM lumen contents at pH 5.6 was insensitive to 1 mM $HgCl_2$. Therefore, the midgut tissue and lumen contents contain different enzymes. PPCP activity in the midgut lumen contents, hypothesized to participate in digestion, was 2.5-fold higher in the AM than in the PM, was observed only at pH 5.6, and was insensitive to 1 mM $HgCl_2$. The activity in the midgut tissue was similar in both midgut parts, was observed at both pH values, and was completely inhibited by mercuric salt.

To examine whether the PPCP from midgut tissue is a membrane-bound enzyme and involved in food digestion, we isolated BBMV from the AM and PM and tested PPCP activity in different cellular fractions (Table 2). Leucine aminopeptidase (LAP) was used as a marker of BBMV integrity, as 75% of the activity of this digestive enzyme is located in the BBMV of the PM in *T. molitor* larvae, and the remainder is in the cytoplasm [17,21]. The majority of PPCP activity in the AM and PM at both pH values was observed in the supernatant S, which contained ribosomes and soluble components. The pellet P2, containing BBMV, lacked PPCP activity, and only traces of the activity were found in the pellet P1 containing the remaining subcellular components and cell debris. LAP activity was predominantly located in BBMV (P2) from the PM, confirming the integrity of BBMV. Therefore, PPCP activity from the midgut tissue was due to soluble proteins and presumably was nondigestive.

3.2. Effect of pH on PPCP activity

The effect of pH on PPCP activity with ZAPpNA was assayed in the AM and PM lumen contents and tissue and was calculated per gut (Fig. 1). Profiles of the effect of pH on PPCP activities in the AM and PM lumen contents were

Table 2
Distribution of PPCP activity with 0.3 mM ZAPpNA and LAP activity with 0.5 mM LpNA in subcellular fractions of AM and PM (mean ± SE)

Subcellular fraction		Activity, nmole/min per gut		
		PPCP		LAP
		pH 5.6	pH 7.9	pH 7.9
AM	Homogenate	2.88 ± 0.28	2.56 ± 0.29	5.23 ± 0.54
	P1[a]	0.10 ± 0.02	0.16 ± 0.02	0.52 ± 0.15
	P2[b]	0	0.05 ± 0.01	0.13 ± 0.05
	S[c]	2.38 ± 0.26	2.20 ± 0.22	2.51 ± 0.25
PM	Homogenate	1.33 ± 0.15	2.38 ± 0.28	73.78 ± 7.28
	P1[a]	0.16 ± 0.02	0.10 ± 0.01	12.95 ± 1.21
	P2[b]	0	0.05 ± 0.01	54.58 ± 5.41
	S[c]	1.23 ± 0.15	2.07 ± 0.27	28.78 ± 2.87

[a] The pellet contained cell debris, subcellular organelles, endoplasmic reticulum and basement–lateral membranes.
[b] The pellet contained BBMV.
[c] The supernatant contained ribosomes and soluble components of the cytoplasm and midgut contents.

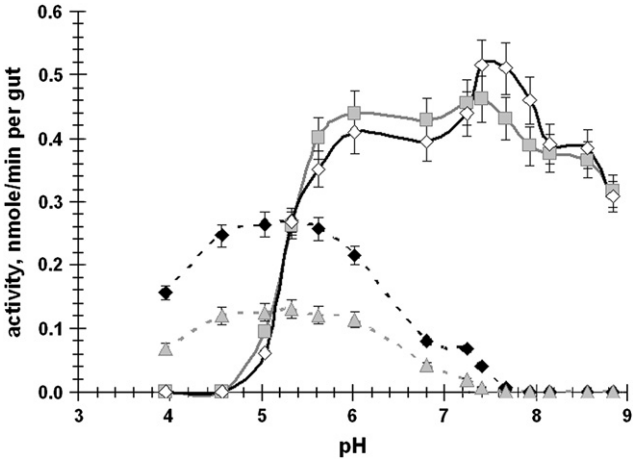

Fig. 1. Effects of pH on the activity of extracts from the lumen contents of AM (---◆---) and PM (---△---), and the tissues of AM (—□—) and PM (—◇—) with 0.3 mM ZAPpNA.

similar and are in agreement with the previous observation of the predominant localization of this activity in the AM. PPCP activity was observed in acidic pH buffers. The highest values were in the pH range 4.6–6.0, with a maximum in pH 5.3 buffer. At pH 7.9, PPCP activity was low.

The effect of pH on PPCP activities from AM and PM tissues also was similar, although these enzymes were active in a broader pH interval than the enzymes from the contents. The activities increased in the pH range 5.6–8.6, with a flat maximum in pH 7.4 buffer. No activity was detected at pH values below 4.6.

3.3. Post-electrophoretic activity testing and analysis

Detailed characteristics of *T. molitor* larvae PPCPs were obtained by a combination of activity electrophoresis and inhibitor analysis of extracts from the AM and PM lumen contents and tissue (Fig. 2). PPCP activity with ZAPpNA in all four extracts was represented by a single electrophoretic fraction migrating to the anode at pH 7.2 (Fig. 2A). Cationic fractions were not observed in any preparation (data not shown). The relative electrophoretic mobilities of enzymes from the lumen contents of the AM and PM were identical. The relative mobilities of PPCPs from the PM and AM tissues also were identical, but the mobility of tissue enzymes was lower than that of PPCPs from the contents. The relative mobilities of PPCP activities did not coincide with any of the previously characterized activities of trypsin-like, chymotrypsin-like or cysteine midgut peptidases of *T. molitor* larvae [12].

Inhibition studies were performed on electrophoretically separated PPCPs to avoid the interference of other midgut peptidases. Specific inhibitors of serine, cysteine, aspartic and metallopeptidases, and a nonspecific inhibitor and activator of SH-dependent peptidases, $HgCl_2$ and DTT, respectively, were used to classify PPCPs. PPCPs from the lumen contents of the AM and PM were insensitive to a cysteine peptidase inhibitor, E-64, an aspartic peptidase inhibitor, pepstatin, and a metallopeptidase inhibitor, EDTA (Fig. 2B). PPCPs from the lumen contents also were insensitive to the nonspecific reagents that react with SH-groups, $HgCl_2$ and DTT. General inhibitors of serine peptidases, PMSF and DFP, as well as a specific inhibitor of POP, ZPPal, partially inhibited both enzymes to similar degrees. Therefore, we predict that the AM and PM lumen contents contain the same enzyme(s), PPCP1.

PPCPs from the AM and PM tissue also were insensitive to E-64, pepstatin and EDTA, but were partially inhibited by PMSF and completely inhibited by DFP and ZPPal and so were classified as serine peptidases (Fig. 2C). PPCPs from the midgut tissue were completely inhibited by $HgCl_2$ and were activated by DTT and therefore were SH-dependent enzymes. Activities from the AM and PM tissue were predicted to be due to the same enzyme(s), PPCP2.

3.4. Dynamics of PPCP activities in the course of food digestion

Enzymatic assays in the course of food digestion were performed with extracts from starved larvae, and after 2, 4, 6 and 8 h from the beginning of feeding, to compare the dynamics of PPCP activities in *T. molitor* larval midgut to the total activity of digestive peptidases. The assays with ZAPpNA for PPCP1 from the AM and PM lumen contents were at pH 5.6. For PPCP2 from the AM and PM tissues, enzymatic assays were at pH 7.9. The dynamics of the total soluble activity of digestive peptidases was monitored with azocasein at physiological pH: 5.6 for the AM lumen contents and 7.9 for the PM lumen contents [12,21] (Fig. 3). The dynamics of PPCP1 was similar to that of the total proteolytic activity both in the AM and PM contents. In the AM, the activities increased 1.5-fold after 2 h of feeding, then decreased gradually, and after 6 h reached the levels of starved animals, but increased again at 8 h. In the PM, PPCP1 and total proteolytic activities gradually decreased, and after 6 h reached a minimum, followed by an increase at 8 h to the level of starved larvae.

The dynamics of PPCP2 from the midgut tissue differed from the dynamics of enzymes in midgut contents. PPCP2 activity, both in the AM and PM, increased at the beginning of feeding and reached a maximum after 4 h in the AM and 6 h in the PM.

4. Discussion

Using the chromogenic peptide substrate ZAPpNA, we demonstrated for the first time two different PPCP activities in the midgut of *T. molitor* larvae. The activity was from soluble proteins, one found in the lumen contents of the midgut, where secreted digestive enzymes are located, and another in the soluble fraction of the midgut tissue. The data on the effect of pH, electrophoretic mobility, and inhibitor analysis indicate that the activities from the AM and PM lumen contents likely represent the same enzyme(s), designated PPCP1, and the activities from the AM and PM tissues represent a distinct enzyme(s), PPCP2. Approximately 70% of the PPCP1 activity was located in the AM, similar to the distribution of cysteine

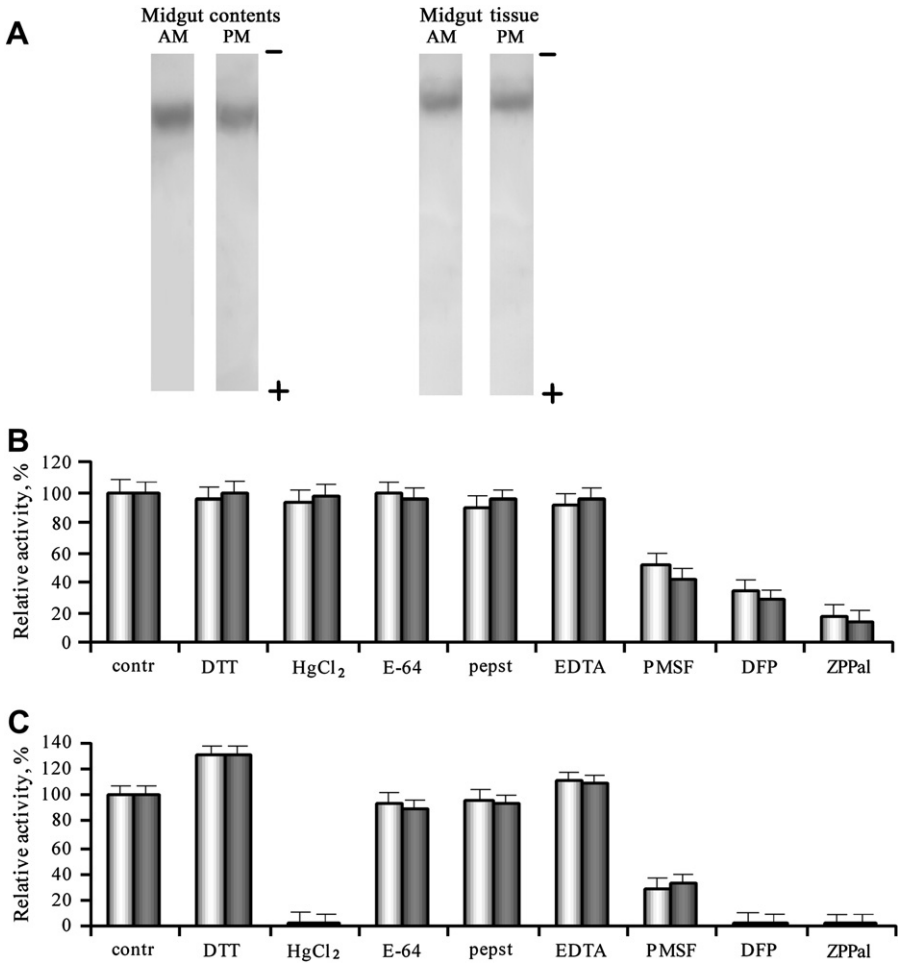

Fig. 2. Post-electrophoretic detection of ZAPpNA hydrolysis by enzymes in AM and PM lumen contents tested at pH 5.6, and in AM and PM tissues tested at pH 7.9 (A), and post-electrophoretic inhibitor analysis of the same activity from midgut lumen contents (B) and midgut tissue (C). AM and PM activities are represented by light and dark columns, respectively. Individual lanes of the corresponding extract were post-electrophoretically incubated with or without inhibitors or activator followed by the detection of the residual proteolytic activity as described in Section 2.5. The relative values of residual activities were expressed as a percentage of the activity of untreated enzyme (control). Each column represents the mean ± S.D. of three independent experiments.

peptidases, the major digestive enzymes in *T. molitor* AM [12]. PPCP2 activity in the midgut tissue was evenly distributed along the midgut.

Both PPCPs were serine peptidases, as was demonstrated by the inhibitor analysis, but their characteristics were different. Tissue enzyme PPCP2 was similar to the strongly conserved and best-characterized post-proline cleaving enzyme, serine peptidase POP (EC 3.4.21.26) [2,22]. The activity of PPCP2 was maximal at pH 7.4, and POPs usually have a pH optimum from 7.0 to 8.0. The PPCP2 activity was completely inhibited by a general inhibitor of serine peptidases, DFP, and a specific inhibitor of POPs, ZPPal and was partially inhibited by PMSF, also characteristics of POPs [1,23]. The sensitivity of enzyme activity to the nonspecific sulfhydryl-modifying reagents, $HgCl_2$ and DTT, again characteristic for PPCP2, also was observed for POP from *T. cruzi* [8] and porcine brain [24] due to a Cys residue situated close to the substrate binding site.

The activity of secreted extracellular PPCP1 was maximal at pH 5.3, within the pH range of the AM contents (5.2–5.6) [12]. PPCP1 was only partially sensitive to DFP and ZPPal, and insensitive to $HgCl_2$ and DTT. Therefore, PPCP1 presumably does not belong to the POP family S9 of clan SC peptidases (http://merops.sanger.ac.uk). In a recent study, the extracellular PPCP from *A. niger*, with an acidic pH optimum, was referred to as a member of the S28 family of clan SC [11]. PPCP1 is localized in the midgut lumen contents of *T. molitor* larvae and is predicted to participate in digestion.

We expected to find digestive PPCP in *T. molitor* midgut, due to the amino acid content of the proteins in the insect's diet. *T. molitor* is a stored product pest and lives primarily on grain products. The major proteins in crop seeds are storage proteins, as much as 72% of the total protein in wheat kernels [25]. Therefore, storage proteins are the main components of the natural protein diet of *T. molitor*. Most storage proteins have an unusually high content of proline, about 15–30% in prolamines, 12–16% in glutelins, and 7% in albumins of wheat grains [25]. To confirm the participation of PPCP1 in food digestion, we compared the dynamics of PPCP activity and the total activity of soluble digestive peptidases in the course of

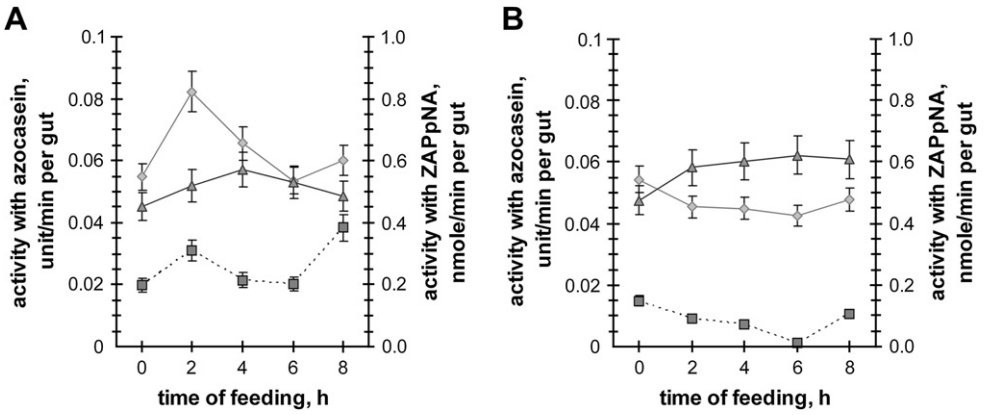

Fig. 3. Dynamics of PPCP1 (---■---), PPCP2 (—△—) and the total proteolytic activitiy of midgut lumen contents (—◇—) from the AM (A) and the PM (B) in the course of food digestion. The assays were performed with ZAPpNA, for PPCP1 from the AM and PM contents at pH 5.6, and for PPCP2 from the AM and PM tissues at pH 7.9. The total soluble activity of digestive peptidases from the midgut contents was monitored with azocasein at the physiological pH for each midgut section: 5.6 for the AM contents, and 7.9 for the PM contents.

food digestion for 8 h after starvation. The dynamics of both PPCP1 and digestive enzymes was similar, and therefore we predict that PPCP1 is a digestive peptidase and is secreted or activated together with the major digestive peptidases of the larvae.

The dynamics of tissue PPCP2 activity in the course of food digestion differed from that of the total proteolytic activity of midgut contents. This enzyme is located within the midgut tissue and is nondigestive.

5. Conclusion

Two different soluble PPCP activities classified as serine proteinases were identified in the midgut of *T. molitor* larvae. PPCP1, with an acidic pH optimum, is a digestive proteinase localized predominantly in the AM contents. PPCP2, with an alkaline pH optimum, is a tissue enzyme and is evenly located along the midgut wall. The characteristics of PPCP2 are similar to those of POP.

Acknowledgements

This work was supported by the Russian Foundation for Basic Research (Grant no. 06-04-49147-a). Mention of trade names or commercial products in this publication is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the US Department of Agriculture. This is Contribution Number 08-39-J from the Kansas Agricultural Experimental Station.

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