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A digestive prolyl carboxypeptidase in *Tenebrio molitor* larvae

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ABSTRACT

Prolyl carboxypeptidase (PRCP) is a lysosomal proline specific serine peptidase that also plays a vital role in the regulation of physiological processes in mammals. In this report, we isolate and characterize the first PRCP in an insect. PRCP was purified from the anterior midgut of larvae of a stored product pest, *Tenebrio molitor*, using a three-step chromatography strategy, and it was determined that the purified enzyme was a dimer. The cDNA of PRCP was cloned and sequenced, and the predicted protein was identical to the proteomic sequences of the purified enzyme. The substrate specificity and kinetic parameters of the enzyme were determined. The *T. molitor* PRCP participates in the hydrolysis of the insect's major dietary proteins, gliadins, and is the first PRCP to be ascribed a digestive function. Our collective data suggest that the evolutionary enrichment of the digestive peptidase complex in insects with an area of acidic to neutral pH in the midgut is a result of the incorporation of lysosomal peptidases, including PRCP.

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1. Introduction

Prolyl carboxypeptidase (PRCP; PCP, lysosomal carboxypeptidase, angiotensinase C, EC 3.4.16.2) belongs to a group of proline specific peptidases (PSP) (Cunningham and O'Connor, 1997) that are involved in the regulation of various metabolic processes (Vanhoof et al., 1995). PSP represent a relatively small group of highly specific exo- and endo-peptidases that cleave bonds formed by proline residues in proteins and peptides. The unique activity of PSP is due to the structural features of proline, the only cyclic imino acid among 20 α-amino acids found in proteins and peptides. Peptide bonds containing proline residues are not hydrolyzed efficiently by most peptidases, and a proline residue in the peptide chain serves to protect against degradation by enzymes with broad specificity. The ability of PSP to hydrolyze this bond determines the specific activity of the enzyme in the regulation of metabolic processes.

PRCP are serine peptidases that catalyze the cleavage of a substrate at the C-terminal amino acid linked to a proline residue. At present, only PRCP from human (Odya et al., 1978; Tan et al., 1993; Shariat-Madar et al., 2002), pig (Yang et al., 1970; Kakimoto et al., 1973), monkey (Suzawa et al., 1995), and bacterium *Xanthomonas maltophilia* (Suga et al., 1995) have been isolated and studied. The primary structure of the isolated enzyme, present as a dimer in solution, has been described only for human PRCP, and the enzyme was assigned to the S28 family of serine peptidases (Tan et al., 1993). The crystal structure was solved recently and revealed that PRCP has a unique peptidase structure, with closest identity to dipeptidyl peptidase 7 (DPP7), containing a conserved α/β hydrolase domain and a novel helical SKS domain that caps the active site with the catalytic Ser-Asp-His triad (Abeywickrema et al., 2010; Soisson et al., 2010).

PRCP is widely distributed in human tissues, but mostly it is localized to the lungs, liver and placenta (Tan et al., 1993). The

Abbreviations: AM, anterior midgut; Bz, benzoyl; DMF, dimethylformamide; DPP, dipeptidyl peptidase; Glp, pyroglutamyl; pNA, *p*-nitroanilide; PM, posterior midgut; PAGE, polyacrylamide gel electrophoresis; POP, prolyl oligopeptidase; PRCP, prolyl carboxypeptidase; PSP, proline specific peptidases; Suc, succinyl; UB, universal buffer; Z, N-benzyloxycarbonyl.

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enzyme was initially found in human lysosomes (Yang et al., 1970; Kumamoto et al., 1981), but later it was found as a membrane-expressed enzyme in cultured human umbilical vein endothelial cells, explained by the association of PRCP with specific membrane proteins after exocytosis (Shariat-Madar et al., 2004).

The exact physiological functions for PRCP are not completely understood. The key role for mammalian PRCP is in regulating blood pressure (Kumamoto et al., 1981; Tamaoki et al., 1994; Kaplan and Ghebrehiwet, 2010; Hagedorn, 2011), but PRCP is also involved in processes of proliferation (Duan et al., 2011), inflammation (Kumamoto et al., 1981; Ngo et al., 2009) and angiogenesis (Mallela et al., 2009). PRCP also regulates food intake by inactivating α-melanocyte stimulating hormone (Wallingford et al., 2009; Shariat-Madar et al., 2010; Diano, 2011; Jeong et al., 2012). The only report of insect PRCP details changes in expression of the PRCP gene in response to magnesium exposure in *Culex quinquefasciatus* larvae (Zhao et al., 2010).

The present research on insect PSP is a part of our studies of digestive enzymes in larvae of the yellow mealworm, *Tenebrio molitor* (Coleoptera: Tenebrionidae), a pest of processed grains and stored products. The complex of digestive peptidases in *T. molitor* larvae differs substantially from human digestive enzymes, although both the beetle and people have grain products as a primary food source. The major digestive organ of the larvae is the midgut, where a sharp pH gradient is found, from 5.6 in the anterior midgut (AM) to 7.9 in posterior midgut (PM) (Terra et al., 1985; Vinokurov et al., 2006a; Elpidina and Goptar, 2007). This gradient restricts the activity of different digestive enzymes in specific compartments of the midgut, which is usually consistent with the pH-optima of their activity (Vinokurov et al., 2006b).

The major digestive peptidases in the AM of *T. molitor* larvae are cysteine peptidases, represented by four to six distinct enzymes (Terra and Cristofoletti, 1996; Vinokurov et al., 2006a,b; Prabhakar et al., 2007). The major cysteine peptidase activity is cathepsin L (Cristofoletti et al., 2005; Beton et al., 2012; Oppert et al., 2012) known as a lysosomal peptidase in eukaryotes (Turk et al., 2012). In the PM of *T. molitor* larvae, digestive enzymes are mostly serine peptidases, including four trypsin-like and five chymotrypsin-like serine peptidases (Tsybina et al., 2005; Elpidina et al., 2005; Vinokurov et al., 2006a,b) as well as a membrane-bound aminopeptidase (Cristofoletti and Terra, 1999, 2000) and soluble carboxypeptidase (Ferreira et al., 1990; Prabhakar et al., 2007).

The major dietary proteins of *T. molitor* larvae, prolamins, contain 30–50% glutamine and 10–30% proline residues (Shewry and Tatham, 1990; Shewry and Halford, 2002). Therefore, we analyzed the major post-glutamine cleaving activities in the *T. molitor* larval digestive complex and found that they were cysteine peptidases (Goptar et al., 2012). Based on the composition of their diet, we also predicted the occurrence of digestive PSP in *T. molitor* larvae. Indeed, we described the first proline-specific digestive peptidase in the midgut, which was a serine peptidase that specifically cleaved after proline, had an acidic pH optimum (5.3), and was found mainly in the AM contents (Goptar et al., 2008a,b), but the enzyme was not identified. In the present study, we identify this enzyme as a PRCP and detail the substrate specificity and kinetic parameters of the first purified PRCP in an insect. Our data suggest that PRCP is a digestive enzyme in *T. molitor* larvae, which is a novel function for PRCP.

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 3–5 days prior to dissection. Oat flakes processed at high temperature were devoid of active peptidases and their inhibitors (data not shown). Midguts were isolated on ice in 0.9% NaCl and divided into two parts, AM and PM. The AM preparations were homogenized at 4 °C in double-distilled water in a glass Downce homogenizer (approximately 50 AM parts in 350 μl of water) and centrifuged for 10 min at 15000 *g*. The supernatants were stored at −70 °C.

2.2. Purification of the peptidase

The clarified extract from 200 pooled AM 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 spectrophotometrically at 280 nm. Enzymatic activity in the fractions was assayed with N-protected peptide substrate Z-FR in 10 μl aliquots, and the chromogenic substrate benzyl-oxycarbonyl-L-alanyl-L-alanyl-L-proline *p*-nitroanilide (Z-AAP-pNA) in 80 μl aliquots as described in the next sections. Active fractions corresponding to the major peak of activity were pooled, concentrated and desalted on Amicon YM3 membranes (Amicon, the Netherlands) at 4 °C and used immediately for further analysis, or were stored at −70 °C.

The pooled fraction was applied to a FPLC Mono Q column (Pharmacia, Sweden), equilibrated with 10 mM phosphate buffer, pH 6.8, and eluted in a 0–1 M NaCl gradient in the same buffer with steps 16% (0.16 M) and 22% (0.22 M) NaCl; the eluate was collected in 1.0 ml fractions. Enzyme activity in the fractions was assessed in 50 μl aliquots with Z-AAP-pNA. Active fractions were pooled, desalted and concentrated, and loaded onto a FPLC Phenyl-Sepharose column equilibrated with 10 mM phosphate buffer, pH 6.8, containing 1.7 M $(NH_4)_2SO_4$. The elution was performed in the same buffer in 1.7–0 M gradient of $(NH_4)_2SO_4$ with steps 60% (1.02 M), 50% (0.85 M) and 40% (0.68 M) of $(NH_4)_2SO_4$. Fractions of 1.0 ml were collected and tested for enzymatic activity using 30 μl aliquots. Fractions active with Z-AAP-pNA were pooled, desalted and concentrated.

2.3. Enzyme assays using chromogenic p-nitroanilide substrates

Activities of peptidases were calculated using the initial rates of hydrolysis of chromogenic *p*-nitroanilide substrates determined spectrophotometrically, according to Erlanger et al. (1961) at 405 nm with a StatFax 2100 microplate reader (Awareness Technology Inc., Westport, CT) using a 492 nm differential filter. Post-proline cleaving activity with chromogenic substrates was assayed with Z-AAP-pNA, Z-AP-pNA, AP-pNA, all in 0.5 mM concentration, Z-GP-pNA, Z-P-pNA P-pNA, all in 0.3 mM concentration. The substrates were synthesized according to standard procedures (Peptide Synthesis Protocols, 1994) and initially dissolved in dimethylformamide (DMF) at 20 mM concentration. Three or five μl of 20 mM substrate were added to each well containing 10–80 μl of enzyme solution at 0.1–8 mg/ml concentration diluted to 197 μl or 195 μl with 0.1 M universal acetate-phosphate-borate buffer (UB) (Frugoni, 1957) adjusted to pH 5.6. Activities of trypsin-like, chymotrypsin-like, cysteine peptidases and aminopeptidases were assessed by the hydrolysis of specific substrates Bz-R-pNA (Tsybina et al., 2005), Glp-AAL-pNA (Lyublinskaya et al., 1987; Elpidina et al., 2005), Glp-FA-pNA (Stepanov et al., 1985; Vinokurov et al., 2006b) and L-pNA, respectively, all in 0.5 mM concentration at pH 5.6 or 7.9 UB. The mixture was incubated at 37 °C, and the absorbance was measured periodically. Enzyme activity was calculated in nmoles/min on the linear part of the time and protein concentration response curves. All assays were run in triplicate.

The post-proline cleaving activity of the purified enzyme with the substrates Suc-AAPF-pNA and Suc-AAPL-pNA was evaluated by chromatography of the hydrolyzate. Seven μl of the enzyme preparation were added to 190 μl of UB, pH 5.6, followed by 2.5 μl 53 mM substrate solutions in DMF. The control contained 7 μl of distilled H_2O instead of enzyme. The reaction mixture was incubated overnight at 37 °C, and 10 μl aliquots were analyzed on a ProntoSil 120-5C18AQ column (2.0 × 75 mm). Elution was performed in 0.1% CF_3COOH and a linear gradient of MeCN 0–80% over 20 min.

2.4. Assay of post-proline cleaving activity with ninhydrin reaction

Post-proline cleaving activity with the substrate Z-PF was assayed by a ninhydrin reaction.

The enzyme solution probe of 2–12 μl was diluted with 100 mM UB, pH 5.6, to a final volume of 53 μl, and 2 μl of 120 mM substrate solution in dimethyl sulfoxide were added. The reaction mixture was incubated for 1–2 h, and 20 μl aliquots of the reaction mixture were withdrawn and diluted with 180 μl of 0.1% ninhydrin solution in ethanol containing 1% acetic acid. The samples were tightly covered and incubated for 1.5 h at 70 °C. After incubation, the absorbance of solutions was measured at 545 nm.

2.5. Gliadin hydrolysis assay

Gliadin hydrolysis was evaluated by a ninhydrin reaction as described previously (Goptar et al., 2012).

2.6. Kinetic studies

All kinetic studies were performed on a spectrophotometer U-2800A (Hitachi, Japan) at 30 °C, with substrate concentrations ranging from 0.01 to 0.5 mM in UB, pH 5.6, containing 2.5% DMF. The K_i of the enzyme with Z-Pro-prolinal was determined with the substrate Z-AAP-pNA at 0.5 mM concentration; inhibitor concentrations were from 2.5 to 19 μM. Kinetic parameters were determined by the Lineweaver–Burk method.

2.7. Electrophoretic studies and postelectrophoretic activity detection

Electrophoresis was carried out under native conditions in 8% polyacrylamide gels containing 35 mM HEPES and 43 mM imidazole at pH 7.2 according to McLellan (1982). An aliquot of enzyme with predetermined activity of 1 nmol/min was added to each well. Electrophoresis was performed toward the anode at a constant current of 20 mA for 90 min at 4 °C. For protein detection, the gel was stained in 0.1% (w/v) Coomassie Brilliant Blue G-250 in 30% ethanol and 10% acetic acid. Gels were destained in the same solution without dye and scanned.

Postelectrophoretic detection of specific proteolytic activity was performed using a nitrocellulose overlay impregnated with specific *p*-nitroanilide substrate Z-AAP-pNA (Vinokurov et al., 2005). After electrophoresis, the resolving gel was washed for 15 min in 100 mM UB, pH 5.6, and was overlaid with a 0.45 μm 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, 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 formation of the pink bands representing proteolytic activity, membranes were placed in heat-sealed plastic bags, scanned and stored at −20 °C.

2.8. Mass spectrometry analyzes

Mass spectrometry was performed as described previously (Prabhakar et al., 2007). The purified peptidase preparation was subjected to native electrophoresis, and the stained protein band, corresponding to the proteolytic activity band with the substrate Z-AAP-pNA in a parallel lane, was excised and subjected to in-gel tryptic hydrolysis. The tryptic peptides were subjected to MALDI-TOF MS and MS/MS analyzes. Mass spectra were recorded on an Ultraflex II MALDI-TOF-TOF mass spectrometer (Bruker Daltonik, Bremen, Germany) equipped with a UV laser (Nd). Mass spectra were obtained in the positive ion mode using the reflectron, and the accuracy of the measured masses after additional calibration using trypsin autolysis peaks was 0.01%. Fragmentation spectra were obtained in the tandem mode LIFT, and the mass measurement accuracy of fragments was 1 Da.

2.9. Cloning of T. molitor PRCP cDNA

Total RNA from the insect gut was isolated using TRIzol Reagent (Invitrogen Life Technologies). A SMART RACE cDNA amplification kit (Clontech, Saint-Germain-en-Laye, France) was used for cDNA synthesis and amplification of 5′ and 3′ ends of PRCP cDNA, as described previously (Matz et al., 1999). Degenerate oligonucleotide primers were designed on the basis of the multiple alignment of PRCP from a closely related tenebrionid *Tribolium castaneum* (XP_971305.1), other insects including *Aedes aegypti* (XP_001651053.1), *Drosophila melanogaster* (NP_610037.1), and *Apis mellifera* (XP_623670.3), as well as *Homo sapiens* (NP_005031.1). Sequences of degenerate primers used for SMART RACE cDNA amplification are in Table 1. Single-stranded cDNA was amplified from total RNA by SMART RACE, and double-stranded cDNA was obtained by Long-Distance PCR (Zhu et al., 2001). 3′- and 5′-fragments of the peptidase cDNA were obtained by 3′- and 5′ RACE. PCR reactions were performed in a 25 μl reaction mixture, containing 1× Advantage KlenTaq Polymerase Mix with provided buffer (Clontech, Saint-Germain-en-Laye, France), 200 μM dNTPs, 0.15 μM of degenerate primers, 0.02 μM of 'heel-carrier' oligo and 0.15 μM of 'heel-specific' oligo (Table 1). PCR was carried out on a MJ Research PTC-200 DNA Thermal Cycler (Ramsey, MN) for 26 cycles. Each PCR cycle included 95 °C for 7 s; 63 °C for 20 s; 72 °C for 1 min. Fragments obtained from 3′- and 5′ RACE were cloned in pGem T-easy vector (Promega, Madison, WI) and sequenced using an Amersham Biosciences MegaBACE 1000 automated sequencer (GE Healthcare Life Sciences, Piscataway, NJ) and FS dye terminator chemistry.

Oligonucleotide primers 'TenDir full' and 'TenRev full' for full-length cDNA amplification were designed on the basis of the

Table 1
Degenerate and cloning primers used to amplify cDNA for PRCP.

TenDir1	5′-GCCGAACAYCGRTAYTAYGG (AEHRYYG)
TenDir2	5′-GGGGGCTCKTAYGGYGGWATG (GGSYGGM)
TenDir3	5′-TTCCAGGCKTGYACYGARATG (FQACTEM)
TenRev1	5′-GGTACARGCYTGRAARTCCCA (WDFQACT)
TenRev2	5′-AGGGTAKGGRTARTTKACCAT (MVNYPYP)
TenRev3	5′-CGCTGCTAACATKCCBCCRTA (YGGMLAA)
SMART PCR primer	5′-AAGCAGTGGTATCAACGCAGAGT
'heel-carrier' primer	5′-GTAATACGACTCACTATAGGGCAAGCAGTGGT ATCAACGCAGAGT
'heel-specific' primer	5′-GTAATACGACTCACTATAGGGC
TenDir-full	5′-ATGGGTCAAGGTTCAACAATC
TenRev-full	5′-GACTGTAACATTACTACCAGC

sequencing data (Table 1). The complete cDNA product was generated by PCR using SMART amplified cDNA as templates. PCR was performed in the same reaction conditions/reagents as before, using gene-specific primers (TenDir/Rev-full), and fragments were cloned in pGem T-easy vector (Promega, Madison, WI) and sequenced.

2.10. Bioinformatics analysis

Nucleotide sequence analysis was performed using NCBI blastx (http://www.ncbi.nlm.nih.gov/blast/). PRCP primary structures used for comparison with the enzyme from *T. molitor* were taken from the NCBI database, and included PRCP from *T. castaneum* (XP_971305.1), *A. aegypti* (XP_001651053.1), *D. melanogaster* (NP_610037.1), *A. mellifera* (XP_623670.3), *Danio rerio* (NP_001002694.1), *Arabidopsis thaliana* (NP_201377.2), *Mus musculus* (AAH55022.1) and *H. sapiens* (NP_005031.1). Multiple alignment of sequences was performed using ClustalW (http://www.ebi.ac.uk/Tools/clustalw2/index.html) (Larkin et al., 2007) and visualized in GeneDoc (Nicholas et al., 1997). Identification of a signal peptide was made with the program SignalP (http://www.cbs.dtu.dk/services/SignalP/) (Petersen et al., 2011).

3. Results

3.1. Purification of T. molitor PRCP

Previously, digestive PSP activity was described in the AM contents of *T. molitor* larvae (post-proline cleaving peptidase, PPCP1) (Goptar et al., 2008a,b). To further characterize this PSP, we used a three-step chromatography process to purify the enzyme from an AM extract. These steps included gel-filtration on Sephadex G-100 column (Fig. 1A), ion-exchange on Mono Q (Fig. 1B), and hydrophobic chromatography on Phenyl-Sepharose columns (Fig. 1C). The elution profile of proteins from the Sephadex G-100 column contained two peaks of post-proline cleaving activity. According to our previous data (Goptar et al., 2008a,b), the digestive PSP (PPCP1) was eluted in the major peak 1 of activity with the chromogenic substrate Z-AAP-pNA. Fractions comprising this peak also displayed activity with the peptide substrate ZPF, selective for PRCP, so the pooled elution from peak 1 was further purified. Due to the simplicity of activity testing with chromogenic substrates, further purification was monitored by the hydrolysis of Z-AAP-pNA. The results of the purification are summarized in Table 2. The enzyme was purified 1200-fold, with an activity yield of 12.5%. An electrophoretic separation of the purified fraction revealed one predominant protein band (Fig. 1D, lane 1), and single activity band with the substrate Z-AAP-pNA (Fig. 1D, lane 2), and the electrophoretic mobilities of both bands were identical.

Table 2
Purification of the PRCP from the AM extract of *T. molitor* larvae. Enzyme activity was determined with 0.5 mM Z-AAP-pNA. Protein was determined spectrophotometrically at 280 nm.

Purification stage	Total protein, mg	Total activity, nmol/min	Specific activity, nmol/min*mg	Fold purification	Yield, %
AM extract	192	120	0.625	1	100
Sephadex G-100	8	81	10.1	16	67.5
Mono Q	0.32	32	100	160	26.7
Phenyl Sepharose	0.02	15	750	1200	12.5

3.2. Cloning of T. molitor PRCP cDNA and deduced amino acid sequence analysis

A complete cDNA sequence encoding PRCP was obtained from cDNA prepared from total RNA isolated from the AM of *T. molitor* larvae, using a PCR-based strategy. Initial fragments were amplified

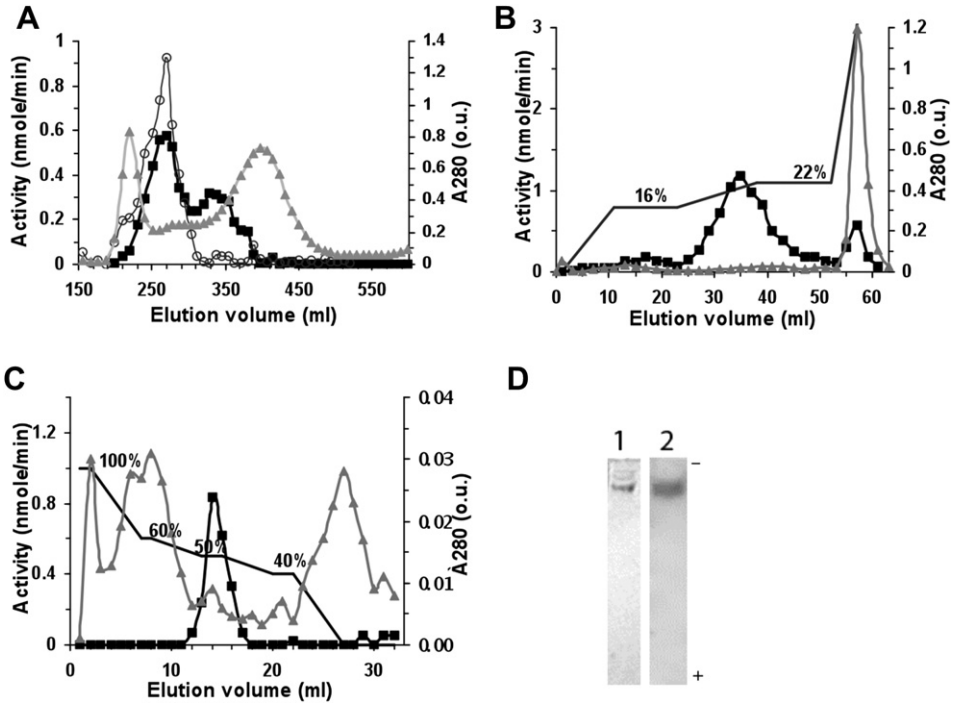

Fig. 1. Purification of the digestive PSP from the extract of *T. molitor* larvae AM. Chromatography of on (A) Sephadex G-100, (B) Mono Q and (C) Penyl-Sepharose columns. Activity was determined in the 0.1 M UB, pH 5.6, with Z-AAP-pNA (1A, B, C, closed squares) or Z-PF (1A, open circles), as substrates. Absorbance at 280 nm is marked with closed triangles. Content of NaCl (1B) or $(NH_4)_2SO_4$ (1C) is shown as a line without markers. (D), native PAGE of the purified preparation: Lane 1 – protein bands were stained with Coomassie G-250, lane 2 – postelectrophoretic detection of the enzyme activity with the Z-AAP-pNA as a substrate, pH 5.6.

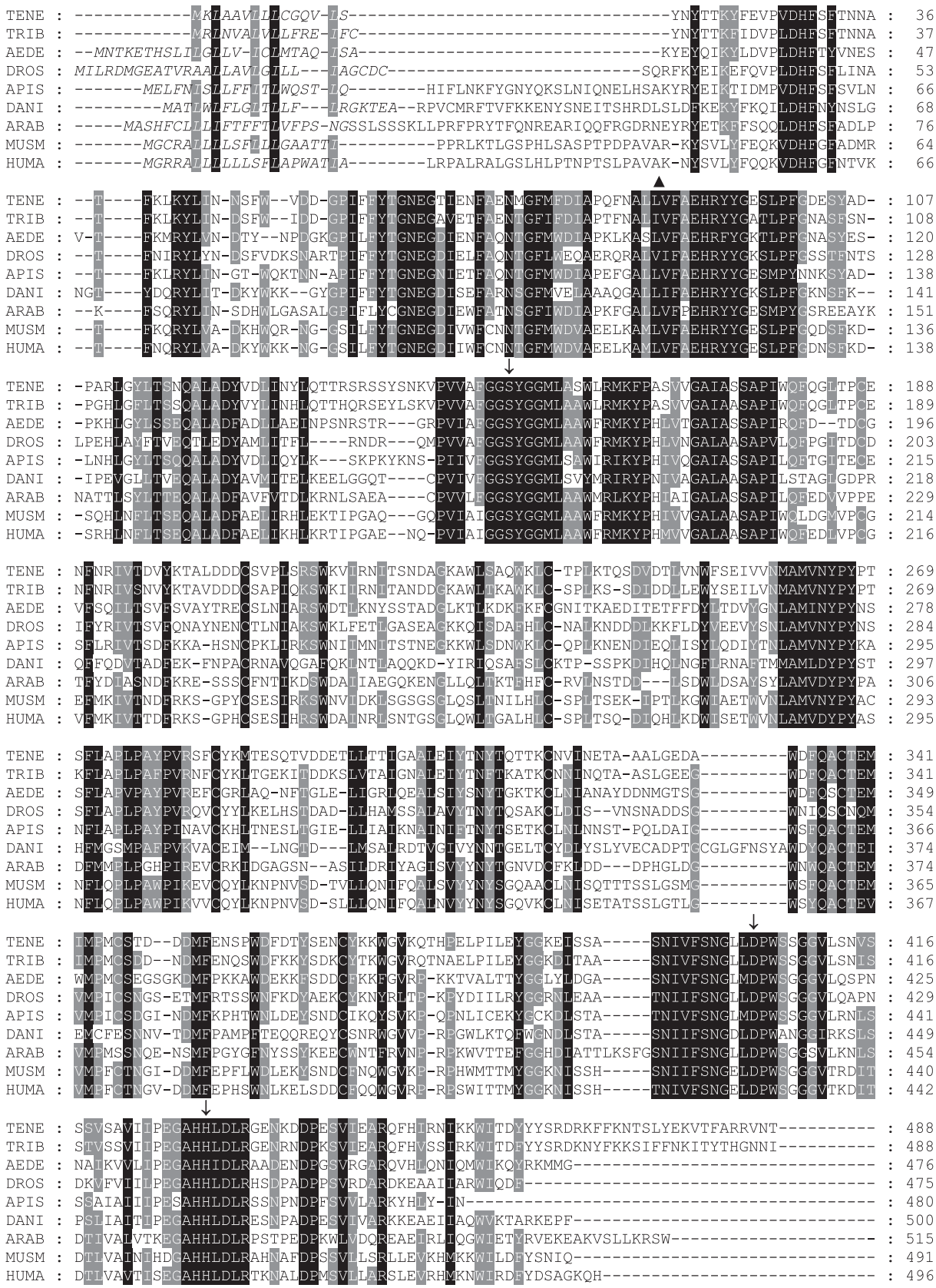

Fig. 2. Alignment of PRCP from *T. molitor* (TENE, ACA09612.1) with sequences from *Tribolium castaneum* (TRIB, XP_971305.1), *Aedes aegypti* (AEDE, XP_001651053.1), *Drosophila melanogaster* (DROS, NP_610037.1), *Apis mellifera* (APIS, XP_623670.3), *Danio rerio* (DANI, NP_001002694.1), *Arabidopsis thaliana* (ARAB, NP_201377.2), *Mus musculus* (MUSM, AAH55022.1) and *Homo sapiens* (HUMA, NP_005031.1). Arrows indicate conserved residues of the catalytic triad – Ser, Asp, His. N-terminal of the mature human PRCP is marked with black triangle (▲). Signal peptides predicted by SignalP program are in italics.

from cDNA by SMART RACE using degenerate primers directed toward conserved amino acid sequences in *T. castaneum* PRCP and those of *A. aegypti*, *D. melanogaster*, *A. mellifera* and *H. sapiens* PRCPs. The cDNA of *T. molitor* larvae PRCP contained a single long open reading frame of 1464 nucleotides, which encoded a predicted polypeptide of 488 amino acids (Supplemental Fig. 1S). The PRCP sequence was deposited in GenBank database with accession #ACA09612.

The predicted amino acid sequence of *T. molitor* PRCP was aligned with annotated PRCP sequences from different organisms (Fig. 2). The PRCP sequence from *T. molitor* was 73% identical to *T. castaneum* PRCP and was from 47 to 72% similar to PRCP sequences from other organisms (S28 family of serine peptidases according to the MEROPS classification (Rawlings et al., 2010)). The predicted signal peptides in the N-terminal of PRCP contained from 15 to 22 amino acid residues, with a large number of hydrophobic residues. The N-terminus of the mature enzyme was determined experimentally only for human PRCP (Tan et al., 1993), and the propeptide included 24 amino acid residues. Predicted propeptides of similar size have been found also in PRCP from species of different classes: insect (Hymenoptera) *A. mellifera*, fish *D. rerio*, mammals *M. musculus* and *H. sapiens*, and plant *A. thaliana.* Interestingly, the propeptide from another predicted insect PRCP, dipteran *D. melanogaster*, contained only two amino acid residues, SQ, and the propeptide was not found in our PRCP from *T. molitor*, as well as in another coleopteran *T. castaneum*, and dipteran *A. aegypti.*

3.3. Comparison of cloned PRCP and purified larval peptidase

To confirm that the sequence of cloned PRCP correlates to the purified protein, the protein band of purified enzyme was excised from a Coomassie G250-stained gel (Fig. 1D, lane 1) and subjected to tryptic digestion and subsequent MS and MS/MS analyzes. The MALDI TOF mass spectrum of tryptic peptides is presented in Fig. 3. The masses of eight peptides from the spectrum coincided with putative masses of peptides from the cloned *T. molitor* PRCP sequence. Sequences of five tryptic peptides, IVTDVYK (residues 193–199), AWLSAQWK (228–235), QTHPELPILEYGGK (375–388), QFHIR (461–465) and NTSLYEKVTFARRVNT (473–488), were verified by tandem mass spectrometry (MS/MS) fragmentation. These data indicate that the sequence of cloned PRCP encodes the purified *T. molitor* PRCP. The calculated molecular mass of the mature enzyme was 53,554 Da, whereas molecular mass determined by gel filtration was 105 kDa, suggesting that the enzyme is a dimer.

3.4. Substrate specificity and kinetic parameters

T. molitor PRCP substrate specificity was evaluated with peptide substrates of different length and structure (Table 3). The enzyme was active with Pro-containing *p*-nitroanilide substrates (I–V) of the general formula A-XP-pNA (where A = H, Z; X = AAP, AP, GP) and Z-PF (VII), a substrate with a free carboxyl group characteristic for carboxypeptidases, but did not hydrolyze the shortest substrate (VI), P-pNA, with an unprotected proline residue. To exclude the endopeptidase activity of PRCP, substrates Suc-AAPF-pNA and Suc-AAPL-pNA, containing a proline residue in the middle of the peptide chain, were used. These substrates were not cleaved by *T. molitor* PRCP, indicating the absence of activity that is characteristic of prolyl oligopeptidase (POP) and prolyl endopeptidase. The enzyme did not hydrolyze specific substrates for peptidases from other classes, Bz-R-pNA (X), Glp-AAL-pNA (XI), Glp-FA-pNA (XII) and L-pNA (XIII). These data indicate the strict specificity of PRCP for the Pro residue, as well as the absence of impurities of trypsin-like, chymotrypsin-like, cysteine peptidases and leucine aminopeptidases, respectively.

Kinetic characteristics (K_m and V_{max}) of the hydrolysis of the Pro-containing *p*-nitroanilide substrates (I–V) were determined (Table 4). Experiments were carried out at pH 5.6 in the range of substrate concentrations from 0.01 to 0.5 mM. The efficiency of hydrolysis decreased in the following series of substrates:

Z-Ala-Ala-Pro-pNA > Ala-Pro-pNA ≈ Z-Ala-Pro-pNA >> Z-Gly-Pro-pNA > Z-Pro-pNA.

3.5. Kinetic parameters of PRCP inhibition by Z-pro-prolinal

Z-Pro-prolinal is a specific inhibitor of some PSP (Cunningham and O'Connor, 1997). The K_i of *T. molitor* PRCP inhibition by Z-Pro-prolinal was determined (Fig. 4). Straight lines of the dependence of the initial reaction rate on the substrate concentration, plotted in double reciprocal Lineweaver–Burk coordinates, crossed in the same point on the ordinate axis, indicating competitive inhibition by Z-Pro-prolinal. The K_i value of *T. molitor* PRCP inhibition by Z-Pro-prolinal was 3.7×10^{-6} M.

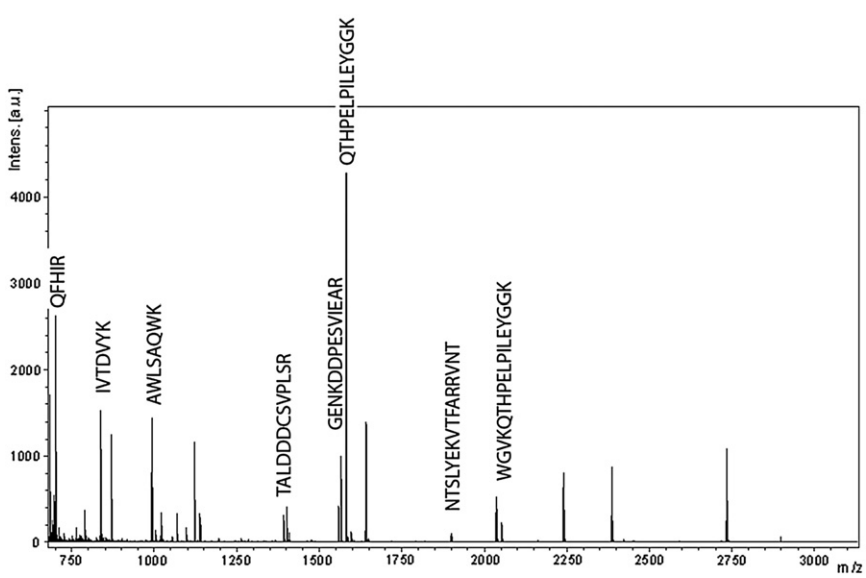

Fig. 3. Analysis of mass spectrum of tryptic hydrolyzate of purified *T. molitor* digestive peptidase using the deduced amino acid sequence of cloned PRCP cDNA.

Table 3
Substrate specificity of digestive PRCP from *T. molitor* larvae.

Substrate	pH	Concentration, mM	Activity, nmole/min
Z-AAP-pNA	5.6	0.5	0.126 ± 0.009
Z-AP-pNA	5.6	0.5	0.048 ± 0.008
AP-pNA	5.6	0.5	0.057 ± 0.006
Z-GP-pNA	5.6	0.3	0.0011 ± 0.0004
Z-P-pNA	5.6	0.3	0.0024 ± 0.0005
P-pNA	5.6	0.3	0
Z-PF	5.6	4.0	6.5 ± 1.3
Suc-AAPF-pNA[a]	5.6	1.5	0
Suc-AAPL-pNA[a]	5.6	1.5	0
Bz-R-pNA	5.6	0.5	0
Bz-R-pNA	7.9	0.5	0
Glp-AAL-pNA	5.6	0.5	0
Glp-AAL-pNA	7.9	0.5	0
Glp-FA-pNA	5.6	0.5	0
L-pNA	5.6	0.5	0
L-pNA	7.9	0.5	0

[a] Hydrolysis of the substrate was detected by HPLC.

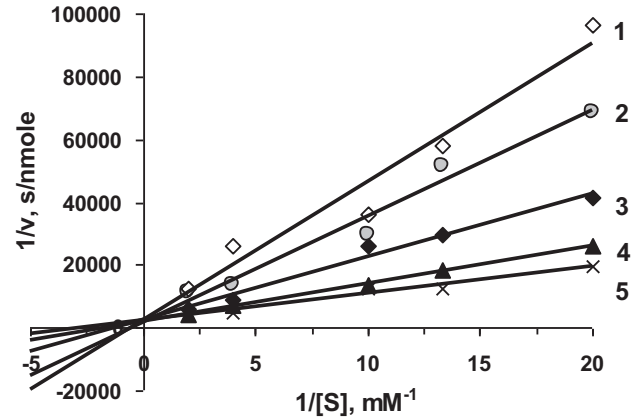

Fig. 4. Inhibition of PRCP from *T. molitor* by Z-pro-prolinal. Inhibitor concentrations used: 1.9×10^{-5} (1), 1.3×10^{-5} (2), 6.3×10^{-6} (3) and 2.5×10^{-6} (4) M, 5 – without the inhibitor.

3.6. Hydrolysis of gliadins by PRCP preparation

We also investigated the effect of the purified PRCP on the major dietary proteins of *T. molitor* larvae – gliadins. Hydrolysis products were detected by ninhydrin reaction with liberated NH_2-groups (Fig. 5). The experiment demonstrated that PRCP cleaved gliadins, and the hydrolysis was decreased by Z-Pro-prolinal (data not shown).

4. Discussion

In this paper, the first PRCP was isolated from an insect, *T. molitor* larvae. For PRCP purification, a three-stage scheme was used. The first stage was by gel filtration. The application of this type of chromatography effectively separated PRCP, with a molecular mass of 105 kDa, from the major digestive serine and cysteine endopeptidases, with molecular masses less than 40 kDa (Vinokurov et al., 2006b) and therefore prevented proteolysis by endopeptidases during purification. Additional purification followed with anion exchange and hydrophobic chromatography, resulting in a relatively pure enzyme. The cDNA of the *T. molitor* larval PRCP was cloned and sequenced, and the identity of the predicted PRCP sequence to the primary structure of the isolated enzyme was confirmed by mass spectrometry. The ratio of the calculated molecular mass of cloned PRCP and that determined by gel filtration suggests that the enzyme is a dimer, and similar data are reported for the human lysosomal PRCP (Odya et al., 1978; Tan et al., 1993). Also similar to the human PRCP, *T. molitor* PRCP displays acidic pH optimum at pH 5.6, correlating with its localization in the acidic AM of *T. molitor* larvae (Goptar et al., 2008b).

Previously, the primary structure of the enzyme was solved only for human PRCP, and the enzyme was assigned to the S28 family of serine peptidases (Tan et al., 1993). When PRCP was compared with annotated sequences of serine carboxypeptidases and POP, there was a low degree of overall identity (10–18%), but a high (67%) identity in active site amino acid residues. The authors concluded that PRCP is an evolutionary link connecting two families, POP and serine carboxypeptidases, because they possess properties characteristic of both families. Like serine carboxypeptidases, PRCP catalyzes the hydrolysis of a C-terminal residue that has a free carboxyl group at acidic pH values. On the other hand, PRCP hydrolyzes bonds formed by the carbonyl group of proline residues as do POP, and a specific inhibitor of POP, Z-Pro-prolinal, inactivates PRCP as well.

Resolution of the 2.8 Å crystal structure of the human lysosomal PRCP (Soisson et al., 2010) delineated the structural basis of the different substrate specificities of the two enzymes comprising the unique S28 family of PSP: carboxypeptidase PRCP and aminopeptidase DPP7. PRCP has an extended active-site cleft that can accommodate proline substrates with multiple N-terminal residues. In contrast, the substrate binding groove of DPP7 is occluded by a short amino acid insertion unique to DPP7 that creates a truncated active site selective for dipeptidyl proteolysis of N-terminal substrates.

The most specific substrate for *T. molitor* PRCP was N-protected peptide Z-PF, a selective substrate for PRCP, but the activity assay with this substrate was rather complicated. Because serine

Table 4
Kinetic parameters of the hydrolysis of selected substrates by PRCP from *T. molitor* larvae.

Substrate	K_m, μM	V_{max}, nM/s	V_{max}/K_m, s^{-1}
Z-AAP-pNA	340 ± 26	14.0 ± 1.2	4.1×10^{-5}
Z-AP-pNA	95 ± 13	2.9 ± 0.7	3.0×10^{-5}
AP-pNA	157 ± 20	5.0 ± 0.7	3.2×10^{-5}
Z-GP-pNA	147 ± 27	0.13 ± 0.04	8.7×10^{-7}
Z-P-pNA	170 ± 28	0.10 ± 0.03	5.9×10^{-7}

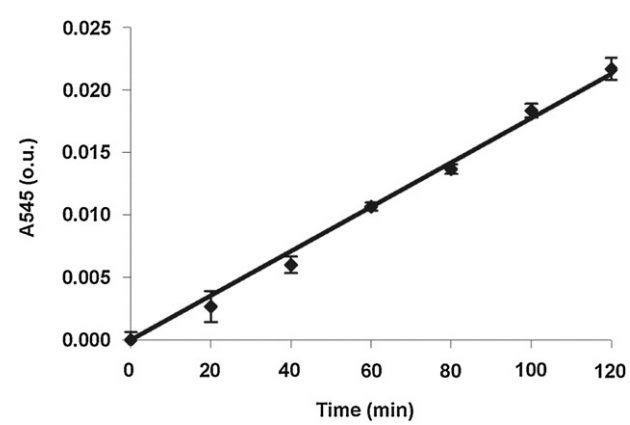

Fig. 5. Increasing hydrolysis of gliadins over time by incubation with *T. molitor* PRCP. Detection by ninhydrin reaction.

carboxypeptidases, unlike metallocarboxypeptidases, are able to hydrolyze chromogenic *p*-nitroanilide substrates with a detectable rate (Scheer et al., 2011), and due to the simplicity of the assay method, we used these substrates for monitoring of the purification process and for the study of the substrate specificity. The best chromogenic peptide substrate was Z-AAP-pNA with the highest V_{max}/K_m value, despite the fact that the substrate had the lowest K_m value. The hydrolysis of substrates Z-AP-pNA and AP-pNA occurred with equal efficiency, although Z-AP-pNA was more tightly bound to the enzyme, and AP-pNA was hydrolyzed faster. Lengthening or shortening the substrate by one Ala residue, substituting Ala at Gly, as well as removing the N-protecting group all led to an increase of the K_m. The maximum rate of hydrolysis of Z-GP-pNA, a specific substrate for POP (Cunningham and O'Connor, 1997) and Z-P-pNA was an order of magnitude lower than for the other substrates.

Kinetic studies revealed complete competitive inhibition of *T. molitor* PRCP by Z-Pro-prolinal. In this type of inhibition, the inhibitor interacts with the same region of the enzyme that binds substrate. Therefore the inhibitor competes with the substrate for interaction with the enzyme, as do other substrate-like inhibitors similar to Z-Pro-prolinal.

In contrast to human PRCP, which specifically functions as a regulatory enzyme affecting the blood system (Hagedorn, 2011; Adams et al., 2011), the PRCP from *T. molitor* is presumably a digestive enzyme. Earlier, we described the localization and functions of two PSPs from *T. molitor* larvae (Goptar et al., 2008a,b). We demonstrated that one of the PSPs had an acidic pH optimum, was localized in the AM contents, and the activity profile changed in the digestive process similar to the general proteolytic activity, but these data were insufficient for conclusive identification of this peptidase. In this report, we identified this enzyme as PRCP and further demonstrate that *T. molitor* larval PRCP participates in gliadin hydrolysis that is reduced by a specific inhibitor of PRCP Z-Pro-prolinal.

Thus, we have isolated, purified, and identified the primary structure and further characterized a digestive PRCP from the larval midgut of an insect pest, *T. molitor*. The unique aspects of this enzyme are that it is the first PRCP isolated from an insect, and the first PRCP found to function as a secreted digestive enzyme. It is unknown if PRCP is involved in digestion in other animals. Specific digestive peptidases in insects that differentiate the mode of gliadin hydrolysis from that of human include cysteine peptidases with post-glutamine cleaving activity (Goptar et al., 2012), and PRCP with post-proline cleaving activity (this report). In eukaryotic organisms, these enzymes participate in the intracellular lysosomal degradation of proteins. Many insects rely on typical serine digestive peptidases, trypsin and chymotrypsin, but some groups of insects with an area of acidic to neutral pH in the midgut use cysteine cathepsins also for digestion (Terra and Ferreira, 1994). Our data suggest that the enrichment of the peptidase digestive complex in such insects during evolution is due to the adaptation of peptidases present in the lysosomes of eukaryotic organisms, such as cysteine cathepsins and PRCP.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ibmb.2013.02.009.

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