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Purification and Characterization of a 25 kDa Cathepsin L-like Protease from the Hemocyte of Coleopteran Insect, *Tenebrio molitor* Larvae

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Insect plasma protein is abundant in the hemolymph of holometabolous insect larvae and is used as a source of amino acids and energy for construction of adult structures during metamorphosis. In order to understand the mechanism of decomposition of larval plasma proteins by hemocyte protease, we tried to purify a cysteine protease from the hemocyte lysate by using Carbobenzoxy-L-Phenylalanyl-L-Arginine-4-Methyl-Coumaryl-7-Amide (Z-Phe-Arg-MCA) as substrate and to identify plasma proteins that are selectively susceptible to the purified protease. Here, we describe the purification and characterization of a cysteine protease that specifically hydrolyzes the plasma protein of the coleopteran insect, *Tenebrio molitor*, larvae. The molecular mass of this enzyme was 25 kDa, as determined by SDS-PAGE under reducing conditions. The amino acids sequence of its NH_2-terminus was determined to be Leu-Pro-Gly-Gln-Ile-Asp-Trp-Arg-Asp-Lys-Gly. This sequence contained Pro, Asp, and Arg residues, conserved in many papain superfamily enzymes. The specific cysteine protease inhibitors, such as E-64 and leupetin, inhibited its hydrolytic activity. One plasma protein with a molecular mass of 48 kDa was selectively hydrolyzed within 3 h when the purified enzyme and plasma proteins were incubated *in vitro*. However, the 48 kDa protein was not hydrolyzed by the purified 25 kDa protease in the presence of E-64. Western blotting analysis at various developmental stages showed that the purified enzyme was detected at larvae, pupae, and adult stages, but not the embryo stage.

Keywords: Cysteine protease, Insect, Metamorphosis, Hemocyte, Plasma proteins.

Introduction

In insects, plasma proteins are major larval serum proteins that are synthesized in the fat body and secreted into the hemolymph during the final larval instar (Wyatt and Pan, 1978). These plasma proteins are used as a source of amino acids and energy for the synthesis of new proteins during metamorphosis (Thomson, 1975). During insect metamorphosis, some of the plasma proteins seems to be degraded *in situ* and the resulting amino acids are released into the hemolymph. Until recently, nothing was known about the decomposition of plasma proteins by hemocyte protease in coleopteran insects.

We are interested in the process of decomposition of plasma proteins by hemocyte protease. With regards the mechanism of decomposition of the plasma proteins, it has been suggested that larval hemocyte will secrete some kind of protease when plasma proteins are needed to be degraded (Liu *et al.*, 1996). Recently, proteases have been implicated in morphogenesis of imaginal discs in *Drosophila* (Pino-Heiss and Schubinger, 1989; Appel *et al.*, 1993; Fessler *et al.*, 1993; Homma and Natori, 1996). These results suggest that hydrolysis of certain target proteins on the surface of imaginal discs by protease is a prerequisite for their transition from eversion to the elongation stage. At the elongation stage, leg discs expand

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markedly to form primordial adult leg structures. Therefore, digestion of the surface proteins by proteases may make it easier for the discs to expand spatially. To provide a clue to the mechanism of decomposition of larval plasma proteins by hemocyte protease, it is essential to purify this protease and examine its decomposition activity against the larval plasma proteins. This paper describes the purification and biochemical characterization of 25 kDa cathepsin L-like protease from *Tenebrio molitor* larval hemocyte. We also identified a larval plasma protein that is susceptible to the purified protease hydrolysis.

Materials and Methods

Collecting of hemolymph and hemocyte *T. molitor* larvae (mealworm) were reared with an artificial diet in a plastic container at 27°C. Before collecting the hemolymph, larvae were anesthetized on ice. To harvest the hemolymph, larvae were injected with 100 μl of modified decoagulation buffer (23 mM citric acid, 15 mM NaCl, 1 mM EDTA, 100 mM glucose, and 30 mM trisodium citrate, pH 6.0) by using a 22G gauge needle. Cutting off the posterior tip of each larva with scissors isolated the hemolymph and the drop of hemolymph that exuded was collected in a test tube on ice. Hemocytes were collected from the hemolymph by centrifugation at 200 $\times$ *g* for 10 min at 4°C, washed with decoagulation buffer, and stored at -80°C. About 3×10^8 packed cells were obtained from 500 ml of hemolymph.

Assay of protease activity Proteinase activity was assayed with various peptidyl-MCA substrates. For Z-Phe-Arg-MCA or Z-Arg-Arg-MCA as a substrate, the enzyme reaction was performed in 0.5 ml of 20 mM citrate buffer (pH 6.0) containing 50 μM substrate, 75 mM NaCl, 1 mM EDTA, 5 mM β-mercaptoethanol, and the sample. For Boc-Phe-Ser-Arg-MCA, Boc-Ile-Glu-Gly-Arg-MCA, or Boc-Gln-Ala-Arg-MCA, 50 mM Tris/HCl (pH 8.0) with 5 mM $CaCl_2$ was used as a reaction buffer. Following incubation at 27°C for 10 min, the reaction was terminated by addition of 0.7 ml of 17% acetic acid. Fluorescence was measured with excitation and emission wavelengths of 380 nm and 460 nm, respectively. One unit of proteinase activity was defined as the amount that hydrolyzed 1 μmol of substrate per 10 min under the reaction conditions.

Effects of inhibitors on protease activity The purified 25 kDa protease (1 μg) was preincubated with several inhibitors in 0.5 ml of 20 mM citrate buffer (pH 6.0) for 15 min at 27°C. Then, 3 μl of 0.1 mM Z-Phe-Arg-MCA was added and the reaction was allowed to proceed for 10 min before it was terminated by the addition of 0.7 ml of 17% acetic acid. Fluorescence was measured with excitation and emission wavelengths of 380 nm and 460 nm, respectively.

Electrophoresis Electrophoresis on SDS–PAGE under denaturing conditions was carried out by the method of Laemmli (1970). Samples were denatured by heating for 20 min at 75°C in 1% SDS and 2% β-mercaptoethanol. After electrophoresis, the gels were stained by the method of Fairbanks *et al.* (1971).

Purification of a protease from larval hemocyte lysate Hemocytes collected from about 5000 larvae were suspended in 10 ml of lysate buffer (20 mM acetate buffer, pH 4.0, containing 50 mM NaCl, 5 mM 2-mercaptoethanol and 0.5% Nonidet P-40) and disrupted by homogenization. The homogenate was centrifuged at 20,000 $\times$ *g* for 30 min at 4°C and the resulting supernatant was used as a hemocyte lysate. The lysate (3 ml) concentrated by ultrafiltration (Amicon, YM10 membrane, Beverly, USA) was applied and fractionated on Sephacryl S-200 (1.8 $\times$ 120 cm) column equilibrated with buffer A (20 mM acetate buffer, pH 4.0, 5 mM β-mercaptoethanol and 1 mM EDTA) containing 100 mM NaCl. The protease activity was eluted as a single peak, and the active fractions were combined and diluted 10 times with buffer A. The diluted solution was subjected to CM-cellulose 52 column (2.8 $\times$ 10 cm) equilibrated with the buffer A and the column was developed with a linear gradient of 0–600 mM NaCl in the same buffer. The active fractions were combined, concentrated, and further purified by Mono-S FPLC column equilibrated with the buffer A. The enzyme was obtained with a linear gradient between 0 and 500 mM NaCl. Protease activity was recovered as a single peak. At this stage, the protease was almost pure on SDS-PAGE.

Antibody and immunoblotting Antiserum against the purified enzyme was raised by injecting 20 μg of the purified protein into a male albino rabbit with complete Freund's adjuvant and giving a booster injection of the same amount of protein 14 days later (McCauley and Racker, 1973). The resulting antiserum was affinity-purified as follows: the purified protease (45 μg) was first electrophoresed on 12% SDS–PAGE and then protein was blotted onto a nitrocellulose filter. The small region of the filter on which protease had been concentrated was excised and treated with a 5% skim milk solution (5% skim milk in 20 mM Tris/HCl, pH 7.9) at 4°C for 1 h. Then, the strip of filter paper was incubated in 10 ml of anti-25 kDa protease IgG solution (1 ml serum was diluted with 1 ml of rinse solution containing 10 mM Tris/HCl, pH 7.9, 150 mM NaCl, 1 mM EDTA, 0.1% Triton X-100, 1% NaN_3, and 0.5% skim milk) at 4°C for 12 h with gentle shaking. The strip was then rinsed well and cut into pieces, and the antibody specifically bound to 25 kDa protease was extracted with 0.2 M glycine/HCl buffer (pH 2.8). The resulting extract was immediately neutralized with 1 M KOH, and bovine serum albumin was added to give a final concentration of 0.1%. This affinity-purified antibody dose-dependently cross-reacted with the 25 kDa protease (data not shown). For immunoblotting, proteins separated on the gel by electrophoresis were transferred electrophoretically to a polyvinylidene difluoride filter (PVDF, Millipore). The filters were immersed in 5% skim milk solution for 12 h and transferred to rinse solution II (20 mM Tris/HCl, pH 7.5, containing 150 mM NaCl, 0.1% Tween 20 and 2.5% skim milk) containing the affinity-purified antibody against 25 kDa protease (50 ng/ml). The treated filters were kept for 2 h at 4°C. The bound antibodies were detected using the ECL Western blotting reagent kit (Amersham Life Science, Buckinghamshire, UK).

***In vitro* incubation of plasma proteins with the purified 25 kDa protease** Plasma protein (100 μg), prepared from the flesh hemolymph of third instar larvae, were incubated with the

purified protease (1 μg) in 250 μl of 20 mM citrate buffer at 27°C. Tenμl aliquots were withdrawn periodically and analyzed by SDS-PAGE. Ten μl of 20 mM E-64 solution was added in the presence of protease as a control.

Results

Purification and characterization of a hemocyte protease When we examined the amidase activity against *T. molitor* hemocyte lysate by using a substrate of cathepsin B and L, Z-Phe-Arg-MCA, a significant protease activity was observed in the crude hemocyte lysate. To purify this protease showing amidase activity against Z-Phe-Arg-MCA, we first subjected hemocyte lysate containing protease activity to Sephacryl S-200 column and assayed for protease activity against each fraction with Z-Phe-Arg-MCA as a substrate. As shown in Fig. 1A, major protease activity was eluted as a single peak. The protease was further purified to near homogeneity by successive CM-cellulose 52 column and Mono-S FPLC column. The elution profiles of CM-cellulose 52 and Mono-S column are shown in Figs. 1B and 1C, respectively. Two major peaks were eluted from Mono-S (Fig. 1C). The protease activity was shown in the former peak, not in the latter one. The coincidence of the major protein peak and protease activity indicates that the purified major protein is a protease. The combined active fractions from the Mono-S column gave a single band with a molecular mass of 25 kDa on SDS-PAGE under reducing conditions as shown in Fig. 1D. A typical purification is summarized in Table 1.

As the protease was purified to homogeneity, we examined its biochemical characters. The optimum pH of this enzyme was pH 6.0 and it showed less than 50% of the

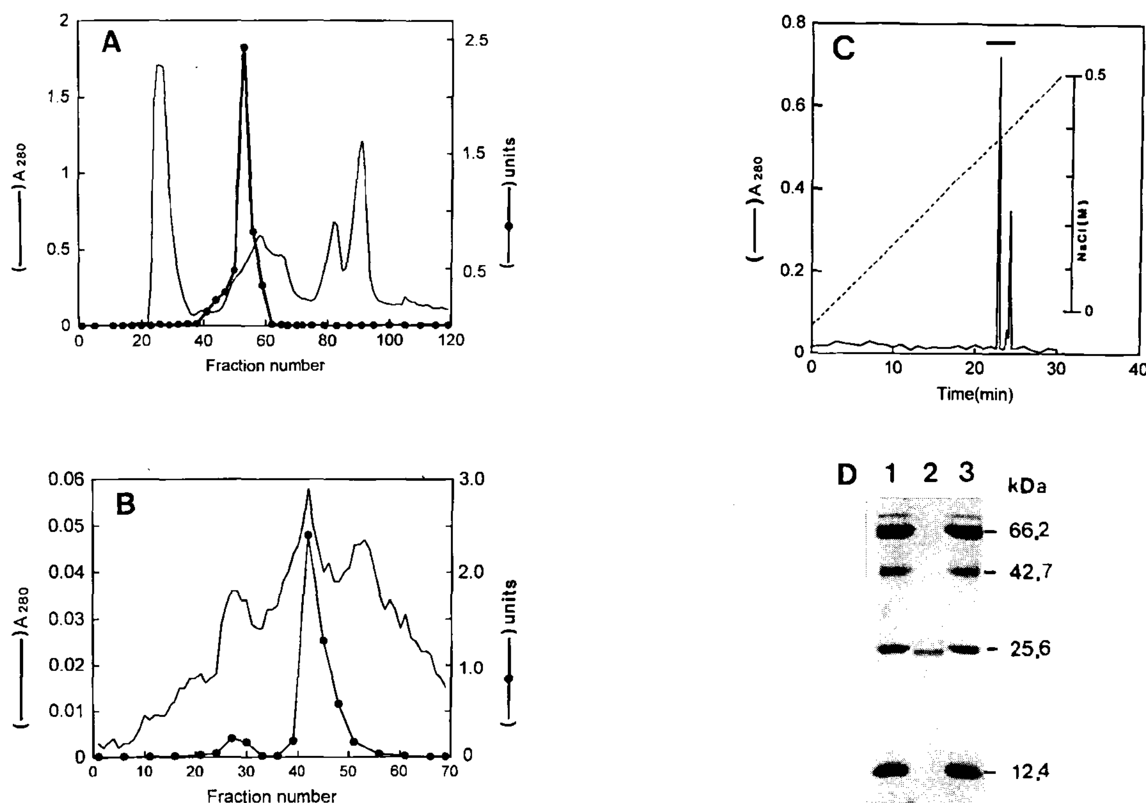

Fig. 1. Purification of 25 kDa cathepsin L-like protease from *T. molitor* larvae. A. Elution profile of Sephacryl S-200 chromatography of a crude hemocyte lysate. A concentrated hemocyte lysate was applied to a Sephacryl S-200 gel filtration column (1.8 × 120 cm) equilibrated with buffer A (20 mM acetate buffer, pH 4.0, 5 mM β-mercaptoethanol, 0.02% Nonidet p-40, and 1 mM EDTA) containing 100 mM NaCl. Fractions of 4 ml were collected with a flow rate of 10 ml/h and assayed for protease activity with Z-Phe-Arg-MCA as substrate. (—●—), protease activity detected by fluorescence; (——), absorbance at 280 nm. B. CM-cellulose 52 chromatography of active fractions from a Sephacryl S-200 gel filtration column (2.0 cm × 20 cm). Fractions containing activity were pooled. (——), absorbance at 280 nm; (—●—), protease activity. C. Elution profile of Mono-S cationic exchange FPLC of active fractions from a CM cellulose 52 column. Bound proteins were eluated with a linear gradient between 0 and 500 mM NaCl. (----), absorbance at 280 nm; (——), NaCl concentration. The bar indicates the active peak against protease activity. D. SDS-PAGE of an active fraction (the former peak) from Mono-S column containing 2 μg protein. Lane 1, size markers; lane 2, purified 25 kDa protease from Mono-S column (the former peak); lane 3, size markers. The gel was calibrated with the following molecular mass markers: bovine serum albumin (66.2 kDa); ovalbumin (42.7 kDa); chymotrypsinogen (25.6 kDa); cytochrome C (12.4 kDa).

Table 1. Summary of purification of hemocyte protease from *T. molitor* larvae.

Purification step	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)	Purification (fold)
Hemocyte lysate	21.7	1.87	0.084	100	1
Sephacryl S-200	2.0	1.38	0.69	73	8
CM-cellulose 52	0.57	0.54	0.94	29	11
Mono-S	0.20	0.34	1.70	18	20

maximal activity at below pH 5.0 or above pH 6.0 (data not shown). The substrate specificity of this enzyme is shown in Table 2. This protease efficiently hydrolyzed Z-Phe-Arg-MCA, a substrate for cathepsin B, L and plasma kallikrein. A substrate for plasmin, Boc-Val-Leu-Lys-MCA, was digested appreciably. However, it had no appreciable activity on Z-Arg-Arg-MCA (substrate of cathepsin B), Suc-Leu-Leu-Val-Tyr-MC (chymotrypsin), and Suc-Ala-Pro-Ala-MCA (elastase). These results suggest that this protease has a unique substrate specificity, different from that of cathepsin B.

To characterize this protease further, we determined the amino terminal sequence of the purified protease and compared it with those of other cysteine proteases. As shown in Fig. 2, a significant similarity was found between the amino acids sequence of this protease and that of cathepsin L, when its first amino acid was aligned with the first amino acid of cathepsin L. Moreover, it also contained the Pro, Asp, and Arg residues, conserved in this in many papain superfamily enzymes (Lee *et al.*, 1997). These findings strongly suggest that this protease is a cysteine protease. Therefore, we examined the effects of various protease inhibitors on the purified enzyme in Table 3. For this, we added 20 μM of inhibitors to a fixed amount of the enzyme and measured the activity with Z-Phe-Arg-MCA. As shown in Table 3, *N*-[*N*-(L,3-*trans*-carboxyoxiran-2-carbonyl)-L-leucyl]agmatine (E-64) strongly inhibited enzyme activity, indicating that this enzyme contains at least one essential cysteine residue (Schaschke *et al.*, 1997). This protease was also strongly inhibited by leupeptin and antipain, which are known as cysteine protease inhibitors (Katunuma and Kominami, 1985). Chymostatin is an inhibitor for chymotrypsin, but it also inhibits some cysteine proteases (Laskowski and Kato, 1980). Pepstatin (aspartic proteinase inhibitors) and phenylmethylsulfonyl fluoride (serine proteinase inhibitors) had essentially no effect on this enzyme. These results also support the conclusion that this purified enzyme is a cysteine protease.

25 kDa proteinase LPGQI**DWR**D**KG**--
cathepsin L IPKTV**DWR**E**KG**--
cathepsin B LPESF**D**A**R**EQW--
cathepsin H YPSSM**DWR**K**KG**--
Papain IPEYV**DWR**Q**KG**—

Fig. 2. Sequence similarity of the amino-terminal region of the 25-kDa protease with those of other cysteine proteases. The sequences are aligned to achieve maximal similarity. Identical residues in all six proteases are in bold type. The amino acids sequences of human cathepsin L (Menard *et al.*, 1998), rat cathepsin B (Cygler *et al.*, 1996), rat cathepsin H (Ishido *et al.*, 1991), and papain (Lee *et al.*, 1997) were obtained from entries in the NCBI protein sequence database (release at November 24, 1997) with Genetyx system (Software Development Co., LTD, Tokyo).

Identification of plasma protein susceptible to the purified enzyme As cathepsin L-like protease was purified to homogeneity from the hemocyte lysate of

Table 2. Specificity of 25 kDa cathepsin L-like protease for the hydrolysis of synthetic substrates.

Substrates	Activity (%)
Z-Phe-Arg-MCA	100
Boc-Phe-Ser-Arg-MCA	0
Suc-Leu-Leu-Val-Tyr-MCA	0
Suc-Ala-Pro-Ala-MCA	0
Boc-Val-Leu-Lys-MCA	20
Boc-Ile-Glu-Gly-Arg-MCA	0
Boc-Val-Pro-Arg-MCA	0
Z-Pyr-Gly-Arg-MCA	0
Z-Arg-Arg-MCA	2

Table 3. Effects of various inhibitors on the activity of 25 kDa cathepsin L-like protease of *T. molitor* larvae.

Inhibitors	Concentration (μM)	Activity (%)
none (control)	20	100
Leupeptin	20	0
Antipain	20	1
E-64	20	0
Pepstatin	20	103
Chymostatin	20	0
PMSF	20	75
PMSF	200	43

T. molitor larvae, we examined the possibility that the purified cathepsin L-like protease may hydrolyze specific plasma proteins. To test this hypothesis, we incubated the plasma proteins with the purified cathepsin L-like protease with various incubation time, and analyzed their proteins by SDS-PAGE. As shown in Fig. 3, the staining intensity of one protein band with molecular mass of 48 kDa appeared to be decreased within 3 h when plasma were incubated with the purified enzyme (lane 5). However, they were not decreased when protease activity was inhibited by E-64, a specific cysteine protease inhibitor, indicating that the 48 kDa protein may be a substrate for the 25 kDa protease (lane 7).

Expression of the 25 kDa protease in *T. molitor* We raised antibody against the purified 25 kDA protein and analyzed the expression of the 25 kDa protein at various developmental stages of this insect. The 25 kDa protein was detected in extracts of larvae, pupae and adult (Fig. 4, lanes 2, 3, 4, and 5). Its content reached a maximum at the early larval stage (lane 2). No appreciable 25 kDa protein was detected in the embryonic stages (lane 1). The antibody cross-reacted with a higher molecular mass bands (35 kDa) in larval and pupal stages (lanes 2, 3, and 4). We suppose that these bands may be a proform of the 25 kDa protease. Therefore, the 25-kDa protease may be needed at larval, pupal, and adult stages.

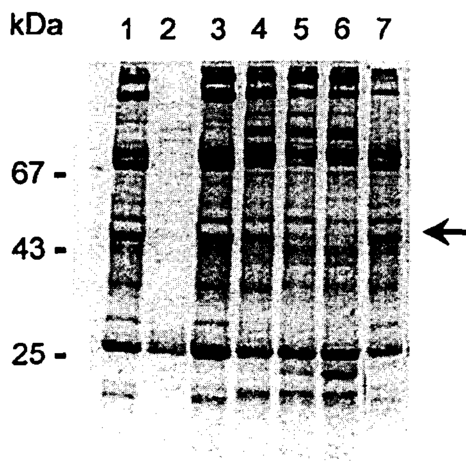

Fig. 3. Analysis of plasma proteins hydrolyzed by 25 kDa protease. Plasma proteins (100 μg) were incubated with 1 μg/ 250 μl 25 kDa protease. The reaction mixture (20 μg) was subjected to SDS-PAGE. Lane 1, plasma protein in the absence of protease; lane 2, the purified 25 kDa protease; lane 3, plasma proteins incubated with protease for 0 min; lane 4, incubated 1 h; lane 5, incubated 3 h; lane 6, incubated 6 h; lane 7, plasma proteins incubated with the protease and E-64. The gel was calibrated with the following molecular mass markers: bovine serum albumin (66.2 kDa); ovalbumin (42.7 kDa); chymotrypsinogen (25.6 kDa).

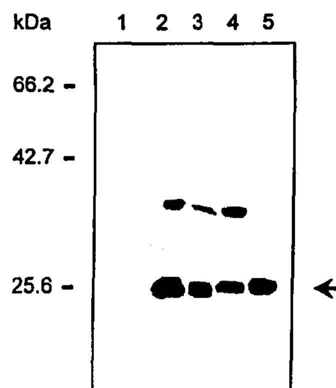

Fig. 4. Immunoblotting analysis of the 25 kDa protease at various developmental stages. Samples were prepared from animals at various developmental stages and subjected to immunoblotting analysis with affinity-purified antibody against the 25 kDa protease. Each lane contained 10 μg protein. Lane 1, eggs; lane 2, early larvae; lane 3, late larvae; lane 4, pupae; lane 5, adults. The gel was calibrated with the following molecular mass markers: bovine serum albumin (66.2 kDa); ovalbumin (42.7 kDa); chymotrypsinogen (25.6 kDa).

Discussion

We have used several buffers (insect saline or insect saline containing serine protease inhibitors) to obtain a large number of hemocytes from the hemolymph. Most buffers (insect saline or insect saline containing serine protease inhibitors) were not effective because they activated prophenoloxidase and clotted the hemolymph. These problems have been overcome in the present work by using modified decoagulation buffer (23 mM citric acid, 30 mM trisodium citrate, 15 mM NaCl, 1 mM EDTA, and 100 mM glucose, pH 6.0) (Saul and Sugumaran, 1987). This buffer made it feasible to obtain a maximum number of hemocytes from hemolymph reproducibly.

In this work, we found that hemocytes of *T. molitor* larvae contain several proteases (data not shown). We purified one of these proteases to near homogeneity. This enzyme had cathepsin L-like substrate specificity and was a 25 kDa protease. Cathepsin L is classified as a cysteine protease and is normally present as a lysozomal protease in mammalian systems. However, we purified cathepsin L-like protease from hemocyte lysate, and we showed that this enzyme specifically degraded a plasma protein of *T. molitor* larvae. This finding suggested that the purified 25 kDa protease may hydrolyze a specific plasma protein during metamorphosis. Nothing is known about the biochemical characteristics of this substrate protein. Further studies on its structure will provide more insight for understanding the biological role of the decomposition of plasma protein by hemocyte cysteine protease.

It is now well known that insect hemocytes are important in the elimination of foreign substances. These

hemocytes are known to pile up around a foreign substance introduced into the abdominal cavity, resulting in nodule formation (Pech and Strand, 1996). Furthermore, nonself proteins seemed to be degraded selectively by a hemocyte protease before their incorporation into the fat body. Kurata *et al.* (1992) previously reported that foreign protein injected into the abdominal cavity of *S. peregrina* (flesh fly) larvae was degraded in the hemolymph by a 29 kDa cysteine protease secreted by the hemocyte. This 29 kDa protease consisted of 26 kDa and 29 kDa subunits with similar substrate specificity to mammalian cathepsin B. This enzyme was shown to be released from hemocytes into the hemolymph of larvae following injection of sheep red blood cells into the larvae. Therefore, a hemocyte protease seems to be important for digestion of nonself proteins. Another cysteine protease, 35 kDa cathepsin L-like protease, has been purified from imaginal discs of *S. peregrina* (Homma *et al.*, 1994). They demonstrated that the 35 kDa protease is essential for the differentiation of imaginal discs of flesh fly. Further study showed that two imaginal disc proteins with molecular masses of 210 and 200 kDa are susceptible to digestion by cathepsin L and selectively hydrolyzed during imaginal disc differentiation (Homma and Natori, 1996).

We found here that cathepsin L-like protease from *Tenebrio* hemocyte may also participate in the decomposition of self-larval plasma proteins, playing an essential role in metamorphosis.

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