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Original article

Expression and characterization of honeybee, *Apis mellifera*, larva chymotrypsin-like protease

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Abstract – Previously, we found three enzyme fractions containing activities for the hydrolysis of royal jelly proteins from honeybee queen larvae. In this study, we identified a honeybee chymotrypsin-like protease (HCLPase) by LC-MS/MS and expressed it as a recombinant protein in *Escherichia coli*. The protease had an estimated molecular weight of around 26 kDa and showed high specificity for succinyl-Ala-Ala-Pro-Phe *p*-nitroanilide as a proteolytic substrate. Furthermore, the protease had an optimal pH of 9, and the activity was markedly inhibited by phenylmethylsulfonyl fluoride but not tosyl phenylalanyl chloromethyl ketone, both of which are irreversible inhibitors of chymotrypsin-like serine proteases. These results suggested that this recombinant protease, HCLPase, was a chymotrypsin-like serine protease with different characteristics from mammalian chymotrypsin.

Apis mellifera **/ queen larva / chymotrypsin-like protease**

1. INTRODUCTION

The honeybee, *Apis mellifera*, is a social insect with several castes. The queen or workers are determined in the caste by food in the larval period. Royal jelly (RJ), a nutrient for the queen and larvae, is secreted from the hypopharyngeal and mandibular glands of worker bees and plays a critical role in the differentiation of the queen. RJ is a protein-rich food (about 40 % of dry mass) and a 57-kDa protein in RJ, royalactin, was recently reported to be crucial for queen differentiation (Kamakura 2011). On the other hand, several functional activities of RJ for human health have also been reported. RJ is commonly used as a health food in many countries. Furthermore, peptides from RJ proteins are produced by commercial proteases and show various properties, including antioxidant (Guo et al. 2008, 2009; Nagai et al. 2006), antihypertensive (Tokunaga et al. 2004; Matsui et al. 2002), antiosteoporotic (Hidaka et al. 2006), and cholesterol-lowering (Nakasa et al. 2003), as well as prevention of sarcopenia (Niu et al. 2013). However, it is not clear how the queen bee and larvae digest RJ in nature. We hypothesized that honeybee queen larvae may have efficient proteases for digestion of RJ to produce functional peptides.

Several digestive proteases, such as trypsin, chymotrypsin, cysteine proteases, aminopeptidases, and carboxypeptidases, have been found in insects (Terra and Ferreira 1994). Several reports have suggested that tryptic or chymotryptic activities play an important role in the digestion of food in the honeybee. Burgess et al. (1996) reported that Kunitz soybean trypsin

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inhibitor (SBTI) ingestion increased mortality of worker bees and decreased trypsin, chymotrypsin, and elastase activities in the bee midgut. Correspondingly, honeybee larvae given 1.0 % SBTI in food showed high mortality rates and low body weight although they developed to adults (Brødsgaard et al. 2003). In addition, Giebel et al. (1971) found four endopeptidases from the midgut of adult worker bees: one of the proteases was similar to bovine trypsin, and the others resembled bovine chymotrypsin. Among them, only one of these proteases was found in adult queen and worker larvae. The protease had chymotrypsin-like characteristics and was inhibited by tosyl-L-phenylalanine chloromethyl ketone (TPCK), a chymotrypsin inhibitor, but could not digest substrates of trypsin and chymotrypsin. The trypsin-like protease obtained from adult worker bees was not found in the queen bee or larvae. Dahlmann et al. (1978) investigated the changes in chymotryptic, tryptic, and aminopeptidyl protease activities occurring during the development of honeybee workers, i.e., in larvae before and after sealing, pupae, and adults. They showed changes in three protease activities in each developmental stage within the same caste. They also showed that chymotryptic activity in the larval stages decreased after sealing when they no longer required feeding.

The above reports suggest that it is possible that the chymotryptic enzyme plays an important role in the digestion of food by bee larvae. There are genes for 44 serine-type proteases and 12 serine-type protease homologs in the honeybee genome (Honeybee Genome Sequencing Consortium 2006; Zou et al. 2006). However, little information is available regarding the expression and function of proteases encoded by these honeybee serine protease genes.

Previously, we found three protease fractions that were capable of digesting RJ protein from 2- to 3-day-old honeybee queen larvae, including carboxypeptidase-A-like activity and chymotrypsin-like activity (Matsuoka et al. 2012). The present study was performed to identify the proteases from honeybee queen larvae and characterize the recombinant chymotrypsin-like protease expressed in *Escherichia coli*.

2. MATERIALS AND METHODS

2.1. Separation and identification of proteases from honeybee queen larvae

2.1.1. Sample preparation

Three-day-old honeybee queen larvae were obtained from Akitaya Honten Co., Ltd. (Gifu, Japan). Honeybee queen larvae were homogenized and filtrated using nylon mesh. The homogenate was diluted in an equal volume of 50 mM phosphate buffer (pH 7.0) and then centrifuged at 10,000×*g*, 4 °C for 20 min. The clear yellowish middle layer was collected and filtrated first through a glass fiber filter (Advantec, Tokyo, Japan) and then a 0.20-μm cellulose acetate filter (Advantec) to obtain the crude enzyme solution.

2.1.2. Column chromatography

The proteases were purified by cation exchange column chromatography and gel filtration column chromatography. The pH value of the cation exchange column chromatography was preliminarily examined for collecting enzymes showing higher specific activities. Aliquots of 5 mL of crude enzyme solution were loaded onto a 1-mL HiTrap SP HP column (GE Healthcare, Little Chalfont, UK) equilibrated with 20 mM phosphate buffer (pH 7.6). The column was washed with five column volumes of 20 mM phosphate buffer (pH 7.6), and then the proteins were eluted with 20 mM phosphate buffer (pH 7.6) containing 0.5 M NaCl. Gel filtration chromatography was performed by the ÄKTAprime system (GE Healthcare). The eluent from the cation exchange column was loaded onto a HiLoad 16/600 Superdex 75 pg column (1.6×60 cm; GE Healthcare) equilibrated with 50 mM phosphate buffer containing 0.15 M NaCl (pH 7.0). Proteins were eluted using the same buffer at a flow rate of 0.7 mL/min with a fraction volume of 2 mL and detected by absorbance at 280 nm.

2.1.3. Chymotryptic and carboxypeptidase-like enzyme assay

Chymotryptic protease activities were determined by hydrolysis of *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (SAAPF*p*NA; Sigma Aldrich, St. Louis, MO) as a substrate. The reaction

mixture contained 386 μL of 50 mM Tris-HCl buffer (pH 9.0), 2 μL of 50 mM SAAPF*p*NA in dimethylformamide, and 10 μL of sample and was incubated at 37 °C for 10 min. The reaction was stopped by adding 20 μL of glacial acetic acid. The absorbance of liberated *p*-nitroaniline was determined at 405 nm. One unit of activity corresponded to the amount of enzyme that released 1 μmol of *p*-nitroaniline per min under standard assay conditions.

The carboxypeptidase activities were determined using *N*-carbobenzoxy-glycyl-L-phenylalanine (Z-Gly-Phe; Sigma Aldrich) as a substrate. The reaction mixture contained 190 μL of 1 mM Z-Gly-Phe in Tris-HCl buffer containing 0.1 M NaCl (pH 9.0) and 10 μL of the sample and was incubated at 37 °C for 30 min. The reaction was stopped by adding 600 μL of 0.4 M acetate buffer (pH 5.0). The absorbance of liberated phenylalanine was determined by a ninhydrin assay (Moore 1968). One hundred microliters of sample was collected in other tubes, and 100 μL of ninhydrin reagent (2 % solution, Sigma Aldrich) was added. The sample was incubated at 100 °C for 15 min. After incubation, 1 mL of ice-cold ethanol was added to the sample. The absorbance of liberated phenylalanine was determined at 570 nm. One unit of activity corresponded to the amount of enzyme that released 1 μmol of phenylalanine per min under standard assay conditions.

2.1.4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis

Proteins of the fractions including carboxypeptidase activity (fraction I) and chymotryptic activity (fraction II) collected by column chromatography were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

2.1.5. Mass spectrometry and database search

After SDS-PAGE, the excised protein spots were subjected to in-gel digestion with trypsin (Shevchenko et al. 1996). The samples were separated using a nanoflow multidimensional HPLC system (Paradigm MS4; Michrom BioResources, Auburn, CA) and analyzed by electrospray ionization ion trap mass spectrometry (ESI-ion trap MS) (LCQDECAXP; Thermo Fisher Scientific, Waltham, MA) under optimum conditions (capillary temperature, 200 °C; capillary voltage, 3.0 V; and spray voltage, 1.90 kV). Peptide sequences were searched against the protein database (MSDB) using Mascot software (Matrix Science Ltd., London, UK).

2.1.6. N-terminal amino acid sequence

After SDS-PAGE, fraction II proteins were transferred onto polyvinylidene difluoride (PVDF) membranes by electroblotting, and each band was separated. The N-terminal amino acid sequence of chymotrypsin-like protein was analyzed using a peptide sequencer (Model 491; Applied Biosystems, Foster City, CA) from blotted PVDF membrane pieces.

2.2. Expression of the enzyme by recombinant *E. coli*

2.2.1. Target DNA amplification and recombinant vector construction

Total RNA of honeybee queen larvae was prepared from whole larvae with a FastPure RNA kit (TaKaRa, Shiga, Japan). Total RNA was stored at −80 °C before use. cDNA was prepared from total RNA by oligo(dT) primer (Toyobo, Osaka, Japan) and reverse transcriptase (ReverTraAce; Toyobo). Aliquots of 1 μg of total RNA, 0.5 μL of oligo(dT) primer, 4 μL of 4× buffer, 8 μL of 2.5 mM dNTP mixture, 1 μL of RNase inhibitor, and 1 μL of ReverTraAce were mixed and incubated at 42 °C for 30 min. The enzyme was deactivated by heating at 99 °C for 1 h. Honeybee chymotrypsin-like protease DNA was amplified by PCR with the sense primer 5′-AAA GTC GAC ATT GTT GGC GGC AGT GAT G-3′ containing a *Sal*I cleavage site and antisense primer 5′-AAAAA GAA TTC TAA GAA ATG TAG AAA CCG TCT GCT G-3′ containing an *Eco*RI cleavage site. The PCR conditions were as follows: 1 cycle of 98 °C for 30 s; 30 cycles of 98 °C for 10 s, 58 °C for 15 s, and 72 °C for 20 s; and 1 cycle of 72 °C for 5 min.

To construct the recombinant *E. coli*, PCR products and Gateway® pENTR™ 1A vector (Life Technologies, Carlsbad, CA) were digested with *Sal*I and *Eco*RI and ligated using T4 DNA ligase (New England Biolabs, Ipswich, MA). The ligated entry clone was transformed into competent *E. coli* DH5α cells and selected on Luria-Bertani (LB) medium plates containing 50 μg/mL kanamycin. The chymotrypsin-like protein-coding region of

the entry clone was transferred into the pDEST15 vector by LR clonase (Invitrogen) in accordance with the manufacturer's instructions. Recombinant pDEST15 vector was transformed into competent *E. coli* DH5α cells and selected on LB medium plates containing 100 μg/mL ampicillin. Positive clones were selected by PCR and sequenced (ABI Genetic Analyzer PRISM3100; Applied Biosystems).

Recombinant protein was expressed after inserting the recombinant vector into *E. coli* BL21 (DE3). Recombinant *E. coli* was cultured in LB medium containing 100 μg/mL ampicillin at 20 °C with 200 μM isopropyl β-D-1-thiogalactopyranoside (IPTG). *E. coli* was collected by centrifugation at 5,000×*g*, 4 °C for 10 min.

2.2.2. Purification of recombinant protein

Expressed protein was refolded using a Rapid GST Inclusion Body Solubilization and Renaturation Kit (Cell Biolabs, San Diego, CA). *E. coli* was resuspended in STE buffer accompanying the kit with 0.2 mg/mL lysozyme and 1 mM dithiothreitol and lysed by sonication on ice. Each *E. coli* lysate was subjected to SDS-PAGE, and recombinant protein expression was confirmed by comparison with the lysate without IPTG. In addition, to confirm the composition of the inclusion body, *E. coli* lysates were separated into soluble and insoluble protein fractions by centrifugation at 12,000×*g*, 4 °C for 15 min, and protein composition was checked by SDS-PAGE. The inclusion body was solubilized and refolded using the kit according to the manufacturer's product manual.

Refolded recombinant GST-protein sample was filtrated through a 0.2-μm membrane filter (Millipore, Billerica, MA). Aliquots of 500 μL of glutathione sepharose 4B beads (50 % slurry; GE Healthcare) were added to 10 mL of the sample solution and incubated at room temperature with rotation. Beads were collected by centrifugation at 500×*g*, room temperature for 5 min. Three milliliters of phosphate-buffered saline (PBS) was added to the beads and centrifuged at 500×*g*, room temperature for 5 min, and the supernatant was discarded. This washing step was repeated a total of three times. Recombinant protein was cleaved out from the GST-tag by 200 μL of 25 U/mL thrombin (GE Healthcare) in PBS at room temperature for 16 h. Recombinant protein was collected by centrifugation at 500×*g*, room temperature for 5 min; 200 μL of PBS was added to the beads followed again by centrifugation, and two supernatants were mixed. The protein concentration in the recombinant protein sample was measured by the Bradford method using the Bio-Rad Protein Assay (Bio-Rad Laboratories, Hercules, CA) and BSA as a standard.

2.3. Characterization of the enzyme

2.3.1. Enzyme assay and substrate specificity

The activities of the recombinant protein toward several serine protease substrates—SAAPF*p* Na, succinyl-alanyl-alanyl-prolyl-leucine *p*-nitroanilide (SAAPL*p* Na), succinyl-alanyl-alanyl-alanine *p*-nitroanilide (SAAA*p* Na), and N-benzoyl-DL-arginine *p*-nitroanilide (BAPA)—were determined. Aliquots of 10 μL of sample solution and 90 μL of 50 mM Tris-HCl buffer (pH 9.0) containing 1 mM of each substrate were added to 96-well plates and incubated at 37 °C. The change in absorbance at 405 nm was measured. One unit of protease activity corresponded to the amount of enzyme that released 1 μmol *p*-nitroaniline per minute.

2.3.2. Optimal pH and temperature

The optimal pH for enzymatic activity was determined using 50 mM citrate buffer at pH 4, 5, and 6; 50 mM phosphate buffer at pH 6, 7, and 8; 50 mM Tris-HCl buffer at pH 8 and 9; and 50 mM glycine-NaOH buffer at pH 9 and 10. Activity was determined using SAAPF*p* Na. The optimal temperature was determined using 50 mM Tris-HCl buffer (pH 9.0) and SAAPF*p* Na at 20, 30, 37, 40, 45, and 50 °C.

2.3.3. Inhibition study

The effects of different protease inhibitors on the activity of the recombinant protein were measured in 50 mM Tris-HCl buffer (pH 9.0) containing 1 mM SAAPF*p* Na at 37 °C. For inhibition assay, phenylmethylsulfonyl fluoride (PMSF), *N*-tosyl-L-lysine chloromethyl ketone (TLCK), pepstatin A, ethylenediaminetetraacetic acid (EDTA) (Nacalai Tesque, Kyoto, Japan), 4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), TPCK (Sigma Aldrich), antipain, leupeptin, and E-64 (Peptide institute Inc., Osaka, Japan) were used.

3. RESULTS

3.1. Separation and identification of proteases from honeybee queen larvae

In the previous report, we found protease fractions including chymotryptic and carboxy peptidyl activities from honeybee queen larvae (Matsuoka et al. 2012). For the mass analysis of honeybee larval proteases, we separated honeybee queen larval proteins by cation exchange and gel filtration chromatography according to our previous report. Activities of fractions including carboxypeptidase activity (fraction I) or chymotryptic activity (fraction II) were detected with substrates, Z-Gly-Phe or SAAPF*p* Na, respectively. The fractions were separated by SDS-PAGE (Figure 1a, b).

Each protein that was obtained from SDS-PAGE gel was analyzed by LC-MS/MS (Table I). The band of number 4 that was about 40 kDa of fraction I (Figure 1a) was identified to a protein carboxypeptidase-B-like protease (XP_623727), and the band of number 1 that was about 25–28 kDa protein of fraction II (Figure 1b) was identified to a chymotrypsin-like protease (XP_394370) from the honeybee genome database. These results were compatible with the results of the enzyme assay for SAAPF*p* Na or Z-Gly-Phe.

3.2. Expression and purification of recombinant chymotrypsin-like protease

The identified chymotrypsin-like protease was expressed by *E. coli* expression system. Recombinant *E. coli* lysate was analyzed by SDS-PAGE (Figure 2a). IPTG induced an expression of a protein with a molecular weight of about 53 kDa. The molecular weight of the recombinant chymotrypsin-like protease was expected to be about 26 kDa and conjugated with a GST-tag of 26 kDa. As recombinant GST-chymotrypsin was expressed as an insoluble protein, it was solubilized and refolded using a Rapid GST Inclusion Body Solubilization and Renaturation Kit. After refolding, the GST-tagged chymotrypsin was collected using glutathione sepharose 4B beads. The GST-tag was

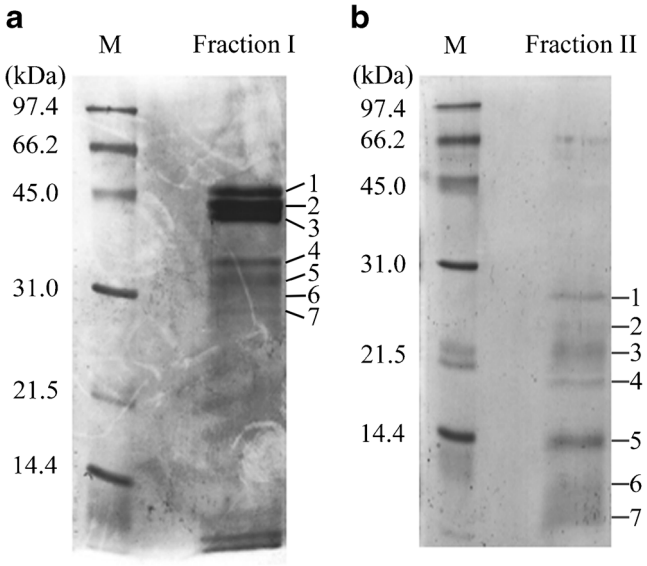

Figure 1. Separation of fractions containing carboxypeptidase-like (fraction I) and chymotrypsin-like (fraction II) activities from honeybee queen larvae. **a** The proteins contained in fraction I were separated by SDS-PAGE, and the *numbers* indicate the protein spots excised for ESI-ion trap MS. **b** The proteins contained in fraction II were separated by SDS-PAGE, and the *numbers* indicate the protein spots excised for ESI-ion trap MS.

Table I. Identification of proteases in carboxypeptidase-like and chymotrypsin-like protease fractions.

Sample	Accession number	Name	Race	Mass
Fraction I-4	XP_623727	Carboxypeptidase-B like	*Apis mellifera*	47,969
Fraction II-1	XP_394370	Chymotrypsin-1	*Apis mellifera*	28,079

cleaved from solubilized chymotrypsin with thrombin (Figure 2b). The size of the obtained protein corresponded to the estimated size of about 26 kDa.

3.3. Optimum pH and temperature for the hydrolysis of SAAPF*p* Na

The pH dependence of recombinant protease activity is shown in Figure 3a. Recombinant protease showed maximum activity for SAAPF*p* Na at pH 9.0. The activity at pH 7 was about half that seen at pH 9. The effects of temperature on recombinant protease activity were shown in Figure 3b. Recombinant protease activity was highest at 40 °C. This recombinant protease showed maximum activity at alkaline pH, and the enzyme did not have cold adaptation or heat resistance.

3.4. Activities of the recombinant protease with different substrates

To investigate the specificity of recombinant protease, its activities against several substrates of serine proteases were investigated as shown in Figure 4. The activities toward elastase (SAAPL*p* Na and SAAA*p* Na) and trypsin (BAPA) were lower than that for the chymotrypsin substrate, SAAPF*p* Na. The relative activities against SAAPL*p* Na, SAAA*p* Na, and BAPA were 32.7, 13.5, and 8.16 % of SAAPF*p* Na, respectively. These results suggested that the recombinant enzyme was a chymotryptic protease.

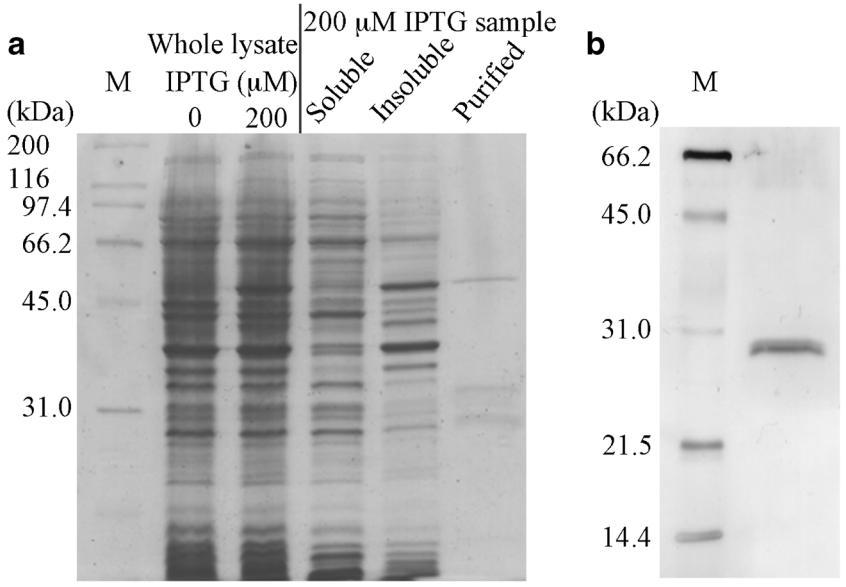

Figure 2. Expression and purification of recombinant chymotrypsin-like protease. **a** SDS-PAGE analysis with CBB staining of recombinant chymotrypsin-like protease extracted from *E. coli* lysate. In the *left panel*, each lane shows whole *E. coli* lysate without and with IPTG stimulation. In the *right panel*, each lane shows soluble and insoluble contents of *E. coli* lysate with IPTG stimulation and proteins purified by glutathione sepharose 4B beads. **b** SDS-PAGE analysis with silver staining of recombinant protein after thrombin treatment.

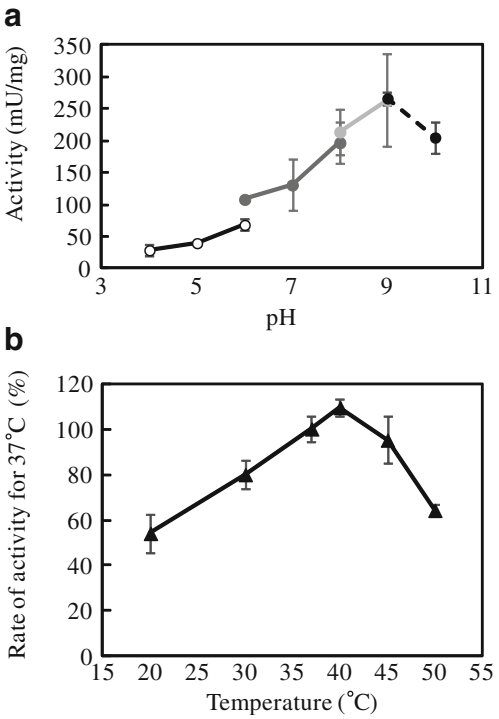

Figure 3. Optimum pH and temperature conditions for chymotryptic activity. **a** Effects of pH on chymotryptic activity. Enzyme activities were measured at various pH values using SAAPF*p* Na as a substrate at 37 °C. For reaction buffer, 50 mM citrate buffer (pH 4 and 5, *white point and black line*), 50 mM phosphate buffer (pH 6, 7, and 8, *thick gray point and line*), 50 mM Tris-HCl buffer (pH 8 and 9, *thin gray point and line*), and 50 mM glycine-NaOH buffer (pH 9 and 10, *black point and broken line*) were used. **b** Effects of temperature on chymotryptic activity. Enzyme activities were measured at various temperatures using SAAPF*p* Na as a substrate at pH 9. Data are presented as mean±SD.

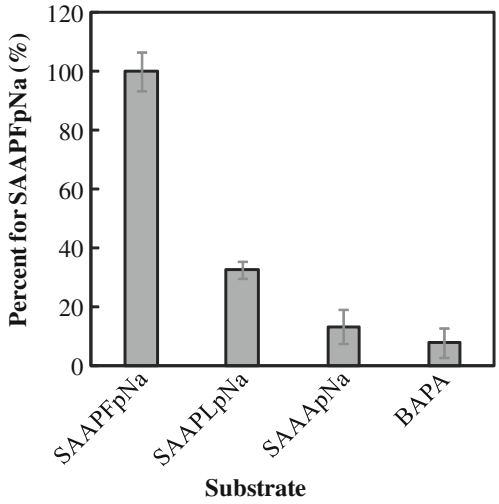

Figure 4. Substrate specificities of the recombinant chymotrypsin-like protease. Enzyme activities were measured with each substrate at pH 9, 37 °C. The data indicate activities relative to that against SAAPF*p* Na (100 %). Data are presented as mean±SD.

3.5. Effects of protease inhibitors on the hydrolysis of SAAPF*p* Na

The effects of protease inhibitors on the hydrolysis of SAAPF*p* Na by the recombinant protease were shown relative to the activity of the sample without inhibitors in Table II. PMSF strongly inhibited the activity. In addition, antipain also showed an inhibitory effect. Both of these inhibitors can inhibit the activities of serine-type proteases. On the other hand, pepstatin A, an aspartic protease inhibitor, and E-64, a cysteine protease inhibitor, did not inhibit the activity of the recombinant protease. Based on these results, the recombinant protease was regarded as a serine protease. EDTA, a metalloprotease inhibitor, showed a weak inhibitory activity, and AEBSF, generally used as a substitute for PMSF, and leupeptin, an inhibitor of several serine- or cysteine-type proteases, had weak inhibitory activities. In addition, neither TPCK nor TLCK inhibited the activity of the recombinant protease. These results suggested that the recombinant chymotrypsin-like protease had an active site that was different from mammalian chymotrypsin.

4. DISCUSSION

The compositions of digestive proteases of honeybee are different depending on the caste, stage (larva or adult), and age mainly because of differences in food. Previous reports regarding honeybee digestive proteases indicated that trypsin-like and chymotrypsin-like proteases are involved in the digestion of food in adult worker bees (Giebel et al. 1971; Dahlmann et al. 1978; Moritz and Crailsheim 1987). However, there

Table II. Effects of protease inhibitors on recombinant chymotrypsin-like protease.

Name	Concentration	Inhibition type	Inhibition (%)
PMSF	1 mM	Serine	78.1±1.62
AEBSF	1 mM	Serine	16.3±9.38
EDTA	1 mM	Metallo	20.9±3.99
TLCK	0.1 mM	Trypsin	0
TPCK	0.1 mM	Chymotrypsin	0
Leupeptin	10 μM	Serine, cysteine	24.7±6.98
Antipain	10 μM	Serine, cysteine	37.6±1.27
Pepstatin A	10 μM	Aspartic	0
E-64	10 μM	Cysteine	0

Data are presented as mean±SD

have been few reports regarding the digestive proteases of the queen or larvae of the honeybee.

In this study, we identified two proteases from the fractions that were separated from the homogenate of 3-day-old honeybee queen larvae that could hydrolyze the substrate for carboxypeptidase, Z-Gly-Phe, or a substrate for chymotrypsin, SAAPF*p* Na, respectively. Mass spectrometry and Mascot search suggested that these activities were derived from proteins similar to carboxypeptidase-B (XP_623727) or chymotrypsin (XP_394370) of *A. mellifera* (Figure 1, Table I). Therefore, we attempted to express the chymotrypsin-like protease in *E. coli*.

The 26-kDa chymotrypsin-like protease produced in *E. coli* (Figure 2) showed chymotryptic activity. The activity was highest at pH 9 and 40 °C (Figure 3). As the temperature of a honeybee colony is maintained at roughly 35 °C for normal brood development (Jones et al. 2004), the optimum temperature of the recombinant protease seemed to coincide with the living environment of honeybee larvae. Furthermore, the optimum pH of the enzyme was in the alkaline range. Most insect chymotrypsin-like enzymes separated from the midgut of adults and larvae have pH optima in the range of pH 8–9 (Terra and Ferreira 1994). Although the intestinal pH of the adult honeybee is weakly acidic and that of larvae is approximately neutral (Herbert and Shimanuki 1983), chymotrypsin-like and trypsin-like enzymes of adult honeybees also showed high levels of activity in the range of pH 7–9 (Moritz and Crailsheim 1987; Jimenez and Gilliam 1989). These reports suggest optimum pH of larval digestive enzymes is in the alkaline range. The result of pH dependence of the recombinant chymotrypsin-like protease from honeybee larvae was consistent with these previous reports.

The recombinant chymotrypsin-like protease hydrolyzed a chymotrypsin substrate, SAAPF*p* Na, more strongly than trypsin and elastase substrates (Figure 4). PMSF and antipain inhibited the activity, but pepstatin A and E-64 did not (Table II). These results suggested that this enzyme was a serine-type protease. Moreover, EDTA showed a weak inhibitory activity. Although EDTA is typically a metalloprotease inhibitor, it has been reported that some chymotrypsin- or trypsin-like digestive enzymes, such as that those of mealworm (Vinokurov et al. 2006; Elpidina et al. 2005; Tsybina et al. 2005), cockchafer (Wagner et al. 2002), beet armyworm (Herrero et al. 2005), and scorpion (Louati et al. 2011), were weakly inhibited by EDTA. On the other hand, TLCK and TPCK did not show inhibitory activity against the recombinant protease. The chymotrypsin-like enzymes of larval mealworms (Elpidina et al. 2005) and fire ants (Whitworth et al. 1998) were unaffected by both TPCK and TLCK. In addition, Botos et al. (2000) reported that the chymotrypsin of the fire ant is markedly similar but showed differences beyond those found among homologs from different mammalian systems. Thus, our protease was considered to have a different reaction mechanism from mammalian chymotrypsin.

Further structural analyses of the protease, such as crystallography, are required to clarify the reaction mechanism.

Giebel et al. (1971) found four endopeptidase fractions from adult honeybee and reported that the adult queen bee and worker larvae lacked three of these proteases. The queen bee and worker larvae only had a protease with a molecular weight of about 30 kDa that did not cleave trypsin substrates (BAEE and BANA) and chymotrypsin substrates (ATEE, APNE, and GlupNa), and its activity was inhibited by PMSF and TPCK. Thus, the protease that they found from the queen bee was different from our chymotrypsin-like protease. In this study, we expressed the chymotrypsin-like protease found in the whole lysate of honeybee queen larvae in *E. coli* , and therefore, we did not confirm whether it was localized in the midgut. Further studies are required to determine the localization of the protein.

From the above results, we designate the recombinant protease as a honeybee chymotrypsin-like protease (HCLPase). This is the first report of the identification of a protease in honeybee larvae. Recently, Kamakura (2011) demonstrated that the RJ protein royalactin played a critical role in honeybee differentiation. However, it is not clear how the honeybee queen and larvae digest RJ and utilize it for their development and physiology. The study of proteases of honeybee larvae may clarify not only the development and differentiation of the honeybee but also the mechanism of action of RJ as health food and allow the development of further functional value.

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Expression et caractérisation d'une protéase analogue à la chymotrypsine chez les larves de l'abeille, *Apis mellifera*

Larve / reine / protéase analogue à la chymotrypsine

Expression und Charakterisierung einer Chymotrypsin-ähnlichen Protease in Larven der Honigbiene, *Apis mellifera*

***Apis mellifera* / Königinnenlarven / Chymotrypsin-ähnliche Protease**

REFERENCES

Botos, I., Meyer, E., Nguyen, M., Swanson, S.M., Koomen, J.M., Russell, D.H., Meyer, E.F. (2000) The structure of an insect chymotrypsin. J. Mol. Biol. **298** , 895–901

Brødsgaard, H.F., Brødsgaard, C.J., Hansen, H., Lövei, G.L. (2003) Environmental risk assessment of transgene products using honey bee (*Apis mellifera*) larvae. Apidologie **34** , 139–145

Burgess, E.P.J., Malone, L.A., Christeller, J.T. (1996) Effect of two proteinase inhibitors on the digestive enzymes and survival of honey bees (*Apis mellifera*). J. Insect Physiol. **42** , 823–828

Dahlmann, B., Jany, K.D., Pfleiderer, G. (1978) The midgut endopeptidase of the honey bee (*Apis mellifica*): comparison of the enzymes in different ontogenetic stages. Insect Biochem. **8** , 203–211

Elpidina, E.N., Tsybina, T.A., Dunaevsky, Y.E., Belozersky, M.A., Zhuzhikov, D.P., Oppert, B. (2005) A chymotrypsin-like proteinase from the midgut of *Tenebrio molitor* larvae. Biochimie **87** , 771–779

Giebel, W., Zwilling, R., Pfleiderer, G. (1971) The evolution of endopeptidases-XII. The proteolytic enzymes of the honeybee (*Apis mellifica* L.). Comp. Biochem. Physiol. **38B** , 197–210

Guo, H., Ekusa, A., Iwai, K., Yonekura, M., Takahata, Y., Morimatsu, F. (2008) Royal jelly peptides inhibit lipid peroxidation in vitro and in vivo. J. Nutr. Sci. Vitaminol. **54** , 191–195

Guo, H., Kouzuma, Y., Yonekura, M. (2009) Structures and properties of antioxidative peptides derived from royal jelly protein. Food Chem. **113** , 238–245

Herbert Jr., E.W., Shimanuki, H. (1983) Effect of diet pH on the consumption, brood rearing, and pH of worker jelly produced by caged honey bees. Apidologie **14** , 191–196

Herrero, S., Combes, E., Van Oers, M.M., Vlak, J.M., de Maagd, R.A., Beekwilder, J. (2005) Identification and recombinant expression of a novel chymotrypsin from *Spodoptera exigua* . Insect Biochem. Mol. Biol. **35** , 1073–1082

Hidaka, S., Okamoto, Y., Uchiyama, S., Nakatsuoma, A., Hashimoto, K., Ohnishi, S.T., Yamaguchi, M. (2006) Royal jelly prevents osteoporosis in rats: beneficial effects in ovariectomy model and in bone tissue culture model. eCAM **3** , 339–348

Honeybee Genome Sequencing Consortium (2006) Insights into social insects from the genome of the honeybee *Apis mellifera* . Nature **443** , 931–949

Jimenez, D.R., Gilliam, M. (1989) Age-related changes in midgut ultrastructure and trypsin activity in the honey bee, *Apis mellifera* . Apidologie **20** , 287–303

Jones, J.C., Myerscough, M.R., Graham, S., Oldroyd, B.P. (2004) Honey bee nest thermoregulation: diversity promotes stability. Science **305** , 402–404

Kamakura, M. (2011) Royalactin induces queen differentiation in honeybees. Nature **473** , 478–483

Louati, H., Zouari, N., Miled, N., Gargouri, Y. (2011) A new chymotrypsin-like serine protease involved in dietary protein digestion in a primitive animal, Scorpio maurus: purification and biochemical characterization. Lipids Health Dis. **10** , 121

Matsui, T., Yukiyoshi, A., Doi, S., Sugimoto, H., Yamada, H., Matsumoto, K. (2002) Gastrointestinal enzyme production of bioactive peptides from royal jelly protein and their antihypertensive ability in SHR. J. Nutr. Biochem. **13** , 80–86

Matsuoka, T., Kawashima, T., Nakamura, T., Kanamaru, Y., Yabe, T. (2012) Isolation and characterization of proteases that hydrolyze royal jelly proteins from queen bee larvae of the honeybee, *Apis mellifera* . Apidologie **43** , 685–697

Moore, S. (1968) Amino acid analysis: aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J. Biol. Chem. **243** , 6281–6283

Moritz, B., Crailsheim, K. (1987) Physiology of protein digestion in the midgut of honeybee (*Apis mellifera* L.). J. Insect Physiol. **12** , 923–931

Nagai, T., Inoue, R., Suzuki, N., Nagashima, T. (2006) Antioxidant properties of enzymatic hydrolysates from royal jelly. J. Med. Food **9** , 363–367

Nakasa, T., Ueda, S., Nakatsuka, M., Okinaka, O. (2003) Effect of protease-treated royal jelly on plasma and liver lipids in rats fed on a high fat plus high cholesterol diet. Nippon Shokuhin Kagaku Kogaku Kaishi **10** , 463–467

Niu, K., Guo, H., Guo, Y., Ebihara, S., Asada, M., Ohrui, T., Furukawa, K., Ichinose, M., Yanai, K., Kubo, Y., Arai, H., Okazaki, T., Nagatomi, R. (2013) Royal jelly prevents the progression of sarcopenia in aged mice in vivo and in vitro. J. Gerontol. A Biol. Sci. Med. Sci. **68** , 1482–1492

Shevchenko, A., Wilm, M., Vorm, O., Mann, M. (1996) Mass spectrometric sequencing of proteins silver-stained polyacrylamide gels. Anal. Chem. **68** , 850–858

Terra, W.R., Ferreira, C. (1994) Insect digestive enzymes: properties, compartmentalization and function. Comp. Biochem. Physiol. **109B** , 1–62

Tokunaga, K., Yoshida, C., Suzuki, K., Maruyama, H., Futamura, Y., Araki, Y., Mishima, S. (2004) Antihypertensive effect of peptides from royal jelly in spontaneously hypertensive rats. Biol. Pharm. Bull. **27** , 189–192

Tsybina, T.A., Dunaevsky, Y.E., Belozersky, M.A., Zhuzhikov, D.P., Oppert, B., Elpidina, E.N. (2005) Digestive proteinases of yellow mealworm (Tenebrio molitor) larvae: purification and characterization of a trypsin-like proteinase. Biochemistry (Moscow) **70** , 370–377

Vinokurov, K.D., Elpidina, E.N., Oppert, B., Prabhakar, S., Zhuzhikov, D.P., Dunaevsky, Y.E., Belozersky, M.A. (2006) Diversity of digestive proteinases in *Tenebrio molitor* (Coleoptera: Tenebrionidae) larvae. Comp. Biochem. Physiol. B **145** , 126–137

Wagner, W., Möhrlen, F., Schnetter, W. (2002) Characterization of the proteolytic enzymes in the midgut of the European cockchafer, *Melolontha melolontha* (Coleoptera: Scarabaeidae). Insect Biochem. Mol. Biol. **32** , 803–814

Whitworth, S.T., Blum, M.S., Travis, J. (1998) Proteolytic enzymes from larvae of the fire ant, *Solenopsis invicta* : isolation and characterization of four serine endopeptidases. J. Biol. Chem. **273** , 14430–14434

Zou, Z., Lopez, D.L., Kanost, M.R., Evans, J.D., Jiang, H. (2006) Comparative analysis of serine protease-related genes in the honey bee genome: possible involvement in embryotic development and innate immunity. Insect Biochem. Mol. Biol. **15** , 603–614