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Apidologie (2012) 43:685–697

DOI: 10.1007/s13592-012-0143-z

Original article

Isolation and characterization of proteases that hydrolyze royal jelly proteins from queen bee larvae of the honeybee, *Apis mellifera*

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Received 25 December 2011 – Revised 1 May 2012 – Accepted 10 May 2012

Abstract – Royal jelly is a nutritious substance secreted from the hypopharyngeal and mandibular glands of worker bees that serves as the only food on which honeybee queen larvae and adults are fed and which causes them to develop into queen bees. Royal jelly is a protein-rich food and one of the most crucial factors for the growth of queen bees. In this study, we characterized the hydrolytic activity of enzymes from the homogenates of honeybee queen larvae on royal jelly proteins. Homogenates of 3-day-old queen bee larvae were capable of hydrolyzing royal jelly proteins under alkaline conditions. Following separation by cation exchange and gel filtration column chromatographies, two proteases of 38 and 28 kDa were found by SDS-PAGE. The protease of 38 kDa had a carboxypeptidase A-like activity and that of 28 kDa a chymotrypsin-like activity. These enzymes may turn out to be useful in the manufacturing of processed royal jelly.

Apis mellifera **/ queen larvae / protease / royal jelly**

1. INTRODUCTION

Royal jelly (RJ), secreted from the hypopharyngeal and mandibular glands of nurse bees of the honeybee, *Apis mellifera*, is provided as food for queen larvae and adults and is widely used as a health food and dietary supplement for humans. RJ has a number of nutritional benefits, especially so the proteins and derived peptides which have various physiological activities.

RJ contains a family of proteins called the major royal jelly proteins (MRJPs), which constitute ~90 % of the total RJ protein (Schmitzová et al. 1998; Drapeau et al. 2006). It is also known that RJ proteins have various physiological activities, such as stimulating cell growth (Watanabe et al. 1996) as well as neurohormonal (Colhoun and Smith 1960) and antibacterial activities (Fujiwara et al. 1990; Casteels et al. 1990; Fontana et al. 2004). Furthermore, Kamakura (2011) recently demonstrated that the monomer of MRJP 1 in RJ, royalactin, plays a critical role in queen differentiation. Thus, RJ is a key factor in caste development, where fertilized eggs give rise to queens or workers and unfertilized eggs to drones. The development of honeybee larvae into either a queen or worker depends on the brood food that is provided by nurse bees. Food for queen larvae consists almost entirely of RJ, and the adult queen bee also feeds only on this RJ-rich brood food during her entire life (Winston 1987).

Commercially available RJ products include those that have been processed with proteases.

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Manuscript editor: Klaus Hartfelder

Several studies have indicated that some dietary protein hydrolyzed by proteases show greater biological effects, e.g., angiotensin I-converting enzyme, inhibitory activity of milk protein hydrolysate (Minervini et al. 2003), and inhibition of cholesterol absorption by soy bean protein hydrolysate (Nagaoka et al. 1997). Similarly, RJ peptides hydrolyzed by various proteases have been reported to show functional activities, such as antioxidant activity (Guo et al. 2008), cholesterol-lowering effect (Nakasa et al. 2003), and anti-hypertensive activity (Matsui et al. 2002; Tokunaga et al. 2004). In these studies, the proteases used for the hydrolysis of proteins were derived from microbes, plants, or animal organs. As RJ proteins consumed by honeybee queen larvae are hydrolyzed by enzymes of the larvae in nature, it is possible that RJ peptides with physiological functions may also be produced by the hydrolytic activity of larval enzymes. Therefore, we propose herein the possibility of producing physiologically active RJ peptides by means of honeybee enzymes.

Proteases of honeybees have been studied for a long time. Giebel et al. (1971) reported four endopeptidases with different cleavage properties from adult honeybee workers. Dahlmann et al. (1978) showed differences in the activities of two endopeptidases between adults and worker larvae. Moritz and Crailsheim (1987) determined the activities of trypsin-like and chymotrypsin-like enzymes in the midgut of honeybees. However, there are no reports yet on how proteases from honeybee may affect RJ proteins

Honeybee larvae are used as traditional food along with honey and RJ in Asian countries, and products of larval homogenates or powders with the addition of RJ are commercially available. If the whole larval body hydrolyzes RJ proteins, crude larval homogenate or powder may thus become useful as an enzyme source for industrial food production. Dahlmann et al. (1978) found chymotryptic activity in whole worker larval homogenate. Therefore, we herein screened for proteases capable of hydrolyzing RJ proteins from whole honeybee queen larvae and investigated their characteristics. We report the isolation and characterization of two proteases exhibiting such activity.

2. MATERIALS AND METHODS

2.1. Preparation of crude enzyme and RJ solution

Three-day-old honeybee queen larvae (average length, 12 mm) and RJ were obtained from Akitaya Honten Co., Ltd. (Gifu, Japan). Honeybee queen larvae were homogenized and filtered with an 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* for 20 min at 4°C. 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 a crude enzyme solution. RJ was diluted in an equal volume of 50 mM phosphate buffer (pH 7.0) and then centrifuged at 25,000×*g* for 20 min at 4°C. The protein concentration of the supernatant was adjusted to 30 mg/mL.

2.2. Determination of protein concentration

Protein concentrations were determined by the Lowry method (Lowry et al. 1951). Briefly, the Lowry reagent consisted of 2 % Na_2CO_3 in 0.1 N NaOH, 1 % $CuSO_4$, and 2 % Rochelle salt in a ratio of 100:1:1 (*v*/*v*/*v*). Aliquots of 50 μL of the sample and 150 μL of the Lowry reagent were mixed and then incubated for 10 min at room temperature, followed by the addition of 15 μL of 1 N Folin & Ciocalteu phenol reagent. The reaction solution was incubated for 30 min at room temperature. Aliquots of 200 μL were pipetted into 96-well plates and absorbance measured at 650 nm. Bovine serum albumin was used as a protein standard.

2.3. Effects of pH and temperature on the hydrolysis of RJ proteins by the crude enzyme solution

The optimum pH for the hydrolysis of RJ proteins by the crude enzyme solution was determined using

different buffers, these being a 50-mM acetate buffer at pH 4 and 5, a 50-mM phosphate buffer at pH 6 and 7, and a 50-mM Tris–HCl buffer at pH 8 and 9. The reaction solutions consisted of 50 μL of the RJ solution (30 mg/mL), 50 μL of the crude enzyme solution (3 mg/mL), and 400 μL of each test buffer. Incubations were done at 37°C for 4, 8, 16, and 24 h, respectively. As controls, 50 μL of the RJ solution (30 mg/mL) and 450 μL of each test buffer were mixed and incubated at pH 4–9 for 24 h at 37°C. The optimum temperature for the hydrolysis of RJ proteins was determined by incubation at 4, 20, 37, or 50°C in 50 mM phosphate buffer (pH 7) or 50 mM Tris–HCl buffer (pH 9) for 4, 16, and 24 h. The hydrolysis of RJ proteins was confirmed by SDS-PAGE.

2.4. Inhibition studies

The serine-type protease inhibitor, phenylmethylsulfonyl fluoride (PMSF), and the metallo-type protease inhibitor, disodium dihydrogen ethylenediaminetetraacetic acid (EDTA), were purchased from Nacalai Tesque (Kyoto, Japan); the cysteine protease inhibitor (antipain), the aspartic protease inhibitor (pepstatin A), and the serine and cysteine-type protease inhibitor (leupeptin) were purchased from Peptide Institute Inc. (Osaka, Japan); the aminopeptidase inhibitor (bestatin) and carboxypeptidase inhibitor from potato tuber (CPI) were purchased from Sigma Aldrich (St. Louis, MO). The hydrolysis of RJ proteins with the protease inhibitors was investigated at 37°C for 24 h in 50 mM phosphate buffer (pH 7.0) or 50 mM Tris–HCl buffer (pH 9.0) in duplicate experiments. Inhibition of the hydrolysis of RJ proteins was also confirmed by SDS-PAGE.

2.5. Enzyme assays

Serine protease activities were determined by the hydrolysis of *N*-succinyl-L-alanyl-L-alanyl-L-prolyl-L-phenylalanine *p*-nitroanilide (SAAPF*p*NA; Sigma Aldrich) as a substrate. The reaction mixture contained 940 μL of 50 mM Tris–HCl buffer (pH 9.0), 10 μL of 50 mM SAAPF*p*NA in dimethylformamide, and 50 μL of the sample and was incubated at 37°C for 20 min. The reaction was stopped by adding 10 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 minute under standard assay conditions. Enzyme activities were determined in duplicate.

The carboxypeptidase activities were determined using *N*-carbobenzoxy-glycyl-L-phenylalanine (Z-Gly-Phe; Sigma Aldrich) as a substrate. The reaction mixture contained 90 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 60 min. The reaction was stopped by adding 300 μL of 0.4 M acetate buffer (pH 5.0). The absorbance of liberated phenylalanine was determined by a ninhydrin assay (Moore 1968). One unit of activity corresponded to the amount of enzyme that released 1 μmol of phenylalanine per minute under standard assay conditions. Enzyme activities were determined in duplicate.

2.6. Column chromatography

The proteases were purified by cation exchange column chromatography and gel filtration column chromatography. Aliquots of 5 mL of crude enzyme solution were loaded onto a 1-mL HiTrap SP HP column (GE Healthcare UK Ltd., 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 proteins were eluted with 20 mM phosphate buffer (pH 7.6) containing 0.5 M NaCl. Gel filtration chromatography was performed by the ÄKTA prime 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 2 mL of fraction volume and detected by absorbance at 280 nm.

2.7. Densitometry

The SDS-PAGE gels were scanned and the intensities of protein bands were determined using ImageJ software (NIH, Bethesda, MD).

3. RESULTS

3.1. Effects of pH and temperature on the hydrolysis of RJ proteins by crude enzyme solution

The RJ protein hydrolytic activities of the crude enzyme solution from honeybee queen larvae were investigated. The activities were stronger in the alkaline range than in the lower pH range (Figure 1). At pH 9, most bands were hydrolyzed at 4 h, except for the main band, and all bands disappeared completely at 16 h. Furthermore, the hydrolysis patterns were different at neutral and alkaline pH. A 46-kDa band, which was the second main band of the RJ, was

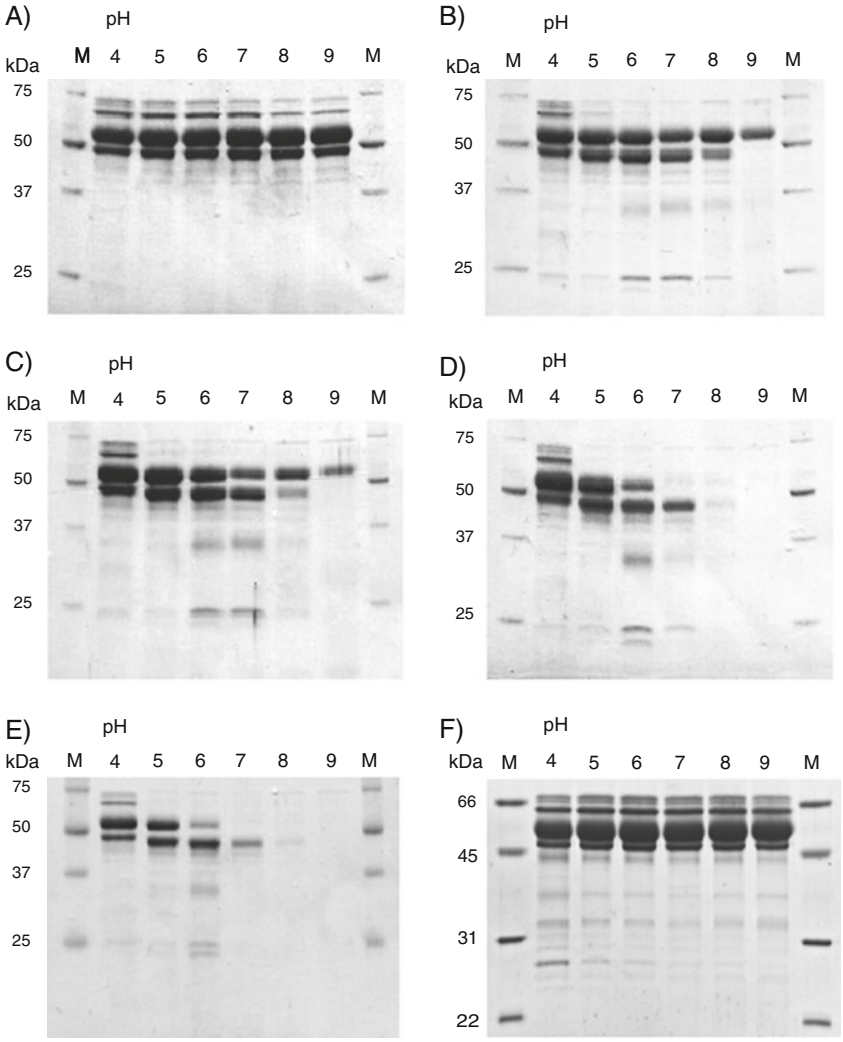

Figure 1. Effects of pH on the hydrolysis of RJ proteins by crude enzyme solutions from honeybee queen larvae. RJ solution (30 mg/mL proteins), crude enzyme solution (3 mg/mL proteins), and buffer (50 mM acetate buffer, pH 4 or 5; 50 mM phosphate buffer, pH 6 or 7; 50 mM Tris–HCl buffer, pH 8 or 9) were mixed at 1:1:8 (*v*/*v*/*v*) and incubated at 37°C for 0 (**a**), 4 (**b**), 8 (**c**), 16 (**d**), and 24 h (**e**), respectively. **f** RJ solution and the buffer without crude enzyme solution mixed and incubated at 37°C for 24 h. *M* protein molecular mass marker.

completely hydrolyzed within 4 h under alkaline conditions, but remained stable until 24 h under neutral conditions. This result suggested that the crude enzyme solution contained a number of proteases. On the other hand, protein hydrolytic activity was weak under acidic conditions. Without the crude enzyme solution, RJ protein was not hydrolyzed to a considerable extent when incubated at 37°C for 24 h (Figure 1f). This result suggested that the hydrolysis of RJ proteins under alkaline and neutral conditions was caused by the crude enzyme solution.

The effects of temperature on the hydrolysis of RJ proteins are shown in Figure 2. The hydrolytic activities increased with temperature when done in the range from 4 to 50°C at both pH 7 and 9. Particularly, a band of 46 kDa was hydrolyzed slowly at 37°C at pH 7, but vanished more quickly than the main band of 51 kDa at 50°C and pH 7. At pH 9 and 50°C, the two main bands disappeared within 4 h.

3.2. Effects of protease inhibitors on hydrolytic activities

To characterize the proteases in the crude enzyme solution from honeybee queen larvae, we examined the effects of various protease/peptidase inhibitors (Figure 3). At both pH 7 and 9, PMSF and CPI resulted in a stronger inhibition than the other five inhibitors examined. At pH 9, the protease activities of the crude enzyme solution were less subject to the inhibitors than at pH 7. This result indicated that the hydrolysis of RJ proteins was mainly by serine proteases and carboxypeptidases and that the proteases acted more strongly under alkaline than under neutral conditions. Carboxypeptidases are classified into three types: metallo, serine, and cysteine types. CPI generally inhibits metallo-type carboxypeptidases, e.g., carboxypeptidase A or B. Furthermore, EDTA showed a lower inhibitory effect than PMSF.

3.3. Purification of proteases by column chromatography

Fractions including the protease activities were purified from the crude enzyme solution by column chromatography. First, the crude enzyme solution was subjected to cation exchange column chromatography (HiTrap SP HP) and then eluted with 0.5 M NaCl. The activities of type A carboxypeptidases in the eluent were determined using Z-Gly-Phe as a substrate and measuring phenylalanine release. Furthermore, the activities of serine proteases in the eluent were also determined using SAAPF*p*NA as a substrate and measuring *p*-nitroaniline release. At the cation exchange column–chromatography purification step, the enzymes that hydrolyzed Z-Gly-Phe were enriched 8-fold and the enzymes that hydrolyzed SAAPF*p*NA enriched 11-fold (Table I). Next, the obtained eluent was subjected to gel filtration column chromatography (HiLoad 16/600 Superdex 75 pg). The activities of the proteases indicated by the two substrates are shown in Figure 4a. Two fractions that hydrolyzed Z-Gly-Phe (fraction I) and hydrolyzed SAAPF*p*NA (fraction II) were obtained. At the gel filtration column chromatography fractionation step, the proteases that hydrolyzed Z-Gly-Phe were enriched 238-fold and the proteases that hydrolyzed SAAPF*p*NA enriched 101-fold from the first crude enzyme solution (Table I). Figure 4 shows that the activity of fraction I detected by SAAPF*p*NA was slight, and no activity was detected in fraction II by Z-Gly-Phe. The proteases in fractions I and II showed different substrate specificities; thus, these fractions contained different types of proteases. In addition, although fractions 18–20 (fraction I) and 26–28 (fraction II) hydrolyzed RJ proteins, fraction 30 also showed hydrolytic activities for RJ proteins (Figure 4b). Figure 4a shows that, regardless of the low protein content in fraction 30, this fraction had SAAPF*p*NA hydrolytic activity. Thus, fraction 30 was designated as fraction III.

The hydrolytic activities of RJ proteins by fractions I, II, and III are shown in Figure 5. All hydrolytic activities for the fractions were stronger under alkaline than under neutral conditions (data not shown). The activities of fractions I and II were inhibited by both PMSF and CPI (Figure 5a, b). In contrast, the activity of fraction III was inhibited only by CPI (Figure 5c). PMSF

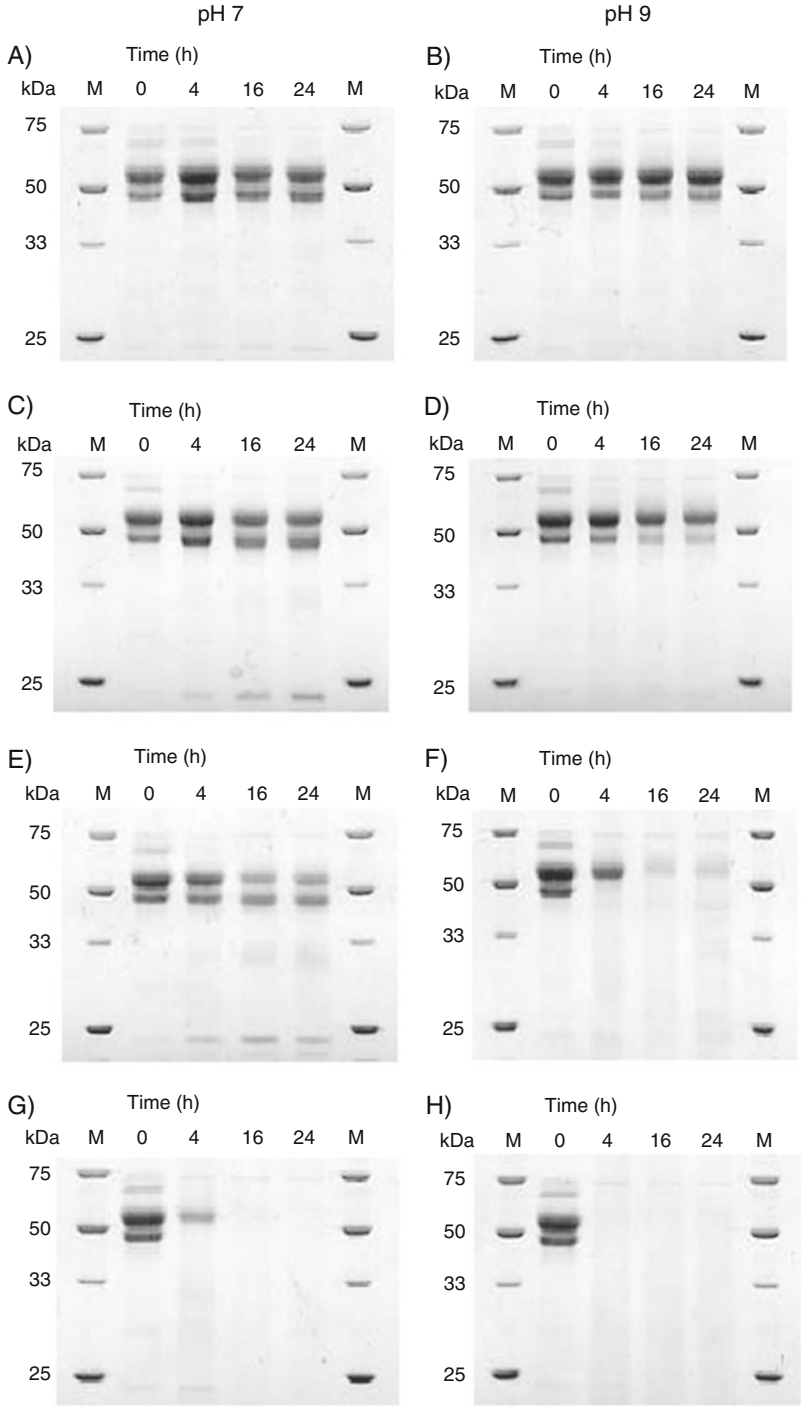

Figure 2 Effects of temperature on the hydrolysis of RJ proteins by crude enzyme solution. RJ solution (30 mg/mL protein), crude enzyme solution (3 mg/mL protein), and buffer (50 mM phosphate buffer, pH 7.0, or 50 mM Tris–HCl buffer, pH 9.0) were mixed at 1:1:8 (*v*/*v*/*v*) and incubated at 4°C (**a**, **b**), 20°C (**c**, **d**), 37°C (**e**, **f**), and 50°C (**h**, **h**), for 0, 4, 16, and 24 h each. *M* protein molecular marker

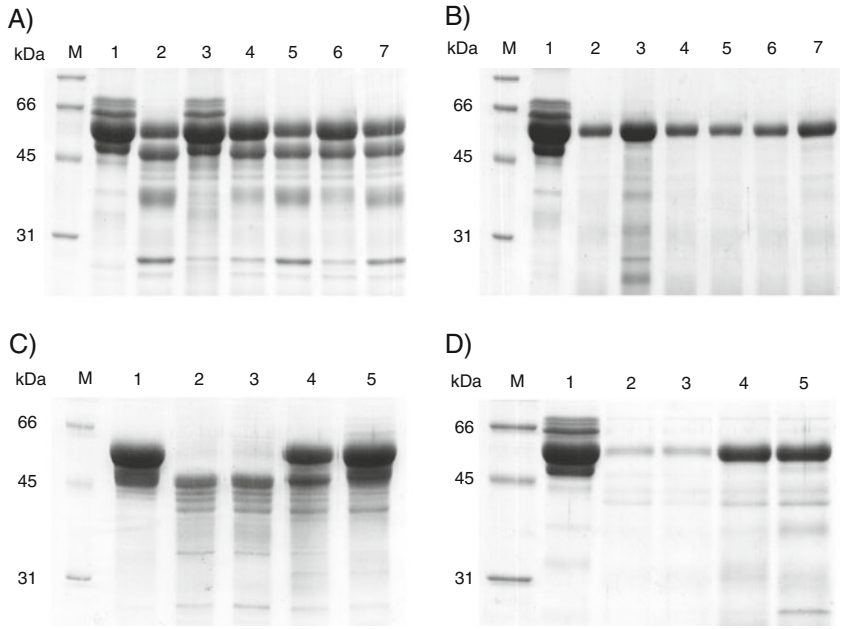

Figure 3. Effects of protease inhibitors on hydrolytic activities of crude enzyme solution. RJ solution (30 mg/mL proteins), crude enzyme solution (3 mg/mL proteins), and buffer (50 mM phosphate buffer, pH 7.0, or 50 mM Tris–HCl buffer, pH 9.0) were mixed at 1:1:8 (*v*/*v*/*v*). The reactions after adding the following inhibitors were carried out at 37°C for 24 h. The results of adding the following protease inhibitors at pH 7.0 (**a**) and at pH 9.0 (**b**) are shown: *1* untreated RJ solution; *2* no inhibitor; *3* 3 mM PMSF; *4* 9 mg/mL antipain; *5* 9 mg/mL pepstatin A; *6* 9 mg/mL leupeptin; *7* 3 mM EDTA. The results of adding the following peptidase inhibitors at pH 7.0 (**c**) and at pH 9.0 (**d**) are shown: *1* untreated RJ solution; *2* no inhibitor; *3* 0.8 mg/mL bestatin; *4* 10 mg/mL CPI; *5* 3 mM PMSF.

did not inhibit hydrolysis by fraction III, and EDTA did not inhibit activity in any fraction. The hydrolysis patterns when using PMSF and CPI were almost the same in fraction I (Figure 5a); therefore, these two inhibitors were considered to act on the same enzyme. In contrast, as shown in Figure 5b, the hydrolysis patterns for fraction II were different when PMSF and CPI were used as inhibitors, suggesting that this fraction contained both serine proteases and carboxypeptidases. The hydrolysis by fraction III was inhibited only by CPI and not

Table I. Purification of protease in crude enzyme solution obtained from honeybee queen larvae.

Step	Total activity (U)	Protein amount (mg)	Specific activity (U/mg)	Yield (%)	Purification factor (fold)
Protease (hydrolyzes Z-Gly-Phe) in fraction I					
Crude extract	1.42	100	0.0142	100	1
Cation exchange	0.0526	0.454	0.116	3.71	8
Gel filtration	0.140	0.0414	3.38	9.88	238
Protease (hydrolyzes SAAPF*p*NA) in fraction II					
Crude extract	2.80	100	0.03	100	1
Cation exchange	0.238	0.511	0.31	8.49	11
Gel filtration	0.215	0.0757	2.84	7.68	101

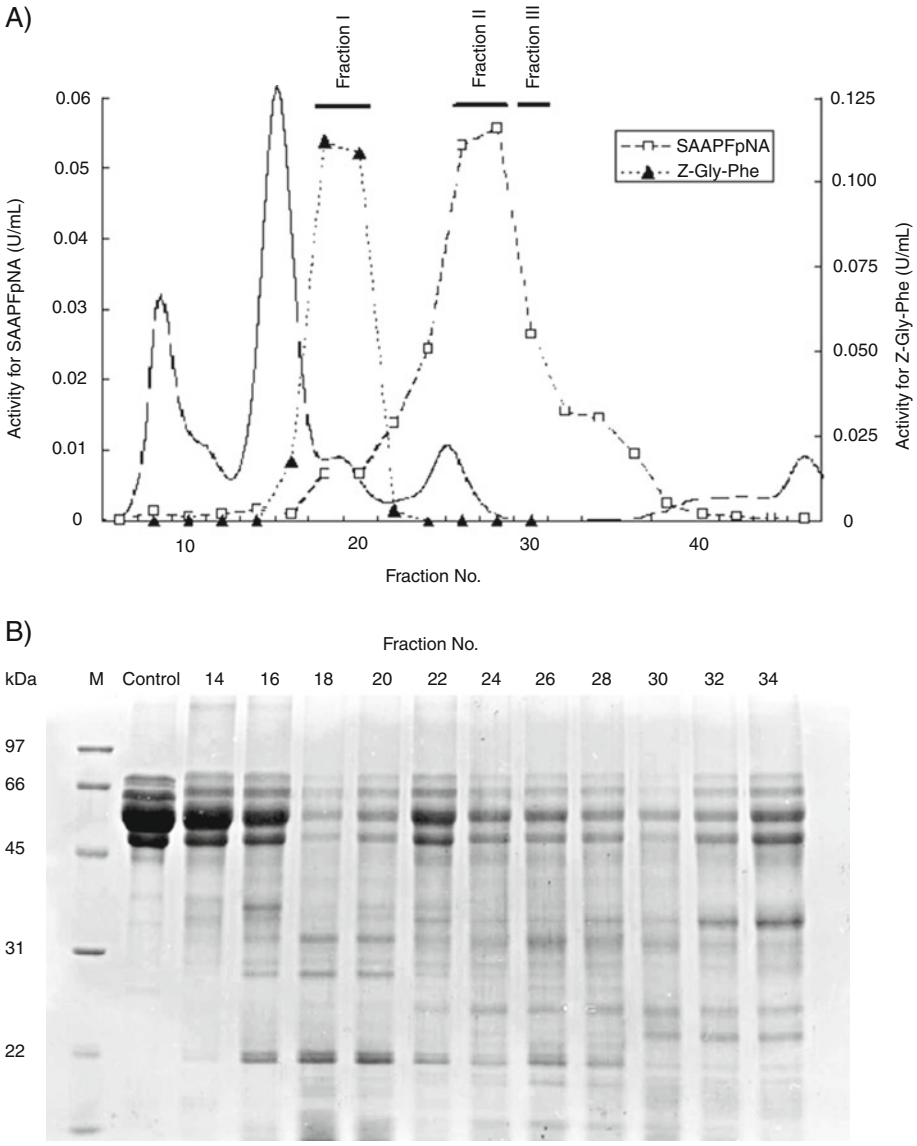

Figure 4. Specific activities of each fraction from gel filtration column chromatography. **a** A chromatogram from gel filtration chromatography is shown with specific activities of each fraction. The eluate was collected every 2 mL for 50 fractions. The *solid line* shows the protein concentration. The *broken line* and *white squares* show hydrolytic activity against SAAPF*p*NA at pH 9.0. The *dotted line* and *black triangles* show hydrolytic activity against Z-Gly-Phe at pH 9.0. **b** Hydrolytic activities of each fraction against RJ proteins are shown. RJ solution (30 mg/mL proteins), each fraction, and buffer (50 mM Tris–HCl buffer, pH 9) were mixed at 1:1:8 and then incubated at 37°C for 16 h. The represented *number* indicates fraction number. *M* marker proteins. **c** Untreated RJ solution.

by PMSF or EDTA, suggesting that the protease in fraction III was carboxypeptidase-like, even though it did not hydrolyze the carboxypeptidase A substrate, Z-Gly-Phe.

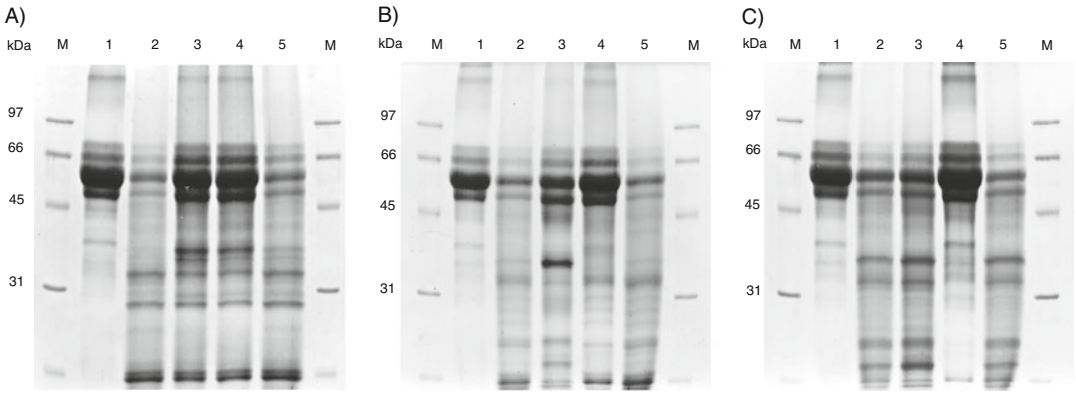

Figure 5. Effects of inhibitors on the hydrolysis of RJ proteins by the fractions obtained from gel filtration chromatography. Inhibitory activities are shown for fraction I (**a**), fraction II (**b**), and fraction III (**c**). RJ solution (30 mg/mL proteins), sample fractions, and buffer (50 mM Tris–HCl buffer, pH 9.0) were mixed at 1:1:8 (*v*/*v*/*v*). The reactions after adding the following inhibitors were carried out at 37°C for 24 h: *1* untreated RJ solution; *2* no inhibitors; *3* 3 mM PMSF; *4* 9 mg/mL CPI; *5* 20 mM EDTA.

To determine the exact source of the protease activities in fractions I and II, proteins in the fractions were further analyzed by SDS-PAGE (Figure 6). By comparing the activities and

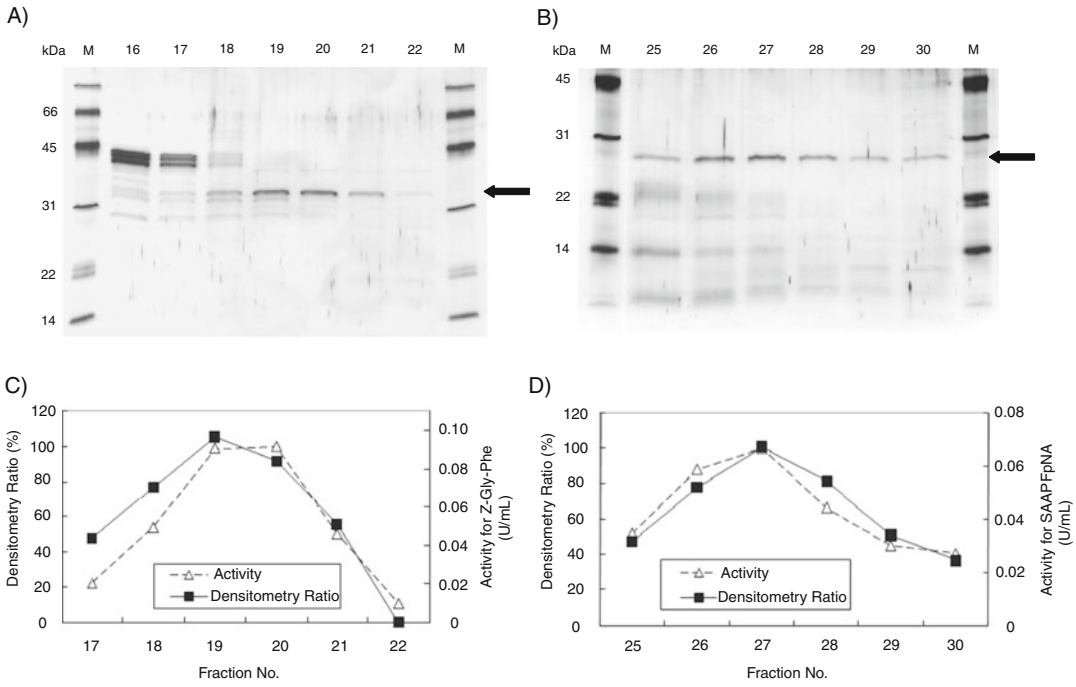

Figure 6. SDS-PAGE analysis of fractions neighboring fractions I and II and the relation of each activity and optical densitometry of 38- or 28-kDa bands. The results of SDS-PAGE for fractions neighboring fraction I (**a**) and II (**b**). The fraction numbers are shown. *Arrows* in (**a**) show the 38-kDa band and in (**b**) show the 28-kDa band. The activities of each fraction and the optical densitometry ratios of 38-kDa (**c**) and 28-kDa (**d**) bands are shown.

densities of the major bands on SDS-PAGE, candidate proteases for the hydrolysis of RJ proteins were predicted: a protein around 38 kDa in fraction I (Figure 6a) and a protein around 28 kDa in fraction II (Figure 6b). Densitometry ratios for the 38- and 28-kDa bands were commensurable with the activities, suggesting that these proteins were likely the proteases responsible for the hydrolysis of RJ proteins. The characteristics of proteases in the three fractions are summarized in Table II.

4. DISCUSSION

RJ is provided to honeybee larvae and the queen and has been confirmed to have multiple biological functions. Also, RJ is widely consumed as a health food. The main components of RJ are proteins, which make up 50 % of its dry mass. Some of the biological functions of RJ proteins have been determined, and the hydrolysates of RJ proteins produced by various proteases have been reported to exhibit even stronger biological activities. As RJ is the only food for queen larvae and and is also used to feed a queen bee, the physiological activities suggest that enzymes from queen larvae may hydrolyze RJ proteins and thus confer stronger physiological activities, including three peptides with cell proliferation activity (Matsuoka et al., unpublished data). In the current study, we investigated the hydrolytic activities of the homogenates of honeybee queen larvae against RJ proteins and then characterized the major proteases in the homogenates.

Table II. Characteristics of three fractions that hydrolyze RJ proteins.

	Substrate	Inhibitor	Molecular weight (kDa)
Fraction I	Z-Gly-Phe	PMSF, CPI	38
II	SAAPF*p*NA	PMSF, CPI	28
III	–	CPI	–

Several reports on differences in the development and nutrition between workers and queen honeybees indicate that proteins fed to larvae come to differ at around 72 h of larval development (Li et al. 2010), suggesting that RJ compounds may stimulate differentiation already in the early larval stages. On the other hand, there have been few reports investigating the hydrolytic enzymes of honeybee queens and larvae (Giebel et al. 1971). In the present study, we used 3-day-old queen larvae obtained as residual products in the process of harvesting RJ.

RJ proteins were hydrolyzed by a crude enzyme solution from whole honeybee queen larvae (Figure 1). The hydrolytic activities of the crude enzyme solution were stronger under alkaline conditions than under neutral conditions. In previous reports, some proteases were detected in RJ (Funakoshi et al. 1993), and proteins in RJ were gradually hydrolyzed during storage (Chen and Chen 1995; Kamakura et al. 2001). Incubations of RJ only, without adding a crude enzyme, did not show any alterations in the pattern of protein hydrolysis when done at pH 7 or pH 9. These results, thus, indicated that the hydrolysis of proteins was caused by the crude enzyme solution. Furthermore, and as expected, the hydrolytic activities were stronger at higher temperatures (Figure 2). Becher and Moritz (2009) reported that the temperature of brood cells during larval development averaged 33.3°C; thus, bee larval enzymes are probably active in this temperature range. Notwithstanding, we showed that the crude enzyme solution also worked well at 50°C.

The hydrolytic activities of the crude enzyme solution against RJ proteins were inhibited by PMSF, a serine protease inhibitor, and CPI, a carboxypeptidase inhibitor (Figure 3). The patterns of hydrolyzed RJ proteins were different between neutral and alkaline conditions. However, the inhibitory effects of PMSF and CPI were confirmed under both conditions (Figure 3), inferring that a serine protease and a carboxypeptidase are the major enzymes involved in the hydrolysis of RJ proteins. Dahlmann et al. (1978) reported that a whole-body homogenate, prepared from worker larvae

before sealing of their cells, had high aminopeptidase activities. In the present study, bestatin, an inhibitor of aminopeptidases, did not inhibit the hydrolysis of the proteases against RJ proteins (Figure 3). Burgess et al. (1996) had shown that bovine trypsin inhibitor and soybean trypsin inhibitor, which inhibit trypsin, chymotrypsin, and elastase, increased bee mortality, but that these inhibitors did not significantly affect aminopeptidase activities. This result suggested that aminopeptidase inhibitors are not crucial for honeybee nutrition and led us to conclude that larval aminopeptidases may not play a role in the hydrolysis of RJ proteins.

Three fractions containing different proteases were obtained by cation exchange and gel filtration column chromatography (Figure 4). Fraction I hydrolyzed Z-Gly-Phe, a substrate for type A carboxypeptidases, and fraction II hydrolyzed SAAPF*p*NA, a substrate for serine proteases, such as chymotrypsin and cathepsin G. Both fractions I and II hydrolyzed RJ proteins, and these activities were inhibited by either PMSF or CPI, but not EDTA (Figure 5). Carboxypeptidases are classified into three types: metallo, serine, and cysteine types. In particular, carboxypeptidase A is known to be a metallo-type protease. Fraction I was, however, not markedly inhibited by EDTA, although it was by PMSF. A carboxypeptidase from *Tineola bisselliella* larvae, reported to show carboxypeptidase A-like activity, was also not inhibited by EDTA, but also by DFP, a serine protease inhibitor (Ward 1976). The proteases in our fraction I, thus, seem to behave like the enzyme reported by this author. Fraction II hydrolyzed SAAPF*p*NA, a substrate for certain serine proteases, such as chymotrypsin and cathepsin G, and was strongly inhibited by both PMSF and CPI. In general, however, CPI does not inhibit serine-type carboxypeptidases (Ryan 1974). As shown in Figure 5b, the products of RJ proteins hydrolyzed by fraction II in the presence of PMSF and CPI were different, suggesting that PMSF and CPI inhibited different proteases. Furhtermore, CPI inhibited fraction III, but PMSF and EDTA did not, and fraction III enzymes did not hydrolyze Z-Gly-Phe or SAAPF*p*NA (Figure 4a, b), but showed greater hydrolysis of RJ proteins than fraction II. However, the hydrolytic activity of this fraction against RJ proteins was inhibited by CPI. Fractions II and III were eluted close to each other in column chromatography, and fraction III contained half the hydrolytic activity for SAAPF*p*NA compared to fraction II. Therefore, fractions II and III may contain a mix of major proteases. In fraction II, the major protease is thought to be a serine protease, such as chymotrypsin, because the fraction showed activity against SAAPF*p*NA and was inhibited by PMSF. In fraction III, the major protease is considered to be a carboxypeptidase because this fraction showed inhibition by CPI, although there was no hydrolytic activity for Z-Gly-Phe. The hydrolytic activities for RJ proteins by fractions I, II, and III were stronger under alkaline than under neutral condition (data not shown).

The proteins in fractions I and II were examined by SDS-PAGE (Figure 6). There were multiple bands in the fractions, but the densest bands in each were proportional to the protease activities for each substrate. Accordingly, we regarded the 38- and 28-kDa proteins as the proteases that hydrolyzed RJ proteins. These proteins should be analyzed by mass spectrometry in future studies.

Furthermore, in each fraction, there were many bands revealed as degradation products (Figure 5), and the pattern of proteins did not seem to be due to digestion by exopeptidases, which hydrolyze proteins from the ends, but by endopeptidases. The major proteases in fraction I are thought to be carboxypeptidases due to the substrate specificity and the effects of inhibitors. In future studies, the degradation products should be analyzed in detail so as to further characterize the proteases.

ACKNOWLEDGMENTS

We thank Dr. Yokichi Hayashi of the Department of Life Science of Asahikawa Medical Collage, Dr. Tomoyuki Mishima of the Gifu University of Medical Science, and Dr. Hisashi Suzuki of the Industrial Technology Center of Gifu Prefectural Government for

helpful suggestions. We also thank Kenji Ota, Suzuyo Watanabe, and Yuri Kashima of Akitaya Honten Co., Ltd. for providing the RJ and larvae samples.

Isolation et caractérisation des protéases hydrolisant les protéines de la gelée royale, obtenues à partir de larves de reines de l'abeille, *Apis mellifera*.

***Apis mellifera* / larve de reine / protéase / gelée royale**

Isolierung und Charakterisierung von Proteasen aus Königinnenlarven der Honigbiene, *Apis mellifera*, die zur Hydrolyse von Gelée royale geeignet sind.

***Apis mellifera* / Königinnenlarven / Protease / Königinnenfuttersaft**

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