

Estimation and characterisation of major royal jelly proteins obtained from the honeybee *Apis mellifera*

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ABSTRACT

Royal jelly (RJ) contains many components, including proteins. We focused on major royal jelly proteins (MRJPs) under natural conditions, and attempted to determine the content ratios and molecular forms of MRJPs by size-exclusion HPLC, SDS–PAGE, 2-DE and MALDI TOF/TOF MS. Soluble RJ proteins were extracted by dialysis followed by several centrifugation techniques. Soluble RJ proteins were universally separated into five peaks (640 kDa, 280 kDa, 100 kDa, 72 kDa and 4.5 kDa) by size-exclusion HPLC on a Superose 12 column. Among these peaks, both the 280 kDa and 72 kDa peaks were major, but the intensity of the 280 kDa peak differed markedly among original RJ samples ($n = 70$). The main 280 kDa protein was separated into a 55 kDa band by reducing and non-reducing SDS–PAGE. This protein was also separated into multiple spots ranging from pH 4.2 to 6.5 by 2-DE. These spots were identified as MRJP 1 by MALDI TOF/TOF MS. From these results, MRJP 1 was thought to comprise an oligomer complex linked by non-covalent bonds under natural conditions. Another major protein, the 72 kDa peak on Superose 12 HPLC, was identified as MRJP 2.

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1. Introduction

Royal jelly (RJ) is the primary food that is secreted from the hypopharyngeal and mandibular glands of nurse honeybees, and it plays a specific and important role in queen honeybee development. The queen honeybee is fed RJ throughout the larval period, while nurse honeybees are fed RJ for only 3 days (Simuth, 2001; Srisuparbh, Klinbunga, Wongsiri, & Sittipraneed, 2003).

RJ contains various components: 60–70% water, 12–15% protein, and 10–16% total sugar, lipids, vitamin, salt and free amino acids (Chen & Chen, 1995; Howe, Dimick, & Benton, 1985; Simuth, 2001). More than 80% of soluble RJ proteins are major royal jelly proteins (MRJPs) (Schmitzova et al., 1998; Simuth, 2001). MRJPs are thought to be the major factor responsible for the specific physiological role of RJ in queen honeybee development, as MRJPs in-

clude numerous essential amino acids, similar to ovalbumin and casein (Schmitzova et al., 1998). In previous reports, the MRJP family, which accounts for most of the soluble proteins (31%), had nine members; MRJPs 1–9 (Albert & Klaudiny, 2004; Drapeau, Albert, Kucharski, Prusko, & Maleszka, 2006; Schonleben, Sickmann, Mueller, & Reinders, 2007). MRJP 1 is a weakly acidic glycoprotein (pI 4.9–6.3, 55 kDa) and forms an oligomer that is estimated to be 350 or 420 kDa (Bilikova et al., 2002; Kimura et al., 2003; Simuth, 2001). MRJP 2, MRJP 3, MRJP 4 and MRJP5 are estimated to be glycoproteins of 49 kDa, 60–70 kDa, 60 kDa and 80 kDa, respectively (Li, Wang, Zhang, & Pan, 2007; Schmitzova et al., 1998). MRJP 2–5 are mainly in the basic pI range (pI 6.3–8.3) (Li et al., 2007; Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005; Schonleben et al., 2007).

The physiological functions of the RJ protein have been reported previously. Crude RJ protein or separated soluble protein, including MRJPs, stimulates cell proliferation (Kamakura, 2002; Kamakura, Suenobu, & Fukushima, 2001; Mishima et al., 2005; Narita et al., 2006; Watanabe et al., 1996; Watanabe et al., 1998). However, it has also been reported that crude RJ protein inhibits the bisphenol A-induced proliferation of human breast cancer cell lines (Nakaya et al., 2007). In addition, MRJP 1 and MRJP 2 stimulate mouse macrophages to release TNF- α (Simuth, Bilikova, Kovacova, Kuzmova, & Schroder, 2004), while MRJP 3 modulates immune responses

Abbreviation: RJ, royal jelly; MRJP, major royal jelly protein; SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; 2-DE, 2-dimensional electrophoresis; pI, isoelectric point; IEF, isoelectric focusing; MALDI TOF/TOF MS, matrix assisted laser desorption/ionisation time of flight/time of flight mass spectrometry; MW, molecular weight; DTT, dithiothreitol; mAU, milliabsorbance units; CBB, Coomassie Brilliant blue.

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by suppressing the production of IL-4, IL-2 and IFN- γ in T cells (Kohno et al., 2004; Okamoto et al., 2003).

Several previous studies have analysed MRJPs by reducing SDS-PAGE, including 2-DE (Li et al., 2007; Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005; Schmitzova et al., 1998; Schonleben et al., 2007). Separated bands on SDS-PAGE do not reflect the data for intact proteins, as samples are boiled and reduced as part of the standard Laemmli method (Laemmli, 1970). It was also reported previously that the 57 kDa protein deteriorates based on storage temperature or time (Kamakura, Fukuda, Fukushima, & Yonekura, 2001), and this degradation of the 57 kDa protein is suppressed by ethylenediaminetetraacetic acid (EDTA) (Kamakura & Fukushima, 2002). It was therefore thought that soluble RJ proteins deteriorate readily under natural conditions.

The present forms of MRJP under natural conditions remain clear. We therefore established a quantitative extraction technique of soluble RJ proteins from original RJ and performed size-exclusion HPLC analysis of numerous original RJ samples ($n = 70$). Moreover, characterisation of MRJP 1 and MRJP 2 was performed by HPLC, SDS-PAGE, 2-DE and MALDI TOF/TOF MS. This paper reports novel biochemical aspects of soluble MRJPs, and the relationship between MRJP 1 and MRJP 2 contents in original RJ samples.

2. Materials and methods

2.1. Materials

Fresh RJ ($n = 70$) was provided by Japan Royal Jelly Co., Ltd., (Tokyo Japan). The samples were obtained from various lots and areas of production, as well as from products produced by other companies in Japan. Samples were preserved at -80°C until analyses.

2.2. Extraction of crude soluble RJ proteins

RJ (3 ml) was injected into dialysis cassettes (molecular weight cut off, 3500; PIERCE, Rockford, Illinois, USA) and was dialysed against distilled water for 1 week at 4°C . After the addition of

1.5 ml of distilled water to dialysed RJ, the solution was incubated for 30 min at 37°C . Next, RJ was centrifuged at 1250g for 30 min at room temperature. The supernatant was then centrifuged at 12,500g for 30 min at 4°C . This supernatant was preserved at 4°C until analysis.

2.3. Measurement of total protein

Total protein concentration in samples was quantified using a Micro BCA protein Assay Kit (PIERCE). Human serum albumin (WAKO, Tokyo, Japan) was used as a standard protein.

2.4. HPLC analysis

Size-exclusion HPLC was performed on a Superose 12 column (10×300 mm, GE Healthcare, Buckinghamshire, England). Sample injection volume was 100 μl . The elution buffer used was phosphate buffered saline (PBS; 20 mM Na_2HPO_4 , 2 mM $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$, 150 mM NaCl, pH 7.5). Flow rate was 0.5 ml/min and fraction volume was 0.8 ml. The Superose 12 column was calibrated using Gel filtration Calibration Kits with low molecular weight and high molecular weight proteins (GE Healthcare).

Anion-exchange HPLC was performed on a Mini Q column (4.6×50 mm, GE Healthcare). Samples were desalted by dialysis with distilled water, followed by concentration with Minicon (Millipore, Billerica, MA, USA). Sample injection volume was 1 ml. Binding buffer was 20 mM Tris-HCl (pH 8.0). Elution buffer was 20 mM Tris-HCl, 1 M NaCl, pH 8.0, with a NaCl gradient from 0 M to 0.5 M. Flow rate was 1.0 ml/min and fraction volume was 1.0 ml.

Both HPLC elution profiles were monitored at 280 nm and were carried out using the Akta Explorer System (GE Healthcare).

2.5. Reducing and non-reducing SDS-PAGE

Protein solutions ranging from 10 to 20 μg protein were mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitro-

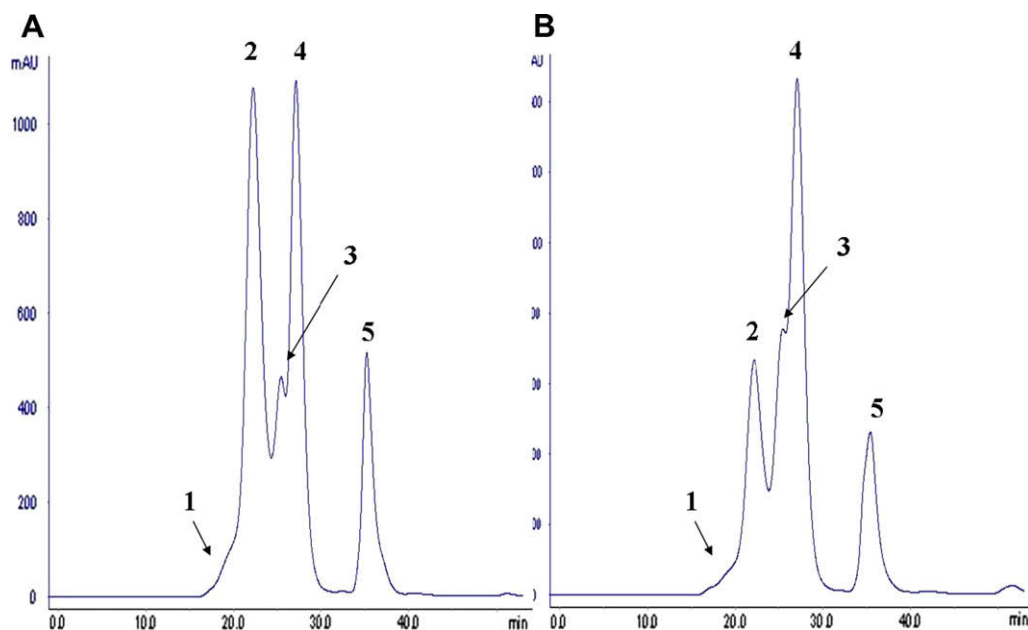


Fig. 1. Typical two-type elution profiles of crude soluble RJ proteins by size-exclusion HPLC on a Superose 12 column. The protein absorbance was monitored at 280 nm. The column was calibrated using Gel filtration Calibration Kits with low molecular weight and high molecular weight proteins (GE Healthcare). Peaks: 1 (640 kDa), 2 (280 kDa), 3 (100 kDa), 4 (72 kDa) and 5 (4.5 kDa). Absorbance of the 280 kDa peak varied widely among RJ samples. (A) Elution pattern of 280 kDa protein rich RJ sample. Absorbance of 280 kDa peak and 72 kDa peak was 1075.632 mAU and 1088.532 mAU, respectively. The injected sample was 0.879 mg protein. (B) Elution pattern of a 280 kDa protein low RJ sample. Absorbances of the 280 kDa peak and 72 kDa peak was 667.329 mAU and 1463.497 mAU. The injected sample was 0.81 mg protein.

gen, Tokyo, Japan), with or without DTT (Invitrogen), followed by boiling at 100 °C for 2 min. Samples were applied to precast NuPAGE 4–12% Bis-Tris polyacrylamide gel (Invitrogen). Running buffer was NuPAGE MOPS or MES SDS running buffer (Invitrogen). SDS-PAGE was then performed at 200 V. After electrophoresis, gels were stained with Coomassie Brilliant blue (CBB) R-250. Mark 12 unstained standard (Invitrogen) was used as a molecular weight marker.

2.6. 2-DE

Twenty-five micrograms of protein were desalted, delipidated and concentrated by deposition with 100% cold acetone. Protein pellets were washed with 80% acetone, and dissolved with protein solubiliser (Invitrogen), carrier ampholytes 3–10 (Invitrogen) and DTT. Protein solution was added to IPG ZOOM strip gel (pH 3–10, Invitrogen) followed by incubation overnight. First-dimension IEF was run under gradient voltage conditions (175 V constant for 20 min, gradient from 175 to 2000 V for 45 min, and 2000 V constant for 30 min). IEF Marker 3–10 SELVA Liquid Mix (SERVA Electrophoresis, Heidelberg, Germany) was used as a pI marker. The IPG strip gel used for IEF was reduced with 50 mM DTT in LDS sample buffer and was alkylated with 125 mM iodoacetamide in LDS sample buffer. The gel was used for second-dimension SDS-PAGE, as described above.

2.7. MALDI TOF/TOF MS

Destaining of gel spots was carried out with 25 mM NH_4HCO_3 containing 50% acetonitrile (ACN). Dehydration was carried out with 100% ACN. Proteins in the gel were digested with trypsin (20 $\mu\text{g}/\mu\text{l}$ in 25 mM NH_4HCO_3 , WAKO) at 37 °C for 18 h. Digested peptides were extracted by sonication for 3 min using extraction buffer (50% ACN, 5% trifluoroacetic acid). Desalting and concentra-

tion was performed with ZipTip C18 (Millipore, Billerica, MA, USA). Final peptides were applied to the target with a α -cyano-4-hydroxy-cinnamic acid matrix. Peptide masses were determined by MALDI TOF/TOF MS (Ultraflex, Bruker Daltonics, Bremen, Germany), and searches conducted using the MASCOT program (http://www.matrixscience.com/search_form_select.html).

3. Results

3.1. Size-exclusion HPLC on Superose 12 column

Crude soluble RJ proteins were subjected to size-exclusion HPLC on Superose 12 column. A representative elution profile is shown in Fig. 1. Crude soluble RJ proteins were separated as five peaks: peak 1 (640 kDa), peak 2 (280 kDa), peak 3 (100 kDa), peak 4 (72 kDa) and peak 5 (4.5 kDa). These five peaks were universally detected in all RJ samples ($n = 70$). It was also found that the 280 kDa peak varied greatly among RJ samples (Figs. 1A and 1B). The minimum of absorbance at peak 2 was 21.85 mAU and the maximum was 2006.45 mAU, which corresponded to a mean $\pm$ SD value of 555.93 ± 538.26 mAU, and a median value of 359.24 mAU (70 RJ samples).

3.2. Purification of 280 kDa peak by anion-exchange HPLC on Mini Q column

The 280 kDa proteins, which were eluted in peak 2 on Superose 12 HPLC, were applied to anion-exchange HPLC on Mini Q column. The injected volume was 1 ml (1.88 mg proteins). The 280 kDa proteins were separated into three peaks, one large elution peak, eluted at 0.27 M NaCl, and two small peaks, at 0.20 and 0.23 M NaCl (Fig. 2). The main peak eluted at 0.27 M NaCl concentration was subsequently analysed by electrophoretic techniques.

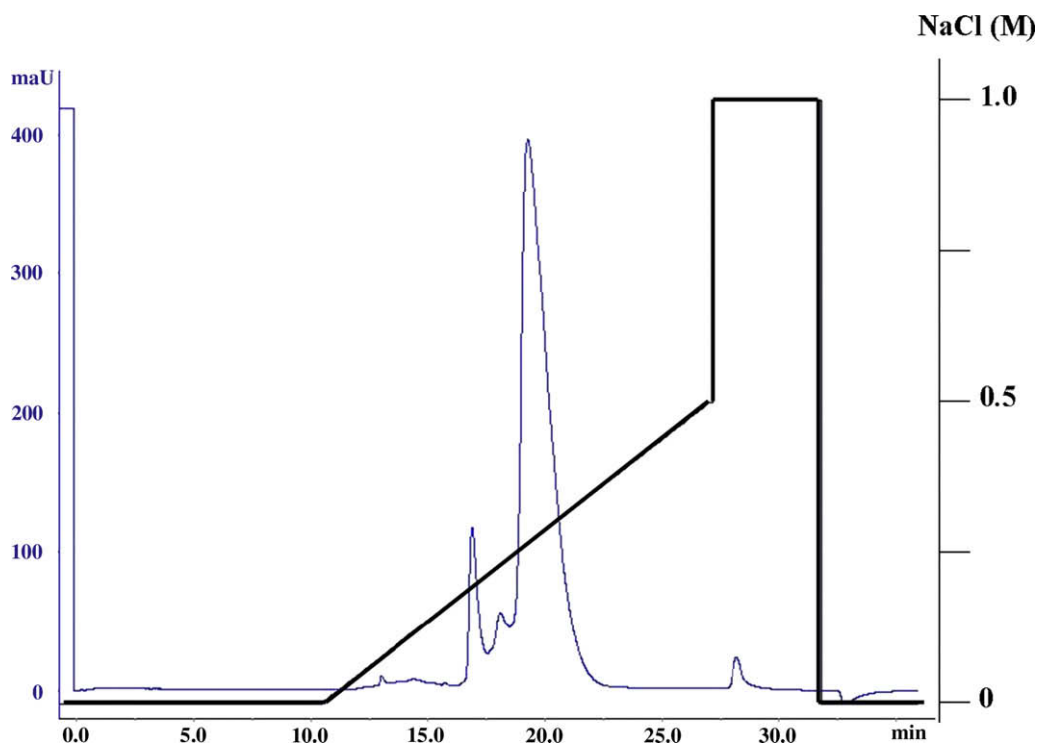


Fig. 2. Separation profile of 280 kDa proteins obtained by Superose 12 HPLC on Mini Q anion-exchange HPLC. The protein absorbance was monitored at 280 nm. One millilitre (1.88 mg protein) of the peak 2 proteins in 20 mM Tris-HCl buffer (pH 8.0) was injected on the Mini Q column equilibrated with the same buffer. The bound proteins were eluted with a linear gradient of up to 0.35 M NaCl at the rate of 1.0 ml/min. The 280 kDa proteins were separated into three peaks eluted at 0.20, 0.23 and 0.27 M NaCl concentration.

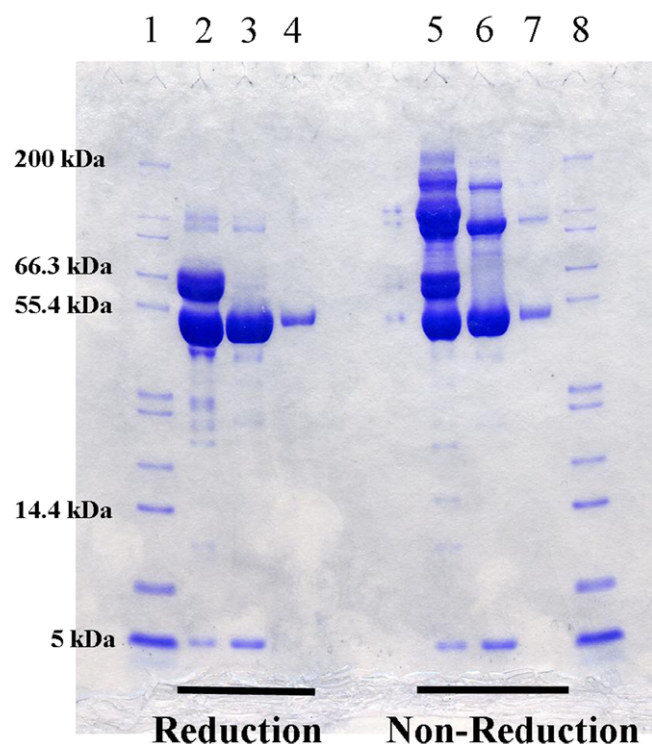


Fig. 3. Reducing and non-reducing SDS-PAGE profiles of 280 kDa proteins. 1 and 8, molecular weight marker proteins using Mark 12 unstained standard kit (Invitrogen); 2, crude soluble RJ proteins with reduction; 3, 280 kDa proteins separated by Superose 12 HPLC with reduction; 4, main peak proteins separated by Mini Q HPLC with reduction; 5, crude soluble RJ proteins with non-reduction; 6, 280 kDa proteins separated by Superose 12 HPLC with non-reduction; 7, main peak proteins separated by Mini Q HPLC with non-reduction. The reduction procedure was performed with 50 mM DTT. The gel was stained with CBB. The main 280 kDa protein was detected by a 55 kDa band with reducing and non-reducing conditions. These results indicated that the main 280 kDa protein was composed of an oligomer consisting of 55 kDa subunits linked by non-covalent bonds.

3.3. SDS-PAGE analysis of 280 kDa peak

Of the crude soluble RJ proteins, the 280 kDa proteins were separated by a Superose 12 column, and the main peak eluted at 0.27 M NaCl on a Mini Q column were subjected to both reducing

and non-reducing SDS-PAGE analysis (Fig. 3). Both the 280 kDa proteins separated by Superose 12 column (Fig. 3, lanes 3 and 6) and the main peak on Mini Q column (Fig. 3, lanes 4 and 7) were detected as a 55 kDa band. Furthermore, this 55 kDa band was similarly detected on both reducing (Fig. 3, lanes 2, 3 and 4) and non-reducing SDS-PAGE (Fig. 3, lanes 5, 6 and 7). These results indicate that the main 280 kDa protein formed an oligomer complex comprising 55 kDa protein subunits under natural conditions. The results also demonstrate that the 55 kDa protein subunits are bound by non-covalent bonds to form an oligomer complex.

3.4. Identification of 55 kDa protein

Proteome analysis by 2-DE and MALDI TOF/TOF MS was then performed in order to identify the 55 kDa protein. On 2-DE, crude soluble RJ proteins were separated into a number of spots ranging from pH 3.8 to 10.7 and from 5 kDa to 100 kDa. These data are shown in Fig. 4A. The 55 kDa protein derived from the main peak purified by Mini Q HPLC was separated into several spots by 2-DE (pH 4.2–6.5). These data are shown in Fig. 4B. Major spots purified Mini Q HPLC step are indicated by the square. These spots were cut from the gel and subjected to MALDI TOF/TOF MS. After a search using the MASCOT program, these spots were identified as a MRJP 1 of *Apis merifera*. An identical score was found to be 92–126, shown Fig. 5.

3.5. Identification of 72 kDa peak eluted in peak 4 on Superose 12 column

The 72 kDa proteins that were eluted in peak 4 on Superose 12 HPLC were also analysed. This peak protein was separated into several spots by 2-DE (pH 6.2–7.9, 51 kDa). These data are indicated in Fig. 4A by the dashed line circle. All of these spots were extracted from the gel and subjected to MALDI TOF/TOF MS. These proteins were then identified as MRJP 2 of *Apis merifera*. An identical score was found to be 127–194, shown Fig. 6.

4. Discussion

First, we found that soluble RJ proteins were universally separated into five peaks at approximately 640 kDa, 280 kDa, 100 kDa, 72 kDa and 4.5 kDa by size-exclusion HPLC on a Superose 12 column. Among these peaks, the 280 kDa and 72 kDa peaks

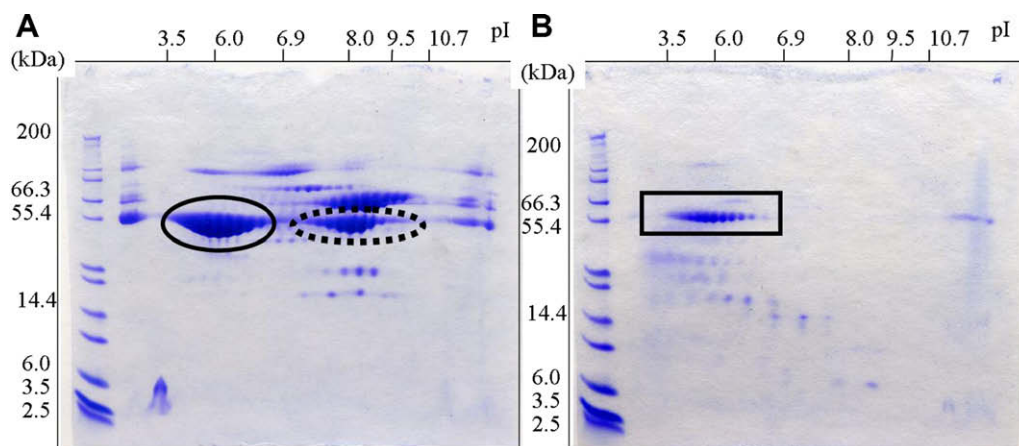


Fig. 4. 2-DE profiles of soluble RJ proteins. Molecular weight marker proteins were used a Mark 12 unstained standard kit (Invitrogen). The gels were stained with CBB. (A) 2-DE profile of crude RJ proteins. Twenty-five micrograms of crude soluble RJ proteins were applied and were separated into numerous spots. The solid line circle indicates the spots (pI, from 4.2 to 6.5, MW, 55 kDa). The dashed line circle indicates the spots (pI, from 6.2 to 7.9, MW, 51 kDa). (B) 2-DE profile of the main peak of Mini Q HPLC. Fifteen micrograms of the main peak proteins obtained by Mini Q HPLC were applied and the major spot indicated by square was detected (pI, from 4.2 to 6.5, MW, 55 kDa).

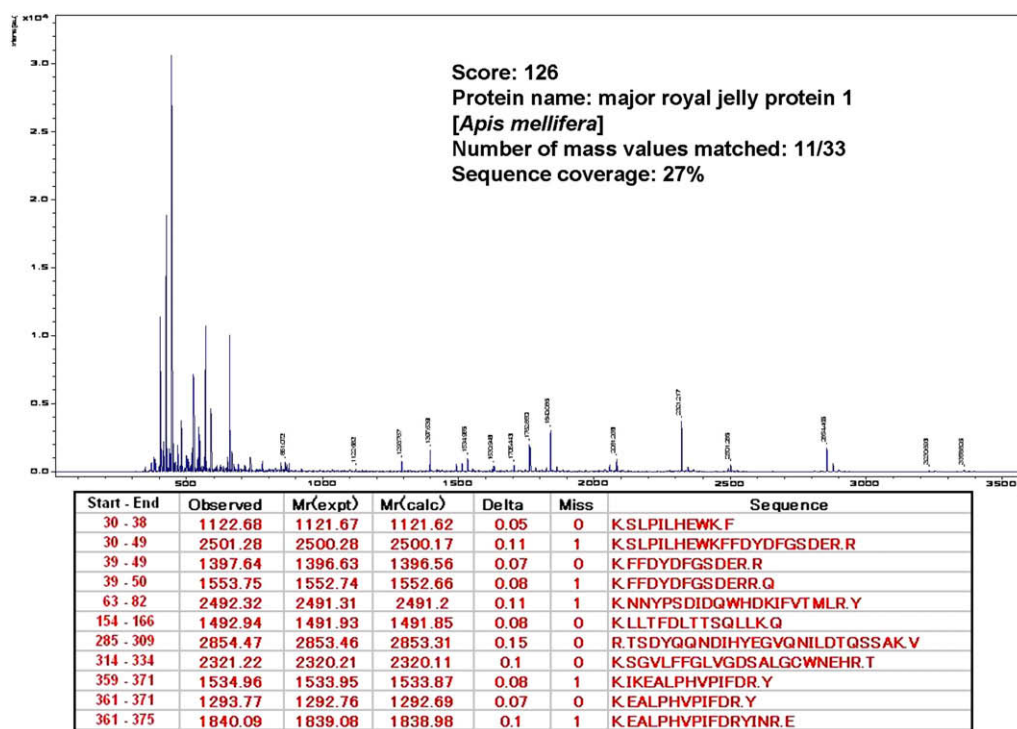


Fig. 5. MALDI TOF MS spectra of spots indicated by square in Fig. 4B. This data resulted from the proteomics analysis of one of the 2-DE spots indicated by square in Fig. 4B. The upper data showed the mass spectra. The lower data showed the matched mass values and amino acid sequences against the MRJP 1 sequence. Eleven mass values were matched against MRJP 1 sequences and their coverage was 27%. From these results, the spot was identified as a major royal jelly protein 1, *Apis mellifera* with a score of 126.

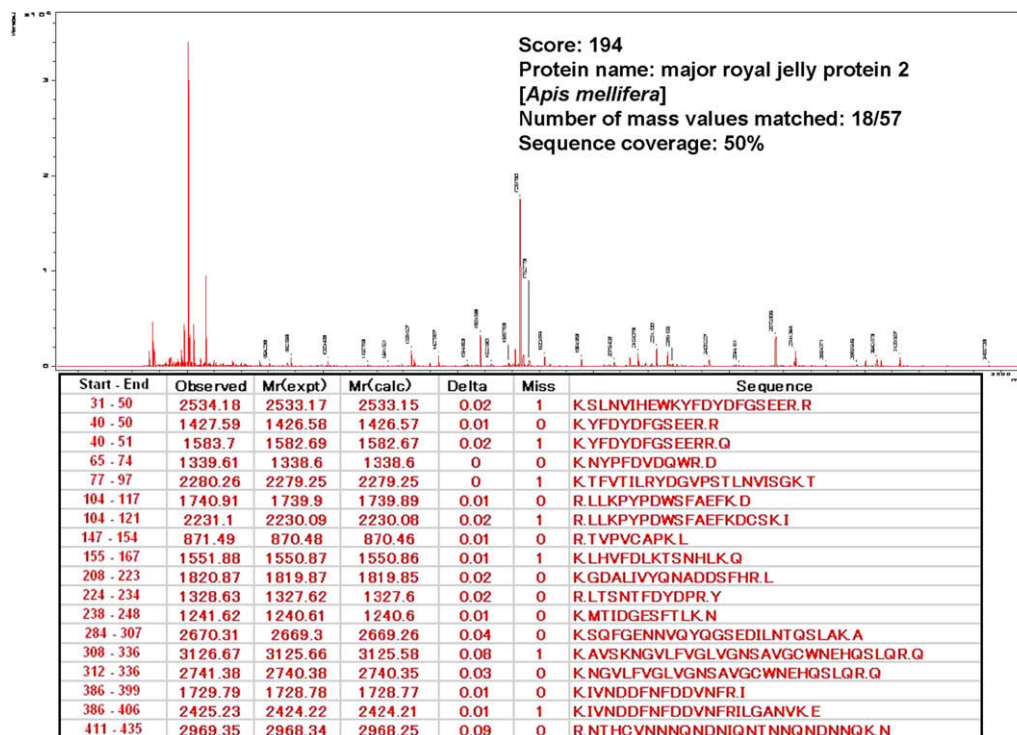


Fig. 6. MALDI TOF MS spectra of spots indicated by the dashed line circle in Fig. 4A. This data resulted from proteomics analysis of one of the 2-DE spots indicated by the dashed line circle in Fig. 4A. The upper data showed the mass spectra. The lower data showed the matched mass values and amino acid sequences against the MRJP 2 sequence. Eighteen mass values were matched against the MRJP 2 sequences and their coverage was 50%. From these results, the spot was identified as a major royal jelly protein 2, *Apis mellifera* with a score of 194.

were the major components. The major proteins were identified as MRJP 1 and MRJP 2 by MALDI TOF/TOF MS analyses, respectively.

However, contents of the 280 kDa peak (MRJP 1) varied remarkably among the lots, areas of production and other available products.

To the best of our knowledge, there have been no reports describing analytical results based on size-exclusion analyses of such a variety of original RJ samples. This study indicates that the contents of MRJP 1 may not be uniform among the available RJ products. Therefore, we must ensure that RJ sources are clearly disclosed when studying the physiological functions of RJ components, as the results of the present study indicate that these components vary significantly. Moreover, RJ is widely used as a functional food and/or supplement. MRJP 1 was thought to be a major RJ protein and numerous *in vitro* studies on its physiological function using RJ samples have been performed to date (Kamakura, 2002; Kamakura, Suenobu, et al., 2001; Kohno et al., 2004; Mishima et al., 2005; Narita et al., 2006; Okamoto et al., 2003; Simuth et al., 2004; Watanabe et al., 1996; Watanabe et al., 1998).

MRJP 1, the main 280 kDa protein, was separated as a 55 kDa band by both reducing and non-reducing SDS-PAGE, and was separated into several spots by 2-DE (pH 4.2–6.5). These results indicate that the MRJP 1 oligomer comprises of 55 kDa protein subunits bound by non-covalent bonds. This data agrees with other reports (Li et al., 2007; Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005), but this is the first study to report the binding pattern of the MRJP 1 oligomer. Based on the results of Superose 12 HPLC and SDS-PAGE, the oligomer is a pentamer of MRJP 1 monomers.

The 72 kDa peak was also characterised on a Superose 12 HPLC. This peak was separated into several spots that were focused at 51 kDa and ranged from pH 6.2 to 7.9. From a proteomics approach, these spots were identified as MRJP 2 with a high score. The data for MRJP 2 basically agreed with previous reports (Li et al., 2007; Sano et al., 2004; Santos et al., 2005; Scarselli et al., 2005). However, this is the first report to compare stable peaks separated by size-exclusion chromatography and identify them as MRJP 2. We believe that the wide pI range of the MRJP proteins is generally due to post-translational modification by glycosylation and/or phosphorylation.

Among the five peaks on Superose 12 HPLC, the absorbance of the 280 kDa peak varied greatly. The mean $\pm$ SD absorbance was 555.93 ± 538.26 mAU, ranging from 21.85 to 2066.45 mAU (70 RJ samples). This indicates that the amount of 280 kDa protein (MRJP 1 oligomer) varies substantially under natural conditions. Two previous reports (Kamakura, Fukuda, et al., 2001; Kamakura et al., 2002) have found that a 57 kDa protein in RJ was decreased depending on temperature and period of storage. Unfortunately, these reports did not confirm whether the 57 kDa protein was MRJP 1. We believe that there are factors other than stability in determining the MRJP 1 content in RJ.

On the other hand, the physiological function of crude RJ protein or MRJP families have been widely studied (Kamakura, 2002; Kamakura, Suenobu, et al., 2001; Kohno et al., 2004; Mishima et al., 2005; Narita et al., 2006; Okamoto et al., 2003; Simuth, 2001; Simuth et al., 2004; Watanabe et al., 1996; Watanabe et al., 1998). However, the results of these studies are not consistent. Therefore, we believe that the crude soluble RJ proteins include numerous proteins, and that the content of the major protein, MRJP 1, differs between products. Purified RJ protein should therefore be used for functional examination. We believe our extraction and purification methods for the MRJP families will contribute to the functional examination of RJ proteins.

5. Conclusion

We extracted soluble RJ proteins and obtained five peaks on by size-exclusion HPLC with a Superose 12 column. These five peaks were approximately 640 kDa, 280 kDa, 100 kDa, 72 kDa and 4.5 kDa. The major proteins, the 280 kDa and 72 kDa peaks, were

identified as MRJP 1 and MRJP 2, respectively, using a proteomics approach. Moreover, SDS-PAGE and 2-DE examination suggested that MRJP 1 is composed of five MRJP 1 monomers, and that they are bound by non-covalent bonds. In addition, we demonstrated that the MRJP 1 pentamer varies substantially among different lots, areas of production and available products. Thus, careful attention should be paid when performing functional examinations using original RJ.

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