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# A rapid method to isolate soluble royal jelly proteins

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Title: A rapid method to isolate soluble royal jelly proteins

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**Abstract**

Soluble royal jelly (RJ) proteins (SRJPs) include the major RJ protein (MRJP) family, which contribute to the physiological actions of RJ. Although SRJPs are prepared using conventional methods involving dialysis and centrifugation, dialysis is a time-consuming process. We have therefore developed a simple method to isolate SRJPs from RJ. This new method produces twenty-fold higher levels of SRJPs than that of the conventional procedure;, hence, the levels obtained by the new and existing methods were compared. A 1-hour ultracentrifugation separated SRJPs in the supernatant into upper, middle and lower layers. Each layer was analyzed by size-exclusion HPLC, SDS-PAGE and 2-DE. The upper and middle layers contained MRJP2 (52 kDa) and MRJP3 (60–70 kDa), while the lower layer contained MRJP1 (290 kDa). In nature, MRJP1 is a monomer and/or oligomer. When the lower layer was analyzed by Superose 12 HPLC, MRJP1 was predominantly an oligomer. Our MRJP isolation method reduces the procedure time by using ultracentrifugation without dialysis to obtain SRJPs and produces layers containing MRJP1 oligomers, MRJP2 and MRJP3.

**Keywords**

royal jelly, ultracentrifugation, soluble royal jelly proteins, MRJP1 oligomer, HPLC

## 1. Introduction

Royal jelly (RJ) is nutritious food that is secreted from the hypopharyngeal and mandibular glands of nurse honeybees. RJ contains 12–15% crude proteins, and it has been reported that 80% of these proteins are members of the major RJ protein (MRJP) family, with MRJP1 accounting for more than 45% (Furusawa, Rakwal, Nam, Shibato, Agrawal, Kim, et al., 2008). MRJP1 is an acidic 55 kDa protein and forms an oligomeric complex; however, its protein structure has not been analyzed yet. RJ is indispensable for the development of a queen bee and MRJP family is thought to be a major factor in queen honeybee development (Kamakura, 2011; Schmitzova, Klaudiny, Albert, Schroder, Schreckengost, Hanes, et al., 1998; Weaver, 1966). Furthermore, the MRJP family is thought to be the main substance involved in the physiological actions of RJ, including cell proliferation, cytokine suppression, and antimicrobial activity (Fujiwara, Imai, Fujiwara, Yaeshima, Kawashima, & Kobayashi, 1990; Kamakura, Suenobu, & Fukushima, 2001; Majtan, Kumar, Majtan, Walls, & Klaudiny, 2010; Oka,

55 Emori, Kobayashi, Hayashi, & Nomoto, 2001; Okamoto, 2003; Shen, Ding, Zhang, Jin,
56 Zhang, & Li, 2010).

57 Previously, soluble RJ proteins (SRJPs) including the MRJP family were prepared
58 from methods that combined dialysis and centrifugation (S. Tamura, Amano, Kono,
59 Kondoh, Yamaguchi, Kobayashi, et al., 2009; Shougo Tamura, Kono, Harada,
60 Yamaguchi, & Moriyama, 2009). Typically, these methods take a long time to produce
61 SRJPs. Most existing methods require seven days of dialysis and produce very low
62 yields because RJ is difficult to dissolve. Therefore, the quantity of MRJPs obtained
63 from SRJPs is a limiting factor for biological research on MRJPs.

64 Ultracentrifugation is known as a useful tool to isolate lipoproteins and whey proteins
65 that are difficult to extract (De Natale, Annuzzi, Bozzetto, Mazzarella, Costabile, Ciano,
66 et al., 2009; Etcheverry, Miller, & Glahn, 2004; Henry, Molle, Morgan, Fauquant, &
67 Bouhallab, 2002; Tanese, 1997; Yee, Pavitt, Tan, Venkatesan, Godsland, Richmond, et
68 al., 2008). Thus, we used ultracentrifugation to isolate SRJPs from RJ and obtained a
69 large quantity of SRJPs within an hour. In this study we describe a simple and rapid
70 method to isolate SRJPs using a novel ultracentrifugal analysis.

71

72 **2. Materials and Methods**

73 **2.1. Materials**

74 Fresh RJ was provided by Japan Royal Jelly (Tokyo, Japan). Samples were stored at
75 -80°C until analysis.

76 **2.2. Extraction of soluble RJ proteins**

77 First, 10 g of RJ was dissoloved in 10 mL of deionized water and vortexed. Next, RJ
78 was ultracentrifuged (Optima™ L-80XP Ultracentrifuge, Beckman Coulter, Tokyo,
79 Japan) at 113400 xg for 1 hour at 4°C. Then, the supernatant was separated into three
80 layers, including the upper, middle, and lower layers. These layers contained defined
81 and layered soluble RJ proteins (LSRJPs). Each layer was recovered with minimal
82 mixing of the layers and then stored at 4°C until further analysis.

83 **2.3. Measurement of total protein levels**

84 The total protein concentration in the samples was quantified using a Micro BCA
85 protein Assay Kit (Thermo Fisher Scientific K. K., Yokohama, Japan). HSA (Wako,
86 Osaka, Japan) was used as a protein standard.

87 **2.4. SDS-PAGE**

88 Proteins in each layer were mixed with NuPAGE lithium dodecyl sulfate (LDS)
89 sample buffer (Invitrogen, Tokyo, Japan) containing 50 mM DTT, and then boiled at
90 100°C for 5 min. The sample mixtures were separated on precast NuPAGE 4-12%

bis-Tris polyacrylamide gels (Invitrogen). Running buffer was prepared using NuPAGE MES SDS Running Buffer (Invitrogen). SDS-PAGE was performed at a constant 200 V. Mark12 Unstained Standard (Invitrogen) was used as molecular weight markers for SDS-PAGE.

### 2.5. 2-DE

Twenty-five micrograms of protein was desalted, delipidated and concentrated by deposition with 100% cold acetone. Protein pellets were washed with 80% acetone and dissolved in a protein solubiliser (Invitrogen), carrier ampholytes 3–10 (Invitrogen) and DTT. The protein solution was added to an IPG ZOOM strip gel (pH 3–10, Invitrogen) and incubated 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 alkylated with 125 mM iodoacetamide in LDS sample buffer. The gel was used for second-dimension SDS–PAGE as described above.

### 2.6. HPLC

Each sample layer was analyzed by size-exclusion and anion-exchange HPLC.

Size-exclusion HPLC was performed on Superose 12 columns (10×300 mm; GE Healthcare). PBS (Invitrogen) was used as the elution buffer for each column. For the Superose 12 column, the sample injection volume was 100 μL and the flow rate was 0.2 mL/min.

Anion-exchange HPLC was performed on a Mini Q column (4.6×50 mm; GE Healthcare). Dialysis with 20 mM Tris-HCl (pH 8.0) was used for sample desalting and buffer exchange, and then the samples were concentrated using a Minicon or Amicon Ultra-4 (Millipore, Billerica, MA, USA). The sample injection volume was 1 mL. The binding buffer contained 20 mM Tris-HCl (pH 8.0), and the elution buffer contained 20 mM Tris-HCl, 1 M NaCl.

## 3. Results

### 3.1. Extraction of LSRJPs by ultracentrifugation

The solution obtained by mixing 10 g of RJ with 10 mL of deionized water was separated into three layers by ultracentrifugation (Fig. 1-A). The supernatant was classified as layered soluble RJ proteins (LSRJPs). In addition, the mixed solution of these layers was classified as crude soluble RJ proteins (CSRJPs). The upper, middle, and lower layers had volumes of 1.5 mL, 7 mL, and 4.5 mL, respectively.

### 3.2.Comparison of the yields obtained by both extraction methods

We recovered the mixture of three LSRJPs layers extracted using the new method to compare with CSRJPs extracted from the existing method. The total amount of protein in CSRJPs extracted from three different RJs was measured. The recovery rate was determined as the percentage of the weight of RJ. The yields obtained from each method are shown in Table 1. Total protein concentrations of supernatant obtained from each RJ using the existing method were 20.52 mg/ml, 18.20 mg/ml, and 24.53 mg/ml; the new method yielded 40.19 mg/ml, 32.79 mg/ml, and 36.03 mg/ml. Recovery rates were 1.267%, 1.041%, and 1.413% using the existing method, while the rates for the new method were 5.225%, 4.263%, and 4.683%. Recovery rates were calculated according to the following equation (formula?): weight of 3 ml RJ/total protein in existing method, and RJ 10 g/total protein in new method.

### 3.3. Comparison of the protein profile from both extraction methods

First, we compared the protein profile of CSRJPs that were extracted using the existing method and the new method. We recovered the mixture of three layers of SRJPs extracted by the new method. It is well established that size-exclusion chromatography of CSRJPs yields the elution pattern presented in Fig. 2. Each major protein peak was identified in a previous study. A peak at 290 kDa represented the MRJP1 oligomer,

while peaks at approximately 70 kDa and 50 kDa were MRJP3 and MRJP2, respectively. There were minimal differences in the major protein peaks between the two extraction methods. The new method yielded greater small molecular peaks than the existing method. Peaks eluted after 15 mL consisted of low molecular constituents and not proteins (data not shown).

### 3.4. Analysis of LSRJPs by electrophoresis

We analyzed the proteins in the three layers that were obtained with the reformed extraction method by SDS-PAGE and 2-DE. SDS-PAGE bands and 2-DE spots were identified by a previous study using MALDI-TOF-MS and standard proteomic analyses. There were minimal differences in both the upper and middle layers. However, a 55 kDa band was predominantly stained in the lower layer.

Additionally, we performed 2-DE and confirmed that MRJP1 predominantly fractionated to the lower layer (Figs. 1-B, C, D).

### 3.5. Analysis of LSRJPs by HPLC

We analyzed the proteins in the three layers that were obtained using the new extraction method using Superose 12 size-exclusion HPLC columns. The chromatograms are shown in Figs. 3-A, B, C. The upper layer contained many peaks corresponding to low molecular weight proteins. There was a small peak representing

163 the MRJP1 oligomer (290 kDa) in the upper layer. In addition, the middle layer
164 contained a small peak corresponding to the MRJP1 oligomer. Peaks for MRJP2 and
165 MRJP3 were higher in the middle than in the upper layer. On the other hand, it was
166 clear that the MRJP1 oligomer predominantly fractionated to the lower layer.
167
168 **4. Discussion**
169 There are numerous reports describing a technically established MRJP purification
170 procedure that includes (i) dialysis to eliminate impurities from RJ and to extract SRJPs
171 by dissolution and (ii) HPLC to isolate the target MRJPs contained in the SRJPs
172 (Fontana, Mendes, de Souza, Konno, Cesar, Malaspina, et al., 2004; Furusawa, et al.,
173 2008; Kimura, Kajiyama, Kanaeda, Izukawa, & Yonekura, 1996; Li, Feng, Zhang,
174 Zhang, & Pan, 2008; Okamoto, 2003; S. Tamura, et al., 2009; Shougo Tamura, Kono,
175 Harada, Yamaguchi, & Moriyama, 2009). However, isolating the target fraction from RJ
176 by dialysis is time-consuming. Similar to previous reports (S. Tamura, et al., 2009;
177 Shougo Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009), the dialysis system at
178 the authors' facility required at least seven days to complete the extraction process.
179 Another disadvantage of this dialysis-based extraction method is the poor yield;
180 processing of dialysis 3 mL of RJ by the conventional procedure yielded only 2 mL of

aqueous solution, with a mean protein concentration of only 21.08 mg/mL on average. In addition, recovery rate (yield of total protein levels/weight of RJ) averaged 1.24%. Thus, one of the major challenges in studying the physiological activities of RJ has been the development of a new method that produces high yields of MRJPs.

The new method described in this report, which processed RJ first by ultracentrifugation, yielded aqueous solutions with a mean volume of 13 mL and a mean protein concentration of 36.33 mg/mL from a starting mixture of 10 g of RJ in 10 mL of dissolution water. Additionally, recovery rate averaged 4.72%. The resulting protein concentration was more than 1.7-fold higher than that obtained by the conventional process, and the resulting recovery rate increased by 3.8-fold. Further, our preliminary experiments suggested that solutions with higher protein concentrations could be obtained by adding smaller volumes of dissolution water (unpublished data). In the case of extensive extraction, products obtained by dialysis-based methods are generally very expensive. The resulting yield of total proteins levels using our method was more than 10-fold higher than that obtained by the conventional method.

MRJPs have been the subject of various research projects on the physiological activities of RJ. Such studies call for a significant amount of highly pure MRJPs. Therefore, we believe that our new technique will contribute to advances in scientific

knowledge of the MRJP family.

We demonstrated that ultracentrifuging RJ for 1 hour resulted in a visible, three-layer separation of the supernatant. The upper and middle layers had high concentrations of MRJP2 (52 kDa) and MRJP3 (60–70 kDa). MRJP1 (55 kDa), which had a similar molecular weight to MRJP2 and MRJP3, was present in the lower layer in the form of an oligomer. Our accomplishment has provided a highly efficient method to purify the target MRJP by collecting the relevant supernatant layer. In particular, MRJP1 exerts cell protective effects (Turkmen, Cavusoglu, Yapar, & Yalcin, 2009; Wagner, Dobler, & Thiem, 1970) and other physiological activities (Majtan, Kovacova, Bilikova, & Simuth, 2006; Matsui, Yukiyoshi, Doi, Sugimoto, Yamada, & Matsumoto, 2002; Narita, Nomura, Ohta, Inoh, Suzuki, Araki, et al., 2006) and exists predominantly as an oligomer in naturally produced RJ (S. Tamura, et al., 2009; Shougo Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009). This underscores the significance of using the MRJP1 oligomer when studying the biological properties of MRJP1.

In conclusion, the novel technique involving ultracentrifugation of RJ described here supersedes conventional methods in terms of technical simplicity and production yields. Despite economic issues related to the high cost of the ultracentrifugation equipment, we expect that this new process will prove to be a new standard method to purify

MRJPs.

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299

**Figure captions**

**Fig.1.** (A) SRJPs after ultracentrifugation. Ten grams RJ was dissolved in 10 mL of deionized water. The supernatant consisted of an upper turbid layer (1.5 mL), clear middle layer (7 mL), and a thick lower layer (4.5 mL). (a) Insoluble layer included RJ. (B), (C) and (D) 2-DE profiles of SRJPs of three layers. (B) 2-DE profile of upper-layer. The spot indicated by a dotted circle was MRJP3. Another spot indicated by a dashed circle was MRJP2. (C) 2-DE profile of middle-layer. The major spot was the same as the upper-layer. (D) 2-DE profile of lower-layer. The major spot indicated by a continuous circle was MRJP1. The amount of protein obtained was 20 μg. The gel was stained with CBB.

**Fig.2.** Two elution profiles of SRJPs 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: a MRJP1 oligomer (290 kDa), b MRJP3 (60-70 kDa), c MRJP2 (52 kDa). With regard to these major peaks, there was not much difference between the two methods (OR No significant differences were found in these major peaks between the two methods.). Lot number of RJ was the same for A and B. (A)

318 Elution pattern of SRJPs obtained by the existing method. Absorbance of MRJP1 peak
319 and MRJP2 peak was 833 mAU and 1426 mAU, respectively. (B) Elution pattern of
320 SRJPs (mixture of upper, middle and lower layers) obtained by the new method.
321 Absorbance of the MRJP1 peak and MRJP2 peak was 831 mAU and 1479 mAU,
322 respectively. Peaks eluted after 15 mL consisted of low molecular constituents and not
323 proteins.
324
325 **Fig.3.** Elution profiles of three layers SRJPs by size-exclusion HPLC on a Superose 12
326 column. The protein absorbance was monitored at 280 nm. (A) Elution pattern of SRJPs
327 of upper layer. MRJP3 peak was 419 mAU and MRJP2 peak was 865 mAU. (B) Elution
328 pattern of SRJPs of middle layer. MRJP3 peak was 624 mAU and MRJP2 peak was
329 1338 mAU. (C) Elution pattern of SRJPs of lower layer. MRJP1 oligomer peak was
330 1660 mAU.

**List of figure captions and a table**

Figure 1. Royal jelly (RJ) after ultracentrifugation and 2-DE profiles of three layers.

Figure 2. Two elution profiles of soluble RJ proteins (SRJPs) by size-exclusion HPLC on a Superose 12 column.

Figure 3. Elution profiles of three layers of SRJPs by size-exclusion HPLC on a Superose 12 column.

Table 1. Comparison of the yields obtained by existing and new extraction methods.

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**Abstract**

Soluble royal jelly (RJ) proteins (SRJPs) include the major RJ protein (MRJP) family, which contribute to the physiological actions of RJ. Although SRJPs are prepared using conventional methods involving dialysis and centrifugation, dialysis is a time-consuming process. We have therefore developed a simple method to isolate SRJPs from RJ. This new method produces twenty-fold higher levels of SRJPs than that of the conventional procedure;, hence, the levels obtained by the new and existing methods were compared. A 1-hour ultracentrifugation separated SRJPs in the supernatant into upper, middle and lower layers. Each layer was analyzed by size-exclusion HPLC, SDS-PAGE and 2-DE. The upper and middle layers contained MRJP2 (52 kDa) and MRJP3 (60–70 kDa), while the lower layer contained MRJP1 (290 kDa). In nature, MRJP1 is a monomer and/or oligomer. When the lower layer was analyzed by Superose 12 HPLC, MRJP1 was predominantly an oligomer. Our MRJP isolation method reduces the procedure time by using ultracentrifugation without dialysis to obtain SRJPs and produces layers containing MRJP1 oligomers, MRJP2 and MRJP3.

**Keywords**

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## 1. Introduction

Royal jelly (RJ) is nutritious food that is secreted from the hypopharyngeal and mandibular glands

of nurse honeybees. RJ contains 12–15% crude proteins, and it has been reported that 80% of these proteins are members of the major RJ protein (MRJP) family, with MRJP1 accounting for more than 45% (Furusawa, Rakwal, Nam, Shibato, Agrawal, Kim, et al., 2008). MRJP1 is an acidic 55 kDa protein and forms an oligomeric complex; however, its protein structure has not been analyzed yet. RJ is indispensable for the development of a queen bee and MRJP family is thought to be a major factor in queen honeybee development (Kamakura, 2011; Schmitzova, Klaudiny, Albert, Schroder, Schreckengost, Hanes, et al., 1998; Weaver, 1966). Furthermore, the MRJP family is thought to be the main substance involved in the physiological actions of RJ, including cell proliferation, cytokine suppression, and antimicrobial activity (Fujiwara, Imai, Fujiwara, Yaeshima, Kawashima, & Kobayashi, 1990; Kamakura, Suenobu, & Fukushima, 2001; Majtan, Kumar, Majtan, Walls, & Klaudiny, 2010; Oka, Emori, Kobayashi, Hayashi, & Nomoto, 2001; Okamoto, 2003; Shen, Ding, Zhang, Jin, Zhang, & Li, 2010).

Previously, soluble RJ proteins (SRJPs) including the MRJP family were prepared from methods that combined dialysis and centrifugation (S. Tamura, Amano, Kono, Kondoh, Yamaguchi, Kobayashi, et al., 2009; Shougo Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009). Typically, these methods take a long time to produce SRJPs. Most existing methods require seven days of dialysis and produce very low yields because RJ is difficult to dissolve. Therefore, the quantity of MRJPs obtained from SRJPs is a limiting factor for biological research on MRJPs.

Ultracentrifugation is known as a useful tool to isolate lipoproteins and whey proteins that are

difficult to extract (De Natale, Annuzzi, Bozzetto, Mazzarella, Costabile, Ciano, et al., 2009; Etcheverry, Miller, & Glahn, 2004; Henry, Molle, Morgan, Fauquant, & Bouhallab, 2002; Tanese, 1997; Yee, Pavitt, Tan, Venkatesan, Godsland, Richmond, et al., 2008). Thus, we used ultracentrifugation to isolate SRJPs from RJ and obtained a large quantity of SRJPs within an hour.

In this study we describe a simple and rapid method to isolate SRJPs using a novel ultracentrifugal analysis.

## 2. Materials and Methods

### 2.1. Materials

Fresh RJ was provided by Japan Royal Jelly (Tokyo, Japan). Samples were stored at -80°C until analysis.

### 2.2. Extraction of soluble RJ proteins

First, 10 g of RJ was dissoloved in 10 mL of deionized water and vortexed. Next, RJ was ultracentrifuged (Optima™ L-80XP Ultracentrifuge, Beckman Coulter, Tokyo, Japan) at 113400 xg for 1 hour at 4°C. Then, the supernatant was separated into three layers, including the upper, middle, and lower layers. These layers contained defined and layered soluble RJ proteins (LSRJPs). Each layer was recovered with minimal mixing of the layers and then stored at 4°C until further analysis.

### 2.3. Measurement of total protein levels

The total protein concentration in the samples was quantified using a Micro BCA protein Assay

Kit (Thermo Fisher Scientific K. K., Yokohama, Japan). HSA (Wako, Osaka, Japan) was used as a protein standard.

### 2.4. SDS-PAGE

Proteins in each layer were mixed with NuPAGE lithium dodecyl sulfate (LDS) sample buffer (Invitrogen, Tokyo, Japan) containing 50 mM DTT, and then boiled at 100°C for 5 min. The sample mixtures were separated on precast NuPAGE 4-12% bis-Tris polyacrylamide gels (Invitrogen). Running buffer was prepared using NuPAGE MES SDS Running Buffer (Invitrogen). SDS-PAGE was performed at a constant 200 V. Mark12 Unstained Standard (Invitrogen) was used as molecular weight markers for SDS-PAGE.

### 2.5. 2-DE

Twenty-five micrograms of protein was desalted, delipidated and concentrated by deposition with 100% cold acetone. Protein pellets were washed with 80% acetone and dissolved in a protein solubiliser (Invitrogen), carrier ampholytes 3–10 (Invitrogen) and DTT. The protein solution was added to an IPG ZOOM strip gel (pH 3–10, Invitrogen) and incubated 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 alkylated with 125 mM iodoacetamide in LDS sample buffer. The gel was used for second-dimension SDS–PAGE as described above.

### 2.6. HPLC

Each sample layer was analyzed by size-exclusion and anion-exchange HPLC. Size-exclusion HPLC was performed on Superose 12 columns (10×300 mm; GE Healthcare). PBS (Invitrogen) was used as the elution buffer for each column. For the Superose 12 column, the sample injection volume was 100 μL and the flow rate was 0.2 mL/min.

Anion-exchange HPLC was performed on a Mini Q column (4.6×50 mm; GE Healthcare). Dialysis with 20 mM Tris-HCl (pH 8.0) was used for sample desalting and buffer exchange, and then the samples were concentrated using a Minicon or Amicon Ultra-4 (Millipore, Billerica, MA, USA). The sample injection volume was 1 mL. The binding buffer contained 20 mM Tris-HCl (pH 8.0), and the elution buffer contained 20 mM Tris-HCl, 1 M NaCl.

## 3. Results

### 3.1. Extraction of LSRJPs by ultracentrifugation

The solution obtained by mixing 10 g of RJ with 10 mL of deionized water was separated into three layers by ultracentrifugation (Fig. 1-A). The supernatant was classified as layered soluble RJ proteins (LSRJPs). In addition, the mixed solution of these layers was classified as crude soluble RJ proteins (CSRJPs). The upper, middle, and lower layers had volumes of 1.5 mL, 7 mL, and 4.5 mL, respectively.

### 3.2.Comparison of the yields obtained by both extraction methods

We recovered the mixture of three LSRJPs layers extracted using the new method to compare with CSRJPs extracted from the existing method. The total amount of protein in CSRJPs extracted from three different RJs was measured. The recovery rate was determined as the percentage of the weight of RJ. The yields obtained from each method are shown in Table 1. Total protein concentrations of supernatant obtained from each RJ using the existing method were 20.52 mg/ml, 18.20 mg/ml, and 24.53 mg/ml; the new method yielded 40.19 mg/ml, 32.79 mg/ml, and 36.03 mg/ml. Recovery rates were 1.267%, 1.041%, and 1.413% using the existing method, while the rates for the new method were 5.225%, 4.263%, and 4.683%. Recovery rates were calculated according to the following equation (formula?): weight of 3 ml RJ/total protein in existing method, and RJ 10 g/total protein in new method.

### 3.3. Comparison of the protein profile from both extraction methods

First, we compared the protein profile of CSRJPs that were extracted using the existing method and the new method. We recovered the mixture of three layers of SRJPs extracted by the new method. It is well established that size-exclusion chromatography of CSRJPs yields the elution pattern presented in Fig. 2. Each major protein peak was identified in a previous study. A peak at 290 kDa represented the MRJP1 oligomer, while peaks at approximately 70 kDa and 50 kDa were MRJP3 and MRJP2, respectively. There were minimal differences in the major protein peaks between the two extraction methods. The new method yielded greater small molecular peaks than the existing method. Peaks eluted after 15 mL consisted of low molecular constituents and not proteins (data not shown).

### 3.4. Analysis of LSRJPs by electrophoresis

We analyzed the proteins in the three layers that were obtained with the reformed extraction method by SDS-PAGE and 2-DE. SDS-PAGE bands and 2-DE spots were identified by a previous study using MALDI-TOF-MS and standard proteomic analyses. There were minimal differences in both the upper and middle layers. However, a 55 kDa band was predominantly stained in the lower layer.

Additionally, we performed 2-DE and confirmed that MRJP1 predominantly fractionated to the lower layer (Figs. 1-B, C, D).

### 3.5. Analysis of LSRJPs by HPLC

We analyzed the proteins in the three layers that were obtained using the new extraction method using Superose 12 size-exclusion HPLC columns. The chromatograms are shown in Figs. 3-A, B, C. The upper layer contained many peaks corresponding to low molecular weight proteins. There was a small peak representing the MRJP1 oligomer (290 kDa) in the upper layer. In addition, the middle layer contained a small peak corresponding to the MRJP1 oligomer. Peaks for MRJP2 and MRJP3 were higher in the middle than in the upper layer. On the other hand, it was clear that the MRJP1 oligomer predominantly fractionated to the lower layer.

## 4. Discussion

There are numerous reports describing a technically established MRJP purification procedure that includes (i) dialysis to eliminate impurities from RJ and to extract SRJPs by dissolution and (ii)

153 HPLC to isolate the target MRJPs contained in the SRJPs (Fontana, Mendes, de Souza, Konno, Cesar,
154 Malaspina, et al., 2004; Furusawa, et al., 2008; Kimura, Kajiyama, Kanaeda, Izukawa, & Yonekura,
155 1996; Li, Feng, Zhang, Zhang, & Pan, 2008; Okamoto, 2003; S. Tamura, et al., 2009; Shougo
156 Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009). However, isolating the target fraction from
157 RJ by dialysis is time-consuming. Similar to previous reports (S. Tamura, et al., 2009; Shougo
158 Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009), the dialysis system at the authors' facility
159 required at least seven days to complete the extraction process. Another disadvantage of this
160 dialysis-based extraction method is the poor yield; processing of dialysis 3 mL of RJ by the
161 conventional procedure yielded only 2 mL of aqueous solution, with a mean protein concentration of
162 only 21.08 mg/mL on average. In addition, recovery rate (yield of total protein levels/weight of RJ)
163 averaged 1.24%. Thus, one of the major challenges in studying the physiological activities of RJ has
164 been the development of a new method that produces high yields of MRJPs.
165 The new method described in this report, which processed RJ first by ultracentrifugation, yielded
166 aqueous solutions with a mean volume of 13 mL and a mean protein concentration of 36.33 mg/mL
167 from a starting mixture of 10 g of RJ in 10 mL of dissolution water. Additionally, recovery rate
168 averaged 4.72%. The resulting protein concentration was more than 1.7-fold higher than that
169 obtained by the conventional process, and the resulting recovery rate increased by 3.8-fold. Further,
170 our preliminary experiments suggested that solutions with higher protein concentrations could be
171 obtained by adding smaller volumes of dissolution water (unpublished data). In the case of extensive

extraction, products obtained by dialysis-based methods are generally very expensive. The resulting yield of total proteins levels using our method was more than 10-fold higher than that obtained by the conventional method.

MRJPs have been the subject of various research projects on the physiological activities of RJ. Such studies call for a significant amount of highly pure MRJPs. Therefore, we believe that our new technique will contribute to advances in scientific knowledge of the MRJP family.

We demonstrated that ultracentrifuging RJ for 1 hour resulted in a visible, three-layer separation of the supernatant. The upper and middle layers had high concentrations of MRJP2 (52 kDa) and MRJP3 (60–70 kDa). MRJP1 (55 kDa), which had a similar molecular weight to MRJP2 and MRJP3, was present in the lower layer in the form of an oligomer. Our accomplishment has provided a highly efficient method to purify the target MRJP by collecting the relevant supernatant layer. In particular, MRJP1 exerts cell protective effects (Turkmen, Cavusoglu, Yapar, & Yalcin, 2009; Wagner, Dobler, & Thiem, 1970) and other physiological activities (Majtan, Kovacova, Bilikova, & Simuth, 2006; Matsui, Yukiyoshi, Doi, Sugimoto, Yamada, & Matsumoto, 2002; Narita, Nomura, Ohta, Inoh, Suzuki, Araki, et al., 2006) and exists predominantly as an oligomer in naturally produced RJ (S. Tamura, et al., 2009; Shougo Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009). This underscores the significance of using the MRJP1 oligomer when studying the biological properties of MRJP1.

In conclusion, the novel technique involving ultracentrifugation of RJ described here supersedes

conventional methods in terms of technical simplicity and production yields. Despite economic issues related to the high cost of the ultracentrifugation equipment, we expect that this new process will prove to be a new standard method to purify MRJPs.

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266

**Figure captions**

**Fig.1.** (A) SRJPs after ultracentrifugation. Ten grams RJ was dissolved in 10 mL of deionized water. The supernatant consisted of an upper turbid layer (1.5 mL), clear middle layer (7 mL), and a thick lower layer (4.5 mL). (a) Insoluble layer included RJ. (B), (C) and (D) 2-DE profiles of SRJPs of three layers. (B) 2-DE profile of upper-layer. The spot indicated by a dotted circle was MRJP3. Another spot indicated by a dashed circle was MRJP2. (C) 2-DE profile of middle-layer. The major spot was the same as the upper-layer. (D) 2-DE profile of lower-layer. The major spot indicated by a continuous circle was MRJP1. The amount of protein obtained was 20 μg. The gel was stained with CBB.

**Fig.2.** Two elution profiles of SRJPs 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: a MRJP1 oligomer (290 kDa), b MRJP3 (60-70 kDa), c MRJP2 (52 kDa). With regard to these major peaks, there was not much difference between the two methods (OR No significant differences were found in these major peaks between the two methods.). Lot number of RJ was the same for A and B. (A) Elution pattern of SRJPs obtained by the existing method. Absorbance of MRJP1 peak and MRJP2 peak was 833 mAU and 1426 mAU, respectively. (B) Elution pattern of SRJPs (mixture of upper, middle and lower layers) obtained by the new method. Absorbance of the MRJP1 peak and

MRJP2 peak was 831 mAU and 1479 mAU, respectively. Peaks eluted after 15 mL consisted of low molecular constituents and not proteins.

**Fig.3.** Elution profiles of three layers SRJPs by size-exclusion HPLC on a Superose 12 column. The protein absorbance was monitored at 280 nm. (A) Elution pattern of SRJPs of upper layer. MRJP3 peak was 419 mAU and MRJP2 peak was 865 mAU. (B) Elution pattern of SRJPs of middle layer. MRJP3 peak was 624 mAU and MRJP2 peak was 1338 mAU. (C) Elution pattern of SRJPs of lower layer. MRJP1 oligomer peak was 1660 mAU.

294 **List of figure captions and a table**

295 Figure 1. Royal jelly (RJ) after ultracentrifugation and 2-DE profiles of three layers.

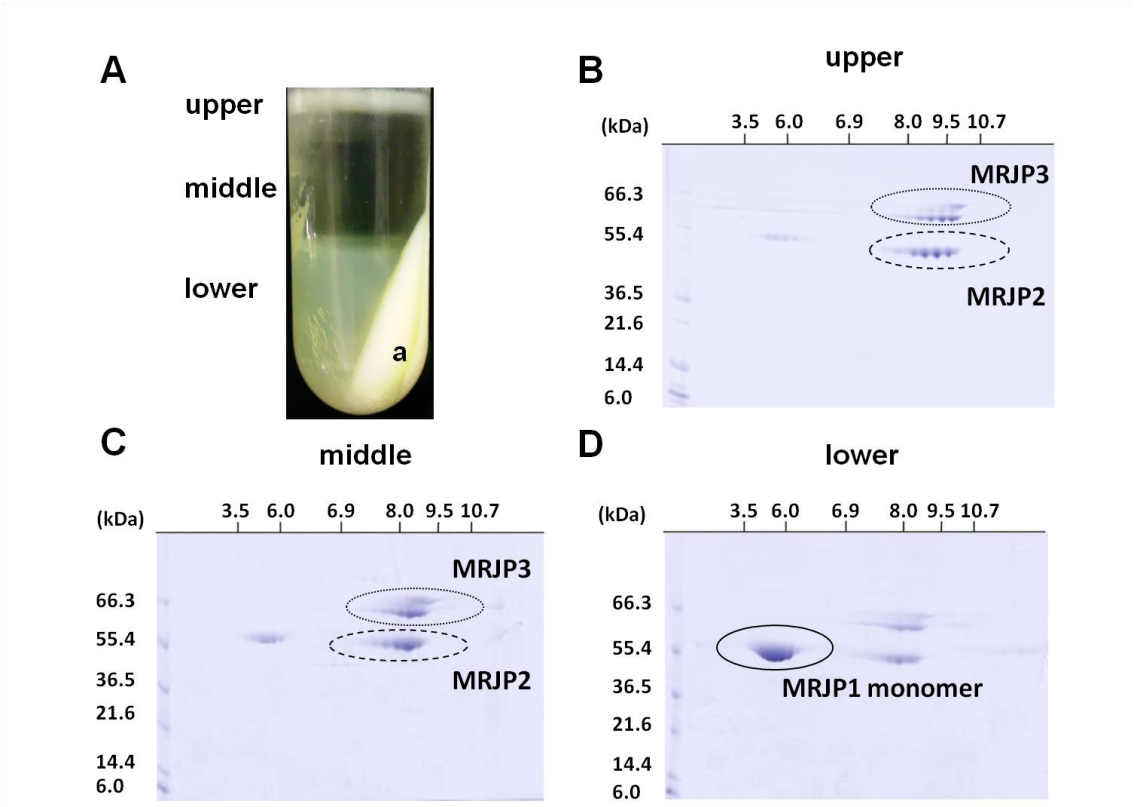
296 Figure 2. Two elution profiles of soluble RJ proteins (SRJPs) by size-exclusion HPLC on a Superose

297 12 column.

298 Figure 3. Elution profiles of three layers of SRJPs by size-exclusion HPLC on a Superose 12 column.

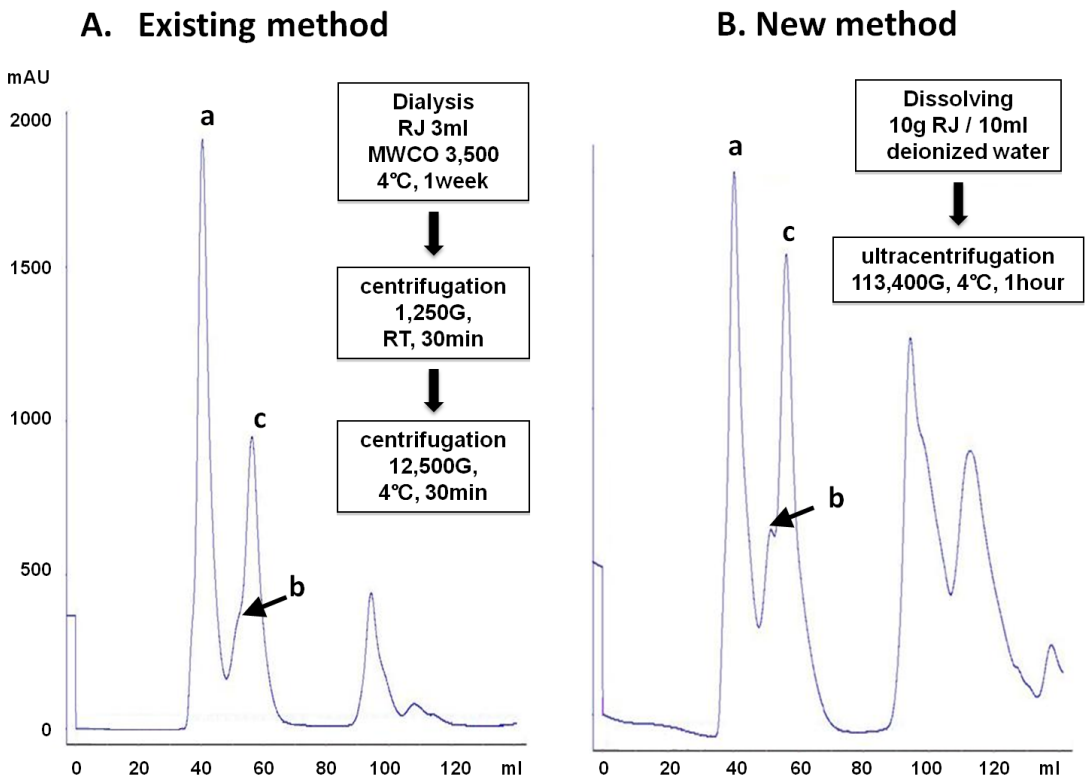
299 Table 1. Comparison of the yields obtained by existing and new extraction methods.

300 Fig.1


A
upper
middle
lower
a
B
upper
(kDa)
3.5 6.0 6.9 8.0 9.5 10.7
MRJP3
MRJP2
66.3
55.4
36.5
21.6
14.4
6.0
C
middle
(kDa)
3.5 6.0 6.9 8.0 9.5 10.7
MRJP3
MRJP2
66.3
55.4
36.5
21.6
14.4
6.0
D
lower
(kDa)
3.5 6.0 6.9 8.0 9.5 10.7
MRJP1 monomer
66.3
55.4
36.5
21.6
14.4
6.0

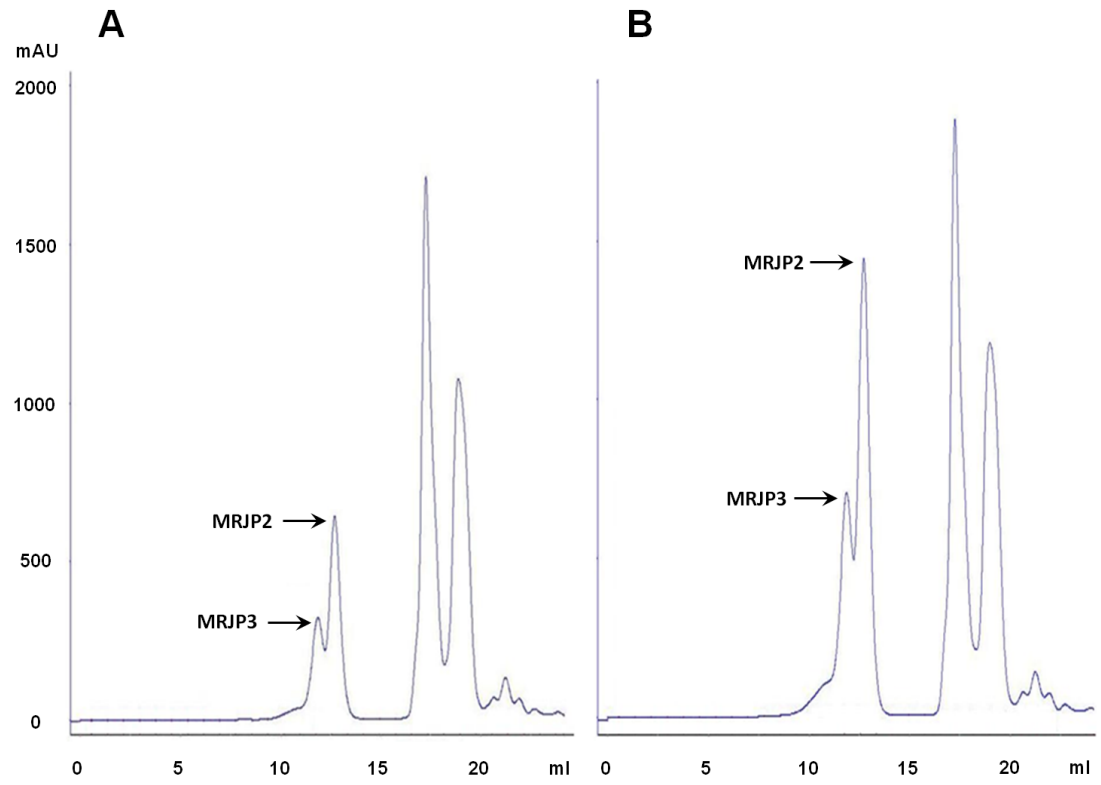
301

302 Fig.2


A. Existing method
B. New method
mAU
2000
1500
1000
500
0
a
b
c
0 20 40 60 80 100 120 ml
Dialysis
RJ 3ml
MWCO 3,500
4℃, 1week
centrifugation
1,250G,
RT, 30min
centrifugation
12,500G,
4℃, 30min
Dissolving
10g RJ / 10ml
deionized water
ultracentrifugation
113,400G, 4℃, 1hour

303

304 Fig.3

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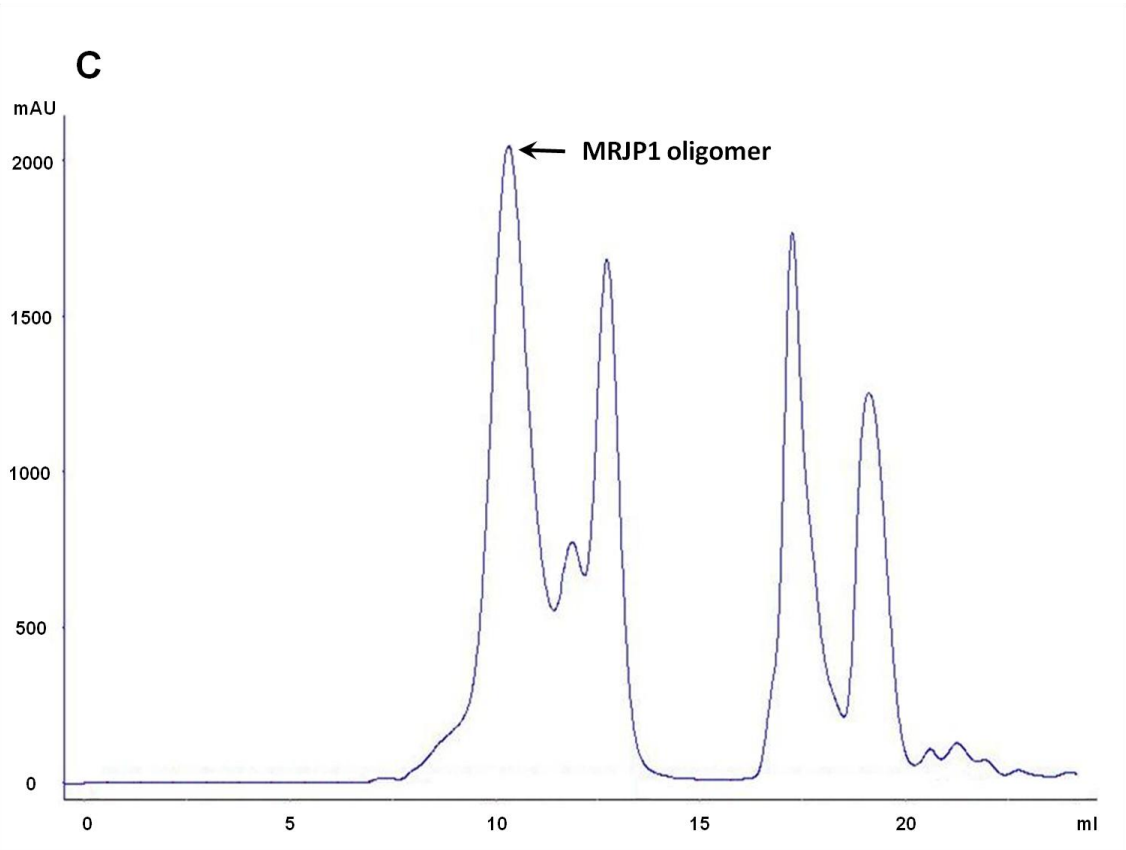


306

**Table 1**
**Comparison of the yields obtained by existing and new extraction methods**

| | Lot | Weight (g) | supernatant after centrifugation (ml) | total protein concentration of supernatant (mg/ml) | yield of total protein levels (mg) | recovery rate (%) |
|---|---|---|---|---|---|---|
| Existing method | a | 3.24 | 2 | 20.52 | 41.04 | 1.27 |
| | b | 3.50 | 2 | 18.20 | 36.40 | 1.04 |
| | c | 3.47 | 2 | 24.53 | 49.06 | 1.41 |
| New method | a | 10 | 13 | 40.19 | 522.50 | 5.22 |
| | b | 10 | 13 | 32.79 | 426.33 | 4.26 |
| | c | 10 | 13 | 36.03 | 468.34 | 4.68 |

307