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A rapid method to isolate soluble royal jelly proteins

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1 Title: A rapid method to isolate soluble royal jelly proteins

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20

21 **Abstract**

22 Soluble royal jelly (RJ) proteins (SRJPs) include the major RJ protein (MRJP) family,  
23 which contribute to the physiological actions of RJ. Although SRJPs are prepared using  
24 conventional methods involving dialysis and centrifugation, dialysis is a  
25 time-consuming process. We have therefore developed a simple method to isolate  
26 SRJPs from RJ. This new method produces twenty-fold higher levels of SRJPs than that  
27 of the conventional procedure; hence, the levels obtained by the new and existing  
28 methods were compared. A 1-hour ultracentrifugation separated SRJPs in the  
29 supernatant into upper, middle and lower layers. Each layer was analyzed by  
30 size-exclusion HPLC, SDS-PAGE and 2-DE. The upper and middle layers contained  
31 MRJP2 (52 kDa) and MRJP3 (60–70 kDa), while the lower layer contained MRJP1  
32 (290 kDa). In nature, MRJP1 is a monomer and/or oligomer. When the lower layer was  
33 analyzed by Superose 12 HPLC, MRJP1 was predominantly an oligomer. Our MRJP  
34 isolation method reduces the procedure time by using ultracentrifugation without  
35 dialysis to obtain SRJPs and produces layers containing MRJP1 oligomers, MRJP2 and  
36 MRJP3.

37

38 **Keywords**

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

40

41 **1. Introduction**

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

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

## 95 **2.5. 2-DE**

96 Twenty-five micrograms of protein was desalted, delipidated and concentrated by  
97 deposition with 100% cold acetone. Protein pellets were washed with 80% acetone and  
98 dissolved in a protein solubiliser (Invitrogen), carrier ampholytes 3–10 (Invitrogen) and  
99 DTT. The protein solution was added to an IPG ZOOM strip gel (pH 3–10, Invitrogen)  
100 and incubated overnight. First-dimension IEF was run under gradient voltage conditions  
101 (175 V constant for 20 min, gradient from 175 to 2000 V for 45 min, and 2000 V  
102 constant for 30 min). IEF Marker 3–10 SELVA Liquid Mix (SERVA Electrophoresis,  
103 Heidelberg, Germany) was used as a pI marker. The IPG strip gel used for IEF was  
104 reduced with 50 mM DTT in LDS sample buffer and alkylated with 125 mM  
105 iodoacetamide in LDS sample buffer. The gel was used for second-dimension  
106 SDS-PAGE as described above.

## 107 **2.6. HPLC**

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

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

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

119

### 120 **3. Results**

#### 121 **3.1. Extraction of LSRJPs by ultracentrifugation**

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

### 127 **3.2.Comparison of the yields obtained by both extraction methods**

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

### 139 **3.3. Comparison of the protein profile from both extraction methods**

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

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

#### 150 **3.4. Analysis of LSRJPs by electrophoresis**

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

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

#### 158 **3.5. Analysis of LSRJPs by HPLC**

159 We analyzed the proteins in the three layers that were obtained using the new  
160 extraction method using Superose 12 size-exclusion HPLC columns. The  
161 chromatograms are shown in Figs. 3-A, B, C. The upper layer contained many peaks  
162 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

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

185 The new method described in this report, which processed RJ first by  
186 ultracentrifugation, yielded aqueous solutions with a mean volume of 13 mL and a mean  
187 protein concentration of 36.33 mg/mL from a starting mixture of 10 g of RJ in 10 mL of  
188 dissolution water. Additionally, recovery rate averaged 4.72%. The resulting protein  
189 concentration was more than 1.7-fold higher than that obtained by the conventional  
190 process, and the resulting recovery rate increased by 3.8-fold. Further, our preliminary  
191 experiments suggested that solutions with higher protein concentrations could be  
192 obtained by adding smaller volumes of dissolution water (unpublished data). In the case  
193 of extensive extraction, products obtained by dialysis-based methods are generally very  
194 expensive. The resulting yield of total proteins levels using our method was more than  
195 10-fold higher than that obtained by the conventional method.

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

199 knowledge of the MRJP family.

200 We demonstrated that ultracentrifuging RJ for 1 hour resulted in a visible, three-layer  
201 separation of the supernatant. The upper and middle layers had high concentrations of  
202 MRJP2 (52 kDa) and MRJP3 (60–70 kDa). MRJP1 (55 kDa), which had a similar  
203 molecular weight to MRJP2 and MRJP3, was present in the lower layer in the form of  
204 an oligomer. Our accomplishment has provided a highly efficient method to purify the  
205 target MRJP by collecting the relevant supernatant layer. In particular, MRJP1 exerts  
206 cell protective effects (Turkmen, Cavusoglu, Yapar, & Yalcin, 2009; Wagner, Dobler, &  
207 Thiem, 1970) and other physiological activities (Majtan, Kovacova, Bilikova, & Simuth,  
208 2006; Matsui, Yukiyoishi, Doi, Sugimoto, Yamada, & Matsumoto, 2002; Narita, Nomura,  
209 Ohta, Inoh, Suzuki, Araki, et al., 2006) and exists predominantly as an oligomer in  
210 naturally produced RJ (S. Tamura, et al., 2009; Shougo Tamura, Kono, Harada,  
211 Yamaguchi, & Moriyama, 2009). This underscores the significance of using the MRJP1  
212 oligomer when studying the biological properties of MRJP1.

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

217 MRJPs.

218

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299

300 **Figure captions**

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

310

311 **Fig.2.** Two elution profiles of SRJPs by size-exclusion HPLC on a Superose 12 column.  
312 The protein absorbance was monitored at 280 nm. The column was calibrated using Gel  
313 filtration Calibration Kits with low molecular weight and high molecular weight  
314 proteins (GE Healthcare). Peaks: a MRJP1 oligomer (290 kDa), b MRJP3 (60-70 kDa),  
315 c MRJP2 (52 kDa). With regard to these major peaks, there was not much difference  
316 between the two methods (OR No significant differences were found in these major  
317 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.

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

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

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

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

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

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

33

#### 34 **Keywords**

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

36

#### 37 **1. Introduction**

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

39 of nurse honeybees. RJ contains 12–15% crude proteins, and it has been reported that 80% of these  
40 proteins are members of the major RJ protein (MRJP) family, with MRJP1 accounting for more than  
41 45% (Furusawa, Rakwal, Nam, Shibato, Agrawal, Kim, et al., 2008). MRJP1 is an acidic 55 kDa  
42 protein and forms an oligomeric complex; however, its protein structure has not been analyzed yet.  
43 RJ is indispensable for the development of a queen bee and MRJP family is thought to be a major  
44 factor in queen honeybee development (Kamakura, 2011; Schmitzova, Klaudiny, Albert, Schroder,  
45 Schreckengost, Hanes, et al., 1998; Weaver, 1966). Furthermore, the MRJP family is thought to be  
46 the main substance involved in the physiological actions of RJ, including cell proliferation, cytokine  
47 suppression, and antimicrobial activity (Fujiwara, Imai, Fujiwara, Yaeshima, Kawashima, &  
48 Kobayashi, 1990; Kamakura, Suenobu, & Fukushima, 2001; Majtan, Kumar, Majtan, Walls, &  
49 Klaudiny, 2010; Oka, Emori, Kobayashi, Hayashi, & Nomoto, 2001; Okamoto, 2003; Shen, Ding,  
50 Zhang, Jin, Zhang, & Li, 2010).

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

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

58 difficult to extract (De Natale, Annuzzi, Bozzetto, Mazzarella, Costabile, Ciano, et al., 2009;  
59 Etcheverry, Miller, & Glahn, 2004; Henry, Molle, Morgan, Fauquant, & Bouhallab, 2002; Tanese,  
60 1997; Yee, Pavitt, Tan, Venkatesan, Godslan, Richmond, et al., 2008). Thus, we used  
61 ultracentrifugation to isolate SRJPs from RJ and obtained a large quantity of SRJPs within an hour.

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

64

## 65 **2. Materials and Methods**

### 66 **2.1. Materials**

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

### 69 **2.2. Extraction of soluble RJ proteins**

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

### 75 **2.3. Measurement of total protein levels**

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

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

#### 79 **2.4. SDS-PAGE**

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

#### 86 **2.5. 2-DE**

87 Twenty-five micrograms of protein was desalted, delipidated and concentrated by deposition with  
88 100% cold acetone. Protein pellets were washed with 80% acetone and dissolved in a protein  
89 solubiliser (Invitrogen), carrier ampholytes 3–10 (Invitrogen) and DTT. The protein solution was  
90 added to an IPG ZOOM strip gel (pH 3–10, Invitrogen) and incubated overnight. First-dimension  
91 IEF was run under gradient voltage conditions (175 V constant for 20 min, gradient from 175 to  
92 2000 V for 45 min, and 2000 V constant for 30 min). IEF Marker 3–10 SELVA Liquid Mix (SERVA  
93 Electrophoresis, Heidelberg, Germany) was used as a pI marker. The IPG strip gel used for IEF was  
94 reduced with 50 mM DTT in LDS sample buffer and alkylated with 125 mM iodoacetamide in LDS  
95 sample buffer. The gel was used for second-dimension SDS-PAGE as described above.

## 96 **2.6. HPLC**

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

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

106

## 107 **3. Results**

### 108 **3.1. Extraction of LSRJPs by ultracentrifugation**

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

### 114 **3.2. Comparison of the yields obtained by both extraction methods**

115 We recovered the mixture of three LSRJPs layers extracted using the new method to compare with  
116 CSRJPs extracted from the existing method. The total amount of protein in CSRJPs extracted from  
117 three different RJs was measured. The recovery rate was determined as the percentage of the weight  
118 of RJ. The yields obtained from each method are shown in Table 1. Total protein concentrations of  
119 supernatant obtained from each RJ using the existing method were 20.52 mg/ml, 18.20 mg/ml, and  
120 24.53 mg/ml; the new method yielded 40.19 mg/ml, 32.79 mg/ml, and 36.03 mg/ml. Recovery rates  
121 were 1.267%, 1.041%, and 1.413% using the existing method, while the rates for the new method  
122 were 5.225%, 4.263%, and 4.683%. Recovery rates were calculated according to the following  
123 equation (formula?): weight of 3 ml RJ/total protein in existing method, and RJ 10 g/total protein in  
124 new method.

### 125 **3.3. Comparison of the protein profile from both extraction methods**

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

134 **3.4. Analysis of LSRJPs by electrophoresis**

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

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

141 **3.5. Analysis of LSRJPs by HPLC**

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

149

150 **4. Discussion**

151 There are numerous reports describing a technically established MRJP purification procedure that  
152 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

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

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

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

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

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

194

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266

267 **Figure captions**

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

276

277 **Fig.2.** Two elution profiles of SRJPs by size-exclusion HPLC on a Superose 12 column. The protein  
278 absorbance was monitored at 280 nm. The column was calibrated using Gel filtration Calibration  
279 Kits with low molecular weight and high molecular weight proteins (GE Healthcare). Peaks: a  
280 MRJP1 oligomer (290 kDa), b MRJP3 (60-70 kDa), c MRJP2 (52 kDa). With regard to these major  
281 peaks, there was not much difference between the two methods (OR No significant differences were  
282 found in these major peaks between the two methods.). Lot number of RJ was the same for A and B.  
283 (A) Elution pattern of SRJPs obtained by the existing method. Absorbance of MRJP1 peak and  
284 MRJP2 peak was 833 mAU and 1426 mAU, respectively. (B) Elution pattern of SRJPs (mixture of  
285 upper, middle and lower layers) obtained by the new method. Absorbance of the MRJP1 peak and

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

288

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

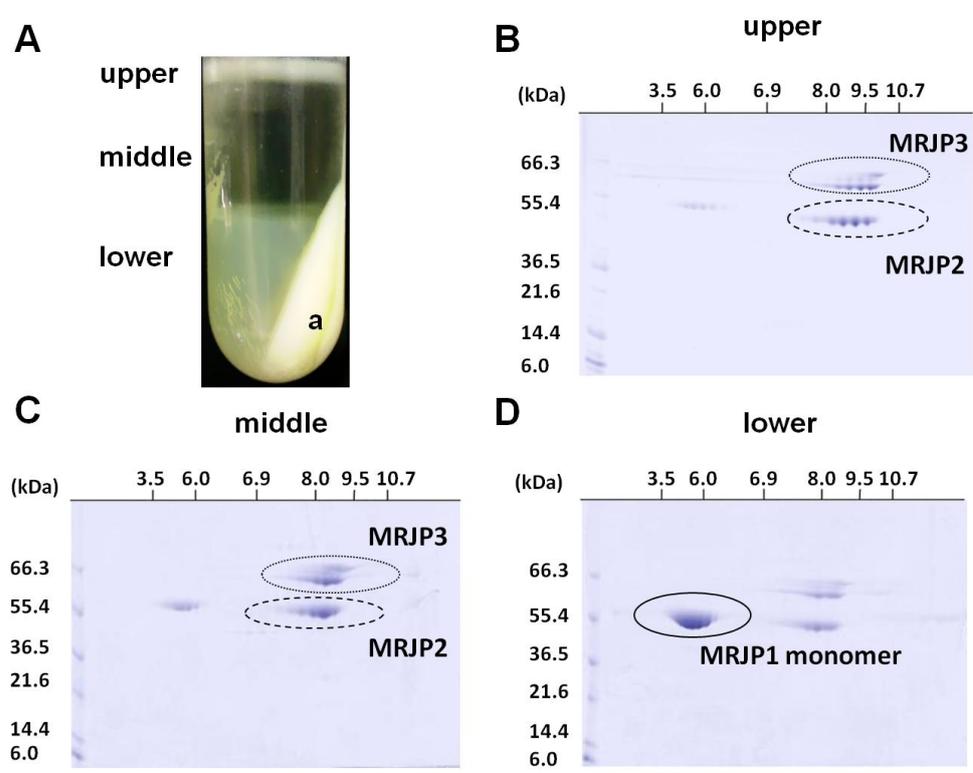
294 **List of figure captions and a table**

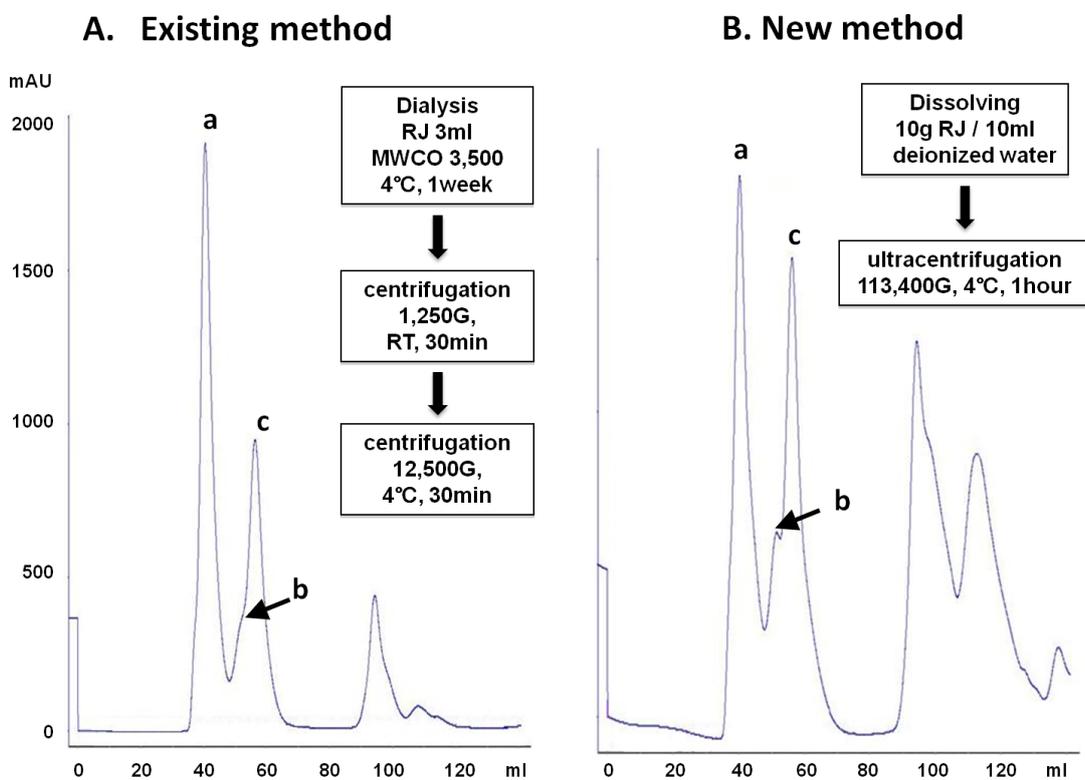
295 Figure 1. Royal jelly (RJ) after ultracentrifugation and 2-DE profiles of three layers.

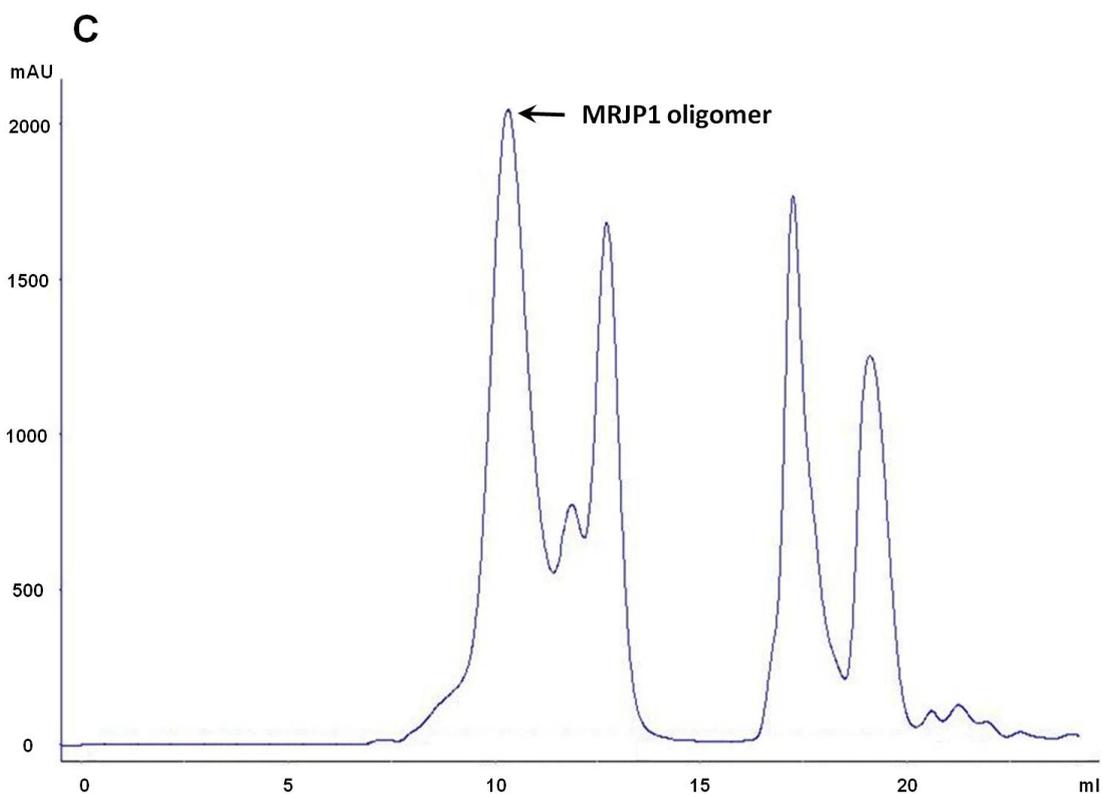
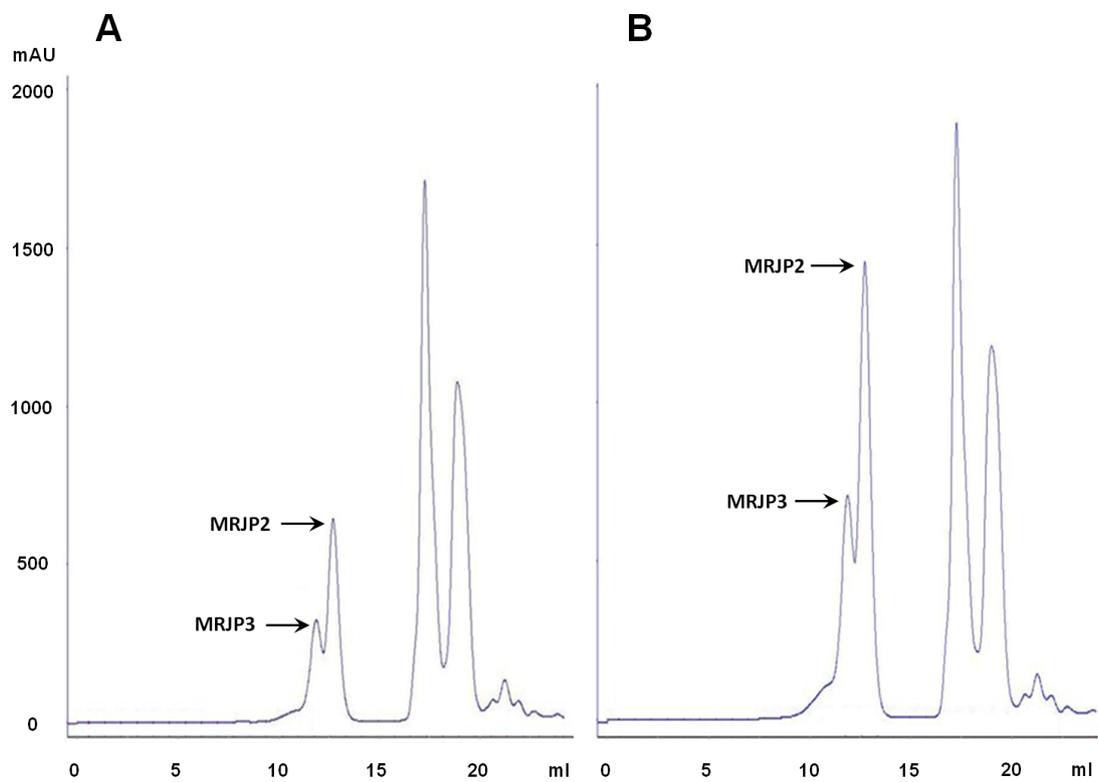
296 Figure 2. Two elution profiles of soluble RJ proteins (SRJPs) by size-exclusion HPLC on a Superose  
297 12 column.

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.







**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