Storage-dependent Degradation of 57-kDa Protein in Royal Jelly: a Possible Marker for Freshness

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In order to find a marker for freshness of royal jelly (RJ), the composition change of RJ during storage was investigated. The contents of 10-hydroxy-2-decanoic acid, a bioactive component of RJ, and several vitamins did not change during storage at 40°C for 7 days. However, a specific protein, designated royal jelly protein-1 (RJP-1), was gradually degraded during storage under various conditions (from 4°C to 50°C for up to 7 days). The specific degradation of RJP-1 was proportional to storage temperature and storage period. RJP-1 was purified to homogeneity and characterized as a monomeric glycoprotein with a molecular mass of 57 kDa. These results suggest that 57-kDa protein in RJ can be used as a marker for freshness of RJ, reflecting the conditions under which RJ has been stored.

Key words: royal jelly; 57-kDa protein; storage-dependent degradation; marker for freshness; quality control

Royal jelly (RJ) is the exclusive food of the queen honey bee (Apis mellifera) larva, and is secreted from the hypopharyngeal and mandibular glands of the worker honey bees mainly between the sixth and twelfth days of their life.¹² So far, RJ has been demonstrated to possess several pharmacological activities in experimental animals, including vasodilative and hypotensive activities,³ increase in growth rate,⁴ disinfectant action,⁵ antitumor activity,⁶⁻⁹ antihypercholesterolemic activity,¹⁰ and anti-inflammatory activity.¹¹ Chemical composition analysis has shown that RJ consisted mainly of proteins, sugars, lipids, vitamins and free amino acids,¹²⁻¹⁴ together with a large number of such bioactive substances as 10-hydroxy-2-decanoic acid,¹⁵ antibacterial protein,¹⁶ a stimulating factor for the development of genital organs in male mice¹⁷ and 350-kDa protein,¹⁸ which stimulates the proliferation of human monocytes. Therefore, RJ has been widely used in commercial medical products, health foods and cosmetics in many countries.

The physical properties and chemical composition of RJ are dependent on the storage conditions. Smith¹⁹,²⁰ has reported that the composition of RJ did not change during storage at 5°C for one year or at −20°C for a few years, but that RJ deteriorated during storage at room temperature for one month. Furthermore, Takenaka et al.²¹ and Chen and Chen²² have demonstrated that the storage of RJ at room temperature caused acceleration of the Mallard reaction, and increases of viscosity, acidity and protein degradation, although no compositional change was observed during storage at 4°C. The antibacterial activity of RJ was also retained during storage. The compositional changes during storage at higher temperature are not influenced by microorganisms, and seem to be attributable to chemical reactions and the actions of enzymes such as protease,²³ α-glucosidase²⁴ and glucose oxidase.²¹ In order to suppress such compositional changes of RJ and to maintain its freshness for an extended period, RJ should be stored at as low a temperature as possible. However, no reliable marker or analysis method for freshness of RJ has so far been established.

In this study, the relationship between the constituents in RJ and the storage conditions was investigated to identify a factor that could be utilized as a marker for freshness. We found that a specific protein, royal jelly protein-1 (RJP-1), underwent storage-dependent degradation, and may be suitable as a marker for freshness of RJ.

Materials and Methods

Materials. Royal jelly (RJ) was purchased from Hangzhou Green Forever Apiculture Company (Hangzhou, China). MiniPlate™ 100 and MiniPlate™ 30 were purchased from MILLIPORE Co. (Bedford, U.S.A.). DEAE-Toyopearl 650M and TSK-gel G3000SW columns were purchased from Tosoh Co. (Tokyo, Japan). HiLoad™ 16/10 Superdex 200 column, Ampholine™ PAG plate, Pharma-

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Abbreviations: RJ, royal jelly; RJP-1, royal jelly protein-1; RJP-2, royal jelly protein-2; 10-HDA, 10-hydroxy-2-decanoic acid
lyte, LMW electrophoresis calibration kit, Gel filtration calibration kit and Isoelectric focusing calibration kit were purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). N-glycosidase F deglycosylation kit was purchased from Boehringer Mannheim (Mannheim, Germany). Cosmosil C18 Econopak column was from Nacalai Tesque (Kyoto, Japan). L-column ODS was from Chemical Evaluation Research Institute (Tokyo, Japan), and Silica 2150-N column was from Senshu Scientific Co. (Tokyo, Japan). All other chemicals were of guaranteed reagent grade.

Preparation of RJ samples. RJ produced by honey bees (*Apis mellifera*) fed with nectar and pollen from rapeseed (*Brassica napus*) was used in this study. RJ samples were harvested, immediately frozen and stored at −20°C. Each RJ sample was dispensed into 35 sterile glass bottles (50 ml). These bottles were then randomly divided into five groups; seven bottles in each group were stored at 4°C, room temperature, 30°C, 40°C and 50°C in well-lit locations in the laboratory. The average room temperature was about 20°C. Sampling was performed every day up to seven days, and each bottle was sampled once only in order to avoid contamination.

Measurement of the color difference of royal jelly during storage. The color differences (ΔE*ab) of RJ stored under various conditions were measured by the method of Takenaka et al., 21) using CR-300 series chroma meters (Minolta, Tokyo). The color of RJ stored at −20°C was used as the standard. ΔE*ab was calculated by the following equation:

\[ \Delta E^{*ab} = [(\Delta L^{*})^2 + (\Delta a^{*})^2 + (\Delta b^{*})^2]^{1/2}. \]

\( \Delta L^{*} \) indicates the lightness, and \( \Delta a^{*} \) and \( \Delta b^{*} \) are the chromaticity coordinates.

Quantitative analysis of several vitamins and 10-hydroxy-2-decenoic acid in RJ. The contents of vitamins in RJ were quantitatively analyzed by the methods described in Standard Methods of Analysis in Food Safety Regulation. 22) Vitamin B1, vitamin B2 and vitamin C in RJ were quantitatively analyzed by high-pressure liquid chromatography (HPLC) with an L-column ODS (4.6 × 150 mm), Cosmosil C18 Econopak column (4.6 × 150 mm) and Silica 2150-N column (4.6 × 100 mm), respectively. Vitamin B6, vitamin B12 and folic acid in RJ were quantitatively analyzed by microbioassays with *Saccharomyces cerevisiae* ATCC 9080, *Lactobacillus delbrueckii* subsp. *lactis* ATCC 7830 and *Lactobacillus rhamnosus* ATCC 7469, respectively. Pantothentic acid, niacinamide and biotin in RJ were also quantitatively analyzed with *Lactobacillus plantarum* ATCC 8014, and 10-hydroxy-2-decenoic acid (10-HDA) in RJ was determined by the method of Yamazaki et al. 23) with some modification.

**Polyacrylamide gel electrophoresis (PAGE).** Native-PAGE was run with 5–20% gradient polyacrylamide gel by the method of Davis. 24) SDS-PAGE was run with 5–20% gradient polyacrylamide gel by the method of Laemmli. 25) To estimate the molecular mass, the following marker proteins were used: lysozyme (14.4 kDa), soybean trypsin inhibitor (20.1 kDa), bovine carbonic anhydrase (30 kDa), ovalbumin (43 kDa), serum albumin (67 kDa), and phosphorylase b (94 kDa). The protein in the gel was stained with Coomassie Brilliant Blue R-250 (CBB).

Purification of royal jelly protein-1 (RJP-1). Protein was monitored in terms of the absorbance at 280 nm during the purification.

**Step 1: Ultrafiltration treatment.** RJ (30 g) was dissolved in 1 L of 20 mM Tris-HCl buffer (pH 7.0). The suspension was centrifuged at 5,000 × g for 10 min, and the supernatant was concentrated to 20 ml by ultrafiltration with MiniPlate™ 100 having a molecular weight (MW) cut-off of 100,000. The concentrate was added to 80 ml of the same buffer and concentrated in the same manner. This concentration procedure was repeated five times, then the final filtrate was further concentrated 12-fold by ultrafiltration with MiniPlate™ 30 having a MW cut-off of 30,000.

**Step 2: DEAE-Toyopearl 650M column chromatography.** The fraction obtained by ultrafiltration treatment was applied to a DEAE-Toyopearl 650M column that had been equilibrated previously with 20 mM Tris-HCl buffer (pH 7.0). The column was washed with the same buffer. The protein was eluted with a linear gradient of NaCl from 0 to 1.0 M at a flow rate of 5 ml/min, and 2.5-ml fractions were collected. A sample of each fraction was taken and electrophoresed on native PAGE and SDS-PAGE.

**Step 3: HiLoad Superdex 200 gel filtration column chromatography.** The fraction containing RJP-1 was put on a HiLoad™ 16/10 Superdex 200 column that had been equilibrated previously with 50 mM phosphate buffer (pH 7.0) containing 0.15 M NaCl. The column was then eluted with the same buffer at a flow rate of 1.0 ml/min, and 2.0-ml fractions were collected. A sample of each protein fraction was taken and electrophoresed on native PAGE and SDS-PAGE. The fraction showing a single band was dialyzed against distilled water and lyophilized.

**Protein determination.** Protein concentration was determined by the method of Lowry et al., 26) using bovine serum albumin as the standard.

**HPLC.** For a quantitative analysis of RJP-1 in RJ and for the molecular mass determination of RJP-1, HPLC was carried out under the following condi-
tions: column, TSK-gel G3000SW (7.5 × 300 mm); solvent, 0.1 M phosphate buffer (pH 7.0) containing 0.3 M NaCl and 0.05% NaN3; flow rate, 0.3 ml/min; temperature, 30°C; pump, Waters 515; and detector, Waters 486 (at 280 nm). RJ (0.15 g) was dissolved in 5 ml of the same solvent to prepare RJ sample for a quantitative analysis. The suspension was centrifuged at 5,000 × g for 10 min, and the supernatant was taken for the analysis. The total nitrogen content in RJ was determined by the Kjeldahl method.30 To determine of molecular mass of RJ-1, the following standard proteins were used to create a calibration curve: ferritin (440 kDa), aldolase (158 kDa), phosphorylase b (94 kDa), serum albumin (67 kDa), ovalbumin (43 kDa) and ribonuclease A (13.7 kDa).

Measurement of isoelectric point (pI). Isoelectric focusing (IEF) was done on an Ampholine PAG plate, using Pharmalyte (pH 3–10) as the carrier. Electrophoresis was done for 2 h at 10 W, and the gel was stained with CBB.

Deglycosylation of RJ-1. Deglycosylation of purified RJ-1 was done with a N-glycosidase F deglycosylation kit. Purified RJ-1 (1 μg) was denatured by heating at 100°C for 3 min, and the denatured protein was incubated with 0.3 U of N-glycosidase F for 50 h at 37°C. The resulting product was analyzed by SDS-PAGE.

Amino acid composition. Purified RJ-1 was hydrolyzed with 6 N HCl at 110°C for 22 h in an evacuated sealed tube. The amino acid composition of the hydrolysate was analyzed with an amino acid analyzer JLC-300 (Jeol, Japan).

Analysis of N-terminal amino acid sequence. The amino acid sequence of the N-terminal region of purified RJ-1 was identified by automated Edman degradation with a gas-phase protein sequencer Model 494 (Applied Biosystems, U.S.A.).

Results

Change in the color difference of RJ during storage

As shown in Table 1, ΔE*ab of RJ increased proportionally to both storage temperature and duration. Similar results were obtained for both Δa* and Δb*. Δa* and Δb* indicate color directions: +Δa* is the red direction, +Δb* is the yellow direction. Therefore, these results indicate that browning of RJ occurred during storage and increased in proportion to storage temperature and storage period. The browning of RJ during storage was confirmed with the naked eye.

Analysis of the contents of several vitamins and 10-hydroxy-2-decenolic acid in RJ

The contents of several vitamins and 10-HDA in RJ samples stored at 4°C and 40°C for 7 days were measured. As shown in Table 2, no significant differences between the two RJ samples were observed in the contents of any of the compounds measured in the present study.

Change in electrophoretic profile of proteins in RJ during storage

To investigate whether compositional changes of proteins in RJ occurred under various storage conditions, the RJ proteins stored at 4°C and 40°C were analyzed by SDS-PAGE and native PAGE. The electrophoretic profile of RJ proteins stored at 4°C for 7 days was almost identical to that of RJ proteins stored at 40°C for 1, 2 and 7 days on SDS-PAGE (Fig. 1A). On the other hand, the results of native PAGE showed that two proteins in RJ, designated as royal jelly protein-1 (RJP-1) and royal jelly protein-2 (RJP-2), were specifically degraded when RJ samples were stored at 40°C (Fig. 1B). Of these two proteins, RJP-1 was degraded proportionally to the length of the storage period.

HPLC Analysis of the proteins in RJ

The proteins in RJ samples stored at 4°C and 40°C for 7 days were quantitatively analyzed by HPLC.

| Table 1. Change in Color Difference of Royal Jelly during Storage |
|-----------------|----------------|----------------|----------------|----------------|
| Storage temperature (°C) | ΔE*ab | ΔL* | Δa* | Δb* |
| Storage period (days) | Storage period (days) | Storage period (days) | Storage period (days) | Storage period (days) |
| 1 | 4 | 7 | 1 | 4 | 7 | 1 | 4 | 7 | 1 | 4 | 7 |
| 4 | 0.02 | 0.67 | 1.40 | 0 | -0.56 | -1.31 | 0.01 | 0.25 | 0.49 | 0 | 0.28 | 0.14 |
| 20 | 1.93 | 2.71 | 3.10 | -1.72 | -2.49 | -1.69 | 0.76 | 1.09 | 1.39 | 0.31 | -0.09 | -2.19 |
| 30 | 2.39 | 2.96 | 2.73 | -2.27 | -2.80 | -2.23 | 0.42 | 0.78 | 0.71 | 0.63 | 0.6 | -1.42 |
| 40 | 4.31 | 5.34 | 6.23 | 3.87 | 3.74 | 4.31 | 1.32 | 2.42 | 3.79 | 1.34 | 2.97 | 2.44 |
| 50 | 6.59 | 7.55 | 9.04 | 5.96 | 3.17 | 2.03 | 2.11 | 5.25 | 6.66 | 1.86 | 4.41 | 5.78 |

The color differences of RJ stored under various conditions were measured with CR-300 series chroma meters. ΔE*ab and ΔL* indicate the color difference and lightness, respectively. Δa* and Δb* are the chromaticity coordinates. Δa* and Δb* indicate the color directions: +Δa* is the red direction, −Δa* is the green direction, +Δb* is the yellow direction and −Δb* is the blue direction. Values are the averages of three replicate experiments.

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Table 2. Contents of Several Vitamins and 10-Hydroxy-2-decenoic Acid in RJ Stored at 4°C and 40°C for 7 Days

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Storage at 4°C for 7 days</th>
<th>Storage at 40°C for 7 days</th>
<th>Relative amount (%)^a</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>mg/RJ 100 g</td>
<td>mg/RJ 100 g</td>
<td></td>
</tr>
<tr>
<td>Vitamin B1</td>
<td>0.34</td>
<td>0.32</td>
<td>94</td>
</tr>
<tr>
<td>Vitamin B2</td>
<td>0.85</td>
<td>0.83</td>
<td>98</td>
</tr>
<tr>
<td>Vitamin B3</td>
<td>0.37</td>
<td>0.33</td>
<td>89</td>
</tr>
<tr>
<td>Vitamin B12</td>
<td>N.D.</td>
<td>N.D.</td>
<td>—</td>
</tr>
<tr>
<td>Vitamin C</td>
<td>N.D.</td>
<td>N.D.</td>
<td>—</td>
</tr>
<tr>
<td>Folic acid</td>
<td>2.70 × 10⁻³</td>
<td>23.0 × 10⁻³</td>
<td>85</td>
</tr>
<tr>
<td>Pantothenic acid</td>
<td>8.46</td>
<td>7.70</td>
<td>91</td>
</tr>
<tr>
<td>Biotin</td>
<td>17.4 × 10⁻³</td>
<td>17.4 × 10⁻³</td>
<td>100</td>
</tr>
<tr>
<td>Nicotinic acid</td>
<td>4.73</td>
<td>4.83</td>
<td>102</td>
</tr>
<tr>
<td>10-Hydroxy-2-decenoic acid</td>
<td>1.67[^b]</td>
<td>1.70[^b]</td>
<td>102</td>
</tr>
</tbody>
</table>

N.D.: not determined.

^a The amount of each ingredient in RJ stored at 40°C for 7 days was expressed as a percentage of that in RJ stored at 4°C for 7 days. Values are the averages of two replicate experiments.

[^b] Values are expressed as a percentage of the weight of RJ.

Fig. 1. Change in Electrophoretic Profile of RJ Proteins during Storage.

Proteins in RJ stored under various conditions were analyzed by SDS-PAGE (A) and native PAGE (B). RJ samples (0.15 g) were dissolved in 5 ml of 50 mM Tris-HCl (pH 7.0) buffer containing 20% glycerin, and the suspensions were centrifugated at 5,000 × g for 10 min. The supernatants were used as the sample for native PAGE. RJ samples were dissolved in 5 ml of 50 mM Tris-HCl (pH 7.0) buffer containing 20% glycerin, 1% SDS and 1% (by vol.) 2-mercaptoethanol for SDS-PAGE. Lane M, marker proteins of which the sizes (in kDa) are indicated; lane 1, RJ stored at 4°C for 7 days; lane 2, RJ stored at 40°C for 1 day; lane 3, RJ stored at 40°C for 2 days; lane 4, RJ stored at 40°C for 7 days. The positions of RJP-1 and RJP-2 are indicated by arrows.

Fig. 2. HPLC Analyses of Water-soluble Proteins in RJ.

The proteins in RJ samples stored at 4°C and 40°C for 7 days were quantitatively analyzed by HPLC with a TSK-gel G3000SW gel filtration column. The sample preparation and HPLC conditions are described in Materials and Methods. (A) RJ stored at 4°C for 7 days and (B) RJ stored at 40°C for 7 days were analyzed by HPLC. Arrows indicate the positions of molecular mass markers: phospholipase b (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), α-chymotrypsinogen (25 kDa) and ribonuclease A (13.7 kDa). Molecular masses are given in kDa.

with a TSK-gel G3000SW gel filtration column. Figure 2A shows an HPLC analysis of RJ samples stored at 4°C for 7 days. Six peaks (from P1 to P6) were detected. In RJ sample stored at 40°C for 7 days, the areas of P2 and P3 were decreased in HPLC analysis (Fig. 2B). RJP-1 and RJP-2 extracted from the gel after native PAGE showed the same retention times as P3 (about 27 min) and P2 (about 25 min), respectively, in HPLC analysis (data not shown). This result indicates that P3 and P2 in this HPLC analysis correspond to RJP-1 and RJP-2, respectively. These results of HPLC analysis were consistent with the results of native PAGE of RJ samples.

Degradation of the proteins in RJ during storage under various conditions
The contents of RJP-1 and RJP-2 in RJ samples stored at several temperatures were analyzed by HPLC. As shown in Fig. 3A, RJP-1 in RJ samples was specifically degraded in proportion to both storage temperature and storage period. RJP-2 was readily degraded in proportion to storage period, but not storage temperature (Fig. 3B).

**Purification of RJP-1**

We next attempted to purify RJP-1 from RJ with column chromatography. RJ proteins with molecular weight above 100,000 were removed by ultrafiltration, and the ultrafiltrate was applied to DEAE-Toyopearl 650M column (Fig. 4A). Each fraction separated by the chromatography was analyzed with native PAGE (Fig. 4B). The peak AIII fraction containing RJP-1 was further purified in a column of Superdex 200 (Fig. 5A). The peak GI fraction containing RJP-1 showed a single band on native PAGE and SDS-PAGE (Figs. 5B and 5C).

**Molecular mass of RJP-1**

The molecular mass of purified RJP-1 was determined to be about 57 kDa by gel filtration. The migration distance of RJP-1 on SDS-PAGE corresponded to a molecular mass of about 57 kDa (Fig. 5C). These results indicated that RJP-1 was a monomeric protein.

**Isoelectric point and deglycosylation of RJP-1**

The apparent isoelectric point of RJP-1, as determined by IEF, was around 5.1. We next treated the purified RJP-1 with N-glycosidase F and subjected the product to SDS-PAGE to determine its molecular mass. The band of native RJP-1 (57 kDa) shifted to the position of 48 kDa after enzyme treatment, indicating that native RJP-1 is a glycoprotein and that the oligosaccharide chains were removed by N-glycosidase F (data not shown).

**Comparison of some properties of RJP-1 with those of other RJ proteins**

The amino acid composition of RJP-1 is shown in Table 3. The amino acid composition and some properties of RJP-1 were compared with those of other RJ proteins (B-4, B-5, A-1 and A-3) reported by Takenaka and Echigo.\(^1\) RJP-1 was not similar to these proteins.

**Analysis of N-terminal amino acid sequence**

The amino acid sequence of the N-terminal region of the purified RJP-1 was determined to be Asn-Ile-Leu-Arg-Gly-Glu-Ser-Leu-Leu-Lys-Lys-Leu-Pro-Ile-Leu-. This N-terminal amino acid sequence of RJP-1 was compared with that of proteins deposited in the

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**Fig. 3.** Changes in the contents of RJP-1 and RJP-2 in RJ stored under various conditions.

The contents of (A) RJP-1 and (B) RJP-2 in RJ during storage were analyzed by HPLC with a TSK-gel G3000SW column. The concentrations of RJP-1 and RJP-2 in RJ were calculated using the following equation: concentration of RJP-1 or RJP-2 (g) = peak area of RJP-1 or RJP-2 × water-soluble protein concentration (g) in RJ (g) × 100/total area. RJ samples were stored at 4°C (●), room temperature (● ●), 30°C (● ● ●), 40°C (○) and 50°C (● ● ●) for 0 to 7 days. Protein concentration was measured by the Kjeldahl method. Each point represents the mean value of two experimental results. Details of the procedure for analysis are given in Materials and Methods.

**Fig. 4.** Elution Profile of RJP-1 on Ion Exchange Chromatography.

(A) The fraction obtained from ultrafiltration treatment was subjected to DEAE-Toyopearl 650M column chromatography. The elution conditions are described in Materials and Methods. Three fractions, A1, AII and AIII, were collected. ○ absorbance at 280 nm; --- NaCl concentration to elute the proteins. (B) Each fraction was subjected to native PAGE. Lane 1, 3% (w/v) RJ solution; lane 2, A1 fraction; lane 3, AII fraction; lane 4, AIII fraction. The position of RJP-1 is indicated by an arrow.
days, indicating that it is stable even at high temperature and may be unsuitable as a marker for freshness. Similar results were obtained for several vitamins in RJ. Takenaka et al.\textsuperscript{21} have previously examined the influence of storage conditions on the composition of RJ and reported that the concentrations of amino acids and sugars in RJ decreased during storage at room temperature for six months, owing to the Maillard reaction. Although browning increased rapidly during initial storage, little further decrease of these constituents owing to the Maillard reaction occurred after the third month of storage, and the compositional change was negligible. They have also reported that the contents of fatty acids and gluconic acid in RJ did not change during storage. This result is consistent with the present analysis. Thus, the quantitative changes of such constituents as 10-HDA, amino acids, sugars and vitamins in RJ during storage seem to be unrelated to the freshness of RJ.

On the other hand, the results of native PAGE and HPLC analyses of RJ samples stored under various conditions showed that two proteins, which were designated as RJP-1 and RJP-2, were specifically degraded when RJ samples were stored at high temperature. RJP-2 was degraded irrespective of storage temperature, even at 4°C. However, RJP-1 was gradually degraded proportionally to both storage temperature and duration. Furthermore, this specific degradation of RJP-1 correlated with the color change (browning) of RJ during storage. These results suggested that RJP-1 could be available as a marker to evaluate the freshness of RJ. Also, when RJ was stored at the higher temperature of 50°C for 4 days, almost all the proteins began to be degraded (data not shown). Thus, RJP-1 should be a good "early-warning" marker for predicting the deterioration of RJ.

Next, we purified RJP-1 from RJ and characterized it. RJP-1 is a glycoprotein with a molecular mass of 57 kDa and an isoelectric point of about 5.1. Takenaka and Echigo isolated four proteins from RJ by column chromatography and determined their molecular masses and isoelectric points.\textsuperscript{30} RJP-1 is distinct from those proteins. The N-terminal amino acid sequence of RJP-1 up to five residues was identical with that of a subunit of 350-kDa protein, which stimulates the proliferation of human monocytes (U 937 cell line)\textsuperscript{39} and human-human hybridomas (HB4C5 cell line).\textsuperscript{52} The 350-kDa protein is a glycoprotein composed of six subunits with a molecular mass of 58 kDa,\textsuperscript{33} whereas RJP-1 is a monomeric glycoprotein with a molecular mass of 57 kDa. These results suggest that RJP-1 might be derived from a subunit of 350-kDa protein in RJ. Further studies will be needed to establish whether this is the case.

It seems reasonable to utilize the quantitative change of a standard substance in a foodstuff during

Fig. 5. Elution Profile of RJP-1 on Gel Filtration Chromatography.

(A) The peak AII fraction containing RJP-1 from DEAE-Toyopearl 650M chromatography was subjected to HiLoad Superdex 200 column chromatography. The elution conditions are described in Materials and Methods. Absorbance at 280 nm. Two fractions, GI and GII, were collected. (B) Each fraction was subjected to native PAGE. (C) Each fraction was subjected to SDS-PAGE. Lane M, marker proteins (sizes in kDa); lane 1, 3% (w/v) RJ solution; lane 2, the fraction obtained by ultrafiltration treatment; lane 3, AII fraction from DEAE-Toyopearl 650M chromatography; lane 4, GI fraction. The GI fraction did not show any protein band on native PAGE or SDS-PAGE. The position of RJP-1 is indicated by an arrow.

SWISS-PROT database and with the sequence of already found proteins in RJ. The first five N-terminal amino acid residues of RJP-1 were identical to those of a subunit of 350-kDa protein in RJ.\textsuperscript{33}

**Discussion**

In this study, we investigated the compositional change of RJ under various storage conditions to identify a marker for freshness of RJ. During storage of RJ at high temperature, a color change (browning) of RJ occurred. This result indicates that the Maillard reaction took place during long-term storage of RJ at high temperature, resulting in a deterioration of RJ. Next, we quantitatively examined the compositional change of RJ during storage. Many physiologically active substances are present in RJ, such as 10-HDA, which shows antitumor activity\textsuperscript{7-10} and antibacterial activity,\textsuperscript{15} and 10-HDA has mainly been used as a standard to control the quality of RJ. However, we found here that 10-HDA did not undergo any change during storage of RJ at 40°C for 7
storage as a marker to evaluate the quality. For example, Yamane et al.34 have reported that the formation of propanethial S-oxide (PSO) in scallions during storage correlated with visible deterioration of scallions, so that the production of PSO can be used as a marker for freshness of scallions. Restani et al.35 have also provided evidence that production of γ-casein fraction released by proteolysis of β-casein during ripening of Grana Padano cheese can be considered as a marker of quality and ripening.

In conclusion, we found that 57-kDa protein in RJ was specifically degraded proportionally to the severity of storage conditions. The correlation of the color change (browning) of RJ and the decrease of 57-kDa protein during storage of RJ under various conditions suggests that it is reasonable to evaluate the freshness and the quality of RJ in terms of the change of 57-kDa protein. RJ has been reported to contain a trypsin-like protease,36 and the possible involvement of this protease in the specific degradation of 57-kDa protein during storage remains to be examined.

References


Table 3. Comparison of Amino Acid Composition and Some Properties of 57-kDa Protein in RJ with Those of Several Proteins Contained in RJ

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>57-kDa protein</th>
<th>B-4</th>
<th>B-5</th>
<th>A-1</th>
<th>A-3</th>
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Molecular mass: 57 kDa
Isoelectric point: 5.1

The amino acid composition of 57-kDa protein in RJ was compared with those of several proteins (B-4, B-5, A-1 and A-3) reported by Takesaka et al. The composition was calculated without taking account residues of tryptophan, and was expressed as mole percent.

N.D.: not determined.
34 From ref. 31.
12) Takenaka, T., Chemical composition of royal jelly. 

13) Echigo, T., Takenaka, T., and Yatsunami, K., Comparative studies on chemical composition of honey, royal jelly and pollen loads. 


15) Blum, M. S., Novak, A. F., and Taber, S., 10-Hydroxy-Δ2-decenolic acid, an antibiotic found in royal jelly. 


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34) Yamane, A., Yamane, A., and Shimamoto, T., Propanethial S-oxide content in scallions (Allium fistulosum L. variety caespitosum) as a possible marker for freshness during cold storage. 

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