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Proteomics Analysis of Major Royal Jelly Protein Changes under Different Storage Conditions

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Protein changes in fresh royal jelly (RJ) were compared when stored at −20, 4 °C, and room temperature (RT) for 12 months. Protein was partially identified using combinations of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption ionization-time-of-flight mass spectrometry (MALDI-TOF/MS), gel filtration chromatography, nanoLC MS/MS, and a protein engine identification tool applied to the honeybee genome. Significantly more protein spots were found in fresh (85 spots) and −20 °C (81 spots) stored RJ than in samples stored at 4 °C (73 spots) and at RT (70 spots) for 1 year. Most identified spots, 56, 57, 51, 46, corresponding to RJ sample of the fresh, −20 °C, 4 °C, and RT, were assigned to major royal jelly proteins (MRJPs). Marked differences were found in the heterogeneity of the MRJPs, in particular, MRJP3. The quantity of MRJP1 decreased significantly following the temperature trend in all images, but MRJP 2 and -3 did not increase or decrease following the temperature trend, thus, suggesting that MRJP 1−3 are sensitive to temperature. However, MRJP4, 5, glucose oxidase (GOD), peroxiredoxin (PRDX), and glutathione *S*-transferase (GST) S1 were clearly absent in all images in samples held at RT for 1 year. This indicates that they are the proteins most sensitive to storage temperature and protein markers for freshness of RJ. Combining chromatography and nanoLC MS/MS results, we tentatively conclude that MRJP5 is a reliable freshness marker and that the best way to maintain quality of RJ is under freezing conditions.

Keywords: major royal jelly proteins (MRJPs) • storage temperature • 2-DE • MALDI-TOF/MS • Nano LC MS/MS

1. Introduction

Royal jelly (RJ) is known as a popular and traditional food for health promotion. It is a viscous substance secreted from the hypopharyngeal gland and mandibular gland of the worker honeybee[1−3] and is the exclusive food for the queen honeybee and the larva.[1,4] RJ has been reported to have such pharmacological characteristics as antitumor,[5] antibacterial,[6] antioxidation,[7] antihypertensive,[8] antiallergic,[9] antifatigue,[10] insulin-like,[11] and wound-healing properties.[12,13] A chemical composition analysis has shown that RJ consists mainly of proteins, sugars, lipids, vitamins and free amino acids.[14,15] In particular, several substances contained in RJ, including 10-hydroxy-2-decenoic acid (10-2-HDA),[5,16,17] royalisin,[6] jelleines[18] and apisin[19,20] have been found to exhibit pharmacological activities.

There is a growing scientific evidence to support the concept that the most attractive compounds of the honeybee products are the proteins of RJ. Proteins account for ∼50% of RJ dry weight and important protein components belong to a family named major royal jelly proteins (MRJPs) with molecular weights of 49−87 kDa.[21,22] However, the physical and chemical composition of RJ are dependent on the storage conditions and undergo various changes such as acceleration of the Maillard reaction and increase of viscosity, acidity, and protein degradation during storage at high temperature.[23] Consequently, the quality control of RJ has gained much more attention in recent years. Particularly, how storage conditions affects its quality. Quality changes of RJ have been reported in RJ stored at −20 °C, 4 °C, and at room temperature (RT) for up to 7 months. It was found that the viscosity of RJ increased remarkably when stored at RT, with the brown intensities increasing rapidly and water-soluble proteins hydrolyzing. This did not occur at −20 °C. When subjected to SDS-PAGE, four to five different protein components, with molecular weights ranging from 50 to 88 kDa, degraded gradually with increase in temperature and time, resulting in the appearance of lower molecular weights (20−40 kDa).[23] The protein components in RJ stored under different conditions have been compared by chromatography and PAGE and a 57 kDa glycoprotein was found and suggested as a freshness marker for RJ.[24] The degradation of the glycoprotein is proportional to storage temperature and time. Glycoprotein might be derived from a subunit of a 350 kDa RJ protein which stimulates the proliferation of human monocytes. Proteinase inhibitor EDTA (ethylenediaminetetraacetic acid) suppresses the degradation of 57 kDa protein during storage at high

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temperature.[25] To suppress such compositional changes and to maintain its freshness for an extended period, RJ should be stored at the lowest temperatures possible. However, no reliable markers and analysis methods for freshness of RJ have so far been established.

Because commercial RJ is most often stored for at least 6−12 months refrigerated at 4 °C, the present study using a proteomics approach investigated the proteome changes of RJ. Fresh RJ was compared to that stored at −20 °C, 4 °C, and RT to gain better understanding of the global protein changes occurring during storage.

2. Materials and Methods

2.1. Chemical Reagents. The immobilized pH gradient (IPG) strip (pH 3−10, Linear), the two-dimensional gel electrophoresis (2-DE) marker, Biolyte (pH 3−10), and mineral oil were purchased from Bio-Rad Laboratories Ltd. Tris-base, ammonium persulfate (AP), sodium dodecyl sulfate (SDS), *N,N,N′,N′*-tetramethylethylene diamine (TEMED) and glycin were from Sigma. Acrylamide, *N,N′*-methylenebisacrylamide, Bromophenol Blue, Coomassie Brilliant Blue (CBB) G-250, thiourea, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS), glycerol, and bovine serum albumin (BSA) were purchased from Amresco. Agarose and urea were from Solarbio. Dithiothreitol (DTT) and iodoacetamide were from Merck. Trypsin was from Roche; trifluoroacetic acid (TFA) and acetonitrile were from J. T. Baker.

2.2. Royal Jelly Samples. RJ was sampled from the apiary of the Bee Research Institute, Chinese Academy of Agricultural Science in June 2006 from queen cell cups where larvae had been grafted 72 h earlier. Five replicated RJ samples from each of 5 colonies were harvested into sterile bottles. These samples were combined to a unique fresh sample which was homogenized and subdivided into the different aliquots to be subjected to the various storage conditions of −20 °C, 4 °C, and RT for 12 months (June 2006−June 2007), respectively. The fresh RJ protein was extracted immediately after collection for further analysis.

2.3. Protein Preparation for 2-DE. Protein preparation was performed by Li et al.[26] RJ (1 mg RJ/10 μL buffer) was mixed in phosphate buffer (PB), pH 7.6, containing 32.5 mM K_2HPO_4, 2.6 mM KH_2PO_4, and 400 mM NaCl. The mixture was homogenized for 5 min on ice and sonicated for 2 min, then centrifuged at 12 000*g* and 4 °C for 10 min, and further centrifuged at 15 000*g* and 4 °C for 10 min. The supernatant was removed to a tube for use. The pellets (1 mg RJ/2 μL buffer) were mixed in the PB, pH 7.6, and then centrifuged at 15 000*g* and 4 °C for 10 min. The supernatant was removed and mixed into a tube containing supernatant as a PB-soluble protein extract, while the pellets (1 mg RJ/10 μL buffer), PB-insoluble proteins, were mixed in lysis buffer (LB, 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, and 2% Biolyte pH 3−10), then the mixture was sonicated for 2 min, and centrifuged at 15 000g and 4 °C for 10 min. The supernatant was removed and mixed into the tube containing PB-soluble proteins extraction, and the debris was discarded. Trichloroacetic acid (TCA) was added to the collected supernatants to a final concentration of 10%, and then the mixture was kept on ice for 10 min for protein precipitation and desalting. Subsequently, the mixture was centrifuged twice at 15 000*g* and 4 °C for 10 min. The supernatant was discarded and the pellets (1 mg RJ/4 μL buffer) were resolved in LB, then the mixture was homogenized for 5 min on ice and sonicated for 2 min, and subsequently adjusted to pH 7.0 with 2 M NaOH. The RJ protein extract mixture was stored at −70 °C for further use. The protein concentration was determined using the BCA Assay (Pierce Biotechnology, Rockford, IL).

2.4. 2-DE. A total of 200 μg of protein sample was suspended in LB and then mixed with rehydration buffer (containing 8 mM urea, 2% CHAPS, 0.001% Bromophenol Blue, 45 mM DTT, and 0.2% Biolyte, pH 3−10). The mixture was loaded on a 17 cm IPG strip (pH 3−10, linear, Bio-Rad, Hercules, CA). IEF was performed at 18 °C (PROTEAN IEF Cell, Bio-Rad, Hercules, CA) according to the following program: active rehydration for 14 h at 50 V; 250 V for 30 min × 4 times; 1000 V for 60 min; 9000 V for 5 h; 9000−60 000 V·h. Before SDS-PAGE, the IPG strips were first equilibrated for 15 min in equilibration buffer 1 (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, and 2% DTT) and then continued in equilibration buffer 2 (6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, and 2.5% iodoacetoamide) for 15 min. After the equilibration, the strip was transferred to SDS-PAGE gel, 12% T separating gel (1.00 mm). Meanwhile, 10 μL of 2-DE marker was loaded into a piece of filter paper and then it was transferred adjacently to the acid tip of the strip when the filter paper was nearly dry. The second-dimension electrophoresis, SDS-PAGE, was performed on PROTEAN xi Cell (Bio-Rad Hercules, CA) at 25 mA/gel for 6.5 h. The gels were stained with CBB G250 and scanned with transparent model, then analyzed with PDQuest V 7.3.0 (Bio-Rad, Hercules, CA) (sensitivity 6.86, scale 9). Each sample was replicated four times and the best five with good reproducibility were subjected to analysis.

2.5. Tryptic Digestion for MALDI-TOF. The CBB stained spots were excised and destained for 30 min using 100 mL of acetonitrile (50%) and 25 mM $(NH_4)HCO_3$, pH 8 (50%) for 3−4 times until the gel was transparent with no color and dried for 10 min with acetonitrile (100%). The gels were dried for 30 min using a Speed-Vac system. Then 2.5 mL of 25 mM $(NH_4)HCO_3$ was added to the 25 μg of trypsin (final concentration 10 ng/μL); 10 μL of this solution was pipetted on each dried protein spot and incubated for 60 min at 4 °C. The supernatant was discarded to minimize autodigestion of trypsin. Then the Eppendorf tube was turned upside down and the sample was incubated for 14 h at 37 °C. To extract the peptide fragments from the tryptic digests, 20 μL of 5% (v/v) TFA was added and incubated for 60 min at 37 °C. Thereafter, 20 μL of 50% (v/v) acetonitrile [containing 2.5% (v/v) TFA] acid was added to the gel and incubated for 60 min at 30 °C. After each step, the supernatants were pooled and dried using a Speed-Vac system.

2.6. MALDI-TOF and Protein Identification. Before obtaining the mass spectra of the peptide mixture, the digested peptides were desalted and cleaned with ZipTip C18 pipet tips (Millipore Corp., Bedford, MA) according to the manufacturer's instructions. All analyses were performed using a Bruker Daltonics Autoflex (Bruker Daltonics, Billerica, MA) operated in the delayed extraction of 190 ns and reflector mode with an accelerating voltage of 20 kV. The peptide mixture was analyzed using a saturated solution of α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics, Billerica, MA) in 50% acetonitrile/0.1% TFA. External calibration was performed with a peptide calibration standard (Bruker Daltonics Billerica, MA, part no.: 206195) and internal calibration with trypsin autoproteolytic fragments. Finally, the masses of proteolytic peptide fragments were obtained by peptide mass fingerprinting (PMF), a mass spectrometry based protein identification technique. To interpret the mass spectra of protein digests, the generated peaks

lists of the tryptic peptide masses were searched against MASCOT (http://www.matrixscience.com/search_form_select.html) and Xproteo (http://xproteo.com:2698). Search parameters for MASCOT were trypsin cleavage; allow up to one missed cleavage; no restriction on protein mass; peptide mass tolerance 100 ppm; quantitative modification: carbamidomethyl (C); nonquantitative modification: oxidation (M); and search parameters for Xproteo were trypsin cleavage; allow up to one missed cleavage; protein mass 0−300 kDa; protein p*I* 1−14; peptide mass tolerance 100 ppm. Proteins identified with a Mowse score greater than 67, 79, and d′ ≥ 4 (significant at 95% confidence interval) are reported with theoretical p*I* and M_r given by protein database.

2.7. Statistics. To accurately compare spot quantities between gels that often have some variation in spot size and intensity not due to differential protein abundance, normalization was done between the samples of the fresh, −20 °C, 4 °C, and RT. ANOVA (Version 6.12, SAS Institute, Cary, NC), a Duncan parametric test, was used to test the significance of the normalized volume in total density of identified proteins in all gels. An error probability of $P < 0.05$ was considered to be statistically significant.

2.8. Protein Preparation for Chromatography. The different RJ samples were dissolved in ultrapure water (1 g RJ/2 mL water) and homogenized at 4 °C for 1 h, respectively. Each sample was precipitated with ice-cold acetone (1:25 v/v) at −20 °C overnight. Then, it was centrifuged at 15 000*g* for 30 min at 4 °C, and each of the pellets was dissolved with buffer (Tris-HCl, 50 mM, pH 8.2) (1:2 m/v). The samples were centrifuged at 15 000*g* for 30 min at 4 °C, and the supernatant was recovered. Each of the collected supernatants was then precipitated with ice-cold acetone and dissolved with Tris-HCl buffer and the RJ protein solution was stored at −20 °C for chromatography analysis.

2.9. Gel Filtration Chromatography. With the use of AKTA purifier 10 system (GE. Healthcare), a 200 μL RJ protein solution was injected on a Superose 12 HR 10/30 column (GE. Healthcare) and eluted with 50 mM-Tris/HCl buffer, pH 8.2, containing 0.15 M NaCl, at flow rate of 0.5 mL/min. The chromatography spectra were acquired under identical conditions and compared with each other in order to find the difference of RJs stored at different conditions. The elution peaks were collected manually and used for nanoLC-MS/MS analysis.

2.10. In-Solution Digestion. A 20 μL collected solution was mixed with 1 μL of 20 mM DTT and incubated at 60 °C for 45 min. Subsequently, 2 μL of iodoacetamide was added to the mixture and incubated at room temperature for 30 min in the dark. Then, 1 μL of DTT was added and the mixture was incubated at 60 °C for 30 min. To ensure the efficient digestion, it is necessary to add 6 μL of acetonitrile. Finally, 10 μL of trypsin (0.02 μg/μL) was added to the mixture and incubated at 37 °C for 12 h. A 1 μL neat formic acid was applied to stop the digestion.

2.11. NanoLC Separation. The digested peptides were separated using an UltiMate nanoscale LC system (Dionex Corporation) with a nano-RP column (C18 PepMap, 75 μm i.d., 15 cm, Dionex Corporation). The flow rate was at 300 nL/min. After injection of 5 μL of digested sample, the column was washed with the equilibrating buffer (100% A, containing 0.1% formic acid) to remove unabsorbed proteins. The gradient elution conditions were as follows: 3% B (buffer B, containing 0.1% formic acid in acetonitrile/water, 95:5, v/v) linearly increased to 50% B in 40 min, then increased up to 95% B in 8 min, further maintained at 95% B in 10 min for washing the column, and then down to 3% B in 1 min and maintain equilibrium for 10 min.

2.12. NanoMS/MS Analysis. The nano-RP column was directly linked to a nano-LC electrospray device. The MS/MS analysis was performed on an ultrOTOF-Q ESI MS equipped with an orthogonal time-of-flight (TOF) mass analyzer (Bruker Daltonios). The instrument was set to perform an MS survey scan of 1 s with m/z range of 50−2000 and operated in positive ion mode. Nitrogen gas was used as the collision gas and the collision energy was 10−40 V. As peptides elute from the nano-RP column into the mass spectrometer, scans are acquired in a data-dependent manner. Any peak with a threshold of 1000 counts/s was automatically detected and the top two precursors from the each MS survey scan were selected by the quadrupole for MS/MS analysis. Dynamic exclusion was applied during the data-dependent acquisition. This prevented an abundant ion from being continually selected for MS/MS. Once selected, the peptide ion is not selected again in 2 min so that other less intense ions can be analyzed.[27] The instrument was calibrated prior to analysis using plasticizer.

2.13. Data Processing and Protein Identification from MS/MS. Peak list files were searched against a nonredundant protein database NCBInr using the MASCOT search engine (http://www.matrixscience.com). Carboxymethyl (C) and Oxidation (M) were selected as a variable modification and no fixed modification was selected. The other parameters used were Taxonomy, all entries; Enzyme, trypsin; Missed cleavages, 1; Peptide tolerance, ±50 ppm; MS/MS tolerance, ±0.1 Da.

3. Results

3.1. Analysis of 2-DE Images of RJ under Four Different Storage Conditions. Figure 1 is a representative gel image of the best 5 runs, showing the soluble proteins extracted from 4 RJ samples, separated by 2-DE on pH 3−10 IPG strips, and stained with CCB. A total of 85 reproducible protein spots from the fresh (Figure 1, Fresh), 81 from −20 °C (Figure 1, −20 °C), 73 from 4 °C (Figure 1, 4 °C), 70 from RT (Figure 1, room temperature), were detected, respectively, with molecular weight ranging from 14.4 to 74.16 kDa and pH 4.98−8.41. A significant higher number of proteins were observed in the fresh and RJ frozen at −20 °C than those at 4 °C and RT, while there were no significant difference between the fresh and −20 °C, as well as 4 °C and RT.

3.2. Comparison of Identified Protein in RJ Stored under Different Conditions. All proteins detected on four RJ samples were selected throughout the molecular mass and isoelectric point (p*I*) ranges of 3−10 gels and analyzed by MALDI-TOF MS. Of these, 61, 62, 56, and 51 spots (Figure 1; Tables 1–4) well-resolved to the images corresponding to the fresh, −20 °C, 4 °C, and RT, were successfully analyzed, respectively. The identified proteins, along with the gene index number and score, are listed in Tables 1–4.

Among the 61 identified proteins in the fresh RJ, 56 belong to the MRJPs family (3 MRJP1, 11 MRJP2, 30 MRJP3, 6 MRJP4, 6 MRJP5), 3 were glucose oxidase (GOD), 1 was peroxiredoxin (PRDX), and 1 was glutathione *S*-transferase (GST S1) (Figure 1, Fresh; Table 1).

One major MRJP1 (M_r 48.86 kDa, p*I* 5.1) was observed (Figure 1, Fresh, spot 1; Table 1), while two minor MRJP1, M_r 48.86 kDa, p*I* 5.10 (Figure 1, Fresh, spots 2 and 3; Table 1), were detected. Eleven different forms of MRJP2, with M_r 51.04 kDa and p*I* 6.83 (Table 1), were identified (Figure 1, Fresh, spots

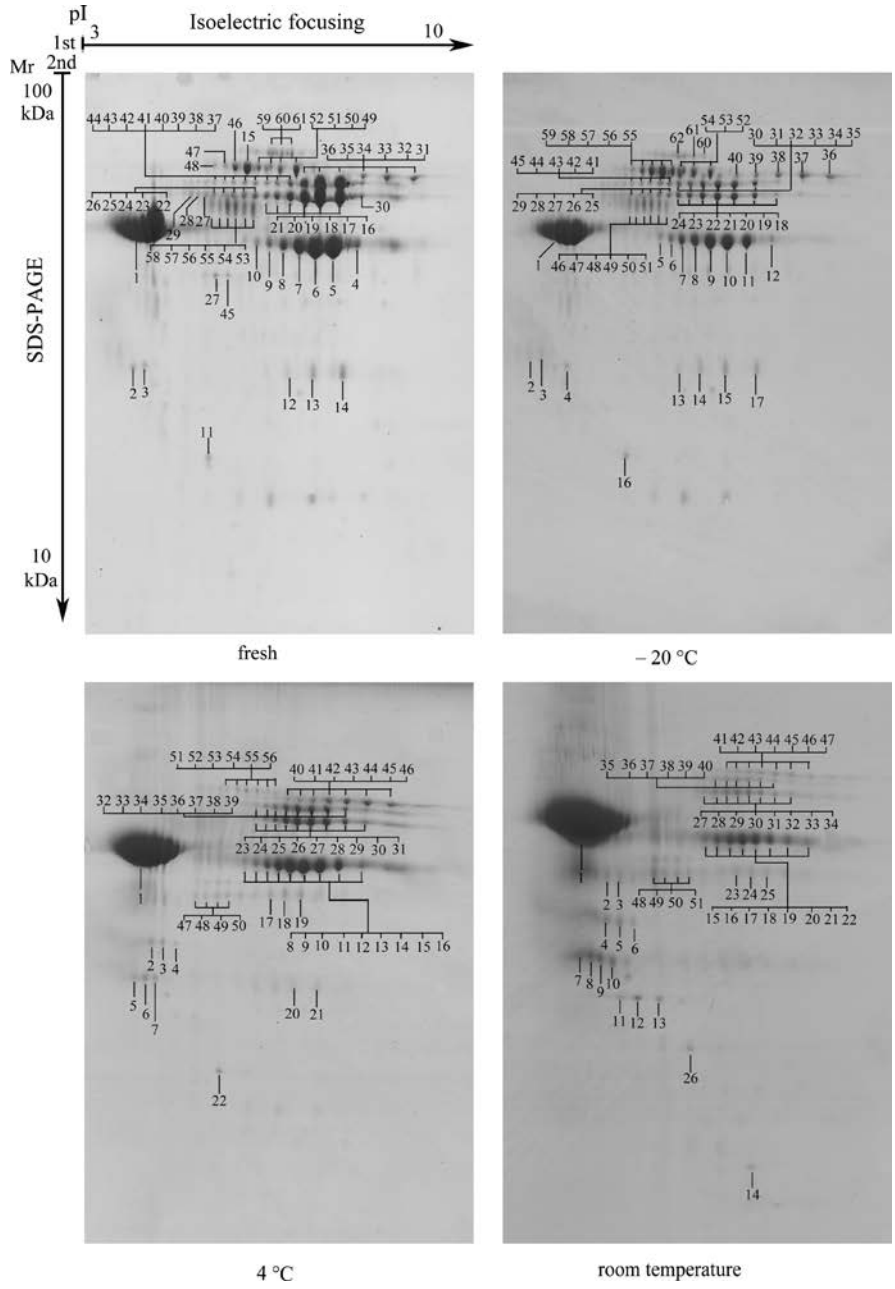

Figure 1. Protein spots subjected to tryptic digestion, MALDI-TOF MS and identification. Shown is a representative profile of royal jelly. A total of 200 μg of royal jelly sample was subjected to 2-DE and stained by CCB G-250. Fresh, −20 °C, 4 °C, and room temperature are images of royal jelly from the fresh to stored at −20 °C, 4 °C, and room temperature, respectively. Number-labeled spots were cut out and subjected to tryptic digestion for mass spectrometry analysis. Proteins identified with a Mowse score greater than 67, 79, and d′ ≥ 4 (significant at 95% confidence interval) are reported with theoretical p*I* and M_r given by MASCOT and Xproteo search engines accordingly.

4−14). Thirty different forms of MRJP3 were identified (Figure 1, Fresh, spots 16−45) with M_r values from 61.62 to 65.66 kDa and p*I* of 6.47−6.90 (Table 1). Six forms (M_r 52.88−52.92 kDa, p*I* 5.89−5.90) of MRJP4 (Figure 1, Fresh, spots 53−58; Table 1) and six different forms (M_r 70.19−70.24 kDa, p*I* 5.90−5.95) of MRJP5 (Figure 1, Fresh, spots 47−52; Table 1) were determined. Three GOD (M_r 67.90, p*I* 6.48, Figure 1, Fresh, spots 59−61; Table 1), 1 GST S1(M_r 17.69, p*I* 5.40, Figure 1, Fresh, spot 15), and 1 PRDX (M_r 25.13, p*I* 5.90, Figure 1, Fresh, spot 46) were observed, respectively.

Thus, a total of 62 protein spots in −20 °C RJ sample were identified, of which 57 belong to the MRJPs family (4 MRJP1, 12 MRJP2, 29 MRJP3, 6 MRJP4, 6 MRJP5), 3 were glucose oxidase, 1 was PRDX, and 1 was GST S1 (Figure 1, −20 °C; Table 2).

One major MRJP1 spot (M_r 48.86 kDa, p*I* 5.10) was observed (Figure 1, −20 °C, spot 1; Table 2), while 3 minor MRJP1 spots (M_r 48.86 kDa, p*I* 5.10) were detected (Figure 1, −20 °C, spots 2−4; Table 2). Twelve different forms of MRJP2, with a M_r range of 51.04−51.07 kDa and p*I* value of 6.83 (Figure 1, −20 °C, spots 5−16; Table 2) and 29 forms of MRJP3 (M_r 61.62−65.70 kDa, p*I* 6.47−6.90) were identified (Figure 1, −20 °C, spots 20−38; Table 2). Six forms (M_r 52.88−52.92 kDa, p*I* 5.89−5.90) of MRJP4 (Figure 1, −20 °C, spots 46−51; Table 2) and 6 MRJP5 were

Table 1. Proteins Identified in the Fresh Royal Jelly[a]

	PMF						
spot number	p*I*	MW (kDa)	sequence coverage	matched peptides (total signals)	score	protein name	accession number
1	5.10	48.86	21.0%	9/13	119	Major royal jelly protein 1 precursor[Apis mellifera]	MRJP1_APIME
2	5.10	48.86	18.0%	10/25	88	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
3	5.10	48.86	18.0%	7/17	76	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
4	6.83	51.04	23.0%	11/24	109	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
5	6.83	51.04	33.0%	15/69	98	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
6	6.83	51.04	24.0%	9/31	74	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
7	6.83	51.04	19.0%	8/18	78	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
8	6.83	51.04	32.0%	15/51	103	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
9	6.83	51.04	21.0%	9/46	68	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
10	6.83	51.04	28.0%	11/59	69	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
11	6.83	51.04	28.0%	12/36	99	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
12	6.83	51.04	23.0%	10/29	81	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
13	6.83	51.04	26.0%	10/35	75	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
14	6.83	51.04	23.0%	10/24	93	Major royal jelly protein 2 precursor) [Apis mellifera]	MRJP2_APIME
15	5.40	17.69	45.0%	8/20	122	Glutathione S transferase S1 CG8938-PA,isoform A, partial [Apis mellifera]	gi|66534655
16	6.47	61.62	19.0%	12/34	78	Major royal jelly protein 3 precursor[Apis mellifera]	MRJP3_APIME
17	6.47	61.62	20.0%	12/21	119	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
18	6.47	61.62	29.0%	15/49	115	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
19	6.47	61.62	42.0%	21/79	136	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
20	6.47	61.62	24.0%	13/33	103	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
21	6.47	61.62	16.0%	11/25	88	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
22	6.50	61.66	18.7%	14/30	$d' = 11.2$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
23	6.47	61.62	19.0%	10/24	76	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
24	6.47	61.62	11.0%	7/17	69	Major royal jelly protein 3 precursor[Apis mellifera]	MRJP3_APIME
25	6.47	61.62	16.0%	8/19	69	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
26	6.47	61.62	25.0%	15/44	110	Major royal jelly protein 3 precursor[Apis mellifera]	MRJP3_APIME
27	6.87	65.66	24.0%	12/30	108	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035
28	6.47	61.62	29.0%	15/38	122	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
29	6.47	61.62	12.0%	6/12	71	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
30	6.47	61.62	20.0%	13/47	76	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
31	6.50	61.66	13.9%	7/164	$d' = 5.2$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
32	6.47	61.62	18.0%	11/17	105	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
33	6.47	61.62	29.0%	14/29	129	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
34	6.50	61.66	18.9%	9/41	$d' = 4.3$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
35	6.47	61.62	21.0%	11/56	73	Major royal jelly protein 3 precursor[Apis mellifera]	MRJP3_APIME

Table 1. Continued

spot number	PMF p*I*	PMF MW (kDa)	PMF sequence coverage	PMF matched peptides (total signals)	PMF score	protein name	accession number
36	6.47	61.62	28.0%	16/35	137	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
37	6.47	61.62	16.0%	9/36	68	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
38	6.50	61.66	19.3%	10/30	$d' = 4.0$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
39	6.90	65.70	14.1%	9/36	$d' = 5.1$	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035|gb|AAV90959.1|
40	6.47	61.62	19.0%	12/22	101	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
41	6.47	61.62	17.0%	10/28	68	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
42	6.47	61.62	16.0%	9/13	92	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
43	6.47	61.62	19.0%	11/24	87	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
44	6.47	61.62	26.0%	14/35	96	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
45	6.47	61.62	13.0%	9/23	72	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
46	5.90	25.13	34.80%	8/16	$d' = 4.2$	Peroxiredoxin 2540 CG11765-PA [Apis mellifera]	gi|66535082|ref|XP_624361.1|
47	5.95	70.19	16.0%	10/17	76	Major royal jelly protein 5 precursor [Apis mellifera]	MRJP5_APIME
48	5.95	70.19	16.0%	9/17	73	Major royal jelly protein 5 precursor [Apis mellifera]	MRJP5_APIME
49	5.90	70.24	26.4%	16/36	$d' = 6.9$	Major royal jelly protein 5 [Apis mellifera]	gi|58585138|ref|NP_001011599.1|
50	5.95	70.19	20.0%	10/17	87	Major royal jelly protein 5 [Apis mellifera]	gi|58585138
51	5.90	70.24	24.4%	15/36	$d' = 6.1$	Major royal jelly protein 5 [Apis mellifera]	gi|58585138|ref|NP_001011599.1|
52	5.90	70.19	18.0%	11/17	84	Major royal jelly protein 5 [Apis mellifera]	gi|58585138
53	5.89	52.88	17.0%	9/34	68	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
54	5.89	52.88	20.0%	9/23	85	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
55	5.89	52.88	22.0%	10/28	92	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
56	5.89	52.88	22.0%	10/28	92	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
57	5.90	52.92	18.3%	8/38	$d' = 4.0$	Major royal jelly protein 4 [Apis mellifera]	gi|58585170|ref|NP_001011610.1|
58	5.90	52.92	15.5%	6/23	$d' = 4.5$	Major royal jelly protein 4 [Apis mellifera]	gi|58585170|ref|NP_001011610.1|
59	6.48	67.90	23.0%	13/37	83	Glucose oxidase [Apis mellifera]	gi|58585090
60	6.48	67.90	32.0%	13/34	120	Glucose oxidase [Apis mellifera]	gi|58585090
61	6.48	67.90	22.0%	10/22	84	Glucose oxidase [Apis mellifera]	gi|58585090

[a] Note: Protein scores greater than 67, 79, and $d' \geq 4$ are significant ($P < 0.05$) in Swiss-Prot, NCBInr (Mascot), and Xproteo database, respectively. Spot number corresponds to the number of a protein spot in Figure 1. MW (molecular weight) and p*I* (isoelectric point) represent the theoretical results identified in Genbank or MSDB or Swiss-Prot. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein. Accession number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e., GenBank).

identified with a M_r value of 70.19−70.24 kDa and p*I* 5.90−5.95 (Figure 1, −20 °C, spots 52−57; Table 2). Three GOD (M_r 67.90, p*I* 6.48, Figure 1, −20 °C, spots 60−62; Table 2), 1 PRDX (M_r 25.13, p*I* 5.90, Figure 1, −20 °C, spot 58, Table 2), and 1 GST S1 (M_r 17.69, p*I* 5.40 (Figure 1, −20 °C, spot 59; Table 2) were identified, respectively.

For 4 °C RJ sample, 56 proteins were identified. Among them, 54 were classed into MRJPs (7 MRJP1, 15 MRJP2, 28 MRJP3, 4 MRJP5). One PRDX and 1 GST S1 were determined (Figure 1, 4 °C; Table 3), respectively. A total of 51 proteins were identified in the RT RJ sample which were all grouped into MRJPs (14 MRJP1, 12 MRJP2, 25 MRJP3) (Figure 1, room temperature; Table 4). Their M_r and p*I* corresponded to those identified above.

As to abundance of MRJP1, 1 major spot (Figure 1, spot 1) resolving in all images showed a significant decrease and 2 minor spots (Figure 1, spots 2 and 3) showed a significant increase (Figure 2A, $P < 0.05$) following the increasing tem-

Table 2. Proteins Identified in Royal Jelly Stored at −20 °C for 1 year[a]

	PMF						
spot number	p*I*	MW (kDa)	sequence coverage	matched peptides (total signals)	score	protein name	accession number
1	5.10	48.86	21.0%	9/13	119	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
2	5.10	48.86	46.0%	19/37	190	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
3	5.10	48.86	18.0%	10/25	88	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
4	5.10	48.86	18.0%	7/17	76	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
5	6.80	51.07	18.5%	11/35	$d' = 5.3$	Major royal jelly protein 2[Apis mellifera]	gi|58585108|ref| NP_001011580.1|
6	6.83	51.04	28.0%	11/59	69	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
7	6.83	51.04	21.0%	9/46	68	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
8	6.83	51.04	32.0%	15/51	103	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
9	6.83	51.04	19.0%	8/18	78	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
10	6.83	51.04	24.0%	9/31	74	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
11	6.83	51.04	33.0%	15/69	98	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
12	6.83	51.04	23.0%	11/24	109	Major royal jelly protein 2 precursor[Apis mellifera]	MRJP2_APIME
13	6.83	51.04	23.0%	10/29	81	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
14	6.83	51.04	26.0%	10/35	75	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
15	6.83	51.04	23.0%	10/24	93	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
16	6.83	51.04	28.0%	12/36	99	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
17	6.47	61.62	13.0%	8/14	78	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
18	6.47	61.62	20.0%	13/47	76	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
19	6.47	61.62	19.0%	12/34	78	Major royal jelly protein 3 precursor (MRJP-3) [Apis mellifera]	MRJP3_APIME
20	6.47	61.62	20.0%	12/21	119	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
21	6.47	61.62	29.0%	15/49	115	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
22	6.47	61.62	42.0%	21/79	136	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
23	6.47	61.62	24.0%	13/33	103	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
24	6.47	61.62	16.0%	11/25	88	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
25	6.50	61.66	18.7%	14/30	$d' = 11.2$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
26	6.47	61.62	19.0%	10/24	76	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
27	6.47	61.62	11.0%	7/17	69	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
28	6.47	61.62	16.0%	8/19	69	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
29	6.47	61.62	25.0%	15/44	110	Major royal jelly protein 3 precursor - Apis mellifera (Honeybee)	MRJP3_APIME
30	6.50	61.66	18.9%	9/41	$d' = 4.3$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
31	6.47	61.62	21.0%	11/56	73	Major royal jelly protein 3 precursor Apis mellifera]	MRJP3_APIME
32	6.47	61.62	28.0%	16/35	137	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
33	6.47	61.62	16.0%	9/36	68	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
34	6.50	61.66	19.3%	10/30	$d' = 4.0$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|

Table 2. Continued

spot number	PMF p*I*	PMF MW (kDa)	PMF sequence coverage	PMF matched peptides (total signals)	PMF score	protein name	accession number
35	6.90	65.70	14.1%	9/36	$d' = 5.1$	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035|gb|AAV90959.1|
36	6.50	61.66	13.9%	7/164	$d' = 5.2$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
37	6.47	61.62	18.0%	11/17	105	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
38	6.47	61.62	29.0%	14/29	129	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
39	6.51	61.66	28.3%	17/30	$d' = 4.9$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
40	6.50	61.66	20.6%	11/36	$d' = 4.8$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
41	6.47	61.62	19.0%	12/22	101	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
42	6.47	61.62	17.0%	10/28	68	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
43	6.47	61.62	16.0%	9/13	92	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
44	6.47	61.62	19.0%	11/24	87	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
45	6.47	61.62	26.0%	14/35	96	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
46	5.89	52.88	17.0%	9/34	68	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
47	5.89	52.88	20.0%	9/23	85	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
48	5.89	52.88	22.0%	10/28	92	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
49	5.89	52.88	22.0%	10/28	92	Major royal jelly protein 4 precursor [Apis mellifera]	MRJP4_APIME
50	5.90	52.92	18.3%	8/38	$d' = 4.0$	Major royal jelly protein 4 [Apis mellifera]	gi|58585170|ref|NP_001011610.1|
51	5.90	52.92	15.5%	6/23	$d' = 4.5$	Major royal jelly protein 4 [Apis mellifera]	gi|58585170|ref|NP_001011610.1|
52	5.90	70.24	26.4%	16/36	$d' = 6.9$	Major royal jelly protein 5 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|
53	5.95	70.19	20.0%	10/17	87	Major royal jelly protein 5 [Apis mellifera]	gi|58585138
54	5.90	70.24	24.4%	15/36	$d' = 6.1$	Major royal jelly protein 5 [Apis mellifera]	gi|58585138|ref|NP_001011599.1|
55	5.90	70.19	18.0%	11/17	84	Major royal jelly protein 5 [Apis mellifera]	gi|58585138
56	5.95	70.19	16.0%	9/17	73	Major royal jelly protein 5 precursor [Apis melifera]	gi|66534655
57	5.95	70.19	16.0%	10/17	76	Major royal jelly protein 5 precursor [Apis melifera]	gi|66535082|ref|XP_624361.1|
58	5.90	25.13	34.80%	8/16	$d' = 4.2$	Peroxiredoxin 2540 CG11765-PA [Apis mellifera]	gi|66535082|ref|XP_624361.1|
59	5.40	17.69	45.0%	8/20	122	Glutathione S transferase S1 CG8938-PA,isoform A, partial [Apis mellifera]	gi|66534655
60	6.48	67.90	22.0%	10/22	84	Glucose oxidase [Apis mellifera]	gi|58585090
61	6.48	67.90	32.0%	13/34	120	Glucose oxidase [Apis mellifera]	gi|58585090
62	6.48	67.90	23.0%	13/37	83	Glucose oxidase [Apis mellifera]	gi|58585090

[a] Note: Protein scores greater than 67, 79, and $d' \geq 4$ are significant ($P < 0.05$) in Swiss-Prot, NCBInr (Mascot), and Xproteo database, respectively. Spot number corresponds to the number of a protein spot in Figure 1. MW (molecular weight) and p*I* (isoelectric point) represent the theoretical results identified in Genbank or MSDB or Swiss-Prot. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein. Accession number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e., GenBank).

perature. In addition, 1, 4, and 11 minor spots were specifically observed in the −20 °C, 4 °C, and RT samples, respectively.

Four of 8 MRJP2 presented on all images (Figure 1, spots 4−7) significantly decreased following the increasing temperature trend, 3 were significantly higher at 4 °C than those of 3 other samples, and 1 had no difference. The others showed an increase or decrease without following the temperature trend (Figure 2B).

MRJP3 was the largest group identified in this study. Sixteen of them occurring on 4 images showed a significant decrease

Table 3. Proteins Identified in Royal Jelly Stored at 4 °C for 1 year[a]

	PMF						
spot number	p*I*	MW (kDa)	sequence coverage	matched peptides (total signals)	score	protein name	accession number
1	5.10	48.86	21.0%	9/13	119	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
2	5.10	48.86	23.0%	9/22	94	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
3	5.10	48.86	20.0%	10/22	95	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
4	5.10	48.86	22.0%	11/26	102	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
5	5.10	48.86	18.0%	7/17	80	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
6	5.10	48.86	18.0%	10/25	88	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
7	5.10	48.86	18.0%	7/17	76	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
8	6.80	51.07	21.6%	10/44	$d' = 4.9$	Major royal jelly protein 2 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|
9	6.80	51.07	18.5%	11/35	$d' = 5.3$	Major royal jelly protein 2[Apis mellifera]	gi|58585108|ref| NP_001011580.1|
10	6.83	51.04	28.0%	11/59	69	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
11	6.83	51.04	21.0%	9/46	68	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
12	6.83	51.04	32.0%	15/51	103	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
13	6.83	51.04	19.0%	8/18	78	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
14	6.83	51.04	24.0%	9/31	74	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
15	6.83	51.04	33.0%	15/69	98	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
16	6.83	51.04	23.0%	11/24	109	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
17	6.80	51.07	40.0%	18/82	$d' = 7.4$	Major royal jelly protein 2 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|
18	6.80	51.07	33.1%	14/48	$d' = 4.2$	Major royal jelly protein 2 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|
19	6.83	51.04	25.0%	12/48	82	Major royal jelly protein 2 [Apis mellifera]	gi|58585108
20	6.83	51.04	26.0%	10/35	75	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
21	6.83	51.04	23.0%	10/24	93	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
22	6.83	51.04	28.0%	12/36	99	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
23	6.90	65.70	32.6%	19/44	$d' = 5.2$	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035|gb|AAV90959.1|
24	6.50	61.66	18.7%	14/30	$d' = 11.2$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
25	6.47	61.62	16.0%	11/25	88	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
26	6.47	61.62	24.0%	13/33	103	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
27	6.47	61.62	42.0%	21/79	136	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
28	6.47	61.62	29.0%	15/49	115	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
29	6.47	61.62	20.0%	12/21	119	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
30	6.47	61.62	19.0%	12/34	78	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
31	6.47	61.62	20.0%	13/47	76	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
32	6.47	61.62	19.0%	12/22	101	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
33	6.90	65.70	14.1%	9/36	$d' = 5.1$	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035|gb|AAV90959.1|
34	6.50	61.66	19.3%	10/30	$d' = 4.0$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
35	6.47	61.62	16.0%	9/36	68	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME

Table 3. Continued

spot number	PMF p*I*	PMF MW (kDa)	PMF sequence coverage	PMF matched peptides (total signals)	PMF score	protein name	accession number
36	6.47	61.62	28.0%	16/35	137	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
37	6.47	61.62	21.0%	11/56	73	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
38	6.50	61.66	18.9%	9/41	$d' = 4.3$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
39	6.47	61.62	15.5%	6/23	$d' = 4.5$	major royal jelly protein 3[Apis mellifera]	gi|58585142|ref|NP_001011601.1|
40	6.50	61.66	25.1%	14/38	$d' = 4.5$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
41	6.50	61.66	16.5%	9/13	$d' = 4.1$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
42	6.50	61.66	24.4%	15/26	$d' = 6.7$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
43	6.50	61.66	21.3%	12/30	$d' = 6.1$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
44	6.47	61.62	29.0%	14/29	129	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
45	6.47	61.62	18.0%	11/17	105	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
46	6.50	61.66	13.9%	7/164	$d' = 5.2$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
47	6.50	61.66	12.8%	7/21	$d' = 5.4$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref| NP_001011601.1|
48	6.87	65.66	24.0%	12/30	108	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035
49	6.47	61.62	13.0%	9/23	72	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
50	6.50	61.66	22.6%	13/41	$d' = 4.7$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
51	5.90	25.13	34.80%	8/16	$d' = 4.2$	Peroxiredoxin 2540 CG11765-PA [Apis mellifera]	gi|66535082|ref|XP_624361.1|
52	5.40	17.69	45.0%	8/20	122	Glutathione S transferase S1 CG8938-PA,isoform A, partial [Apis mellifera]	gi|66534655
53	5.95	70.19	20.0%	10/17	87	Major royal jelly protein 5 [Apis mellifera]	gi|58585138
54	5.90	70.24	24.4%	15/36	$d' = 6.1$	Major royal jelly protein 5 [Apis mellifera]	gi|58585138|ref|NP_001011599.1|
55	5.90	70.19	18.0%	11/17	84	Major royal jelly protein 5 [Apis mellifera]	gi|58585138
56	5.90	70.24	26.4%	16/36	$d' = 6.9$	Major royal jelly protein 5 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|

[a] Note: Protein scores greater than 67, 79, and $d' \geq 4$ are significant ($P < 0.05$) in Swiss-Prot, NCBInr (Mascot), and Xproteo database, respectively. Spot number corresponds to the number of a protein spot in Figure 1. MW (molecular weight) and p*I* (isoelectric point) represent the theoretical results identified in Genbank or MSDB or SwissProt. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein. Accession number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e., GenBank).

as temperature increased (Figure 2C). Three disappear at RT but resolved to 3 other samples which also showed a significantly decreased amount as temperature increased. Seven were detected in both the fresh and −20 °C samples. Four of these decreased significantly, while the other 3 did not. Six detected in both the 4 °C and RT samples were not found in the fresh and −20 °C samples. Four significantly decreased with 2 showing no difference. In the 4 images arranged according to the temperature trend, 2, 3, 1, and 1 MRJP3, respectively, were specifically detected (Figure 2C; Table 1–4).

Six MRJP4 and 3 GOD were detected only in the fresh and −20 °C samples but disappeared at 4 °C and RT (Figure 1). Abundance showed either significant decrease or no difference between the fresh and −20 °C samples (Figure 2D,F). Three of 4 MRJP5, observed in the samples of the fresh, −20 °C, and 4 °C but absent at RT sample, showed a significant decrease in amount. Only 2 identified in the 4 °C and RT samples significantly decreased (Figure 2E). PRDX and GST S1 showed a significant decrease in abundance following the temperature trend in the samples of the fresh, −20 °C, and 4 °C and were absent in the RT sample (Figure 2F).

3.3. Gel Filtration Chromatography and NanoLC-MS/MS Analysis. The gel filtration chromatography analysis showed that 6 main peaks occurred in the spectrum of protein samples of the fresh and −20 °C RJ (Figure 3A,B), while only five main peaks were detected in the 4 °C and RT RJ sample (Figure 3C, D). The peak 5 could clearly be observed in the fresh and −20 °C RJ sample, but it could not be identified by nanoLC-MS/MS analysis and protein identification. However, some MRJPs were identified in peaks 2 and 3 (Table 5). It was interesting that MRJP5 was only identified in peak 3 of the fresh and −20 °C RJ.

Table 4. Proteins Identified in Royal Jelly Stored at Room Temperature for 1 year[a]

			PMF				
spot number	p*I*	MW (kDa)	sequence coverage	matched peptides (total signals)	score	protein name	accession number
1	5.10	48.86	21.0%	9/13	119	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
2	5.10	48.86	35.0%	16/46	125	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
3	5.10	48.86	31.0%	14/26	165	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
4	5.10	48.86	23.0%	9/22	94	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
5	5.10	48.86	20.0%	10/22	95	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
6	5.10	48.86	22.0%	11/26	102	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
7	5.10	48.86	18.0%	10/25	88	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
8	5.10	48.86	18.0%	7/17	76	Major royal jelly protein 1 precursor [Apis mellifera]	MRJP1_APIME
9	5.10	48.86	21.0%	11/31	79	Major royal jelly protein 1 precursor [Apis melifera]	MRJP1_APIME
10	5.10	48.86	21.0%	10/31	78	Major royal jelly protein 1 precursor[Apis melifera]	MRJP1_APIME
11	5.10	48.86	21.0%	10/21	96	Major royal jelly protein 1 [Apis mellifera]	gi|58585098
12	5.10	48.86	16.0%	6/16	68	Major royal jelly protein 1 precursor[Apis melifera]	MRJP1_APIME
13	5.10	48.86	18.0%	9/35	67	Major royal jelly protein 1 precursor [Apis melifera]	MRJP1_APIME
14	5.10	48.89	23.1%	14/38	$d' = 5.2$	Major royal jelly protein 1 [Apis mellifera]	gi|58585098|ref|NP_001011579.1|
15	6.80	51.07	18.5%	11/35	$d' = 5.3$	Major royal jelly protein 2[Apis mellifera]	gi|58585108
16	6.83	51.04	28.0%	11/59	69	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
17	6.83	51.04	21.0%	9/46	68	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
18	6.83	51.04	32.0%	15/51	103	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
19	6.83	51.04	19.0%	8/18	78	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
20	6.83	51.04	24.0%	9/31	74	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
21	6.83	51.04	33.0%	15/69	98	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
22	6.83	51.04	23.0%	11/24	109	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
23	6.80	51.07	40.0%	18(82)	$d' = 7.4$	Major royal jelly protein 2 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|
24	6.80	51.07	33.1%	14/48	$d' = 4.2$	Major royal jelly protein 2 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|
25	6.83	51.04	25.0%	12/48	82	Major royal jelly protein 2 [Apis mellifera]	gi|58585108|ref| NP_001011580.1|
26	6.83	51.04	28.0%	12/36	99	Major royal jelly protein 2 precursor [Apis mellifera]	MRJP2_APIME
27	6.90	65.70	32.6%	19/44	$d' = 5.2$	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035|gb|AAV90959.1|
28	6.50	61.66	18.7%	14/30	$d' = 11.2$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
29	6.47	61.62	16.0%	11/25	88	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
30	6.47	61.62	24.0%	13/33	103	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
31	6.47	61.62	42.0%	21/79	136	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
32	6.47	61.62	29.0%	15/49	115	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME

Table 4. Continued

spot number	PMF p*I*	PMF MW (kDa)	PMF sequence coverage	PMF matched peptides (total signals)	PMF score	protein name	accession number
33	6.47	61.62	20.0%	12/21	119	Major royal jelly protein 3 [Apis mellifera]	gi|58585142
34	6.47	61.62	19.0%	12/34	78	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
35	6.47	61.62	19.0%	12/22	101	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
36	6.90	65.70	14.1%	9/36	$d' = 5.1$	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035|gb|AAV90959.1|
37	6.50	61.66	19.3%	10/30	$d' = 4.0$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
38	6.47	61.62	16.0%	9/36	68	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
39	6.47	61.62	28.0%	16/35	137	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
40	6.47	61.62	21.0%	11/56	73	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
41	6.50	61.66	13.9%	7/164	$d' = 5.2$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
42	6.50	61.66	25.1%	14/38	$d' = 4.5$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
43	6.50	61.66	16.5%	9/13	$d' = 4.1$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
44	6.50	61.66	24.4%	15/26	$d' = 6.7$	Major royal jelly protein 3 [Apis melifera]	gi|58585142|ref|NP_001011601.1|
45	6.50	61.66	21.3%	12/30	$d' = 6.1$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|
46	6.47	61.62	29.0%	14/29	129	Major royal jelly protein 3 precursor [Apis melifera]	MRJP3_APIME
47	6.47	61.62	18.0%	11/17	105	Major royal jelly protein 3 precursor [Apis mellifera]	MRJP3_APIME
48	6.50	61.66	12.8%	7/21	$d' = 5.4$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref| NP_001011601.1|
49	6.87	65.66	24.0%	12/30	108	Major royal jelly protein 3 [Apis mellifera carnica]	gi|56422035
50	6.47	61.62	13.0%	9/23	72	Major royal jelly protein 3 precursor[Apis melifera]	MRJP3_APIME
51	6.50	61.66	22.6%	13/41	$d' = 4.7$	Major royal jelly protein 3 [Apis mellifera]	gi|58585142|ref|NP_001011601.1|

[a] Note: Protein scores greater than 67, 79, and $d' \geq 4$ are significant ($P < 0.05$) in Swiss-Prot, NCBInr(Mascot), and Xproteo database, respectively. Spot number corresponds to the number of a protein spot in Figure 1. MW (molecular weight) and p*I* (isoelectric point) represent the theoretical results identified in Genbank or MSDB or SwissProt. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein. Accession number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e., GenBank).

4. Discussion

We investigated the major proteins change of RJ from fresh to various storage conditions to look for potential freshness markers of RJ and find the best way to store RJ. All RJ used in our experiment was from the apiary of our laboratory. This guaranteed the sample accuracy and RJ quality. The molecular weight of MRJPs in this experiment was within the range of 48.86−70.24 kDa (Tables 1–4), which was almost consistent to the 49−87 kDa reported previously.[28]

That MRJP1 may present variant forms with different MW and p*I* is probably due to the presence of potential glycosylation sites as revealed by the NCBI protein databank (http://www.ncbi.nlm.nih.gov/).[29] The MW of MRJP1 identified in this study (Figure 1; Tables 1–4) differs from that estimated by Hanes and Simuth[21] but was in accordance with Santos et al.[29] MRJP1is likely to promote liver regeneration and may have a cytoprotective action on hepatocytes.[30] Interestingly, the abundance of one major MRJP1 significantly decreased following the temperature trend (Figure 2A). Some minor spots which are thought to be degradation products from MRJP1[24] can be clearly detected in 4 °C and RT samples (Figure 1). This indicates that MRJP1 is sensitive to both storage temperature and time.

MRJP 2 and MRJP3 seem to function as a store of a processable form of nitrogen and MRJP3 can also exhibit potent immunoregulatory effects.[31] The MW and p*I* of MRJP2 identified in this study were in accordance with that of Santos et al.[29] NCBI databank reveals two hypothetical glycosylation sites for this protein that could explain the observed heterogeneity.[29] Because of the extensive repetitive regions in the C-terminal region and the various sugar chains attached to the protein,[28] the MW (61.62−65.70 kDa) and p*I* (6.47- 6.90) of MRJP3 in this study (Figure 1; Tables 14) were lower than those identified by Santos et al.[29] MRJP3 has the most isoforms in RJ protein

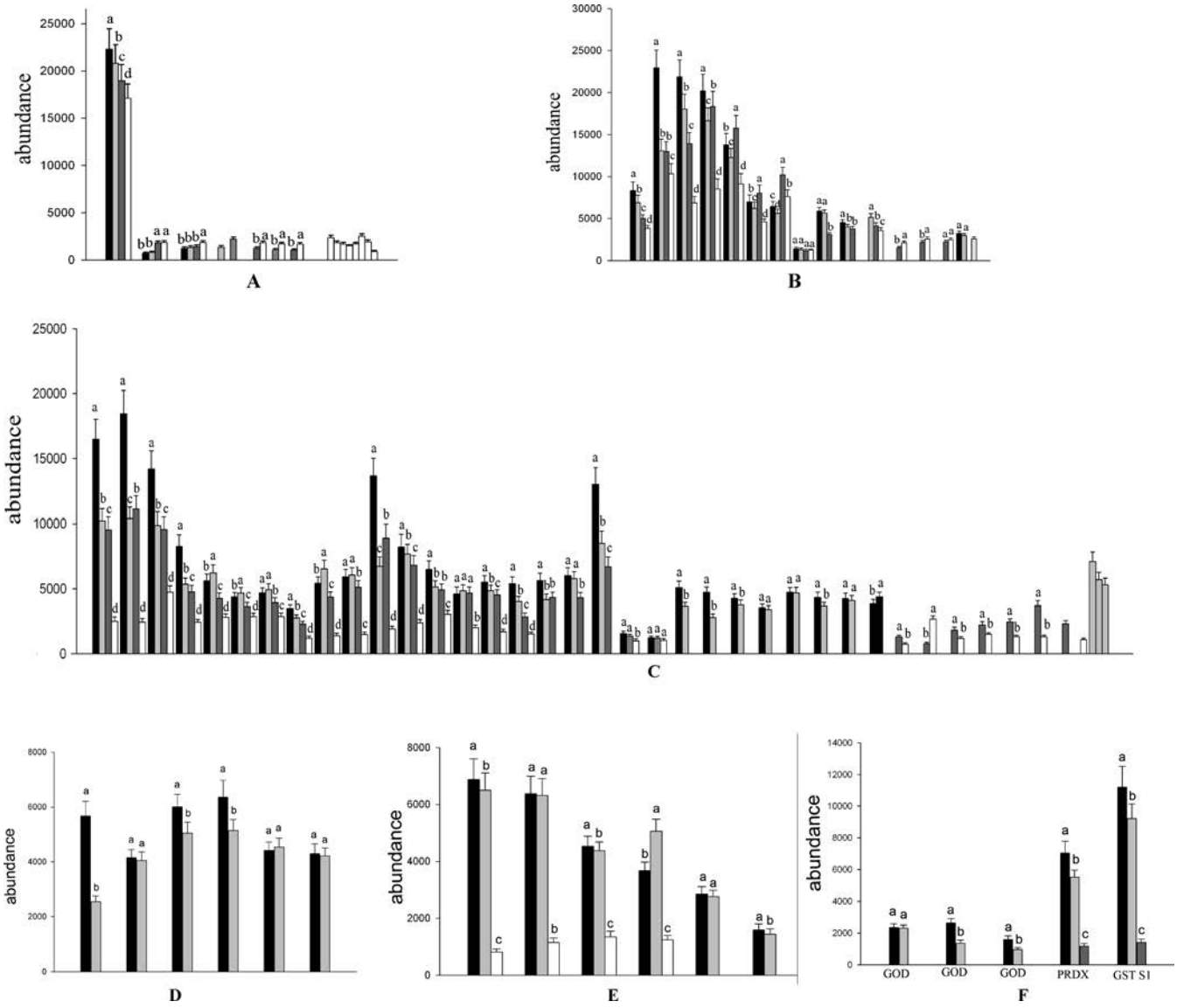

Figure 2. Abundance analysis of identified proteins from the fresh to stored at −20 °C, 4 °C, and room temperature. The black, the light gray, the dark gray, and the white bars represent identified proteins from the fresh to stored at −20 °C, 4 °C, and room temperature, respectively. A−F show proteins of major royal jelly protein (MRJP) 1, MRJP2, MRJP3, MRJP4, MRJP5, and Glucose oxidase (GOD), Peroxiredoxin (PRDX), glutathione *S*-transferase (GST) S1, respectively.

constituents as shown in the present report. This could be attributable to its polymorphism in a region with a variable number of tandem repeats (VNTR) located at the C-terminal part of the coding region.[31] The abundance of MRJP2 and MRJP3 detected in all images did not always follow a regular trend. Some of them showed a significant regular decrease, while others were significantly higher at higher temperature (Figure 2B,C), possibly because the proteins polymerized during storage.[23] This indicates that these two protein families are also sensitive to storage conditions.

On the contrary, the spots of MRJP4 and GOD were clearly detected in the fresh and −20 °C RJ stored for 1 year (Figure 1; Tables 1 and 2), but not in all under the other two conditions. This indicates that MRJP4 and GOD are sensitive to storage temperature. In terms of its sensitivity to temperature and molecular weight range of 52.88−52.91 kDa (Tables 1 and 2), MRJP4 is very similar to a 57 kDa glycoprotein suggested as a freshness marker for RJ.[24,25] But MRJP4 was not detected by 2-DE until 2004.[28] In the 2D gel of Scarselli et al.,[32] some spots not identified seem to match those of the MRJP4 found in this work. Possibly their volume was not sufficient for identification since the IPG strip they used (11 cm) was shorter than the one we used (17 cm) even though their RJ was fresh. Spots of MRJP5, PRDX, and GST S1 were detected in the fresh RJ, −20 °C, and 4 °C stored for 1 year (Figure 1; Tables 1–3), but not in the RT group. Furthermore, the quantity of MRJP5, PRDX, and GST S1 at 4 °C was significantly lower than those of the fresh and that at −20 °C. This indicates that they are also sensitive to storage temperature. So, we believe that MRJP4 and MRJP5, both of which supply nutritive components as essential amino acids,[31,33] and GOD, PRDX, and GST S1 can be used as a reference in the assessment of RJ freshness.

The results indicate that all of MRJPs and PRDX and GST S1 are sensitive to storage temperature, whereas MRJP4, MRJP5, GOD, PRDX, and GST S1 are the most sensitive. MRJP1−3, however, are not suitable as markers for the freshness of RJ because they can be detected under every condition even with changes in quantity. In contrast, MRJP4, MRJP5, GOD, PRDX,

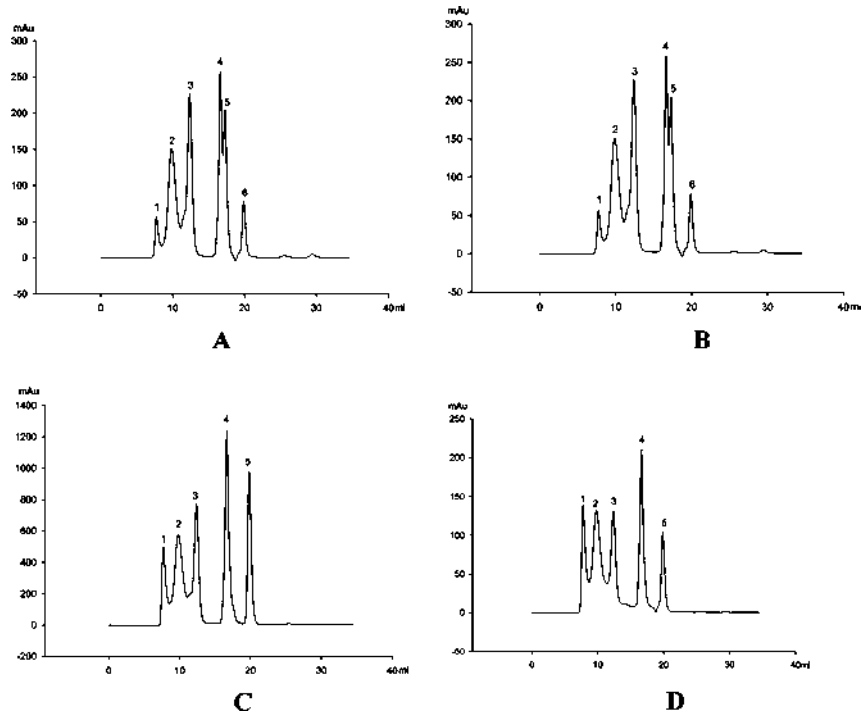

Figure 3. Gel filtration chromatography pattern of royal jelly protein samples. A−D show separation from the fresh to stored at −20 °C, 4 °C, and room temperature, respectively.

Table 5. Proteins Identified by NanoLC-MS/MS[a]

storage condition	peak no.	accession number	protein name	total ion score	M_r	p*I*	no. of peptides matched	sequence coverage
−20 °C	2	gil58585098	major royal jelly protein 1	563	48855	5.1	18	36%
		gil56422035	major royal jelly protein 3	255	65656	6.87	6	10%
		gil58585108	major royal jelly protein 2	77	51041	6.83	3	6%
−20 °C	3	gil58585098	major royal jelly protein 1	681	48855	5.1	19	55%
		gil58585108	major royal jelly protein 2	520	51041	6.83	14	36%
		gil62198227	major royal jelly protein 7	306	50509	4.9	8	30%
		gil56422035	major royal jelly protein 3	295	65656	6.87	10	25%
		gil58585138	major royal jelly protein 5	169	70189	5.95	3	4%
4 °C	2	gil58585098	major royal jelly protein 1	906	48855	5.1	25	51%
		gil56422035	major royal jelly protein 3	52	65656	6.87	2	4%
4 °C	3	gil58585098	major royal jelly protein 1	515	48855	5.1	14	35%
		gil58585108	major royal jelly protein 2	494	51041	6.83	13	30%
		gil62198227	major royal jelly protein 7	251	50509	4.9	7	19%
		gil56422035	major royal jelly protein 3	175	65656	6.87	6	14%

[a] Note: Protein scores greater than 37 are significant ($P< 0.05$) in NCBInr (Mascot). Peak number corresponds to the number in Figure 3. MW (molecular weight) and p*I* (isoelectric point) represent are the theoretical results identified in Genbank. Sequence coverage is the ratio of the number of amino acids in every peptide that matches with the mass spectrum divided by the total number of amino acids in the protein sequence. Matched peptide is the number of paring an experimental fragmentation spectrum to a theoretical segment of protein. Accession number is the unique number given to mark the entry of a protein sequence to a primary or secondary database (i.e., GenBank).

and GST S1 are clearly absent at RT. Therefore, MRJP4, MRJP5, GOD, PRDX, and GST S1 could be used as protein markers in the assessment of RJ freshness. We conclude that RJ frozen at −20 °C represents the best way to maintain quality. On the bases of the results from gel filtration chromatography (Figure 3), we could definitely detect peak 5 in the fresh and −20 °C samples. This should be the freshness marker for RJ. Unfortunately, it cannot be easily identified by the present method. Also, the peaks 1, 2, and 6 could not be effectively identified. The potential 2D markers, MRJP4, GOD, PRDX, and GST S1, may be contained in these peaks. During the determination of the peak 2 and peak 3, however, MRJP5 was identified only in the fresh and −20 °C (Table 5). Considering the above results, we believe that MRJP5 is a reliable marker for assessing the freshness of RJ.

To date, many physiologically active substances are present in RJ, such as 10−2-HDA, which shows antitumor activities[16] and antibacterial activity.[6] 10-2-HDA has mainly been used as a standard to control the quality of RJ. However, 10-2-HDA does not undergo any change during storage of RJ at 40 °C for 7 days indicating it is stable at high temperature and is unsuitable as a marker for freshness. In addition, changes in amino acids, vitamins, and sugars in RJ during storage seem to be unrelated to the freshness of RJ.[24] However, Marconi et al.[34] reported that furosine, which is used to assess the development of the Maillard reaction, is a suitable marker for assessing the freshness of RJ since its content increased significantly after 10 months of storage at RT while it increased to a much lower level when RJ was stored at 4 °C. Whether our protein markers or furosine markers are better and more practical to assess the

freshness of RJ still needs to be validated. However, our 2D markers are more useful than the 57 kDa glycoprotein freshness marker for RJ suggested by Kamakura et al.,[24] since these makers were identified to a specific member of MRJP family. This provides a basic theory to develop a test paper based on antigen antibody binding that could be more convenient than quantitate markers. This study employing complementary strategies of 2D and gel filtration has identified freshness markers of RJ by a different approach, and the results could validate each other. So, more specific markers could be screened for RJ by our present protocol.

Abbreviations: MRJP, major royal jelly protein; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time-of-flight mass spectrometry; GOD, glucose oxidase; PRDX, peroxiredoxin; GST, glutathione *S*-transferase.

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