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# Proteomic Analysis of Royal Jelly from Three Strains of Western Honeybees (*Apis mellifera*)

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To compare the protein complement of royal jelly (RJ) from high RJ producing honeybees (*Apis mellifera* L.), a strain of *A. mellifera* artificially selected for increased RJ production from Italian honeybees in China for more than two decades was compared to those of native Italian honeybees (*A. mellifera* L.) and Carnica honeybees (*A. mellifera* C.); the protein in RJ from these three strains of honeybees was partially identified by using a combination of two-dimensional polyacrylamide gel electrophoresis (2D-PAGE), matrix-assisted laser desorption ionization-time of flight mass spectrometry (MALDI-TOF/MS), and a protein engine identification tool applied to the honeybee genome. The results showed that 152, 157, and 137 proteins were detected in the three species of RJ; among which 57, 57, 51 high abundant proteins ere identified, respectively. Most identifited spots, 45, 45, 41, were assigned to major royal jelly proteins (MRJPs). Remarkable differences were found in the heterogeneity of the MRJPs, in particular, MRJP3. Also, 3-glucose oxidase, 1-peroxiredoxin (PRDX), and 1-glutathione *S*-transferase (GST) S1 were identified in three RJ samples. Furthermore, during the determination of the peptides mass fingerprinting (PMF) of each spot, for the first time, PRDX and GST S1 proteins have been identified in RJ. Thus, the results suggest that the protein complement of high RJ producing honeybees is not different compared to native Italian honeybees, while a difference remains between Carnica honeybees.

**KEYWORDS: Royal jelly (RJ); major royal jelly protein (MRJP); honeybee (*Apis mellifera*); proteome**

## INTRODUCTION

Royal jelly (RJ), which is secreted by the hypopharyngeal and mandibular glands of worker honeybees mainly between the 6th and the 12th day of their image life, is fed to worker larvae within three days and to queens and plays a key role in honeybee caste determination (*1–5*). RJ is a white-yellow colloid with a pH between 3.6–4.2, and it is a compound with several constituents, containing water, proteins, lipids, carbohydrates, free amino acids, vitamins, and minerals (*6–9*). Proteins account for ~50% of RJ dry weight, and important protein components belong to a family named major royal jelly proteins (MRJPs), now named apalbumins, with a molecular weight of 49–87 kDa (*10, 11*). Apalbumin 1 is likely to promote liver regeneration and may have a cytoprotective action on hepatocytes (*12*). Apalbumins 2 and 3 seem to function as a store of a processable form of nitrogen, and apalbumin 3 can exhibit potent immunoregulatory properties (*13*). Both apalbumin 4 and apalbumin 5 supply nutritive components as essential amino acids (*13, 14*).

RJ has nutritional, health, and pharmacological functions, such as hypotensive activities, antitumor activities, anti-inflammatory activities, and anti-diabetes activites with its insulin-like peptide (*15–17*). So far, the biological functions of some component proteins in RJ have been reported (*7*). Royalisin is an antimicrobial peptide against Gram-positive bacteria and fungi (*18, 19*); jelleines are an antimicrobial peptide family against Gram-positive, Gram-negative bacteria, and yeasts (*20*); and apisin is a 350 kDa glycoprotein that can stimulate proliferation of human monocytes (*21, 22*). RJ proteins are detected by two-dimensional gel electrophoresis (2-DE), mass spectrometry, and de novo sequencing, and all the identified proteins belonged to the *Apis mellifera* genome (*6*). RJ proteins in both Africanized and European honeybees (*A. mellifera*) are characterized using 2-DE and N-terminal amino acid sequencing, and remarkable differences are found in the heterogeneity of the MRJPs, in particular, MRJP3, in terms of molecular weights and isoelectric points between the two species of RJ; at the same time, the existence of MRJP4 is identified for the first time in 2-DE images (*23*).

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Since the Italian honeybee was introduced into China as early as the 1930s, China's honeybee scientists have paid close attention to selecting bees for increased RJ production. With nearly half a century's effort, China has now selected the highest production RJ honeybees (*A. mellifera* L.) from the Italian honeybees (*A. mellifera* L.) in the world. To date, this strain of honeybee is the most important RJ producer around the globe, which can produce 6 kg of RJ a colony a year, thus making China the biggest RJ producing country with an annual production of more than 2000 tons, accounting for more than 90 percent of the world's total output (*24, 25*). Since then, a wide spectrum of studies have been done on this honeybee. Phenotypic analysis has proved that RJ production of high RJ producing honeybees significantly exceeds that of native Italian honeybees (*26*). Further research shows that RJ production is a quantitative trait dominated by genetic components (*27*). DNA microsatellite analysis indicates that seven alleles are likely molecular markers of the high RJ producing honeybees (*28*).

The recent availability of the honeybee genome (*29*) encourages a proteomic approach to detect whether the artificially selected high RJ producing honeybees (*A. mellifera* L.) have protein changes in RJ compared to its counterpart native Italian honeybees (*A. mellifera* L.) and Carnica honeybees (*A. mellifera* C.). So, this work could be relevant to the food industry and the RJ industry.

## MATERIALS AND METHODS

**Chemicals.** Immobilized pH gradient (IPG) strips (pH 3–10, linear), two-dimensional gel electrophoresis (2-DE) marker, Bio-lyte (pH 3–10), 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, and trifluoroacetic acid (TFA) and acetonitrile were from JT Baker.

**RJ Samples.** A total of 100 g of RJ from five colonies of high RJ producing honeybees (*A. mellifera* L.), native Italian honeybees (*A. mellifera* L.), and Carnica honeybees (*A. mellifera* C.) were collected from Apiary of the Institute of Apicultural Research, Chinese Academy of Agricultural Science, Beijing, China. The RJ was harvested into sterile bottles when the larvae were grafted into the queen cell cups for 72 h, and a unique sample was homogenized.

**Preparation of Protein Samples.** The approach of sample preparation was slightly modified according to Zhong et al (*30*). The RJ (1 mg of RJ/10 $\mu$L of buffer) was mixed in phosphate buffer (PB) pH 7.6, containing 32.5 mM $K_2HPO_4$, 2.6 mM $KH_2PO_4$, 400 mM NaCl. The mixture was homogenized for 5 min in ice and sonicated for 2 min, then centrifuged at 12000*g* and 4 °C for 10 min, and further centrifuged at 15000*g* and 4 °C for 10 min. The supernatant was removed to another tube for use. The precipitate (1 mg of RJ/2$\mu$L of buffer) was mixed in the PB pH 7.6, and then centrifuged at 15000*g* and 4 °C for 10 min. The supernatant was removed and mixed into the tube containing supernatant above as a PB-soluble protein extract, while the precipitate (1 mg of RJ/10 $\mu$L of buffer), PB-insoluble proteins, were mixed in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT, 2% Bio-lyte pH 3–10), and then the mixture was sonicated for 2 min and centrifuged at 15000*g* and 4 °C for 10 min. The supernatant was removed and mixed into the above-mentioned tube containing PB-soluble proteins extraction, and the debris was discarded. Trichloroacetic (TCA) was added to the collected supernatants to a final concentration of 10%, and then the mixture was kept in ice for 10 min to precipitate proteins and desalting. Subsequently, the mixture was twice centrifuged at 15000*g* and 4 °C for 10 min. The supernatant was discarded, and the precipitate (1 mg of RJ/4 $\mu$L of buffer) was dissolved in foregoing lysis buffer; then the mixture was homogenized for 5 min in ice and sonicated for 2 min and subsequently adjusted to pH 7.0 with 2 M NaOH. The mixture, the protein extraction of the RJ, was stored at −70 °C for further use. The protein concentration was determined according to the method of Bradford (1976) using bovine serum albumin as reference.

**2-DE.** A 150 $\mu$g protein sample was suspended in LB and then mixed with rehydration buffer (containing 8 M urea, 2% CHAPS, 0.001% Bromophenol Blue, 45 mmol/L DTT, 0.2% Bio-lyte, pH 3–10). The mixture was loaded on a 17-cm IPG strip (pH 3–10, linear, Bio-Rad Hercules, CA, USA). IEF was performed at 18 °C (PROTEAN IEF Cell, Bio-Rad Hercules, CA, USA) 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 V for 60000 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, 2% DTT) and then continued in equilibration buffer 2 [6 M urea, 0.375 M Tris-HCl (pH 8.8), 20% glycerol, 2% SDS, 2.5% iodoacetoamide] for 15 min. After the equilibration, the strip was transferred to SDS–PAGE gel, 12% T separating gel (1.00 mm, 3.0% C). Meanwhile, 15 $\mu$L 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, USA) at 25 mA/gel for 6.5 h. The gel was stained with CBB G250 and scanned with transparent model, at 32-bit red-green-blue colors and dpi resolution for documentation. The image was analyzed with PDQuest V 7.3.0 (Bio-Rad Hercules, CA, USA) (sensitivity 6.86, scale 9). Each sample was replicated five times, and the best three with good reproducibility were subjected to analysis. ANOVA (version 6.12, SAS Institute, Cary, NC, USA) was used to compare the mean normalized volume of selected spots in four treatments. In all statistical analysis a probability of $P < 0.05$ was considered to be statistically significant.

**Tryptic Digestion.** The CBB stained spots were excised from the 2-DE gels and destained for 30 min × 3–4 times until the gel was transparent with no color, using a decoloring solution consisting of 50% acetonitrile and 25 mM $(NH_4)HCO_3$, and then they were immersed in acetonitrile (100%) for 10 min. The gels were dried for 30 min using a Speed-Vac system. 2.5 mL of 25 mM $(NH_4)HCO_3$ was added to 25 $\mu$g of trypsin (final concentration 10 ng/$\mu$L); 10 $\mu$L of this trypsin solution was pipetted on each dried protein spot and the sample was incubated at 4 °C for 60 min. The supernatant was discarded to minimize autodigestion of trypsin. Then the Ep tubes were placed upside down and incubated at 37 °C for 14 h. To extract the peptide fragments from the tryptic digest, 20 $\mu$L of 5% (v/v) TFA was added to the digest and the sample was incubated at 37 °C for 60 min; then the supernatant was transferred into another Ep tube. Thereafter, 20 $\mu$L of 50% (v/v) acetonitrile [containing 2.5% (v/v) TFA] was added to the gel and the sample was incubated at 30 °C for 60 min. The supernatants were pooled together and dried for 2 h heating in a Speed-Vac system.

**MALDI-TOF/MS and Database Search.** Before obtaining the mass spectra of the peptide mixture, the digested peptides were desalted and cleaned with ZipTip C18 pipette tips (Millipore Corporation, Bedford, MA, USA) according to the manufacturer's instructions. All analyses were performed using a Bruker Daltonics Autoflex (Bruker Daltonics Billerica, MA, USA) 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, USA) in 50% acetonitrile/0.1% trifluoroacetic acid. External calibration was performed with a peptide calibration standard (Bruker Daltonics Billerica, MA, USA, Part No.: 206195) and internal calibration with trypsin autoproteolytic fragments. To interpret the MS 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).

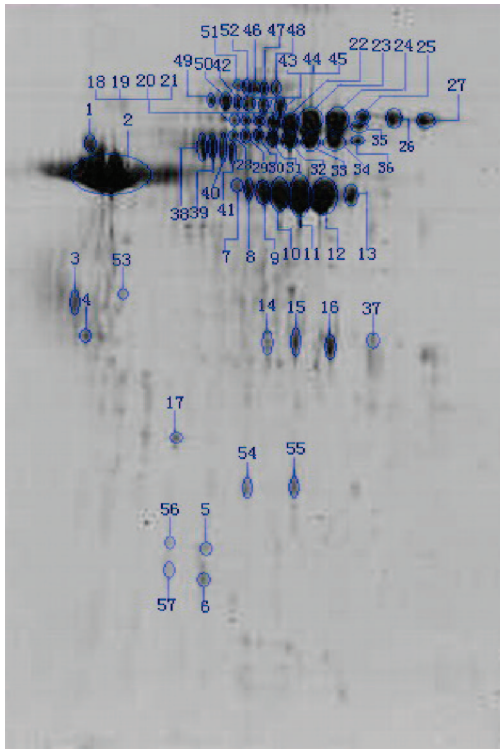

**Figure 1.** Protein spots subjected to tryptic digestion, MALDI-TOF/MS, and identification. Shown is a representative RJ protein profile from high RJ producing bees (*A. mellifera* L.). A total of 150 $\mu$g of an RJ sample was subjected to 2-DE and stained by CCB G-250.

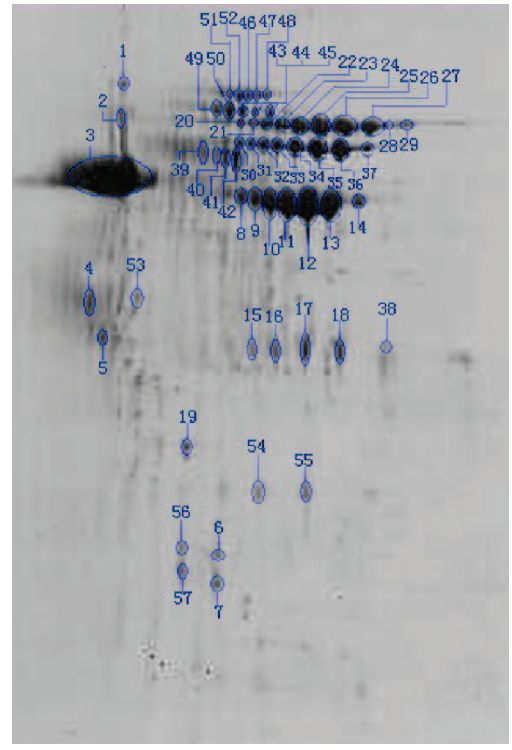

**Figure 2.** Protein spots subjected to tryptic digestion, MALDI-TOF/MS, and identification. Shown is a representative RJ protein profile from native Italian bees (*A. mellifera* L.). A total of 150 $\mu$g of an RJ sample was subjected to 2-DE and stained by CCB G-250.

## RESULTS

**2-DE Images of RJ from the Three Honeybee Strains.** Each RJ sample procedure was repeated several times to obtain at least five 2-DE images with high reproducibility. **Figures 1–3** show the representatives of RJ protein complement from three species of western honeybees. The total detected spots were 152 in RJ from high RJ producing honeybees, 157 in RJ from native Italian honeybees, and 137 in RJ from Carnica honeybees, with a molecular weight range of 12.38–100.77 kDa and pH 4.43–8.60. There was no significant difference between high RJ producing bees and native Italian bees in protein number ($P > 0.05$), while significant differences ($P < 0.05$) were observed between Carnica honeybees and high RJ producing bees and native Italian bees.

**Protein Identification.** A total of 57 protein spots with a high abundance of RJ from high RJ producing honeybees were subjected to identification; 45 belong to the MRJPs family (6 MRJP1, 11 MRJP2, 20 MRJP3, 4 MRJP4, 4 MRJP5), 3 were glucose oxidase (GOX), 1 was peroxiredoxin (PRDX), 1 was glutathione *S*-transferase (GST S1), and 7 were not identified due to a deficiency of protein (**Figure 1**; **Table 1**).

One major MRJP1 spot ($M_r$ 56.27 kDa, pI 5.47) was observed (**Figure 1**, spot 2; **Table 1**), while five minor MRJP1 spots, $M_r$ 17.35–62.40 kDa, pI 4.93–6.30 (**Figure 1**, spots 1, 3–6; **Table 1**), were detected. Eleven different forms of MRJP2, with an $M_r$ range of 26.29–54.62 kDa and pI value of 6.04–7.51 (**Table 1**), were identified (**Figure 1**, spots 7–17). Twenty different forms of MRJP3 were identified (**Figure 1**, spots 18–37) with $M_r$ values from 35.17 to 68.93 kDa and pI of 6.50–8.25 (**Table 1**). Four different forms ($M_r$ 74.89–79.87 kDa, pI 6.53–6.81) of MRJP5 were determined (**Figure 1**, spots 42–45; **Table 1**).

The following proteins were identified with the same numbers in the three species of RJ. Four different forms of MRJP4 were identified with $M_r$ 60.71–61.73 kDa and pI 6.28–6.48 (**Figures 1–3**, spots 38–41, spots 39–42, spots 34–37; **Tables 1–3**,

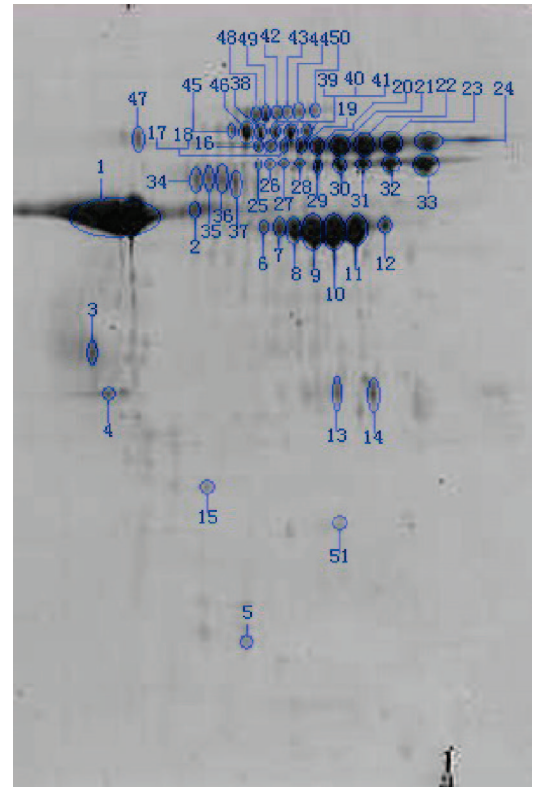

**Figure 3.** Protein spots subjected to tryptic digestion, MALDI-TOF/MS, and identification. Shown is a representative RJ protein profile from Carnica bees (*A. mellifera* C.). A total of 150 $\mu$g of an RJ sample was subjected to 2-DE and stained by CCB G-250.

**Table 1.** Proteins Identified in RJ from High RJ Producing Honeybees[a]

| | 2-D gel | | PMF | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| spot no. | pI | MW (kDa) | pI | MW (kDa) | sequence coverage (%) | matched peptides (total signals) | score | protein name | accession no. |
| 1 | 5.10 | 62.40 | 5.10 | 48.86 | 21.00 | 9/13 | 119 | major royal jelly protein 1 precursor (MRJP-1) | MRJP1_APIME |
| 2 | 5.47 | 56.27 | 5.10 | 48.86 | 35.00 | 16/46 | 125 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 3 | 4.93 | 39.1 | 5.10 | 48.86 | 31.00 | 14/26 | 165 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 4 | 5.06 | 35.4 | 5.10 | 48.86 | 24.00 | 11/29 | 83 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 5 | 6.30 | 19.05 | 5.10 | 48.86 | 25 | 10/25 | 101 | major royal jelly protein 1 precursor (MRJP-1) (bee-milk protein) [contains: jellein-1 (jelleine-I); jellein-2 (jelleine-II); jellein-4 (jelleine-IV)] - *A. mellifera* (honeybee) | MRJP1_APIME |
| 6 | 6.28 | 17.35 | 5.10 | 48.86 | 21.00 | 11/36 | 76 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 7 | 6.51 | 54.62 | 6.83 | 51.04 | 33.00 | 15/69 | 98 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 8 | 6.60 | 54.30 | 6.83 | 51.04 | 24.00 | 9/31 | 74 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 9 | 6.69 | 54.26 | 6.83 | 51.04 | 19.00 | 8/18 | 78 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 10 | 6.81 | 53.9 | 6.83 | 51.04 | 32.00 | 15/51 | 103 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 11 | 6.98 | 53.36 | 6.83 | 51.04 | 21.00 | 9/46 | 68 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 12 | 7.21 | 53.01 | 6.83 | 51.04 | 28.00 | 11/59 | 69 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 13 | 7.51 | 53.54 | 6.83 | 51.04 | 31.00 | 14/43 | 106 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 14 | 6.72 | 34.59 | 6.83 | 51.04 | 23.00 | 10/29 | 81 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 15 | 6.93 | 34.68 | 6.83 | 51.04 | 26.0 | 10/35 | 75 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 16 | 7.29 | 34.12 | 6.83 | 51.04 | 14.00 | 7/13 | 79 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 17 | 6.04 | 26.29 | 6.80 | 51.07 | 10.6 | 5/18 | $d' = 5.2$ | major royal jelly protein 2 [*A. mellifera*] | gi 58585108 ref NP_001011580.1 |
| 18 | 6.50 | 67.42 | 6.47 | 61.62 | 19.00 | 12/22 | 101 | major royal jelly protein 3 precursor (MRJP-3) [*A. mellifera*] | MRJP3_APIME |
| 19 | 6.57 | 67.55 | 6.90 | 65.70 | 14.10 | 9/36 | $d' = 5.1$ | major royal jelly protein 3 [*A. mellifera* carnica] | gi 56422035 gb AAV90959.1 |
| 20 | 6.67 | 67.17 | 6.50 | 61.66 | 19.30 | 10/30 | $d' = 4.0$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 ref NP_001011601.1 |
| 21 | 6.76 | 66.24 | 6.47 | 61.62 | 16.00 | 9/36 | 68 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 22 | 6.87 | 66.2 | 6.47 | 61.62 | 28.00 | 16/35 | 137 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 23 | 7.10 | 67.55 | 6.47 | 61.62 | 21.00 | 11/56 | 73 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJ57-1) [*A. mellifera*] | MRJP3_APIME |
| 24 | 7.35 | 68.21 | 6.50 | 61.66 | 18.9 | 9/41 | $d' = 4.3$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 ref NP_001011601.1 |
| 25 | 7.63 | 68.93 | 6.47 | 61.62 | 29 | 14/29 | 129 | major royal jelly protein 3 precursor (MRJP-3) (bee-milk protein) (royal jelly protein RJP57-1) - *A. mellifera* (honeybee) | MRJP3_APIME |
| 26 | 7.92 | 68.28 | 6.47 | 61.62 | 18.00 | 11/17 | 105 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 27 | 8.25 | 66.68 | 6.50 | 61.66 | 13.90 | 7/164 | $d' = 5.2$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 ref NP_001011601.1 |
| 28 | 6.50 | 63.77 | 6.50 | 61.66 | 18.70 | 14/30 | $d' = 11.2$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 29 | 6.58 | 63.64 | 6.47 | 61.62 | 16.00 | 11/25 | 88 | major royal jelly protein 3 precursor (MRJP-3) (bee-milk protein) (royal jelly protein RJP57-1) - *A. mellifera* (honeybee) | MRJP3_APIME |
| 30 | 6.67 | 63.83 | 6.47 | 61.62 | 24.00 | 13/33 | 103 | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 31 | 6.76 | 63.19 | 6.47 | 61.62 | 42.00 | 21/79 | 136 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 32 | 6.87 | 63.19 | 6.47 | 61.62 | 29.00 | 15/49 | 115 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 33 | 7.08 | 63.23 | 6.47 | 61.62 | 20.00 | 12/21 | 119 | major royal jelly protein 3 | gi 58585142 |
| 34 | 7.34 | 63.10 | 6.47 | 61.62 | 19.00 | 12/34 | 78 | major royal jelly protein 3 precursor (MRJP-3) (bee-milk protein) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 35 | 7.59 | 65.38 | 6.47 | 61.62 | 18.00 | 12/25 | 84 | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |

**Table 1.** Continued

| | 2-D gel | | PMF | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| spot no. | pI | MW (kDa) | pI | MW (kDa) | sequence coverage (%) | matched peptides (total signals) | score | protein name | accession no. |
| 36 | 7.58 | 62.47 | 6.47 | 61.62 | 20.00 | 13/47 | 76 | major royal jelly protein 3 precursor (MRJP-3) (bee-milk protein) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 37 | 7.72 | 35.17 | 6.47 | 61.62 | 13.00 | 8/14 | 78 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 38 | 6.28 | 61.73 | 5.89 | 52.88 | 22.00 | 10/28 | 92 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 39 | 6.36 | 61.32 | 5.89 | 52.88 | 22.00 | 10/28 | 92 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 40 | 6.43 | 61.12 | 5.89 | 52.88 | 20.00 | 9/23 | 85 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 41 | 6.48 | 60.71 | 5.89 | 52.88 | 17.00 | 9/34 | 68 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 42 | 6.53 | 78.58 | 5.90 | 70.24 | 10.0 | 5/41 | 3726 | major royal jelly protein 5 precursor (MRJP-5) [*A. mellifera*] | O97432 |
| 43 | 6.63 | 79.87 | 5.90 | 70.24 | 10.0 | 5/41 | 3726 | major royal jelly protein 5 precursor (MRJP-5) [*A. mellifera*] | O97432 |
| 44 | 6.70 | 77.11 | 5.90 | 70.24 | 10.0 | 5/41 | 3726 | major royal jelly protein 5 precursor (MRJP-5) [*A. mellifera*] | O97432 |
| 45 | 6.81 | 74.89 | 5.90 | 70.24 | 10.0 | 5/41 | 3726 | major royal jelly protein 5 precursor (MRJP-5) [*A. mellifera*] | O97432 |
| 46 | 6.63 | 88.87 | 6.48 | 67.90 | 23.00 | 13/37 | 83 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 47 | 6.71 | 88.34 | 6.48 | 67.90 | 32.00 | 16/34 | 120 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 48 | 6.78 | 89.19 | 6.48 | 67.90 | 20.00 | 10/22 | 84 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 49 | 6.35 | 80.11 | 5.90 | 25.13 | 34.80 | 8/16 | $d' = 4.2$ | peroxiredoxin 2540 CG11765-PA [*A. mellifera*] | gi 66535082 ref XP_624361.1 |
| 50 | 6.44 | 78.10 | 5.40 | 17.69 | 45.00 | 8/20 | 122 | glutathione *S*-transferase S1 CG8938-PA, isoform A, partial [*A. mellifera*] | gi 66534655 |
| 51 | 6.52 | 90.76 | | | | | | not identified | |
| 52 | 6.57 | 90.89 | | | | | | not identified | |
| 53 | 5.58 | 40.10 | | | | | | not identified | |
| 54 | 6.58 | 22.70 | | | | | | not identified | |
| 55 | 6.90 | 22.77 | | | | | | not identified | |
| 56 | 5.98 | 19.29 | | | | | | not identified | |
| 57 | 5.96 | 17.87 | | | | | | not identified | |

[a] Protein scores greater than 79 and $d' \geq 4$ are significant ($P < 0.05$) in Mascot and Xproteo databases, respectively. Spot number is the number of protein spots in **Figure 2**. PMF represents the peptide mass fingerprinting. MW and pI in the 2-D gel mean the values on 2-D gel analyzed by PDQuest; those in PMF are the results identified in the database on-line. Sequence coverage refers to the fraction of the complete protein sequence analyzed by a method. Matched peptides are the ratio of the number of peptide mass values matched to that of searched. Accession number is a unique number or code given to mark the entry of a protein sequence to a primary or secondary database.

**Table 2.** Proteins Identified in RJ from Native Italian Honeybee[a]

| | 2-D gel | | PMF | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| spot number | pI | MW (kDa) | pI | MW (kDa) | sequence coverage (%) | matched peptides (total signals) | score | protein name | accession number |
| 1 | 5.49 | 99.77 | 5.10 | 48.89 | 18.0 | 9/25 | $d' = 4.5$ | major royal jelly protein 1 [*A. mellifera*] | gi 58585098 ref NP_001011579.1 |
| 2 | 5.46 | 62.59 | 5.10 | 48.86 | 29.00 | 13/33 | 109 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 3 | 5.47 | 56.27 | 5.10 | 48.86 | 35.00 | 16/46 | 125 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 4 | 4.93 | 39.1 | 5.10 | 48.86 | 31.00 | 14/26 | 165 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 5 | 5.06 | 35.4 | 5.10 | 48.86 | 24.00 | 11/29 | 83 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 6 | 6.30 | 19.05 | 5.10 | 48.86 | 25 | 10/25 | 101 | major royal jelly protein 1 precursor (MRJP-1) *A. mellifera* (honeybee) | MRJP1_APIME |
| 7 | 6.28 | 17.35 | 5.10 | 48.86 | 21.00 | 11/36 | 76 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 8 | 6.51 | 54.62 | 6.83 | 51.04 | 33.00 | 15/69 | 98 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 9 | 6.60 | 54.30 | 6.83 | 51.04 | 24.00 | 9/31 | 74 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 10 | 6.69 | 54.26 | 6.83 | 51.04 | 19.00 | 8/18 | 78 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 11 | 6.81 | 53.9 | 6.83 | 51.04 | 32.00 | 15/51 | 103 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 12 | 6.98 | 53.36 | 6.83 | 51.04 | 21.00 | 9/46 | 68 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 13 | 7.21 | 53.01 | 6.83 | 51.04 | 28.00 | 11/59 | 69 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 14 | 7.51 | 53.54 | 6.83 | 51.04 | 31.00 | 14/43 | 106 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 15 | 6.56 | 34.77 | 6.83 | 51.04 | 23.00 | 10/24 | 93 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 16 | 6.72 | 34.59 | 6.83 | 51.04 | 23.00 | 10/29 | 81 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 17 | 6.93 | 34.68 | 6.83 | 51.04 | 26.0 | 10/35 | 75 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 18 | 7.29 | 34.12 | 6.83 | 51.04 | 14.00 | 7/13 | 79 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 19 | 6.04 | 26.29 | 6.80 | 51.07 | 10.6 | 5/18 | $d' = 5.2$ | major royal jelly protein 2 [*A. mellifera*] | gi 58585108 ref NP_001011580.1 |
| 20 | 6.50 | 67.42 | 6.47 | 61.62 | 19.00 | 12/22 | 101 | major royal jelly protein 3 precursor (MRJP-3) [*A. mellifera*] | MRJP3_APIME |
| 21 | 6.57 | 67.55 | 6.90 | 65.70 | 14.10 | 9/36 | $d' = 5.1$ | major royal jelly protein 3 [*A. mellifera* carnica] | gi 56422035 gb AAV90959.1 |
| 22 | 6.67 | 67.17 | 6.50 | 61.66 | 19.30 | 10/30 | $d' = 4.0$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 ref NP_001011601.1 |
| 23 | 6.76 | 66.24 | 6.47 | 61.62 | 16.00 | 9/36 | 68 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 24 | 6.87 | 66.2 | 6.47 | 61.62 | 28.00 | 16/35 | 137 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 25 | 7.10 | 67.55 | 6.47 | 61.62 | 21.00 | 11/56 | 73 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJ57-1) [*A. mellifera*] | MRJP3_APIME |
| 26 | 7.35 | 68.21 | 6.50 | 61.66 | 18.9 | 9/41 | $d' = 4.3$ | major royal jelly protein 3 | gi 58585142 ref NP_001011601.1 |
| 27 | 7.63 | 68.93 | 6.47 | 61.62 | 29 | 14/29 | 129 | major royal jelly protein 3 precursor [*A. mellifera*] | MRJP3_APIME |
| 28 | 7.74 | 69.08 | 6.47 | 61.62 | 14.00 | 6/11 | 68 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJ57-1) [*A. mellifera*] | MRJP3_APIME |
| 29 | 7.92 | 68.28 | 6.47 | 61.62 | 18.00 | 11/17 | 105 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 30 | 6.50 | 63.77 | 6.50 | 61.66 | 18.70 | 14/30 | $d' = 11.2$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 31 | 6.58 | 63.64 | 6.47 | 61.62 | 16.00 | 11/25 | 88 | major royal jelly protein 3 precursor (MRJP-3) *A. mellifera* | MRJP3_APIME |
| 32 | 6.67 | 63.19 | 6.47 | 61.62 | 24.00 | 13/33 | 103 | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 33 | 6.76 | 63.19 | 6.47 | 61.62 | 42.00 | 21/79 | 136 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 34 | 6.87 | 63.19 | 6.47 | 61.62 | 29.00 | 15/49 | 115 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 35 | 7.08 | 63.23 | 6.47 | 61.62 | 20.00 | 12/21 | 119 | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 36 | 7.34 | 63.10 | 6.47 | 61.62 | 19.00 | 12/34 | 78 | major royal jelly protein 3 precursor (MRJP-3) *A. mellifera* (honeybee) | MRJP3_APIME |
| 37 | 7.58 | 62.47 | 6.47 | 61.62 | 20.00 | 13/47 | 76 | major royal jelly protein 3 precursor (MRJP-3) *A. mellifera* | MRJP3_APIME |
| 38 | 7.72 | 35.17 | 6.47 | 61.62 | 13.00 | 8/14 | 78 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |

**Table 2.** Continued

| | 2-D gel | | PMF | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| spot number | pI | MW (kDa) | pI | MW (kDa) | sequence coverage (%) | matched peptides (total signals) | score | protein name | accession number |
| 39 | 6.28 | 61.73 | 5.89 | 52.88 | 22.00 | 10/28 | 92 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 40 | 6.36 | 61.32 | 5.89 | 52.88 | 22.00 | 10/28 | 92 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 41 | 6.43 | 61.12 | 5.89 | 52.88 | 20.00 | 9/23 | 85 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 42 | 6.48 | 60.71 | 5.89 | 52.88 | 17.00 | 9/34 | 68 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 43 | 6.53 | 78.58 | 5.90 | 70.24 | 26.4 | 16/36 | $d' = 6.9$ | major royal jelly protein 5 [*A. mellifera*] | gi 58585108 |
| 44 | 6.63 | 79.87 | 5.90 | 70.24 | 26.4 | 16/36 | $d' = 6.9$ | major royal jelly protein 5 [*A. mellifera*] | gi 58585108 |
| 45 | 6.70 | 77.11 | 5.90 | 70.24 | 26.4 | 16/36 | $d' = 6.9$ | major royal jelly protein 5 [*A. mellifera*] | gi 58585108 |
| 46 | 6.57 | 90.89 | 6.48 | 67.90 | 23.00 | 13/37 | 83 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 47 | 6.63 | 88.87 | 6.48 | 67.90 | 32.00 | 16/34 | 120 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 48 | 6.71 | 88.34 | 6.48 | 67.90 | 20.00 | 10/22 | 84 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 49 | 6.35 | 80.11 | 5.90 | 25.13 | 34.80 | 8/16 | $d' = 4.2$ | peroxiredoxin 2540CG11765-PA [*A. mellifera*] | gi 66535082 ref XP_624361.1 |
| 50 | 6.44 | 78.10 | 5.40 | 17.69 | 45.00 | 8/20 | 122 | glutathione *S*-transferase S1 CG8938-PA, isoform A, partial [*A. mellifera*] | gi 66534655 |
| 51 | 6.52 | 90.76 | | | | | | not identified | |
| 52 | 6.57 | 90.89 | | | | | | not identified | |
| 53 | 5.58 | 40.1 | | | | | | not identified | |
| 54 | 6.58 | 22.7 | | | | | | not identified | |
| 55 | 6.90 | 22.77 | | | | | | not identified | |
| 56 | 5.98 | 19.29 | | | | | | not identified | |
| 57 | 5.96 | 17.87 | | | | | | not identified | |

[a] Note: Protein scores greater than 79 and $d' \geq 4$ are significant ($P < 0.05$) in Mascot and Xproteo databases, respectively. Spot number is the number of protein spots in **Figure 2**. PMF represents the peptide mass fingerprinting. MW and pI in 2-D gel mean the values on 2-D gel analyzed by PDQuest; those in PMF are the results identified in the database on-line. Sequence coverage refers to the fraction of the complete protein sequence analyzed by a method. Matched peptides are the ratio of the number of peptide mass values matched to that of searched. Accession number is a unique number or code given to mark the entry of a protein sequence to a primary or secondary database.

**Table 3.** Proteins Identified in RJ from Carnica Honeybee[a]

| | 2-D gel | | PMF | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| spot number | pI | MW (KDa) | pI | MW (KDa) | sequence coverage (%) | matched peptides (total signals) | score | protein name | accession number |
| 1 | 5.47 | 56.27 | 5.10 | 48.86 | 35.00 | 16/46 | 125 | major royal jelly protein 1 precursor(MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 2 | 6.00 | 57.11 | 5.10 | 48.86 | 24.00 | 11/29 | 86 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 3 | 4.93 | 39.1 | 5.10 | 48.86 | 31.00 | 14/26 | 165 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 4 | 5.06 | 35.4 | 5.10 | 48.86 | 24.00 | 11/29 | 83 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 5 | 6.28 | 17.35 | 5.10 | 48.86 | 21.00 | 11/36 | 76 | major royal jelly protein 1 precursor (MRJP-1) [*A. mellifera*] | MRJP1_APIME |
| 6 | 6.51 | 54.62 | 6.83 | 51.04 | 33.00 | 15/69 | 98 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 7 | 6.60 | 54.30 | 6.83 | 51.04 | 24.00 | 9/31 | 74 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 8 | 6.69 | 54.26 | 6.83 | 51.04 | 19.00 | 8/18 | 78 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 9 | 6.81 | 53.9 | 6.83 | 51.04 | 32.00 | 15/51 | 103 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 10 | 6.98 | 53.36 | 6.83 | 51.04 | 21.00 | 9/46 | 68 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 11 | 7.21 | 53.01 | 6.83 | 51.04 | 28.00 | 11/59 | 69 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 12 | 7.51 | 53.54 | 6.83 | 51.04 | 31.00 | 14/43 | 106 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 13 | 6.93 | 34.68 | 6.83 | 51.04 | 26.0 | 10/35 | 75 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 14 | 7.29 | 34.12 | 6.83 | 51.04 | 14.00 | 7/13 | 79 | major royal jelly protein 2 precursor (MRJP-2) [*A. mellifera*] | MRJP2_APIME |
| 15 | 6.04 | 26.29 | 6.80 | 51.07 | 10.6 | 5/18 | $d' = 5.2$ | major royal jelly protein 2 [*A. mellifera*] | gi 58585108 ref NP_001011580.1 |
| 16 | 6.50 | 67.42 | 6.47 | 61.62 | 19.00 | 12/22 | 101 | major royal jelly protein 3 precursor (MRJP-3) [*A. mellifera*] | MRJP3_APIME |
| 17 | 6.57 | 67.55 | 6.90 | 65.70 | 14.10 | 9/36 | $d' = 5.1$ | major royal jelly protein 3 [*A. mellifera* carnica] | gi 56422035 gb AAV90959.1 |
| 18 | 6.67 | 67.17 | 6.50 | 61.66 | 19.30 | 10/30 | $d' = 4.0$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 ref NP_001011601.1 |
| 19 | 6.76 | 66.24 | 6.47 | 61.62 | 16.00 | 9/36 | 68 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 20 | 6.87 | 66.2 | 6.47 | 61.62 | 28.00 | 16/35 | 137 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 21 | 7.10 | 67.55 | 6.47 | 61.62 | 21.00 | 11/56 | 73 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJ57-1) [*A. mellifera*] | MRJP3_APIME |
| 22 | 7.35 | 68.21 | 6.50 | 61.66 | 18.9 | 9/41 | $d' = 4.3$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 ref NP_001011601.1 |
| 23 | 7.63 | 68.93 | 6.47 | 61.62 | 29 | 14/29 | 129 | major royal jelly protein 3 precursor (MRJP-3) *A. mellifera* (honeybee) | MRJP3_APIME |
| 24 | 7.92 | 68.28 | 6.47 | 61.62 | 18.00 | 11/17 | 105 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 25 | 6.50 | 63.77 | 6.50 | 61.66 | 18.70 | 14/30 | $d' = 11.2$ | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 26 | 6.58 | 63.64 | 6.47 | 61.62 | 16.00 | 11/25 | 88 | major royal jelly protein 3 precursor (MRJP-3) *A. mellifera* (honeybee) | MRJP3_APIME |
| 27 | 6.67 | 63.83 | 6.47 | 61.62 | 24.00 | 13/33 | 103 | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 28 | 6.76 | 63.19 | 6.47 | 61.62 | 42.00 | 21/79 | 136 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 29 | 6.87 | 63.19 | 6.47 | 61.62 | 29.00 | 15/49 | 115 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 30 | 7.08 | 63.23 | 6.47 | 61.62 | 20.00 | 12/21 | 119 | major royal jelly protein 3 [*A. mellifera*] | gi 58585142 |
| 31 | 7.34 | 63.10 | 6.47 | 61.62 | 19.00 | 12/34 | 78 | major royal jelly protein 3 precursor (MRJP-3) *A. mellifera* (honeybee) | MRJP3_APIME |
| 32 | 7.58 | 62.47 | 6.47 | 61.62 | 20.00 | 13/47 | 76 | major royal jelly protein 3 precursor (MRJP-3) *A. mellifera* (honeybee) | MRJP3_APIME |
| 33 | 7.9 | 62.84 | 6.47 | 61.62 | 14.00 | 8/23 | 70 | major royal jelly protein 3 precursor (MRJP-3) (royal jelly protein RJP57-1) [*A. mellifera*] | MRJP3_APIME |
| 34 | 6.28 | 61.73 | 5.89 | 52.88 | 22.00 | 10/28 | 92 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 35 | 6.36 | 61.32 | 5.89 | 52.88 | 22.00 | 10/28 | 92 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 36 | 6.43 | 61.12 | 5.89 | 52.88 | 20.00 | 9/23 | 85 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |

**Table 3.** Continued

| spot number | 2-D gel pI | 2-D gel MW (KDa) | PMF pI | PMF MW (KDa) | PMF sequence coverage (%) | PMF matched peptides (total signals) | PMF score | protein name | accession number |
|---|---|---|---|---|---|---|---|---|---|
| 37 | 6.48 | 60.71 | 5.89 | 52.88 | 17.00 | 9/34 | 68 | major royal jelly protein 4 precursor (MRJP-4) (royal jelly protein RJP57-2) [*A. mellifera*] | MRJP4_APIME |
| 38 | 6.53 | 78.58 | 5.90 | 70.24 | 26.4 | 16/36 | $d' = 6.9$ | major royal jelly protein 5 [*A. mellifera*] | gi 58585108 |
| 39 | 6.63 | 79.87 | 5.90 | 70.24 | 26.4 | 16/36 | $d' = 6.9$ | major royal jelly protein 5 [*A. mellifera*] | gi 58585108 |
| 40 | 6.70 | 77.11 | 5.90 | 70.24 | 26.4 | 16/36 | $d' = 6.9$ | major royal jelly protein 5 [*A. mellifera*] | gi 58585108 |
| 41 | 6.81 | 74.89 | 5.90 | 70.24 | 26.4 | 16/36 | $d' = 6.9$ | major royal jelly protein 5 [*A. mellifera*] | gi 58585108 |
| 42 | 6.63 | 88.87 | 6.48 | 67.90 | 23.00 | 13/37 | 83 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 43 | 6.71 | 88.34 | 6.48 | 67.90 | 32.00 | 16/34 | 120 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 44 | 6.78 | 89.19 | 6.48 | 67.90 | 20.00 | 10/22 | 84 | glucose oxidase [*A. mellifera*] | gi 58585090 |
| 45 | 6.35 | 80.11 | 5.90 | 25.13 | 34.80 | 8/16 | $d' = 4.2$ | peroxiredoxin 2540 CG11765-PA [*A. mellifera*] | gi 66535082 ref XP_624361.1 |
| 46 | 6.44 | 78.10 | 5.40 | 17.69 | 45.00 | 8/20 | 122 | glutathione *S*-transferase S1 CG8938-PA, isoform A, partial [*A. mellifera*] | gi 66534655 |
| 47 | 5.58 | 76.54 | | | | | | not identified | |
| 48 | 6.52 | 90.76 | | | | | | not identified | |
| 49 | 6.57 | 90.89 | | | | | | not identified | |
| 50 | 6.83 | 91.10 | | | | | | not identified | |
| 51 | 6.90 | 22.77 | | | | | | not identified | |

[a] Protein scores greater than 79 and $d' \geq 4$ are significant ($P < 0.05$) in Mascot and Xproteo databases, respectively. Spot number is the number of protein spots in **Figure 3**. PMF represents the peptide mass fingerprinting. MW and pI in 2-D gel mean the values on 2-D gel analyzed by PDQuest; those in PMF are the results identified in the database on-line. Sequence coverage refers to the fraction of the complete protein sequence analyzed by a method. Matched peptides are the ratio of the number of peptide mass values matched to that of searched. Accession number is a unique number or code given to mark the entry of a protein sequence to a primary or secondary database.

respectively); three different forms of glucose oxidase were identified with $M_r$ values of 88.34–89.19 and pI of 6.63–6.78 (**Figures 1–3**, spots 46–48, spots 46–48, spots 42–44; **Tables 1–3**, respectively). Both PRDX ($M_r$ 80.11 kDa, pI 6.35) (**Figures 1–3**, spot 49, spot 49, spot 45; **Tables 1–3**, respectively) and GST S1 ($M_r$ 78.10 kDa, pI 6.44) (**Figures 1–3**, spot 50, spot 50, spot 46; **Tables 1–3**, respectively) were identified in RJ protein complement for the first time, to our knowledge.

Also, a total 57 protein spots in RJ from native Italian honeybees were identified, of which 45 belong to the MRJPs family (7 MRJP1, 12 MRJP2, 19 MRJP3, 4 MRJP4, 3 MRJP5), 3 were glucose oxidase, 1 was PRDX, 1 was GST S1, and 7 were not identified due to a deficiency of protein (**Figure 2**, **Table 2**).

One major MRJP1 spot ($M_r$ 56.27 kDa, pI 5.47) was observed (**Figure 2**, spot 3; **Table 2**), while 6 minor MRJP1 spots ($M_r$ of 17.35–99.77 kDa, pI of 4.93–6.30, **Figure 2**, spots 1, 2, 4–7, **Table 2**) were detected. Twelve different forms of MRJP2, with an $M_r$ range of 26.29–54.62 kDa and pI value of 6.04–7.51 (**Figure 2**, spots 8–19; **Table 2**) and 19 forms of MRJP3 ($M_r$ 35.17–69.08 kDa, pI 6.50–7.92) were identified in RJ of native Italian honeybees (**Figure 2**, spots 20–38; **Table 2**). Three MRJP5 were identified with an $M_r$ value of 77.11–79.87 kDa and pI 6.53–6.70 (**Figure 2**, spots 43–45; **Table 2**).

For the Carnica bees, a total of 51 protein spots in RJ were subject to identification, of which 41 belong to the MRJPs family (5 MRJP1, 10 MRJP2, 18 MRJP3, 4 MRJP4, 4 MRJP5), 3 were glucose oxidase, 1 was similar to PRDX, 1 was similar to GST S1, and 4 were not identified due to a deficiency of protein (**Figure 3**; **Table 3**).

One major MRJP1 spot ($M_r$ 56.27 kDa, pI 5.47) (**Figure 3**, spot 1; **Table 3**) and four minor MRJP1 spots with $M_r$ values of 17.35–57.11 kDa, pI of 4.93–6.28 (**Figure 3**, spot 2–5; **Table 3**) were observed. Ten different forms of MRJP2, with an $M_r$ range of 26.29–54.62 kDa and pI value of 6.04–7.51 (**Figure 3**, spots 6–15; **Table 3**), 18 forms ($M_r$ 62.47–68.93 and pI 6.50–7.92) of MRJP3 (**Figure 3**, spots 16–33; **Table 3**), and 4 different forms ($M_r$ 74.89–79.87 kDa, pI 6.53–6.81) of MRJP5 were determined (**Figure 3**, spots 38–41; **Table 3**).

## DISCUSSION

On the basis of 2-DE patterns of RJ from high RJ producing bees, native Italian bees, and Carnica bees, the detected number of proteins are significant higher in high RJ producing bees and native Italian bees than in Carnica bees, indicating that RJ from the former two types of bees contain more proteins than the latter one.

One major MRJP1 was observed in all three RJ samples (**Figures 1–3**, spot 2, spot 3, spot 1, respectively), while five minor MRJP1 spots (**Figure 1**, spots 1, 3–6; **Table 1**), six minor MRJP1 spots (**Figure 2**, spots 1, 2, 4–7; **Table 2**), and four minor MRJP1 spots (**Figure 3**, spots 2–5; **Table 3**) were also detected in RJ of three breeds of bees, respectively. This demonstrates that MRJP1 may present variant forms with different $M_r$ and pI values, which is probably due to its potential glycosylation sites revealed by the NCBI protein databank (http://www.ncbi.nlm.nih.gov/) (*31*). To date, only two forms and one form of this protein was identified in RJ of Africanized honeybee and European honeybees (*23*), respectively, which is possibly a consequence of a difference between honeybee species or by virtue of the relative lower resolution of a 13-cm IPG strip compared to a 17-cm strip used in this experiment. Six different forms of MRJP1 were identified in the protein complement of the hypopharyngeal gland of Africanized nurse

bees with an $M_r$ of 48.81–60.00 kDa and pI of 4.23–5.50 (*31*) that are narrower than the $M_r$ and pI ranges demonstrated in this experiment, which may also be due to the shorter IPG strips or to structural changes suffered by this protein, such as proteolysis, glycosylation, or deglycosylation after being secreted from the gland.

A total of 11, 12, and 10 different forms of MRJP2 were detected in RJ of high RJ producing honeybees (**Figure 1**, spots 7–17), native Italian honeybees (**Figure 2**, spots 8–19), and Carnica honeybees (**Figure 3**, spots 6–15), respectively. NCBI databank reveals two hypothetical glycosylation sites for this protein that could explain the observed heterogeneity. A total of 15 and 12 different forms of this protein were observed in the RJ from Africanized and European honeybees, respectively (*23*), while eight forms exist in the proteome complement of the hypopharyngeal gland of Africanized nurse bees with an $M_r$ of 50.67–60.00 kDa and pI of 4.92–7.02 (*31*). The larger scale of $M_r$ and pI values in this experiment is probably attributed to the longer IPG (17-cm) strips used compared to IPG I previous experiments (13 cm).

Twenty different forms of MRJP3 were identified in RJ of high RJ producing honeybees (**Figure 1**, spots 18–37; **Table 1**); nineteen forms (**Figure 2**, spots 20–38; **Table 2**) were identified from RJ of native Italian honeybees and 18 forms of this protein were identified in RJ of Carnica honeybees (**Figure 3**, spots 16–33; **Table 3**). In RJ of Carnica honeybees, spot 50 (**Figure 3**) corresponding to a lower molecular weight MRJP3 spot in two other RJ samples was not determined due to a deficiency of protein. A total of 5, 10, and 24 different forms of this protein were reported in the protein complement of the hypopharyngeal gland of nurse bees (*31*), and RJ of Africanized and European honeybees (*23*), respectively. MRJP3 has the most isoforms in RJ protein constituents as shown in the present report, which could be attributable to its polymorphism of a region with a variable number of tandem repeats (VNTR) located at the C-terminal part of the coding region (*13*). PCR analyses have confirmed the presence of an extensive repetitive region that showed inter- and intraspecific polymorphisms in size and sequence in four honeybee species (*A. mellifera*, *A. cerana*, *A. dorsata*, and *A. florae*), and the repetition is suggested to be due to a selection for an increase in nitrogen storage for more efficient nutrition for queens and larvae (*32*).

MRJP4 has been confirmed by 2-DE analysis for the first time by Sano et al. (*23*), and five spots and two spots of this protein were identified, respectively, in the RJ of Africanized and European honeybees (*23*). In all three RJ samples of this research, four different forms of MRJP4 were identified (**Figure 1**, spots 38–41, **Figure 2**, spots 39–42, **Figure 3**, spots 34–37; **Tables 1–3**, respectively), while none was found in proteomic analysis of the secretion of the hypopharyngeal gland (*31*). The difficulty in detecting MRJP4 in RJ may be due to its sensitivity to storage temperature (*33*).

Four different forms of MRJP5 were determined in RJ of both high RJ producing honeybees (**Figure 1**, spots 42–45; **Table 1**) and Carnica honeybees (**Figure 3**, spots 38–41; **Table 3**), and three of them were found in RJ of native Italian honeybees (**Figure 2**, spots 43–45; **Table 2**). Taking into account of a single copy of the MRJP5 gene, the slight heterogeneity in $M_r$ and pI may be from post-translational modifications. Three of them were found in the protein complement of the secretion from the hypopharyngeal gland of nurse bees (*31*), while seven and four of them were found in RJ from Africanized and European honeybees (*23*), respectively.

No MRJP6, -7, and -8 was detected in this work, neither in Africanized nor European honeybee RJ, whereas a single form of these proteins was identified in the proteome complement of the secretion from the hypopharyngeal gland of Africanized nurse honeybees (*31*). To our present knowledge, MRJP6, -7, and -8 have been only implied by cloning of the honeybee cDNA sequence, but they have not been identified in RJ until now. Therefore, whether these three proteins exist in RJ remains unsubstantiated.

Three different forms of glucose oxidase (GOX) were identified in the three RJ samples (**Figure 1**, spots 46–48; **Figure 2**, spots 46–48; **Figure 3**, spots 42–44; **Tables 1–3**, respectively), while five of them were confirmed in Africanized honeybee RJ and one was found in the proteome complement of the secretion from the hypopharyngeal gland of Africanized nurse honeybees (*23, 31*). GOX catalyzes the oxidation of glucose to glucono-1,5-lactone (which spontaneously hydrolyzes nonenzymically to gluconic acid) using molecular oxygen and releasing hydrogen peroxide ($H_2O_2$). GOX is used for the biological production of gluconic acid and for the removal of either glucose or oxygen from foodstuffs to improve their storage capability (*34*). GOX is of interest in relation to antibacterial properties in honey since hydrogen peroxide is the main agent responsible for the antibacterial activity in most honeys, and gluconic acid is the main acid found in honey and usually accounts for most of the acidity of honey. Similarly, GOX may partly contribute to the acid pH and antiseptic trait of RJ.

To date, PRDX (**Figure 1**, spot 49; **Figure 2**, spot 49; **Figure 3**, spot 45; **Tables 1–3**, respectively) and GST S1 (**Figure 1**, spot 50; **Figure 2**, spot 50; **Figure 3**, spot 46; **Tables 1–3**, respectively) were identified in RJ protein complement for the first time. PRDXs have been identified as a large family of peroxidases able to reduce $H_2O_2$ and alkyl hydroperoxides (*35–39*). PRDXs are part of the enzymatic antioxidant system, collaborating in cells with well-characterized catalase, superoxide dismutases, and selenium glutathione peroxidases (*40*). It could play a major protective role in animal cells against reactive oxygen. In addition to their protective antioxidant role, it has been suggested that PRDXs are involved in cell signaling, apoptosis, cell differentiation, and other regulatory processes (*41–43*). Thus, this class of enzymes has a wide variety of functions that are vital for metabolism and cellular integrity by protecting lipids, enzymes, and DNA against peroxides. GST represents a group of detoxification enzymes to catalyze the conjugation of a diverse array of electrophilic compounds with glutathione. GST induction represents part of an adaptive response mechanism to chemical stress caused by electrophiles (*44*). In insects, GSTs play an important role in the resistance against several classes of insecticides including organophosphate (OP) (*45*). *Drosophila melanogaster* GST S1 plays a central role in the lipid peroxidation product 4-hydroxynonenal (4-HNE) metabolism of *Drosophila* as it accounts for more than two-thirds of the insect's capacity to conjugate 4-HNE, and it may have alternative and/or additional functions in detoxification, protection against oxidative injury, and perhaps in signaling processes (*46*). The biological activities of PRDX and GST possibly partly contribute to the longer longevity of queens compared to workers and surprising ability to lay eggs, as queens are fed RJ throughout their lives. Meanwhile, these functional properties of PRDX and GST may partly explain RJ's pharmacological and/or cosmetic traits for human beings.

In summary, there is no significant difference in RJ protein complements between high RJ producing honeybees and native Italian honeybees, while a significant difference remains compared

to Carnica honeybees. Among the identified proteins with high abundance, most of them are assigned to MRJPs. Remarkable differences are found in the heterogeneity of the MRJPs, in particular, MRJP3. Besides glucose oxidase, for the first time, PRDX and GST S1 have been identified in RJ. We preliminarily assume that the major components of identified RJ proteins among three species have no differences. However, due to the low abundance they are not identified; further research is necessary to complete this project.

## ABBREVIATIONS USED

MRJP, major royal jelly protein; RJ, royal jelly; 2-DE, two-dimensional electrophoresis; PRDX, peroxiredoxin; GST, glutathione *S*-transferase; MALDI-TOF/MS, matrix-assisted laser desorption ionization-time of flight mass spectrometry; VNTR, variable number of tandem repeats; PCR, polymerase chain reaction; OP, organophosphate.

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