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Novel Royal Jelly Proteins Identified by Gel-Based and Gel-free Proteomics

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ABSTRACT: Royal jelly (RJ) plays an important role in caste determination of the honeybee; the genetically same female egg develops into either a queen or worker bee depending on the time and amount of RJ fed to the larvae. RJ also has numerous health-promoting properties for humans. Gel-based and gel-free proteomics approaches and high-performance liquid chromatography−chip quadruple time-of-flight tandem mass spectrometry were applied to comprehensively investigate the protein components of RJ. Overall, 37 and 22 nonredundant proteins were identified by one-dimensional gel electrophoresis and gel-free analysis, respectively, and 19 new proteins were found by these two proteomics approaches. Major royal jelly proteins (MRJPs) were identified as the principal protein components of RJ, and proteins related to carbohydrate metabolism such as glucose oxidase, α-glucosidase precursor, and glucose dehydrogenase were also successfully identified. Importantly, the 19 newly identified proteins were mainly classified into three functional categories: oxidation−reduction (ergic53 CG6822-PA isoform A isoform 1, Sec61 CG9539-PA, and ADP/ATP translocase), protein binding (regucalcin and translationally controlled tumor protein CG4800-PA isoform 1), and lipid transport (apolipophorin-III-like protein). These new findings not only significantly increase the RJ proteome coverage but also help to provide new knowledge of RJ for honeybee biology and potential use for human health promotion.

KEYWORDS: royal jelly, proteome, one-dimensional gel electrophoresis, gel-free

INTRODUCTION

Royal jelly (RJ) is secreted by the hypopharyngeal gland and the mandibular gland of nurse honeybees and serves as the exclusive food for the queen bee throughout her life; however, for the worker and the drone larvae RJ is only fed within the first 3 days after hatching.[1] The significant role of RJ is to provide both nutrition and protection for fast developing honeybee larvae.[2,3] RJ is also the key driving force in honeybee caste determination; a fertile egg develops into either a sexually perfect queen or a sexually immature worker depending strictly on the time and amount of RJ fed to the larvae.[4−6] More recently, it has been reported that the minor 57 kDa protein of RJ royalactin could influence honeybee caste determination.[7] Moreover, RJ has been accepted and broadly used as a health-promoting substance for human beings.[8−11] Except for conventional nutrition constituents such as carbohydrates, proteins, free amino acids, lipids, and trace and mineral elements, RJ has some special components such as the lipidic fraction, 10-hydroxy-2-decenoic acid (10-HDA),[12] which has antibiotic and immunomodulatory activities.[13−15] Nevertheless, because proteins account for 12−15% of fresh RJ and up to 50% of dried RJ, it is evident that proteins contribute significantly to honeybee biology and the health promotion properties for mankind. Some major proteins contained in RJ, such as major royal jelly proteins (MRJPs),[16] jelleines.[17] and royalisin,[18] have been attributed various physiological properties of antimicrobial, antifungal, antitumor, antidiabetes, and anti-hypertension activities.[19−22] Still, a glycoprotein in RJ could suppress allergic reactions and stimulate the proliferation of human monocytes.[23,24]

To date, proteomic analysis of RJ has proven that >80% of the total proteins belonging to MRJPs[25,26] contain MRJP1−9 families.[27−29] Currently, the most commonly used techniques to analyze the RJ proteome are sodium dodecyl sulfonate−polyacrylamide gel electrophoresis (SDS-PAGE, 1-DE) and two-dimensional gel electrophoresis (2-DE) in combination with mass spectrometry, and with the advent of these new techniques and more precise instruments, many new proteins have been successfully identified. This includes some important enzymes in RJ involved in carbohydrate metabolism, such as α-glucosidase, glucose oxidase, and glucose dehydrogenase isoform 1, which are thought to help digest RJ sugar components for larva feeding and protection.[29−31] Two other important antioxidant proteins, peroxiredoxin (PRDX) and glutathione *S*-transferase (GST) S1, have been identified in RJ by application of 2-DE and matrix-assisted laser desorption ionization−time-of-flight mass spectrometry (MALDI-TOF/MS).[32] More recently, some other proteins such as venom protein 2, ferritin, retinoid, and fatty acid binding protein have been identified in RJ with tandem mass spectrometry (MS/MS).[29,30]

Although these findings significantly improve proteome coverage of RJ, using complementary gel-based and gel-free proteomic approaches to try to identify possible new proteins in RJ is still missing. To this effect, gel-based 1-DE was used to separate all of the proteins according to their molecular weight then cut the entire lane into 12 slices for further protein identification. Simultaneously, a gel-free technique, in which we digested all RJ proteins in solution for analysis, was also used to increase the

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efficiency of protein identification. The combination of this approach with high-performance liquid chromatography−chip quadruple time-of-flight tandem mass spectrometry (HPLC-Chip-QTOF MS/MS) for protein identification, which is one of the most powerful proteomics approaches for low-abundance protein identification,[33−35] allowed us to comprehensively characterize and increase coverage of RJ proteome. This may help us to get a better understanding of the roles RJ plays both in honeybee biology and in human health promotion.

MATERIALS AND METHODS

Chemical Reagents. Tris-base, ammonium persulfate (AP), sodium dodecyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), sodium bicarbonate (NH_4HCO_3), glycin, agarose, urea trichloroacetic acid (TCA), and formic acid were from Sigma (St. Louis, MO). Acrylamide, *N*,*N*′-methylenebisacrylamide, bromophenol blue, thiourea, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), glycerol, and bovine serum albumin (BSA) were purchased from Amresco (Solon, OH). Dithiothreitol (DTT) and iodoacetamide were from Merck (Darmstadt, Germany). Trypsin was from Roche (Basel, Switzerland), and trifluoroacetic acid (TFA) and acetonitrile were from J. T. Baker (Phillipsburg, NJ). Silver nitrate, sodium carbonate, dipotassium phosphate (K_2HPO_5), monopotassium phosphate (KH_2PO_4), sodium chloride (NaCl), sodium hydroxide (NaOH), and formaldehyde were from Beijing Shiji Co. (Beijing, China).

Royal Jelly Samples. RJ was collected from five colonies of Italian honeybees (*Apis mellifera* L.) in the apiary of the Bee Research Institute, Chinese Academy of Agricultural Science (Beijing, China). RJ samples from queen cell cups where larvae had been grafted 72 h before were harvested into sterile bottles. The fresh RJ protein was extracted immediately after collection for further analysis.

Protein Preparation. Protein preparation was performed as described by Li et al.[32] Briefly, RJ (1 mg of RJ/10 μL of buffer) was mixed with 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 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 a tube for use. The pellets (1 mg of pellets/2 μL of buffer) were mixed with the PB, pH 7.6, and then centrifuged at 15000*g* and 4 °C for 10 min. The supernatant was removed and suspended into a tube containing supernatant as a PB-soluble protein extract, whereas the pellets (1 mg of pellets/10 μL of buffer), PB-insoluble proteins, were mixed with lysis buffer (LB, 8 M urea, 2 M thiourea, 4% CHAPS, 20 mM Tris-base, 30 mM DTT); then the mixture was sonicated for 2 min and centrifuged at 15000*g* and 4 °C for 10 min. The supernatant was removed and suspended into the tube containing PB-soluble protein extraction, and the debris was discarded. 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 15000*g* and 4 °C for 10 min. The supernatant was discarded, and the pellets (1 mg of RJ/4 μL of buffer) were resolved in LB, and then the mixture was homogenized for 5 min on ice, sonicated for 2 min, and subsequently adjusted to pH 7.0 with 2 M NaOH. The protein extract mixture was stored at −70 °C for further use. Protein concentration was determined according to the Bradford method.[36] BSA was used as standard, and the absorption was measured at 595 nm with spectrophotometer DU800 (Beckman Coulter, Los Angeles, CA).

One-Dimensional Gel Electrophoresis (1-DE). A protein sample aliquot (6 μL) was mixed with 2 μL of 4× protein loading buffer [62 mM Tris (pH 6.8) containing 10% (v/v) glycerol, 2.5% (w/v) SDS, and 5% (v/v) 2-mercaptoethanol, pH 6.8]. The mixture was centrifuged and the supernatant was used for 1-DE [4% T (total acrylamide concentration), 2.6% C (degree of cross-linking) stacking gels, pH 6.8, and 12.5% T, 2.6% C separating gels, pH 8.8]. Gel staining was accomplished by MS compatible silver-staining method.[37] Entire lanes were cut into 12 fractions and transferred to sterile 1.5 mL microcentrifuge tubes, washed, and subjected to tryptic in-gel digestion and MS/MS analysis. Three replications were performed for the protein sample.

In-Gel Digestion. The silver-stained bands were destained and dried for 10 min with acetonitrile (100%). The gels were dried for 30 min using a Speed-Vac system (Marin Christ., Osterode, Germany). Then 2.5 mL of 25 mM NH_4HCO_3 was added to 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 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.

In-Solution Digestion. Protein sample aliquots (50 μL) were subjected to 200 μL of ice cold acetone, and then the mixture was kept on ice for 30 min for protein precipitation and desalting. Subsequently, the mixture was centrifuged at 15000*g* and 4 °C for 5 min, the resulting supernatants were discarded, and the pellets were dried. The dried pellets were dissolved in 100 mM NH_4HCO_3, and the proteins were reduced with 10 mM DTT and were alkylated with 50 mM iodoacetamide. Proteins were digested using trypsin (0.5 mg/mL) in the ratio 1:50 enzyme/protein w/w at 37 °C for 14 h. After digestion, 1 μL of formic acid was added to the solution to stop the reaction and then dried by a Speed-Vac system.

Protein Identification by MS. Proteins were identified by HPLC-Chip/ESI-QTOF-MS (QTOF G6530, Agilent Technologies, Santa Clara, CA), equipped with capillary pump G1382A, nanopump G2225A, autosampler G1377D, and Chip Cube G4240A. The LC-Chip used (Agilent Technologies) to analyze protein bands was constituted of a Zorbax 300SB-C18 enrichment column (160 nL, 5 μm) and a Zorbax 300SB-C18 analytical column (75 μm × 43 mm, 5 μm). The loading flow rate was 4 μL/min, and the loading mobile phase was water with 0.1% formic acid. Elution from the analytical column was performed by a binary solvent mixture composed of water with 0.1% formic acid (solvent A) and acetonitrile with 0.1% formic acid (solvent B). The following gradient program was used: from 3 to 8% B in 1 min, from 8 to 40% B in 5 min, from 40 to 85% B in 1 mi,n and 85% B for 1 min. The chip flow rate was 300 nL/min. The mass spectrometric conditions were performed as follows: positive ion mode; Vcap, 1900 V; drying gas flow rate, 5 L/min; drying gas temperature, 350 °C; fragmentary voltage, 175 V; skimmer voltage, 65 V; reference masses, *m*/*z* 149.02332 and 1221.02332. The digested samples were diluted in 20 μL of water with 0.1% formic acid; all of the samples were centrifuged for 10 min at 15000*g*, and about 15 μL of upper solution was analyzed from which 8 μL of sample was injected. Tandem mass spectra were retrieved and stored in a combined mgf file using MassHunter software (Agilent Technologies). MS/MS data were searched using the Mascot search engine online. Search parameters carboxymethyl (C) and oxidation (M) were selected as variable modifications, and no fixed modification was selected. The other parameters used were as follows: taxonomy, all entries; enzyme, trypsin; missed cleavages, 1; peptide tolerance, 0.8 Da; MS/MS tolerance, 0.6 Da. Protein identifications were accepted if they could be established at >95% probability and contained at least two identified peptides. If the protein was identified on the basis of one peptide, we set a conservative threshold to minimize false-positive protein identifications. We set the theoretical probability of obtaining a false-positive protein identification to < 1 in 1000, which meant that

these peptides must have a (false-positive) Mascot expectation value of ≤0.001. When a given peptide could have originated from more than one protein, those proteins were considered to be positively identified as long as they were identified using at least one unique peptide.

RESULTS

To further explore the RJ proteome, total proteins were extracted and separated by 1-DE followed by using MS compatible silver staining to visualize the protein bands, even the low abundant proteins (Figure 1). The entire lane was sliced into 12 fractions (marked by red boxes and numbered 1−12), and proteins in each fraction were identified by HPLC-Chip-QTOF-MS/MS. As shown in Table 1, a total of 134 proteins were identified in all 12 fractions. The numbers of proteins identified in fractions 1−12 were 23, 13, 15, 13, 11, 14, 7, 9, 10, 7, 6, and 6, respectively. These 134 proteins corresponded to 37 nonredundant proteins and, importantly, 17 proteins were identified in RJ for the first time. Because the MRJP family is the main component of RJ, MRJP1−MRJP7 were identified by 1-DE analysis in each fraction (Table 1). No identification of MRJP8 and MRJP9 was possible in this study; it is likely that our present approaches were not suitable to identify them or their abundance was too low to be identified.

In addition, 22 nonredundant proteins were identified by gel-free proteomics approach (Table 2). These included five newly found proteins, and three of them, regucalcin (RC) (senescence marker protein 30), apolipophorin-III-like protein, and Y4C6B.6, were overlapped with the protein identified by 1-DE, but only two other proteins, plasma glutamate carboxypeptidase isoform 1 and yellow-e2 CG17044-PA, were uniquely identified in this approach. However, the total protein number identified by the gel-free method was less than that identified by 1-DE analysis; it is probably the existence of the high-abundance peptides of MRJPs that influenced the signal intensity of some low-abundance peptides.

In total, 41 nonredundant proteins were identified by both gel-based and gel-free techniques (Table 3). As shown in Table 3, 11 MRJPs accounted for 26.8% of the total identified proteins, including 8 from *A. mellifera*, whereas MRJP1 precursor, MRJP4 precursor, and MRJP5 precursor were from *A. cerana*. Proteins related to protein biosynthesis were the second most represented (19.5%); they are ribosomal protein S5 isoform 1, ribosomal protein L3 CG4863-PA, isoform A, ribosomal protein S7 CG1883-PA, isoform A, elongation factor 2 (EF-2) isoform 1, translation elongation factor 1-γ isoform 2, ergic53 CG6822-PA, isoform A isoform 1, Sec61 CG9539-PA, and ADP/ATP translocase. Proteins involved in carbohydrate metabolism accounted for 17.1%, followed by oxidation−reduction (9.8%), hydrolase (9.8%), and protein binding (7.4%). The remaining proteins were involved in lipid transport (2.4%), lipid metabolism (2.4%), immunity (2.4%), and unknown (2.4%) categories. Interestingly, there were 18 protein overlapped by two methods, whereas 19 and 4 proteins were exclusively identified by both 1-DE and gel-free approaches, respectively (Figure 2).

DISCUSSION

Digging out as many proteins as possible that exist in RJ is a persistent pursuit for biologists. To this end, we employed gel-based and gel-free proteomic techniques as well as highly sensitive MS to increase coverage of RJ proteome. Fortunately, among those 41 nonredundant proteins, 19 novel proteins were identified by this complementary proteomics strategy. These newly found proteins not only improve proteome coverage of RJ but also help us get a better understanding of its biofunction for honeybee biology and human health promotion.

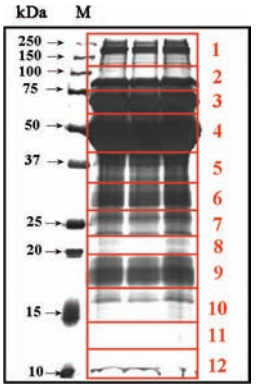

Figure 1. Separation of royal jelly proteins by one-dimensional gel electrophoresis. The proteins were stained according to a mass spectrometry compatible silver-staining method and separated into 12 fractions (marked by red boxes and labeled 1−12). Three replications were performed in each gel lane. The molecular weight markers (M) are indicated on the left. Protein was identified by high-performance liquid chromatography−chip quadruple time-of-flight tandem mass spectrometry.

As the most abundant protein family in RJ, MRJPs have multiple biological functions both for honeybees and for humans. Apart from supplying nutritive amino acids for honeybees,[25] MRJP1 can form to 420 kDa oligomeric subunits that are used in cosmetic products for moisturizing.[38] Still, it has cell proliferation functions in the human lymphoid cell[39] and could accelerate the wound-healing process through stimulating keratinocytes to produce cytokines.[40] Royalactin, a monomer of MRJP1, has effects of enhancing proliferation of rat hepatocytes and could possibly be used as a cytoprotective agent to prevent deterioration in human liver transplantation.[41,42] It is also established that MRJP1 and MRJP2 stimulate mouse macrophages to release tumor necrosis factor (TNF) for potential therapeutic functions in the areas of arthritis and cancer.[43,44] Moreover, MRJP2 and MRJP3 have been proved to have antimicrobial and anti-inflammatory functions.[45,46] Despite these advancements, information on the effects of other MRJPs in regard to health promotion is not yet evidently available.

Honeybees have evolved an extremely carbohydrate-rich diet of nectar and pollen, and they rely strongly on the energy produced from the carbohydrate metabolism to decide caste determination and energize foraging activities.[47] Although enzymes involved in carbohydrate metabolism such as glucose oxidase, α-glucosidase precursor, glucose dehydrogenase, α-amylase, and glucose dehydrogenase isoform 1 have been individually identified previously, all of these enzymes were found for the first time in one RJ sample in this study. This indicates that our complementary proteomics strategy has high efficiency in protein identification (Table 3; Figure 2). All seven identified proteins are related to carbohydrate metabolism including the previously identified five proteins and the newly identified proteins CG14476-PB, isoform B isoform 2, and Y4C6B.6. They work together to ensure the fast-developing queen and worker larvae meet their high requirement of carbohydrate metabolism during caste determination. In addition, glucose oxidase can catalyze glucose to form hydrogen peroxide, which may have an important role in antibacterial properties in RJ.[31]

Table 1. Proteins Identified by One-Dimensional Gel Electrophoresis[a]

protein accession no.	protein name	score	*Mr*	p*I*	no. of unique peptides	sequence coverage (%)	gel fraction (Figure *1*)	*E* value
gi|58585098	major royal jelly protein 1	15389	49311	5.1	25	73	1−12	
gi|110758964	regucalcin (RC) (senescence marker protein 30) (SMP-30)	1089	10211	8.76	4	61	6, 7, 9, 10	
gi|58585108	major royal jelly protein 2 precursor	2714	51441	6.83	17	50	1−10, 12	
gi|56422035	major royal jelly protein 3	19933	66055	6.87	21	50	1−5, 8−12	
gi|58585090	glucose oxidase	5036	68352	6.48	20	43	1−6, 8, 9, 11	
gi|66511547	glucocerebrosidase precursor	711	58315	5.19	10	34	3, 4	
gi|58585170	major royal jelly protein 4 precursor	920	53225	5.89	9	31	1−8, 12	
gi|284812514	MRJP5	2390	70477	6.11	12	31	1, 3−8, 10−12	
gi|58585144	α-amylase	99	56482	6.94	1	30	1	1.20×10^{-4}
gi|58585138	major royal jelly protein 5 precursor	70531	70531	5.95	12	25	2, 3, 6, 9	
gi|62198227	major royal jelly protein 7	1507	50851	4.9	7	24	1−6, 8, 9	
gi|58585164	α-glucosidase precursor	737	65694	5.06	8	23	1−6	
gi|48094573	hypothetical protein	398	19479	6.6	2	23	10, 11	
gi|40557703	major royal jelly protein MRJP1 precursor	1956	49394	5.5	5	21	1	
gi|110762641	ferritin 1 heavy chain homologue CG2216-PE, isoform E	104	17454	5.78	2	21	9	
gi|110757098	CG9451-PA	121	41533	7.99	4	20	6	
gi|110772962	glucose dehydrogenase, partial	5315	12543	4.31	1	19	1−8	2.30×10^{-12}
gi|166795901	apolipophorin-III-like protein	168	21335	5.48	2	16	9, 10	
gi|110756961	glucose dehydrogenase isoform 1	270	69933	5.58	4	15	3	
gi|42601246	major royal jelly protein MRJP5 precursor	434	68585	8.66	6	13	4, 6	
gi|110764063	ribosomal protein S7 CG1883-PA, isoform A	85	22348	9.8	1	13	1	6.70×10^{-6}
gi|66515987	translationally controlled tumor protein CG4800-PA isoform 1	75	19830	4.57	1	13	5	3.10×10^{-5}
gi|48120555	ribosomal protein S5 isoform 1	219	24345	9.51	1	12	1	3.40×10^{-11}
gi|110768510	protein disulfide-isomerase precursor (PDI), partial	170	24438	4.74	1	11	1	1.40×10^{-6}
gi|60115688	venom protein 2 precursor	59	24830	4.51	1	11	6	1.60×10^{-4}
gi|110749126	CG9518-PA	60	70764	6.58	3	10	3	
gi|58531215	ADP/ATP translocase	147	33193	9.71	1	9	1	1.30×10^{-9}
gi|66566113	ribosomal protein L3 CG4863-PA, isoform A	200	47520	10.26	1	7	1−3	1.70×10^{-6}
gi|110758966	CG7430-PA isoform 1	72	55118	7.55	1	6	1, 2	1.10×10^{-4}
gi|110761214	translation elongation factor 1-γ isoform 2	57	49240	6.02	1	5	1	9.80×10^{-5}
gi|110758966	Sec61 CG9539-PA	74	52811	8.64	1	5	1	1.70×10^{-4}
gi|66508439	elongation factor 2 (EF-2) isoform 1	132	95547	6.11	1	4	1	5.10×10^{-5}
gi|66500170	CG14476-PB, isoform B isoform 2	57	107169	5.84	1	4	2	9.10×10^{-5}
gi|66522931	ergic53 CG6822-PA, isoform A isoform 1	57	57838	5.87	1	3	1	3.10×10^{-4}
gi|48118838	Y4C6B.6	81	58933	7.03	1	3	4	3.10×10^{-7}
gi|110758214	Tudor-SN CG7008-PA	128	98935	8.09	1	3	1	1.40×10^{-5}
gi|58585188	major royal jelly protein 6	711	50154	5.89	1	2	1−9, 12	1.00×10^{-6}

[a] All of the identified proteins are from *A. mellifera* except major royal jelly protein MRJP1 precursor (gi|40557703) and major royal jelly protein MRJP5 precursor (gi|42601246), which are from *A. cerana*. Accession number is the unique number given to mark the entry of a protein in the database NCBInr. Protein name is given when proteins were identified by HPLC-Chip/ESI-QTOF-MS. Score is search against the database NCBInr. Theoretical molecular weight (*Mr*) and isoelectric point (p*I*) of the identified proteins were retrieved from the protein database of NCBInr. No. of unique peptides is the number of the unique peptide. 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. Gel fraction indicates from which fraction of the gel (Figure 1) the protein was identified, and if one protein was identified from more than one fraction only the highest score and sequence coverage and the maximal number of unique peptides are given. *E* value is the theoretical probability of obtaining false-positive protein identification; if the protein was identified on the basis of one peptide, this item was given and the figure should be less than 1 in 1000.

Protein biosynthesis plays a major role in ontogenesis of organisms. Among the novel proteins identified, several proteins, ribosomal protein S5 isoform 1, ribosomal protein L3 CG4863-PA isoform A, and ribosomal protein S7 CG1883-PA isoform A, function as architectural components of ribosomes responsible for the translational fidelity.[48−50] Elongation factor 2 (EF-2) isoform 1 and translation elongation factor 1-γ isoform 2 participate in aminoacyl-tRNA binding and translocation.[51] Simultaneously, ergic53 CG6822-PA isoform A isoform 1, Sec61 CG9539-PA, and ADP/ATP translocase play a great part in

Table 2. Proteins Identified by Gel-free Analysis[a]

peptide	score	sequence coverage (%)	accession no.	protein name	*Mr*	p*I*	matched sequence	matched sequence no.	*E* value
526	15356	79	gi|58585098	major royal jelly protein 1	49311	5.1	SLPILHEWK, FFDYDFGSDER, FFDYDFGSDERR, RQDAILSGEYDYK, QDAILSGEYDYK, NNYPSDIDQWHDK, NNYPSDIDQWHDKIFVTMLR, YNGVPSSLNVISK, KVGDGGPLLQPYPDWSFAK, VGDGGPLLQPYPDWSFAK, YDDCSGIVSASK, LAIDKCDR, LLTFDLTTSQLLK, QVEIPHDVAVNATTGK, LSSLAVQSLDCNTNSDTMVYIADEK, LSSLAVQSLDCNTNSDTMVYIADEK, LSSLAVQSLDCNTNSDTMVYIADEKGEGLIVYHNSDDSFHR, GEGLIVYHNSDDSFHR, LTSNTFDYDPK, MTIDGESYTAQDGISGMALSPMTNNLYYSPVASTSLYYVNTEQFR, TSDYQQNDIHYEGVQNILDTQSSAK, VVSKSGVLFFGLVGDSALGCWNEHR, SGVLFFGLVGDSALGCWNEHR, TVAQSDETLQMIASMK, EALPHVPIFDR, EYILVLSNK, MVNNDFNFDDVNFR, IMNANVNELILNTR	27	
291	11114	63	gi|110776421	similar to yellow-e2 CG17044-PA	39815	8.29	LTSNTFDYDPR, LTVAGESFTVK, NGICGIALSPVTNNLYYSPLSSHGLYYVDTEQFR, NGICGIALSPVTNNLYYSPLSSHGLYYVDTEQFRDPQYEENNVQYEGSQDILNTQSFGK, DPQYEENNVQYEGSQDILNTQSFGK, NGVLFLGLVGNSGIACVNEHQVLQR, ESFDVVAQNEETLQMIVSMK, ESFDVVAQNEETLQMIVSMKIMENLPQSGR, IMENLPQSGR, INDPEGNEYMLALSNR, IINNDFNFNDVNFR, ILGANVDDLMR, YHNQNAGNQNAGNQNAGNQNADNQNVDNQNANNQNADNQNANK	13	
337	9630	39	gi|40557703	major royal jelly protein MRJP1 precursor	49394	5.5	YNGVPSSLNVISK, LAIDKCDR, LLTFDLTTSQLLK, QVEIPHDVAVNATTGK, LSSLAVQPLDCNINGDTMVYIADEKGEGLIVYHDSDNSFHR, MTINGESFTTQSGISGMALSPMTNNLYYSPVASTSLYYVNTEQFR, VVSKSGVLFFGLVGDSALGCWNEHR, SGVLFFGLVGDSALGCWNEHR, EALPHVPIFDR	9	
279	7671	62	gi|58585108	major royal jelly protein 2 precursor	51441	6.83	YFDYDFGSEER, YFDYDFGSEERR, QAAIQSGEYDHTK, NYPFDVDQWR, DKTFVTILR, YDGVPSTLNVISGK, LLKPYPDWSFAEFK, IAIDKFDR, LHVFDLK, QIEIPHDIAVNATTGK, GGLVSLAVQAIDLANTLVYMADHK, GGLVSLAVQAIDLANTLVYMADHKGDALIVYQNADDSFHR, GDALIVYQNADDSFHR, LTSNTFDYDPR, MTIDGESFTLK, SQFGENNVQ	22	
165	6373	43	gi|58585170	major royal jelly protein 4 precursor	53225	5.89	YLDYDFDNDER, YLDYDFDNDERR, QAAIQSGEYDR, IAIDEYER, LFAFDLNTSQLLK, GELVSLTVQAMDSTNTMVYMVDNK, NTLIIYQNADDSFHR, LSSHTLNHNSDK, VYGMALSPVTHNLYYNSPSSENLYYVNTESLMK, SENQGNDVQYER, VQDVFDSQLTVK, NEDTLQMVVSMK, NEYLLALSDR, NEYLLALSDRNQNVLNNDLNLEHVNFQILGANVNDLIR, NQNV	15	
27	931	25	gi|284812514	MRJP5	70477	6.11	YLDYDFGSDER, YLDYDFGSDERR, QAAIQSGEYDHTK, NYPFDVDQWR, LLQPYPDWSWANYK, IAIDKFDR, LHVFDLNTSHQLK, LTSNTFDYDPK, SEYGANNVQYQGVQDIFNTESIAK, ENMDMVAQNEETLQTVVAMK, FINNDYNFNEVNFR, ILGANVNDLIMNTR	12	
17	635	14	gi|42601246	major royal jelly protein MRJP5 precursor	68585	8.66	QAAIQSGEYDHTK, NYPFDVDR, IAIDKFDR, LHVFDLNTSQQIK, LTSNTFDYDPK, QNMDMVAQNEETLQTVVAMK, FINNDYNFNEVNFR	7	
24	414	26	gi|62198227	major royal jelly protein 7	50851	4.9	NYPFDVDQWR, LLVFDLNSSQLIK, QVDIPHEIAVNTTTEQGR, SLAVQAISSVNTLVYIADNK, SLAVQAISSVNTLVYIADNKGDGLIVYQNSDDSFHR, GDGLIVYQNSDDSFHR, LTSNTFNYDPR, ILNNDLNFNDINFR, ILIGGVSDLLENTR	9	
14	189	18	gi|42601244	major royal jelly protein MRJP4 precursor	55279	6	QAAIQSGEYDR, TGNGGPLLQPYPDWSFAK, IAIDKFDR, LWVLDSGLVNNIQPMCSPK, QVEIPHDVAVNATTGK, DDALIVYQNADDSFHR	6	

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Table 2. Continued

peptide	score	sequence coverage (%)	accession no.	protein name	*Mr*	p*I*	matched sequence	matched sequence no.	*E* value
55	1867	19	gi|110772962	glucose dehydrogenase, partial	12543	4.31	IPDLSYDFIVVGGGAAGAVVAGR	1	2.30×10^{-12}
47	1293	26	gi|58585090	glucose oxidase	68352	6.48	ATGVNVLINGR, INGFTVAQTISR, AAEEAGFGVSEDLSGDR, AADFIKEDWGELLQLL, EVILSAGSVNTPQLLMLSGIGPK, REVILSAGSVNTPQLLMLSGIGPK, VADASVQPQVISGNPVASVNMVGER, ITLNSKDPLDPPVIWSNDLATEHDR, AAEEAGFGVSEDLSGDRINGFTVAQTISR, VLLLEAGPDEPAGAEIPSNLQLYLGGDLDWK	10	
17	1116	27	gi|254910938	defensin preproprotein	11052	6.28	VTCDLLSFK, GQVNDSACAANCLSLGK	2	
22	715	11	gi|58585164	α-glucosidase precursor	65694	5.06	EDLIVYQVYPR, IILDFVPNHTSDQHEWFQLSLK, YYDYGADFPFNFAFIK, VSALGFFILISQDAK	4	
14	410	53	gi|110758964	regucalcin (RC) (senescence marker protein 30) (SMP-30)	10211	8.76	GLGVYGLVANSFK, GLGVYGLVANSFKI, ELTEKPYSGYVFAIK, VTSCTFGGPLLDTLFVTTSSR	4	
4	221	28	gi|166795901	apolipophorin-III-like protein	21335	5.48	DQSANFVNNIQDYIK, QIQEQWNIPDQDTIVK, QAVDIAVQATQNLNNQLQTAATQK	3	
6	213	19	gi|48094573	hypothetical protein	19479	6.6	ASTPIENLIEIFDTIK, EQVANALLEGSSDLIGALK	2	
2	103	3	gi|110757098	CG9451-PA	41533	7.99	LDELAVYDILIR	1	6.60×10^{-6}
1	101	3	gi|48118838	Y4C6B.6	58933	7.03	IIGFGGAFTDATGINIAK	1	9.70×10^{-8}
5	100	6	gi|66511547	glucocerebrosidase precursor	58315	5.19	GTFHWYVSSR, LGLPWFVNEIFK, FFSAAWSAPTWMK	3	
3	94	3	gi|110756961	glucose dehydrogenase isoform 1	69933	5.58	EIIVSGGAVNSPQILLLSGIGPK	1	1.80×10^{-5}
4	82	6	gi|60115688	venom protein 2 precursor	24830	4.51	KNVDTVLVLPSIER, NVDTVLVLPSIER	2	
1	61	3	gi|66564326	plasma glutamate carboxypeptidase isoform 1	53028	5.26	IPVASITVEDATLLR	1	3.50×10^{-4}

[a] All of the identified proteins are from *A. mellifera* except major royal jelly protein MRJP1 precursor (gi|40557703), major royal jelly protein MRJP4 precursor (gi|42601244), and major royal jelly protein MRJP5 precursor (gi|42601246) are from *A. cerana*. Peptide is the number of pairing an experimental fragmentation spectrum to a theoretical segment of protein, and searched is the total searched peptide. Score is search against the database NCBInr. 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. Accession number is the unique number given to mark the entry of a protein in the database NCBInr. Protein name is given when proteins were identified by HPLC-Chip/ESI-QTOF-MS. Theoretical molecular weight (M_r) and isoelectric point (p*I*) of the identified proteins were retrieved from the protein database NCBInr. Matched sequence is all the unique peptides identified in the protein. Matched sequence number is the number of the unique peptide. *E* value is the theoretical probability of obtaining false-positive protein identification; if the protein was identified on the basis of one peptide, this item was given and the figure should be <1 in 1000.

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Table 3. Total Nonredundant Identified Proteins from Gel-Based and Gel-free Analyses[a]

accession no.	protein name	gel-based	gel-free	functional category	percentage
gi|58585098	major royal jelly protein 1	yes	yes	major royal jelly protein family	26.8
gi|40557703	major royal jelly protein MRJP1 precursor	yes	yes		
gi|58585108	major royal jelly protein 2 precursor	yes	yes		
gi|56422035	major royal jelly protein 3	yes			
gi|58585170	major royal jelly protein 4 precursor	yes	yes		
gi|42601244	major royal jelly protein MRJP4 precursor		yes		
gi|284812514	MRJP5	yes	yes		
gi|58585138	major royal jelly protein 5 precursor	yes			
gi|42601246	major royal jelly protein MRJP5 precursor	yes	yes		
gi|58585188	major royal jelly protein 6	yes			
gi|62198227	major royal jelly protein 7	yes	yes		
gi|48120555	ribosomal protein S5 isoform 1	yes		protein biosynthesis	19.5
gi|66566113	ribosomal protein L3 CG4863-PA, isoform A	yes			
gi|66508439	elongation factor 2 (EF-2) isoform 1	yes			
gi|110764063	ribosomal protein S7 CG1883-PA, isoform A	yes			
gi|110761214	translation elongation factor 1-γ isoform 2	yes			
gi|58531215	ADP/ATP translocase	yes			
gi|110758966	Sec61 CG9539-PA	yes			
gi|66522931	ergic53 CG6822-PA, isoform A isoform 1	yes			
gi|58585090	glucose oxidase	yes	yes	carbohydrate metabolism	17.1
gi|58585164	α-glucosidase precursor	yes	yes		
gi|110772962	glucose dehydrogenase, partial	yes	yes		
gi|58585144	α-amylase	yes			
gi|110756961	glucose dehydrogenase isoform 1	yes	yes		
gi|66500170	CG14476-PB, isoform B isoform 2	yes			
gi|48118838	Y4C6B.6	yes	yes		
gi|110762641	ferritin 1 heavy chain homologue CG2216-PE, isoform E	yes		oxidation−reduction	9.8
gi|110749126	CG9518-PA	yes			
gi|110768510	protein disulfide-isomerase precursor (PDI), partial	yes			
gi|110758966	CG7430-PA isoform 1	yes			
gi|110758214	Tudor-SN CG7008-PA	yes		hydrolase	9.8
gi|110757098	CG9451-PA	yes	yes		
gi|60115688	venom protein 2 precursor	yes	yes		
gi|66564326	plasma glutamate carboxypeptidase isoform 1		yes		
gi|110758964	regucalcin (RC) (senescence marker protein 30) (SMP-30)	yes	yes	protein binding	7.4
gi|66515987	translationally controlled tumor protein CG4800-PA isoform 1	yes			
gi|110776421	yellow-e2 CG17044-PA		yes		
gi|166795901	apoliporphorin-III-like protein	yes	yes	lipid transport	2.4
gi|66511547	glucocerebrosidase precursor	yes	yes	lipid metabolism	2.4
gi|254910938	defensin preproprotein		yes	immunity	2.4
gi|48094573	hypothetical protein	yes	yes	unknown	2.4

[a] All of the nonredundant proteins identified by both gel-based and gel-free techniques are classified into functional groups. The table lists accession number, protein name, functional category, and percentage.

protein transportation.[52] The heteromeric Sec61 complex can form a channel across the endoplasmic reticulum (ER) membrane, through which newly synthesized secretory proteins can be translocated, whereas misfolded proteins can be dislocated from the ER and into the cytosol.[53] Ergic53 is a cargo receptor protein operating in the transport of newly synthesized glycoproteins from the ER to the ER-Golgi intermediate compartment.[54] Most of the time, the function of these proteins is intracellular, so the

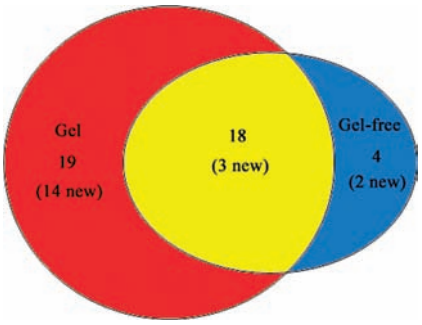

Figure 2. Comparison of the nonredundant protein number identified by both gel-free and gel-based techniques. Venn diagram shows the taxonomical distribution of 41 nonredundant proteins. Bold numbers indicate the amount of proteins in each partition. Numbers in parentheses are numbers of newly identified proteins in this study.

origination of these proteins in RJ cannot be fully excluded from the disruption of some hypopharyngeal gland cells during the RJ secretory process. However, honeybee larvae develop very quickly in both size and weight after hatching, and because queen larvae continue to be fed with RJ, they gain weight more rapidly and grow larger.[55,56] Therefore, this suggests that these proteins may partly contribute to the development of honeybee larvae, especially queen larvae. Although there is no definite evidence on human health promotion, these proteins may help in cell growth and tissue reparation and renewal, which makes RJ a popular supplement for professional athletes for better performance and injury recuperation.[57]

There were two proteins involved in protein binding identified for the first time, regucalcin (RC) (senescence marker protein 30) and translationally controlled tumor protein CG4800-PA isoform 1. RC is a calcium-binding protein and plays an important role in the maintenance of intracellular Ca^{2+} homeostasis.[58,59] Moreover, because Ca^{2+} is a second messenger for signal transduction, it is essential for various cellular functions, thus indicating that regucalcin is involved in the regulation of protein kinases and protein phosphatasesand possesses gluconolactonase activity and activity against oxidative damage.[59−61] This protein, also known as senescence marker protein, as the name implies, has a close relationship with age-associated deterioration of cellular functions in tissues and organs.[62−64] Translationally controlled tumor protein also has calcium-binding activity, which is implicated in cell growth, cell cycle progression, and the protection of cells against various stress conditions and apoptosis.[65−67] The biological function of these two proteins may partly explain the longer longevity of queens and the morphology difference between the queen and worker bees.[68] Furthermore, these two proteins may be why RJ could be used against gastric and duodenal ulcers and improve regeneration of skin in wounds for human beings.[69]

Apolipophorin-III-like protein was also identified in RJ for the first. It is a lipid-binding protein that can form protein−lipid complexes, transport lipids in the aqueous environment, and regenerate high-density lipoprotein.[70,71] Triacylglycerols constitute a subgroup of lipids, hydrolyzed into free fatty acids when adipokinetic action occurs, whereas apolipophorin-III plays a crucial role in stabilization purposes during this process.[71] In addition, apolipophorin-III has been reported to increase antibacterial activity and participate in cellular immune responses in several insects.[72−75] Therefore, this protein may potentially have a function in the involvement of larvae development and immune responses. Particularly, due to its lipid transport ability, this is better for treatment of atherosclerosis.[76]

As to several other proteins identified, protein disulfide-isomerase precursor (PDI) is a multifunctional protein, which catalyzes the isomerization of intramolecular disulfide bridges and helps the proper folding of other proteins.[77,78] CG9518-PA and CG7430-PA isoform 1 participate in ion transfer during oxidation−reduction reaction. Because reactive oxygen species are produced by respiration of a fast-growing organism causing oxidative damage, we suppose these two proteins probably function in elimination of the oxygen radical and protection against oxidative injury for the developing honeybee larvae and also support the idea that RJ has antioxidant activities for humans.[79]

In conclusion, our complementary gel-based and gel-free proteomic platform is efficient to analyze protein components of RJ. There were 19 proteins identified in RJ for the first time, and most of them were related to protein biosynthesis, carbohydrate metabolism, and oxidation−reduction processes. This new finding significantly expands coverage of the RJ proteome and opens a new way for further understanding and development of RJ. To identify more proteins in RJ, further study is required to remove the high-abundance proteins such as MRJPs to allow identification of more low-abundant proteins. In addition, it is necessary to separate and purify these novel proteins for in vitro experiments to confirm their biological function and better appreciate their potential use for human health.

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