



Isolation and characterization of proteins and lipids from honeybee (*Apis mellifera* L.) queen larvae and royal jelly



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ABSTRACT

Honeybee queen larvae (QL), the byproduct of royal jelly (RJ) production, have not been fully utilized as a food resource. Proteins in QL were characterized by gel electrophoresis and identified by matrix-assisted laser desorption/ionization time of flight mass spectrometry. Qualitative and quantitative analysis of fatty acid and sterol composition in QL and RJ were performed. The molecular weight of proteins in QL was generally lower than those in RJ. QL were dominated by major royal jelly protein 1 (MRJP1) and MRJP2 as well as their degradation proteins. High amounts of triacylglycerols (797.5 mg/g lipid) and phospholipids (89.8 mg/g lipid) were presented in QL, whereas the main fatty acids in RJ were composed of free middle carbon chain fatty acids (>91%). Twelve sterols were identified and clerosterol, Δ^7 -sitosterol and cycloartenol were reported for the first time in QL. Noteworthy, little difference was observed in the sterol profiles of QL and RJ. Sterols were found to be integrated with MRJP1 oligomer in QL and RJ. This study provides new insight into the nutritional values of QL. The results suggest overall that proteins and lipids of QL compare favorably with those of RJ and could be potential used in food industry.

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1. Introduction

Recently, natural products from insects have been of considerable interest in food and medicine industries (Ratcliffe, Mello, Garcia, Butt, & Azambuja, 2011). Insects commonly consist of high amount of proteins and lipids, accounting for up to 80% of the dry matter (Avila, Sirot, LaFlamme, Rubinstein, & Wolfner, 2011). Proteins from insects are often used as food ingredients for the high net protein utilization and high proportion of lysine and threonine (DeFoliart, 1992). Moreover, some extracts from insect body had been traditionally used in folk medicine, for treating inflammatory and immune diseases, cancer and other diseases (Lai & Gallo, 2009; Lee et al., 2007; Ratcliffe et al., 2011). Insects have been playing a crucial role in human nutrition for a long time in Asia and other parts of the world.

Royal jelly (RJ), a nutritional reservoir for the growth and development of larvae of honeybee (*Apis mellifera* L.) queen, is secreted from the hypopharyngeal and mandibular glands of worker bees (Huang et al., 2012). It is a rich source of proteins which are composed of a major royal jelly protein (MRJP) family (Li, Wang, Zhang, & Pan, 2007; Nozaki et al., 2012). In addition, RJ contains a unique hydroxy fatty acid, trans-10-hydroxy-2-decenoic acid (HDA) (Terada, Narukawa, & Watanabe, 2011). Substantial evidence indicated that these bioactive components might have many beneficial effects, including antibacterial, antiinflammatory, antioxidant, immunostimulatory and liver regeneration promoting activities (Fujiwara et al., 1990; Majtán, Kováčová,

Bíliková, & Simúth, 2006; Kamakura, Suenobu, & Fukushima, 2001; Ramadan & Al-Ghamdi, 2012). Although the cause and effect relationship has not been established between the consumption of RJ and the physiological functions up to now (Agostoni et al., 2011), RJ has been used extensively for improving human health in food and nutraceutical industry.

China is the biggest RJ producing country with an output of greater than 90% of the world's total productions (Li et al., 2007). Queen larvae (QL) are the byproduct of royal jelly production. During the production, QL are usually unwanted and might be finally discarded by bee keepers. In fact, QL consume RJ as the exclusive food and it could serve as a new rich source of protein. To date, the abundant QL have not been fully utilized as an insect food resource.

The composition of proteins and lipids in QL determines its properties, which would dominate its nutraceutical function as functional food. However, this information has not clearly been defined yet, particularly about the relationship between proteins and lipids of QL. The present work was therefore undertaken to explore the protein and lipid profiles of QL with RJ as a reference. In addition, the relationship between proteins and sterols in lipids was studied.

2. Materials and methods

2.1. Chemicals

Low molecular weight protein markers were purchased from Fermentas. Bovine serum albumin and coomassie brilliant blue (CBB) R-250 were from Amresco. Phenylmethanesulfonyl fluoride (PMSF),

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cholesterol, campesterol, stigmasterol, β -sitosterol, 5- α -cholestane (internal standard), pyridine, and bis-(trimethylsilyl)-trifluoroacetamide with 1% trimethylchlorosilane (BSTFA) were purchased from Sigma-Aldrich Chemical Co. Acetonitrile used in liquid chromatography analysis was of HPLC grade; all other chemicals were of analytical grade, unless otherwise stated.

2.2. Materials

The 3 day (72 h) queen honeybee larvae and royal jelly were collected from queen cells in the beehive in June 2011 at Bee Research Institute, Chinese Academy of Agricultural Science. The honeybee (*A. mellifera* L.) eggs were laid by the queen bee in the frame and kept for incubation. Then, the young larvae were transferred to the queen cells in the frame for 3 days to sample queen larvae and royal jelly. After washed by 0.9% saline solution, QL samples were stored at -80°C until used.

2.3. Preparation of proteins from QL and RJ

For water soluble (WS) protein extraction, samples of QL (10.0 g) were stirred with 50 mL of water (containing 0.1 mM PMSF) at 4°C for 20 min. The slurry was then centrifuged at 25,000 g for 20 min at 4°C . Sequential extraction of salt soluble (SS) fraction, ethanol soluble (ES) fraction and alkaline soluble (AS) fraction were carried out by using 2% NaCl and 70% ethanol, followed by 0.05 M NaOH. The extraction processes were repeated twice for each fraction. All the extracts were concentrated and desalted extensively using Amicon® Ultra-15 Centrifugal Filter Devices. The extracts were lyophilized for further separation. WS protein of RJ was prepared in the same manner as above.

2.4. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

The WS, SS, ES, and AS protein fractions of QL and WS protein fraction of RJ were analyzed by SDS-PAGE on a discontinuous buffered system using 12% SDS polyacrylamide gel (1.50 mm). For each sample, aliquots (10 μL) containing 30 μg of protein were applied to each lane. Electrophoresis was conducted at a constant voltage of 80 V for 2 h. Gels were stained with coomassie brilliant blue (CBB) R-250.

2.5. Purification of QL proteins with column chromatography

The albumin of QL was purified by anion-exchange chromatography on a Macro-Prep High Q ion exchange column. In detail, albumin was dissolved in Tris-HCl buffer (20 mM, pH 8.6) and 5 mL of solution (10 mg/mL) was loaded on the column (20 cm $\times$ 1.5 cm) which had previously been equilibrated with the same Tris-HCl buffer. The column was initially eluted with Tris-HCl buffer at a flow rate of 1.0 mL/min for 60 min to remove unbound materials. It was then eluted with a gradient of 0–1 M NaCl in Tris-HCl buffer (20 mM, pH 8.6) over 120 min. The eluate was monitored at 280 nm. Seven fractions were collected, desalted and freeze-dried. Determination of protein concentration was performed according to the method of Bradford using BSA as a standard (Bradford, 1976).

2.6. Gel filtration chromatography (GFC)

The GFC experiment was performed on a Shimadzu LC-20 high performance liquid chromatography (HPLC) system (Shimadzu Co., Japan). Separation of albumin was carried out with a Shim-pack DIOL-300 column (250 mm $\times$ 7.9 mm i.d.). The mobile phase was consisted of 0.01 M phosphate buffer (pH 7.0) containing 0.2 M sodium sulfate. The system was operated at 1 mL/min and the absorbance was recorded at 280 nm in a diode array detector. Standard proteins used were thyroglobulin

(669 kDa), catalase (232 kDa), bovine serum albumin (67 kDa), myoglobin (16.7 kDa) and lysozyme (14.4 kDa).

2.7. Reversed phase high performance liquid chromatography (RP-HPLC) purification

Albumin fractions with high protein concentration were separated by reversed phase high performance liquid chromatography (RP-HPLC) with a ZORBAX 300SB C8 column (150 mm $\times$ 4.6 mm i.d., 5 μm) for further purification. The column was kept at 35°C . Solvent A was 0.1% trifluoroacetic acid (TFA) in water/acetonitrile (95:5, v/v), and solvent B was 0.85% TFA in 95% water/acetonitrile (5:95, v/v). Elution was carried out with a linear gradient from 10% to 70% of solvent B in 30 min at a flow rate of 1 mL/min. The detection was monitored at 280 nm. The purified protein subfractions were analyzed by SDS-PAGE.

2.8. Native PAGE

Fractions P3-7 and P6-6 purified by gel filtration chromatography (GFC) as well as WS fractions of QL and RJ were analyzed by native PAGE. The protein samples were solubilized in 0.125 M Tris-HCl buffer (pH 6.8), containing 25% (v/v) glycerol and 1% (w/v) bromophenol blue. Electrophoresis was run with 8% polyacrylamide gel (1.50 mm) at 4°C . Gels were stained with coomassie brilliant blue (CBB) R-250.

2.9. Matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF/MS)

Peptide mass fingerprinting was performed to identify the purified protein. The bands in the gels were destained with 25 mM NH_4HCO_3 in 50% acetonitrile and then dried completely. The proteins were then digested with trypsin (10 ng/ μL) for 16 h at 37°C in 50 mM NH_4HCO_3 . The peptide fragments were extracted by 50% acetonitrile containing 1% trifluoroacetic acid (TFA) and dried in a Speed-Vac system. The peptides were spotted onto the MALDI plate preloaded with 1 μL of CHCA (α -cyano-4-hydroxycinnamic acid, 10 $\mu\text{g}/\mu\text{L}$) and allowed to air dried prior to the MALDI analysis. The spectra were recorded in the linear, positive mode ion mode with an accelerating voltage of 20 kV and 100 laser shots were accumulated to obtain the final spectrum. The peak lists of the peptide mass values were searched against MASCOT.

2.10. Lipid extraction and composition analysis

QL and RJ were freeze-dried for lipid extraction. The lipid was extracted according to the procedure described by Yoshida, Tomiyama, Tanaka, and Mizushima (2007). Briefly, QL powder was triple-extracted with chloroform/methanol (2:1 v/v) and filtrated with a folded filter paper to recover the liquid phase. The filtrates were concentrated with a rotary vacuum evaporator. The extract was dissolved in the extraction solvent (100 mL) and washed with 20 mL of 0.75% sodium chloride solution and vigorously mixed. After phase separation, the lipid in the lower chloroform phase was obtained through drying with anhydrous sodium sulfate and desolventizing. The extracts were then weighted to obtain the yields of lipids. The total lipids were separated by preparative TLC into different fractions. Fatty acid composition and the sterols were determined according to our previous methods (Xu, Dong, Mu, & Sun, 2011).

2.11. Statistical analyses

All measurements were conducted in triplicate. The results were presented as the means $\pm$ SD. Data were analyzed by one way analysis of variance (ANOVA) using SPSS 17.0 and differences were considered to be significant at $P < 0.05$.

3. Results and discussion

3.1. SDS-PAGE

Molecular weight profiles of proteins in QL extracted by different solvents and water soluble proteins in RJ were analyzed by SDS-PAGE (Fig. 1). RJ had six bands at 85, 79, 68, 60, 56 and 49 kDa, representing glucose oxidase, MRJP5, MRJP3, MRJP4, MRJP1 and MRJP2, respectively (Li et al., 2007). MRJP1, the dominant protein in RJ, was indistinct in the gel since it was a typical glycoprotein with non-protein substituents (Furusawa et al., 2008). Albumin and globulin from QL consisted of a number of bands with different electrophoretic mobilities that were quite distinct from the water soluble proteins of RJ. The molecular weights of most proteins in the albumin and globulin of QL were much lower than MRJPs. Furthermore, the albumin had a slightly different protein composition profiles from the globulin. Intensive protein bands of albumin appeared between 30 and 50 kDa, while globulin spreaded from 25 to 40 kDa. The ES fraction only contained some low molecular weight peptides. AS fraction had low molecular bands below 14.4 kDa which were similar to the peptide composition of the ES fraction. The extraction yields of WS, SS, ES and AS fractions were 24.7%, 13.2%, 1.1% and 6.1%, respectively. WS fraction of QL was therefore employed for further purification.

3.2. Comparison of WS fractions of QL and RJ

Water extract solutions of queen bee larvae and royal jelly were analyzed by gel filtration chromatography for determination of molecular weight (MW) distribution based on the molecular weight of protein standards (Fig. 2). The MWs of four protein peaks in royal jelly were approximately 290 kDa, 130 kDa, 60 kDa and 28.2 kDa. This is in accordance with the results of separation of RJ water soluble proteins with a TSK G3000SW column (Kamakura, Fukuda, Fukushima, & Yonekura, 2001). The evidence pointed to the 290 kDa protein as MRJP1 complex (Tamura, Amano, et al., 2009; Tamura, Kono, Harada, Yamaguchi, & Moriyama, 2009). In QL sample, the molecular weight of the peak (6.252 min) was calculated to be about 270 kDa, which is slightly smaller than the oligomer of MRJP1 in RJ. The protein of QL was predominant in the peak with retention time of 7.516 min (about 40 kDa).

3.3. Purification of major proteins in WS fraction of QL

A lyophilized WS fraction was chromatographed on a high Q support anion exchange column. The eluate was divided into seven major

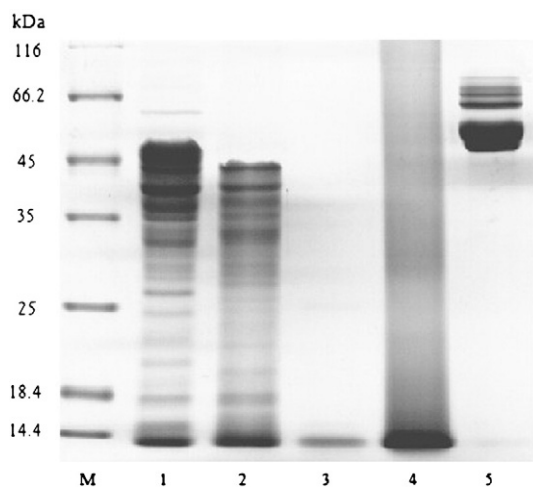


Fig. 1. SDS-PAGE analysis of queen larvae (QL) and royal jelly (RJ) protein fractions. M: molecular weight marker; lanes 1–4: protein fractions of QL: 1—water soluble (WS) fraction; 2—salt soluble (SS) fraction; 3—ethanol soluble (ES) fraction; and 4—alkaline soluble (AS) fraction; and lane 5—WS fraction of RJ.

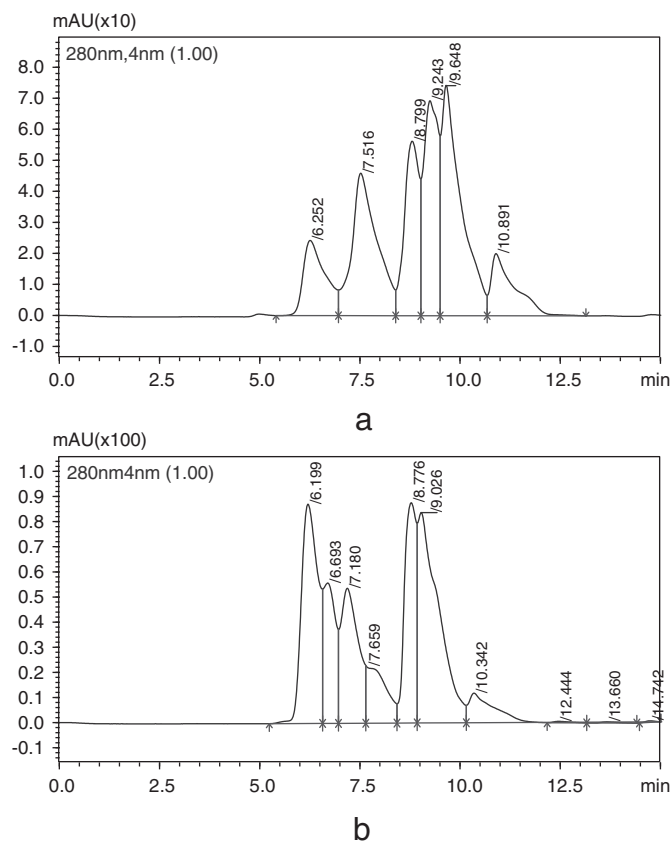


Fig. 2. Separation profiles of albumin from queen larvae (a) and royal jelly (b) by gel filtration chromatography on DIOL-300 column.

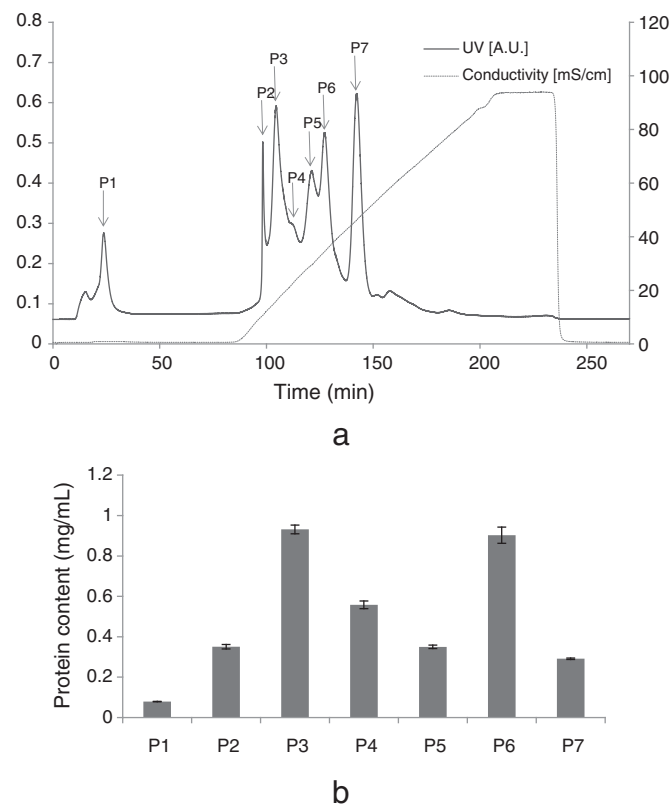


Fig. 3. Separation profiles of the albumin from queen larvae by anion exchange chromatography (a) and the protein content of the eluates P1–P7 respectively (b).

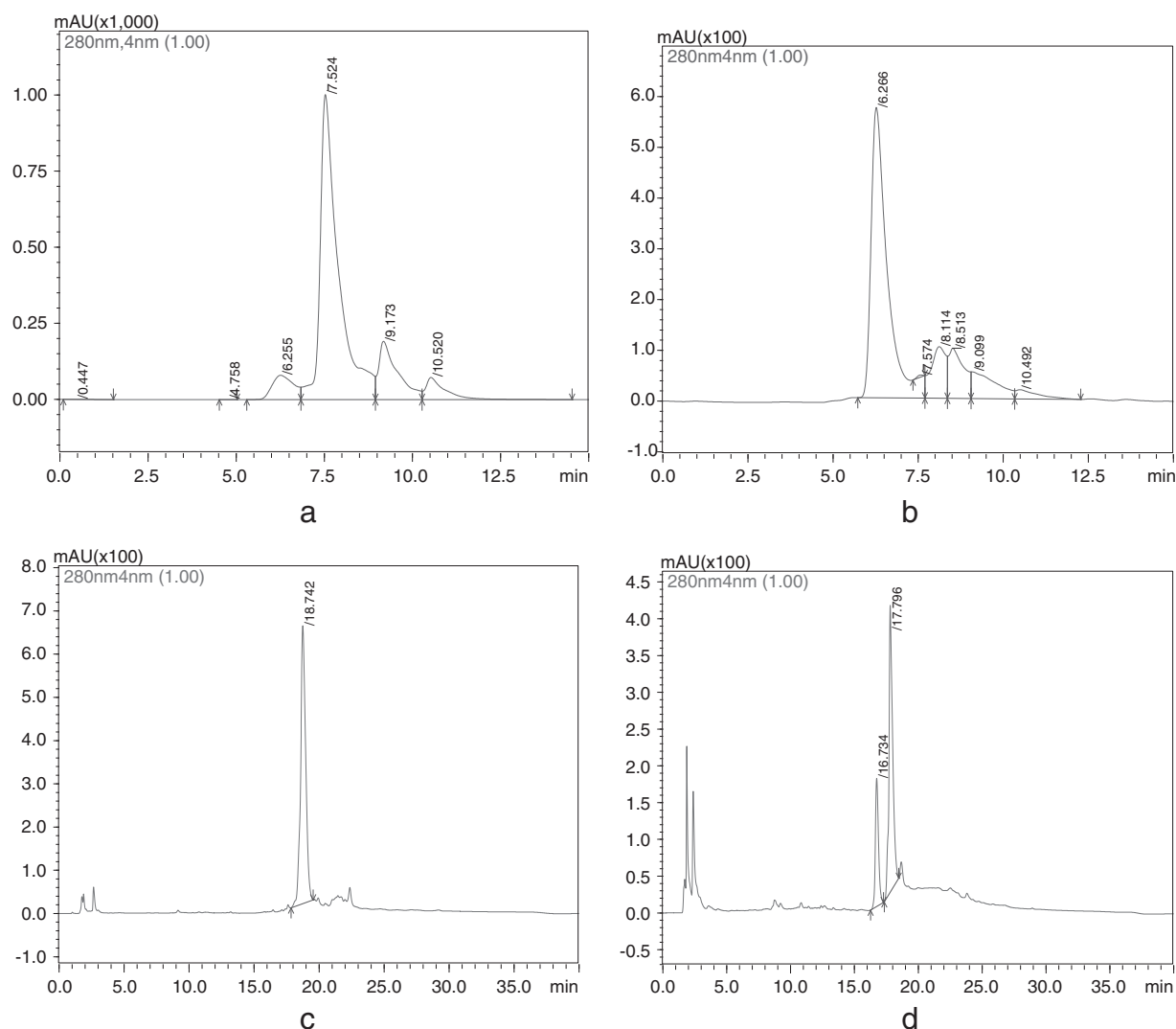


Fig. 4. Purification of major proteins in the albumin from queen larvae. (a) Gel filtration chromatogram of P-3 on DIOL-300 column; subfraction P3-7 (7.524 min) was collected; (b) gel filtration chromatogram of P-6 on DIOL-300 column; subfraction P6-6 (6.266 min) was collected; (c) RP-HPLC chromatogram of P3-7 on 300 Å SB-C8 column; new subfraction P3-7-18 was collected; and (d) RP-HPLC chromatogram of P6-6 on 300 Å SB-C8 column; new subfractions of P6-6-16 and P6-6-17 were collected.

fractions according to ultraviolet absorbance at 280 nm. The content of protein was the highest in the P3, followed by P6 fraction (Fig. 3). Additionally, P3 and P6 were found to correspond to the peaks of 7.5 min and 6.2 min, respectively. Thereafter, both fractions were fractionated by size exclusion chromatography (Fig. 4(a) and (b)). Subfraction P3-7 (7.5 min) was collected from P3 fraction, pooled and subjected to RP-HPLC (Fig. 4(c)). The chromatography profile displayed a major peak (P3-7) which contained 2 bands as confirmed by SDS-PAGE analysis (Fig. 5, lane 3). Similarly, subfraction P6-6 (6.2 min), which collected from P6 fraction, split up into two new subfractions (P6-6-16 and P6-6-17) during the reversed phase chromatography (Fig. 4(d)). SDS-PAGE profile showed that both subfractions appeared as one band with an apparent molecular mass of 35 kDa and 56 kDa, respectively (Fig. 5, lanes 5 and 6).

3.4. Native PAGE analysis

The P3-7 and P6-6 fractions isolated from the complex groups of polypeptides in QL were examined by native PAGE to verify the homogeneity of the protein. Both P3-7 and P6-6 migrated as a single diffuse band on the gels (Fig. 6). In contrast, water soluble fractions of QL gave 6 major peaks. Three bands of proteins were distributed in the range of 440–669 kDa. The two main bands around 140 kDa corresponded to

P6-6 and P3-7. Other two bands of lower MW proteins were also present. According to Kamakura, Fukuda, et al. (2001), MRJP1 oligomer and royalactin in RJ sample were dominated in native PAGE. The results also

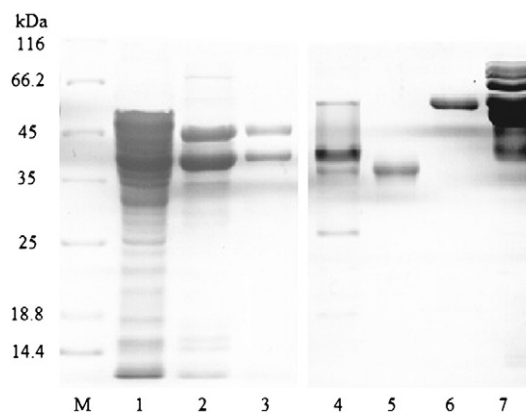


Fig. 5. SDS-PAGE analysis of protein fractions from queen larvae. M: molecular weight marker; lane 1: water soluble (WS) fraction of queen larvae; lanes 2 and 4: P3-7 and P6-6 fractions purified by gel filtration chromatography (GFC); lanes 3, 5 and 6: P3-7-18, P6-6-16 and P6-6-17 subfractions purified by GFC followed by RP-HPLC; and lane 7: WS fraction of royal jelly.

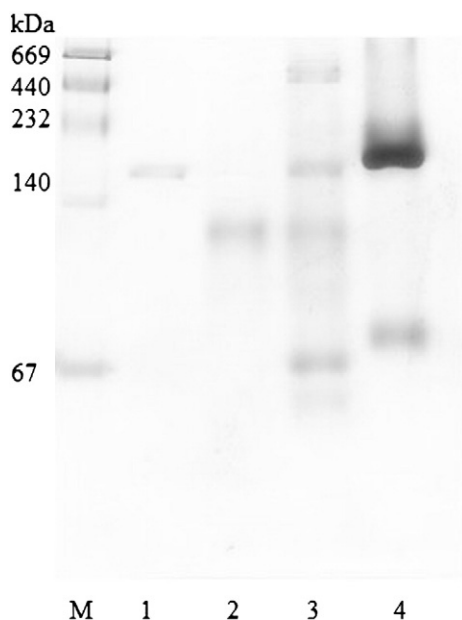


Fig. 6. Native PAGE analysis of protein fractions purified by gel filtration chromatography (GFC). M: molecular weight marker; lanes 1 and 2: P3-7 and P6-6 fractions purified by gel filtration chromatography (GFC); and lanes 3 and 4: water soluble (WS) fractions of queen larvae and royal jelly.

showed that QL proteins were structurally complex compared with RJ which was in agreement with the SDS-PAGE results.

3.5. Identification the proteins by MALDI-TOF/MS

The proteins in P3-7-18 were identified by peptide mass fingerprinting (PMF) as isoforms of MRJP2 (Table 1). The 35 kDa protein was a degraded MRJP2. MRJP2 was found to be relatively stable at room temperature after a long time storage (Li, Feng, Zhang, Zhang, & Pan, 2008). Our results implied that the MRJP2 was more resistant to digestion in QL compared with other MRJPs. In contrast, P6-6-17 was identified as MRJP1, while P6-6-16 was the degraded protein and detaching a polypeptide of 20 kDa from MRJP1. According to Tamura, Kono, et al. (2009), MRJP1 oligomer in RJ is a pentamer of MRJP1 monomers. The peak area of Fig. 4(d) showed that the relative proportion of P6-6-16 and P6-6-17 was approximately 1:4. It demonstrated that the P6-6 was also an pentamer of MRJP1 containing one modified monomer. The results demonstrated that the main components of QL proteins were still MRJP1 and MRJP2; however, they were quite distinct from MRJPs of RJ in the composition and structure.

3.6. Lipids and fatty acid composition analysis

QL yielded higher amount of lipid ($3.43 \pm 0.20\%$) than RJ ($3.06 \pm 0.06\%$). Lipid components in QL included hydrocarbons, triacylglycerols, free fatty acids, sterols, diacylglycerols and phospholipids. Triacylglycerols were the principal of QL lipid, which covered over 79% of the

Table 2

Lipid components in queen larvae (QL) and royal jelly (RJ).

Lipid	Content (mg/g lipid)	
	QL	RJ
Hydrocarbons	$26.47 \pm 9.21a$	$12.15 \pm 3.70b$
Triacylglycerols	$797.51 \pm 27.08a$	$16.98 \pm 0.85b$
Free fatty acids	33.24 ± 1.78	–
Sterols	$23.29 \pm 4.84a$	$25.69 \pm 8.73a$
Diacylglycerols	$29.73 \pm 3.44a$	$16.03 \pm 3.03b$
Middle carbon chain fatty acids	–	919.6 ± 18.85
Phospholipids	$89.85 \pm 19.80a$	$9.6 \pm 2.54b$

Values are mean $\pm$ SD, n = 3; –, not detected.

total lipids. As shown in Table 3, the main fatty acid distributions in triacylglycerols of QL were palmitic, stearic and oleic acids. Phospholipids were the second most abundant constituents, followed by free fatty acids and diacylglycerols. On the whole, the fatty acid pattern of the different lipid classes in QL was similar as that of triacylglycerols, containing significant quantities of saturated fatty acids (>50%).

In contrast, RJ showed a different lipid composition and fatty acid profiles. The predominant components were middle carbon chain fatty acids (Table 2). It is easily accountable due to the high HDA content in RJ (about 2% of fresh weight) (Antinelli et al., 2003). The hydrocarbons, triacylglycerols, diacylglycerols and phospholipids were present in small quantities, which were significantly different ($P < 0.05$) from the lipid contents of QL. The fatty acid composition of triacylglycerols, diacylglycerols and phospholipids in RJ was featured by a high amount of polyunsaturated fatty acids (PUFAs), which accounted for more than 39% of the total fatty acids (Table 3). Among PUFAs, linoleic acid and α -linolenic acids dominated. Moreover, 7, 10, 13-hexadecatrienoic acid was identified in triacylglycerols of RJ. The data provide new evidence for the biological activities of RJ on human health. Both QL and RJ are the abundant sources of lipids that are rich in health-promoting lipophilic components. QL contained a high level of phospholipids while RJ was rich in hydroxy fatty acids. Furthermore, it is interesting that no significant difference ($P > 0.05$) was found only in the content of sterols between QL and RJ.

3.7. Comparison of sterol composition

The sterol fractions in QL and RJ were further characterized by gas chromatography (Table 4). The primary sterol in both samples was 24-methylencholesterol, with the content over 5.7 mg/g lipid. 24-methylencholesterol in RJ had been investigated with respect to the estrogenic activity (Suzuki et al., 2008). Two 4-desmethylsterols, β -sitosterol and Δ^5 -avenasterol, were the next major sterols, which were consistent with the results of sterols in RJ reported by Svoboda, Herbert, Thompson, and Feldlaufer (1986). Cholesterol, desmosterol, campesterol, as well as stigmaterol and Δ^7 -avenasterol were detected at low concentrations, less than 0.1 mg/g lipid. These sterols in worker bee larvae have been investigated (Vanderplanck, Michez, Vancraenenbroeck, & Lognay, 2011), which were in the equivalent level compared with the contents of QL in the present study. Besides these constituents, clerosterol, Δ^7 -sitosterol and cycloartenol were identified in small quantities in both RJ and QL. To the best of our

Table 1

Proteins identified in the fractions purified from queen larvae.

Protein	SDS-PAGE MW (kDa)	PMF			Name	Accession no.
		Sequence coverage (%)	Matched peptides (total signals)	Score		
P3-7-18-1	45	29	12/30	99	Major royal jelly protein 2 (<i>Apis mellifera</i>)	MRJP2_APIME
P3-7-18-2	40	30	15/30	166	Major royal jelly protein 2 (<i>Apis mellifera</i>)	MRJP2_APIME
P6-6-16	56	28	8/14	78	Major royal jelly protein 1 (<i>Apis mellifera</i>)	MRJP1_APIME
P6-6-17	35	45	16/31	146	Major royal jelly protein 1 (<i>Apis mellifera</i>)	MRJP1_APIME

Table 3

Distribution (%) of fatty acids in lipids of queen larvae (QL) and royal jelly (RJ).

	Triacylglycerols		Free fatty acids		Diacylglycerols		Phospholipids	
	QL	RJ	QL	RJ	QL	RJ	QL	RJ
Fatty acids								
Capric acid (C10:0)	–	–	1.23 ± 0.20	–	1.78 ± 0.22	–	–	–
(E)-2-decenoic acid (C10:1)	–	–	–	–	–	9.93 ± 0.17	–	–
Myristic acid (C14:0)	1.06 ± 0.25	1.51 ± 0.33	1.13 ± 0.01	–	1.30 ± 0.01	–	–	–
Myristoleic acid (C14:1)	–	6.08 ± 0.85	–	–	–	–	–	–
Palmitic acid (C16:0)	45.05 ± 2.71	33.51 ± 0.56	35.70 ± 0.78	–	41.59 ± 0.34	14.86 ± 0.29	50.15 ± 2.35	17.19 ± 0.98
Palmitoleic acid (C16:1)	3.01 ± 0.66	–	3.99 ± 0.18	–	4.30 ± 0.78	–	1.71 ± 0.57	–
7,10,13-hexadecatrienoic acid (C16:3)	–	0.92 ± 0.25	–	–	–	–	–	–
Margaric acid (C17:0)	–	2.18 ± 0.08	–	–	–	3.92 ± 0.11	–	–
Stearic acid (C18:0)	18.40 ± 2.33	10.72 ± 0.47	12.06 ± 0.85	–	13.81 ± 1.55	10.39 ± 0.22	27.61 ± 2.31	10.69 ± 0.20
Oleic acid (C18:1)	31.72 ± 3.17	4.91 ± 0.60	44.58 ± 2.09	–	36.60 ± 1.91	14.92 ± 1.27	19.50 ± 1.40	27.24 ± 0.87
Linoleic acid (C18:2)	0.46 ± 0.00	3.56 ± 0.31	0.27 ± 0.06	–	0.42 ± 0.03	5.26 ± 0.04	0.79 ± 0.29	33.57 ± 0.41
α-Linolenic acid (C18:3)	0.29 ± 0.05	34.92 ± 1.64	0.62 ± 0.18	–	0.20 ± 0.04	39.20 ± 0.54	0.24 ± 0.02	5.65 ± 0.27
Nonadecanoic acid (C19:0)	–	1.69 ± 0.07	–	–	–	–	–	–
Arachidic acid (C20:0)	–	–	0.41 ± 0.02	–	–	–	–	–
Monounsaturated fatty acid (MUFA)	34.73 ± 3.68	10.64 ± 0.15	48.58 ± 1.90	–	40.90 ± 1.68	24.84 ± 0.45	21.21 ± 1.62	27.24 ± 0.87
Polyunsaturated fatty acid (PUFA)	0.75 ± 0.04	40.02 ± 1.29	0.89 ± 0.12	–	0.62 ± 0.10	44.46 ± 0.38	1.03 ± 0.30	39.22 ± 0.49
Saturated fatty acid (SFA)	64.51 ± 2.87	49.34 ± 1.30	50.53 ± 1.78	–	58.48 ± 1.67	30.69 ± 0.84	77.76 ± 2.75	27.88 ± 0.92

–, not detected.

knowledge, such phytosterols have not been previously reported in RJ or honeybee larvae.

The differences found in the relative percentages of sterols between prepupae and RJ collected over a 6-day period indicated their participation in sterol metabolism and ecdysteroid synthesis (Svoboda et al., 1986; Svoboda, Thompson, Herbert, Shortino, & Szczepanik-Vanleeuwen, 1982). Surprisingly, the sterol composition was roughly similar between QL and RJ. These data implied that although lipid metabolized in a high level, most sterols remained the same in the larvae during the initial three days of larval development. There was a coincidence of previous results of the major QL proteins. QL still contained a considerable amount of MRJP1 and MRJP2, which were the dominant proteins in RJ. Therefore, it is speculated that sterols might be combined with MRJP1 or MRJP2.

3.8. The sterols in P3-7 and P6-6 fractions

To check for the association between sterol and protein in QL and RJ, the sterol profile of P3-7, P6-6 and water extract of RJ containing MRJP1 were examined. Fig. 7 illustrated the GC chromatogram of

different samples. Sterols were observed in P6-6 and water extract of RJ as expected. It can be inferred that sterols conjugate with MRJP1 but not MRJP2. Consequently, MRJP1 is actually a sterol carrier glycoprotein and plays a vital role in the selective sterol transfer mechanism in the honeybee (Svoboda et al., 1986). Based on the lipid analysis of QL and RJ, the sterol compounds were dominated by free forms. Other than that, four fatty acids were coexistent with sterols (Fig. 7(b)), suggesting that a small part of sterols in MRJP1 formed ester linkages with fatty acids.

As the physiologically functional protein, MRJP1 can enhance the release of tumor necrosis factor α (TNF α) in macrophages (Majtán et al., 2006), significantly stimulate proliferation of hepatocyte cell (Kamakura, Fukuda, et al., 2001; Kamakura, Suenobu, et al., 2001) and mediate the cytokine in human keratinocyte (Majtán, Kumar, Majtán, Walls, & Klaudiny, 2010). Additionally, the health-promoting effects of sterols have been particularly well documented during the last two decades. Long term intake of sterols seems to be necessary owing to the beneficial roles in cholesterol level reduction and lowering the risk of hyperlipemia, atherosclerosis, and cardiovascular chronic diseases (Jones, MacDougall, Ntanos, & Vanstone, 1997; Moreau,

Table 4

Fragmentation ions in mass spectra of the sterols (as TMSi ethers) and contents in the lipid of queen larvae (QL) and royal jelly (RJ).

Compd.	Identification	RRT ^a	Major fragmentation ions (m/z, relative abundance %)						Content (mg/g lipid)	
			M+	M-15	M-90	M-105	M-129	Others	QL	RJ
1	Cholesterol	1.35	458 (13)	443 (5)	368 (38)	353 (23)	329 (60)	129 (100), 95 (35), 73 (50)	0.06 ± 0.01	0.10 ± 0.01
2	Desmosterol	1.41	456 (11)	441 (10)	366 (15)	351 (19)	327 (25)	343 (51), 253 (36), 129 (100), 69 (97)	0.07 ± 0.00	0.08 ± 0.01
3	24-methylenecholesterol	1.52	470 (6)	455 (5)	380 (14)	365 (10)	341 (17)	386 (20), 343 (13), 296 (17), 257 (14), 213 (10), 129 (100)	5.71 ± 0.10	6.06 ± 0.27
4	Campesterol	1.53	472 (16)	457 (5)	382 (40)	367 (19)	343 (52)	315 (3), 261 (9), 255 (14), 129 (100)	0.77 ± 0.04	0.97 ± 0.05
5	Stigmasterol	1.59	484 (11)	469 (2)	394 (18)	379 (5)	355 (12)	255 (24), 213 (9), 159 (21), 129 (50), 83 (100)	0.04 ± 0.01	0.03 ± 0.00
6	Clerosterol	1.68	484 (19)	469 (2)	394 (22)	379 (8)	355 (17)	386 (2), 255 (10), 213 (15), 129 (100), 55 (66)	0.03 ± 0.00	0.08 ± 0.01
7	β-Sitosterol	1.71	486 (15)	471 (5)	396 (39)	381 (18)	357 (46)	275 (9), 255 (12), 129 (100), 121 (30)	2.43 ± 0.06	3.01 ± 0.12
8	Δ ⁵ -avenasterol	1.75	484 (4)	469 (4)	394 (3)	379 (5)	355 (8)	386 (69), 296 (62), 257 (29), 211 (20), 129 (100)	2.13 ± 0.12	3.64 ± 0.20
9	Gramisterol ^b	1.81	484 (12)	469 (17)	394 (2)	379 (11)		357 (37), 296 (29), 159 (56), 75 (100)	0.03 ± 0.00	0.02 ± 0.00
10	Δ ⁷ -sitosterol	1.85	486 (73)	471 (17)	396 (5)	381 (11)		255 (100), 229 (29), 213 (41), 75 (87)		
11	Cycloartenol ^b	1.86	498 (1)	483 (2)	408 (38)	393 (25)		365 (25), 339 (17), 286 (5), 189 (14), 75 (47), 69 (100)		
12	Δ ⁷ -avenasterol ^b	1.89		469 (7)	394 (6)	379 (5)		386 (58), 343 (100), 296 (7), 255 (13), 213 (20), 75 (52)		
Total									11.28 ± 0.29	13.99 ± 0.60

^a Relative retention time to cholestane (8.543 min) using HP-5MS column.^b Trace.

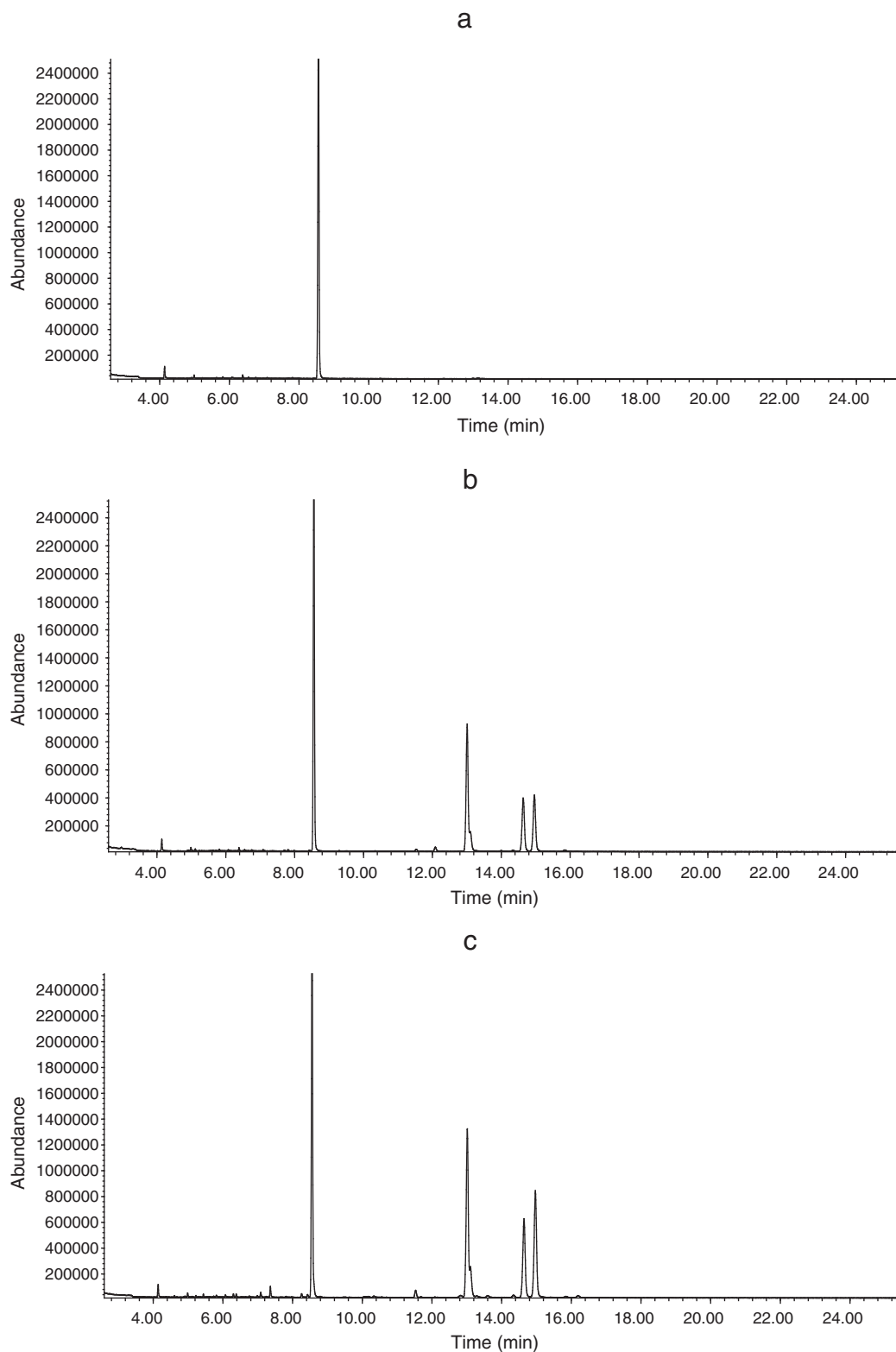


Fig. 7. GC–MS profiles of TMSi ethers of sterols presented in P3-7 (a) and P6-6 (b) protein fractions and water soluble (WS) fraction of royal jelly (c). Peaks and relative retention time were illustrated in Table 4.

Whitakerb, & Hicksa, 2002). On the other hand, sterols are naturally present in vegetable oils (Moreaua et al., 2002). It implies that MRJPs possess excellent emulsification property. The results provided insights into the composition of MRJP1 in QL and RJ which may influence its nutraceutical and medicinal properties and the practical application in the food industry.

RJ from 72 hour time-point is commercially used for consumption, while the QL are usually discarded as waste by beekeepers. The possible production yield of QL is about a quarter of RJ output. The current study showed that QL could be utilized as a new rich source of MRJPs. The main components in QL proteins were water soluble proteins which mainly consisted of MRJP1 and MRJP2. MRJP1 in QL was quite

distinct from that in RJ in the composition. The lipid composition of QL and RJ showed great differences except for the pattern of the sterols. Further analysis showed that sterols were integrated with MRJP1 oligomer both in QL and RJ. Thus, this study has revealed, for the first time, the biological function of MRJP1 as a sterol carrier in the honeybee. It also emphasized the nutraceutical properties and potential application of QL as functional ingredients.

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