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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, Δ7-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.

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, Kovácová, 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 lyophilizated 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 × 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 × 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 × 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 μg/μL) 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 Mizushina (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 ± SD. Data were analyzed by one way analysis of variance (ANOVA) using SPSS 17.0 and differences were considered to be significant at $P < 0.05$.