JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Immunochemical Approach to Detection of Adulteration in Honey: Physiologically Active Royal Jelly Protein Stimulating TNF-α Release Is a Regular Component of Honey

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The presence of royal jelly (RJ) proteins in honey collected from nectars of different plants, origin, and regions and in honeybee's pollen was detected by Western-blot analysis using polyclonal antibodies raised against water-soluble RJ-proteins. The most abundant RJ-protein in honeybee products corresponded to a 55 kDa protein. The N-terminal amino acid sequence of 55 kDa protein was N−I−L−R−G−E. This sequence is identical to the apalbumin-1, the most abundant protein of RJ. Apalbumin-1 is a regular component of honeybee products and thus is a suitable marker tool for proving adulteration of honey by means of immunochemical detection. Its presence in all tested samples of honeys and honeybee pollen was confirmed also by Western-blot analysis using polyclonal antibodies raised against recombinant apalbumin-1. It has been found that major RJ-proteins, apalbumin-1, and apalbumin-2, stimulate mouse macrophages to release TNF-α, which demonstrates that physiologically active proteins of honey could be used for its biological valuation.

KEYWORDS: Honey; adulteration; major royal jelly protein; honeybee products; immunochemical analysis; TNF-α release

INTRODUCTION

Honey is of limited supply and is relatively expensive; therefore, it has often been a target for adulteration with inexpensive sweeteners. Different methods have been applied to detect adulteration of honey by complex of isoglucose syrups (*1*−*3*). Recently, differential scanning calorimetry has been described to be used in testing of adulteration of honey (*4*). Another approach to detect authenticity of honey is based on protein analysis. At least 19 protein bands were detected by silver-staining sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) in honeys of different plant origin (*5*). Honey contains proteins only in minute quantities with an average content of 0.7% (*6*). Among these proteins, "technological enzymes" are the most important components of honey, because they play a vital part in the biochemical processing of honey from plants nectar. Several enzymes such as α-glucosidase (*7*), glucose oxidase (*8*), β-glucosidase (*9*), and amylase (*10*) are regular components of honey. Monitoring the levels of enzymes is sometimes useful to test for authenticity of honey, but because their activity due to heating and prolonged storage is lost, they cannot be used for detection of adulterants in honey. Until now, not a single protein of honeybee origin was applied for a practical use as a marker for adulteration of honey.

At present, honey is being utilized in food commodities as a "natural" sweetener and it has also occasionally been used as a medicine rather than food (*11*). Recently, it has been discovered that 1% honey stimulates release of tumor necrosis factor-α (TNF-α) in human monocytic cell line, though artificial honey (mixture of glucose and fructose similar to those found in natural honeys) failed to elicit TNF-α release (*12*). Although it was found that honeybees secreted physiologically active proteins into royal jelly (RJ) (*13*−*14*), no study has ever been conducted to confirm their presence in honey. RJ is a multiple-substance system containing water (60−70%), proteins (12−15%), carbohydrates (10−12%), and lipids (3−7%), as well as minerals, amino acids, and vitamins (*15*). A substantial part of RJ is made of proteins, which constitute about 50% of its dry mass (*16*). Major proteins accounting for 90% of total RJ-proteins with molecular masses of 49−87 kDa have been assigned to one protein and gene family (*17*, *18*). The minor part of RJ-proteins is composed of proteins and peptides with different functions, including antibacterial and antifungal properties (*19*−*21*). It is expected that the protein with the highest probability to be present in honeybee products is apalbumin-1, the most abundant

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10.1021/jf034777y CCC: $27.50
Published on Web 03/25/2004

protein of RJ, because it is expressed in the hypopharyngeal glands of foragers (*22*, *23)* of nectar and flower pollen. It represents 48% of the water-soluble proteins of RJ, with a molecular weight of 55 kDa (*24*).

The overall goal of this study was to immunochemically identify RJ-proteins in honey and in honeybee pollen (honeybee pollen pellet and honeybee pollen bread). The specific objectives included: (1) Identification and characterization of the protein honeybee origin in honey as a potential protein marker for preliminary immunochemical testing of adulteration of honey, and (2) determination of immunomodulatory effects RJ-proteins as the tools for biological valuation of honey.

MATERIALS AND METHODS

Samples. Honeys from different regions of Slovakia were harvested by the Institute of Apiculture Research, Liptovský Hrádok (Slovak Republic) and stored at 6 °C. The botanical origin of the samples of honey was confirmed by sensory analysis. Honey from Greece, Germany, USA, and Czech Republic were obtained as commercial goods and stored at room temperature. Samples of the honeybee pollen pellet and honeybee pollen bread were stored at −20 °C. Honeybee (*Apis mellifera* L.) RJ was collected 48 h after accepting of 1-day larvae and was supplied from an apiary of A. Kostrian (Slovakia).

Preparation of Samples. The samples of honey were prepared by dilution with water (1:1 w/w) (MilliQ, Millipore, UK). The water-soluble fraction of honeybee pollen pellet or honeybee pollen bread was obtained as supernatant upon centrifugation (20 000xg for 10 min) of a mixture of honeybee pollen pellet or honeybee pollen bread with water in a ratio of 1:2 (w/w) after vortexing for 5 min at 20 °C. The supernatants were dialyzed for 24 h at 4 °C against water. For quantification of proteins, the samples (honey or water-soluble fraction of honeybee pollen) were dialyzed for 24 h at 4 °C against water and subsequently lyophilized.

Protein Quantification. The protein content was determined using the Bradford assay (*25*). Bovine serum albumin (Sigma, St. Loius, MO) was used as reference protein.

Immunochemical Detection of RJ proteins in Honeybee Products by Western-Blot Analysis. Samples were processed in 12% SDS-polyacryalamide gels (*26*) calibrated with Gibco protein molecular weight standards (BRL, Life Technologies, Germany). Gels were either stained with Coomassie Brilliant Blue G-250 (Serva Electrophoresis, GmbH, Heidelberg, Germany) or were electroblotted onto poly-(vinylidene difluoride) (PVDF) membrane (ProBlott, Applied Biosystems, Foster City, CA) using the tank method (MiniTrans-Blot Electrophoretic Transfer Cell, Bio-Rad Laboratories, Hercules, CA). The PVDF membranes were first incubated overnight in a buffer solution (20 mM NaCl, 50 mM Tris-HCl, pH 7.4) containing 10% powdered nonfat milk with polyclonal rabbit antibody against water-soluble proteins of RJ (*17*), or with polyclonal rabbit antiserum against recombinant apalbumin-1 (*27*) at a dilution of 1:1000 and subsequently for 2 h in the same buffer with peroxidase-conjugated porcine anti-rabbit IgG (SwAR Px, Institute of Sera and Vaccines, Czech Republic) at a dilution of 1:2000. Visualization of the immunoactive protein bands was performed by incubation of the membrane with 0.33% 3,3-diaminobenzidine tetrahydrochloride and 30 $\mu g \cdot mL^{-1}$ hydrogen peroxide (Fluka, Switzerland) in 50 mM Tris-HCl, pH 7.4 for 5 min.

N-Terminal Amino Acid Sequencing. The samples of honeys and honeybee pollen were separated by 12% SDS-PAGE and electroblotted onto PVDF membranes as described above. The transfer was performed in electroblotting buffer consisting of 10 mM CAPS (3-[cyclohexylamino]-1-propanesulfonic acid) in 10% methanol, according to the procedure recommended by manufacturer. Staining was performed using Coomassie Blue R 250 (Serva, Germany). The 55 kDa bands were excised and subjected to sequencing by automated Edman degradation on a gasphase sequencer (Applied Biosystems, model 473A).

Stimulants of TNF-α Release. Oligomeric (appr. 420 kDa) and monomeric (55 kDa) forms of apalbumin-1 (previously designated as MRJP 1) (*16*), apalbumin-2, a 49 kDa RJ protein (previously designated as MRJP 2) (*28*), and apisimin, a RJ peptide with MW 5540.4 Da (*21*), were used as stimulants of TNF-α release, and human serum albumin (Sevapharma, Czech Republic) was used as negative control.

Preparation of Mouse Macrophages Used for Monitoring of TNF-α Release. Peritoneal BALBc mouse macrophages were prepared from 8 to 12 weeks old BALBc mice according to Park and Rikihisha (*29*). Cells ($n = 5 \times 10^6$) were resuspended in 0.5 mL RPMI 1640 with l-glutamine (PAA Laboratories GmbH, Austria) supplemented with 10% heat-inactivated fetal bovine serum (FCS, Gibco BRL, Life Technologies, Germany) and placed into each well of 24-well plates (Sarstadt, Germany). The samples were incubated at 37 °C in 5% CO_2 for 2 h. Nonadherent cells were removed, and 0.5 mL of complete RPMI 1640 medium (10% FCS) and 50 μg of stimulants were added to each well. Supernatants were collected after 3 h of incubation and stored at −80 °C. Released TNF-α was quantified by using an enzyme-linked immunosorbent assay kit ELISA (DUO Set, Mouse TNF-α Europe Ltd., R&D systems, UK) according to manufacturer's instructions. A recombinant mouse TNF-α was used as standard. The assay was repeated three times. Values represent the means ± standard deviation (SD).

Microscopic Examination. Microscopic examination of apalbumin-1 was performed using a scanning electron microscope (model JSM-580, JEOL, Japan). The samples were subsequently negatively stained with Cu in a vacuum chamber at 10^{-3} Pa and examined. Formation of spherical and membranous-filamentous structures of oligomeric apalbumin-1 was examined by using light microscope (Axiophot, Carl Zeiss, Germany).

RESULTS AND DISCUSSION

Immunochemical Detection of RJ Proteins in Honeybee Products. Until now, the proteins secreted by honeybees into honey were recognized as enzymes necessary for conversion of nectar to honey. The finding that mRNA coding for apalbumin-1 is present in higher amounts in hypopharyngeal glands of foragers (*22*, *23*) simultaneously with mRNA of α-glucosidase (*30)*, an enzyme that splits sucrose in nectar into fructose and glucose, indicated that apalbumin-1 could be a candidate protein which is secreted by honeybee foragers into honey and honeybee pollen (pollen pellet and pollen bread). The protein analysis of honeys of different origins and honeybee pollen was performed by SDS-PAGE and staining with Coomassie Brilliant Blue. The abundance of protein band with a molecular weight of 55 kDa was revealed in all samples of honey (**Figure 1***A*, lanes 3−7) and honeybee pollen (**Figure 1A**, lanes 8−9). The same samples as those used in this SDS-PAGE were later used in the subsequent Western-blot analyses. The presence of the RJ-proteins in honey and honeybee pollen (**Figure 1B**, lanes 3−9) was detected using Western-blot analysis of SDS-PAGE, applying antibodies raised against water-soluble proteins of RJ. The most intensive bands correspond to a 55 kDa protein with identical mobility to apalbumin-1 (**Figure 1B**, lane 2). The faster mobility of 55 kDa protein in the samples of honeybee pollen than in honey samples was influenced by higher amounts of proteins in analyzed samples (**Figure 1A**, lanes 8−9) in comparison to honey samples (**Figure 1A**, lanes 3−7). Honey and honeybee pollen also contained other immunoactive RJ-proteins with the molecular weight in the range of 60 to 75 kDa, although in smaller amounts in comparison with that of the 55 kDa protein (**Figure 1B**, lanes 3−9). A 20 kDa protein that was immunoactive against polyclonal antibodies raised against water soluble RJ-protein was detected in trace amount in honeys and RJ (**Figure 1B**). The presence of proteins immunoactive against apalbumin-1 with MW under 55 kDa was observed mainly in RJ (**Figure 1C**), which implied that apalbumin-1 was partially degraded.

The identity of the 55 kDa bands in honey and honeybee pollen was confirmed as apalbumin-1 by Western-blot analysis

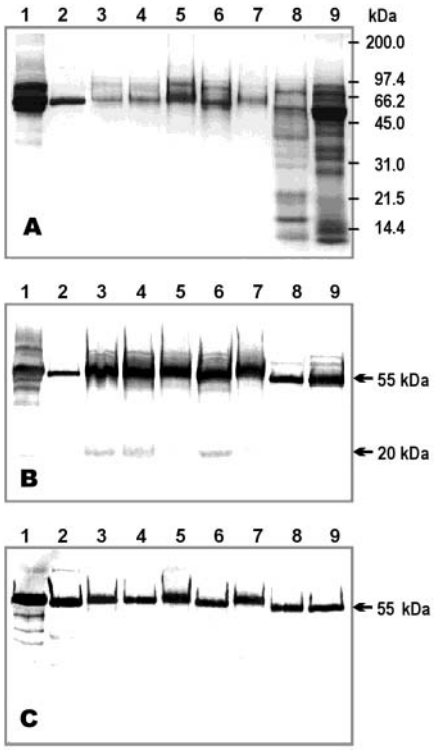

Figure 1. Immunochemical detection of RJ proteins in honeybee products. (**A**) Electrophoretic analysis of SDS-PAGE (12%) of honeybee proteins visualized by Coomassie Brilliant Blue staining. Lane 1 = RJ, 4 μg of the proteins; lane 2 = native apalbumin-1, 1 μg; lane 3 = honey, apple tree, (Slovakia); lane 4 = honey, rape (Germany); lane 5 = honey, mixed flowers (Greece); lane 6 = honey, mixed flowers (USA); lane 7 = honey, mixed flowers (Czech Republic); lane *8* = honeybee pollen pellet; lane 9 = honeybee pollen bread. The 10 μL of 50% honey or 5 μL water-soluble fraction of the honeybee pollen in the sample buffer were applied per lane. (**B**) Western-blot analysis of RJ-proteins as described in panel **A** probed with antibodies against water-soluble RJ-proteins with the following differences: the samples used in panel **A**: RJ (lane 1), apalbumin-1 (lane 2), and honeybee pollen pellet (lane 8) were diluted 1:5 (v/v), while honeybee pollen bread (lane 9) was used in dilution of 1:10 (v/v) with sample buffer. (**C**) Western-blot analysis of RJ-proteins, as described in panel **A** (with differences mentioned in panel **B**), using antibody against recombinant apalbumin-1.

using polyclonal antibodies raised against recombinant apalbumin-1 (**Figure 1C**). The ultimate evidence for the presence of apalbumin-1 in honey and honeybee pollen was provided by N-terminal amino acid sequence analysis of 55 kDa bands (**Figure 1A**, lanes 3−9). All analyzed 55 kDa protein bands contained sequence N−I−L−R−G−E, identical to that previously characterized in apalbumin-1 (*17*, *23*).

These experimental data confirmed that honeys of different plant and territorial origins contained apalbumin-1, the most abundant protein of RJ (**Figure 1C**, lines 3−7). It could be proposed that the major 56 kDa protein constituents of different Australian honeys (*5*) identified by silver staining of SDS-PAGE protein pattern and by two-dimensional electrophoresis was apalbumin-1. These results indicate that foragers secrete apalbumin-1, the most abundant protein of RJ, into honey and honeybee pollen, and therefore it is proposed that this protein as the regular component of honey. The evidence on presence of apalbumin-1 in honeys of heterogeneous plant origins, different regions, and conditions of storage could prove useful for a simple immunochemical Western immunoblot monitoring of the authenticity of honey using polyclonal antibodies raised against recombinant apalbumin-1. Results obtained by immunochemical analysis of honey and those obtained by other techniques such as differential scanning calorimetry (*4*) could be compared for validation.

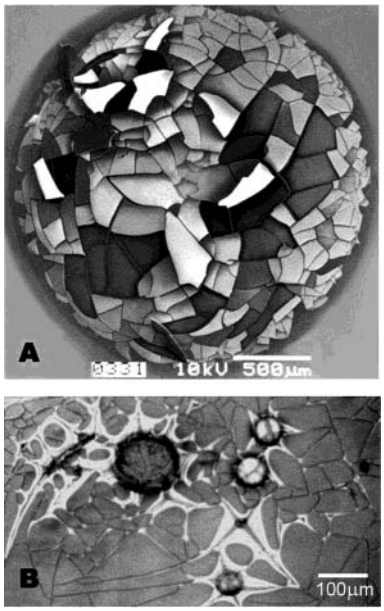

Figure 2. The structures of free-assembling apalbumin-1. (**A**) The scanning electron microscopy view of self-assembled structures of oligomeric apalbumin-1 (300 mg · mL^{-1} in water). (**B**) The optical microscopy visualizing was performed 20 min after applying a drop (3 μL) of oligomeric apalbumin-1 (100 mg · mL^{-1} in water) on cover slip and subsequently covered with another cover slip.

Possible Role of Apalbumin-1 in Processing of Honeybee Products. To date, apalbumin-1 as a component of RJ has been considered to be a major nutritional protein in the honeybee larval food. However, its function in honey and honeybee pollen appears to be other than nutritional. To identify the possible role of apalbumin-1 in honeybee products, we have characterized its structural and molecular properties. To obtain apalbumin-1 in the most natural state, we fractionated RJ by ultracentrifugation and succeeded in obtaining apalbumin-1 in a form of a semisolid gel (*16*). In this gel, apalbumin-1 was present as oligomer (app. 420 kDa) and monomer (55 kDa) (*16*). Scanning electron microscopic (**Figure 2A**) and visual microscopic (**Figure 2B**) examinations of oligomeric apalbumin-1 showed formation of various self-assembled repeating structures. Mechanical properties of apalbumin-1 as a viscoelastic gel, formation of thin films (**Figure 2**) on air, and its hydrophobicity (*16*) predestinated it as a substance participating in physical processing of honey (e.g., formation of thin film that provides an improved surface for evaporation of water from nectar) (*31*). Complex filamentous networks with globular particles that were formed on cover glass immediately after solvation of apalbumin-1 in water were observed by light microscopy (**Figure 2B**). The self-assembly of apalbumin-1 and its ability to change shape (**Figure 2B**) suggest that apalbumin-1 may participate in immobilization of the flower pollen dust and formation of pollen pellet (*32*). Thus, apalbumin-1 could be a factor participating in physical−mechanical processing of honey and honeybee pollen.

Physiological Evaluation of Honey. The quality of honey has long been evaluated based on its static properties that can be determined by chemical, physical, and instrumental analyses. Nowadays, it is generally understood that quality of honey should be defined in terms of a variety of physiological functions

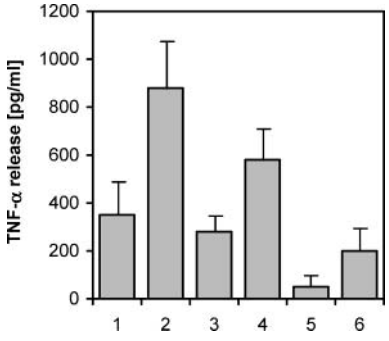

Figure 3. TNF-α release from mice macrophages by honeybee RJ-proteins Stimulants: column 1, apalbumin-1 (oligomeric app. 420 kDa); column 2, apalbumin-1 (monomeric form 55 kDa); column 3, apisimin (a RJ peptide 5540.4 Da); column 4, apalbumin-2 (49 kDa); column 5, control (without stimulant); column 6, negative control (human serum albumin). Each column representsmeans ± SD of three indipendent experiments.

of its individual components, mainly RJ-proteins, which are present in honey (**Figure 1**). The finding that 1% honey stimulated production of TNF-α, while artificial honey (mixture of sugars similar to those found in natural honeys) failed to elicit TNF-α release in human monocytic cell line (*12*) raised a question of what ingredients of honey were responsible for such physiological effect. We have found that RJ-proteins stimulate mouse macrophages to release TNF-α (**Figure 3**). The pronounced stimulation of TNF-α release was observed by native apalbumin-1 in monomeric form (55 kDa) (**Figure 3**, column 2) and the second most abundant RJ-protein apalbumin-2 (*28*) (**Figure 3**, column 4). It has been previously described that these RJ-proteins possess interesting physiological activity (*13*, *14*). The stimulating effect of apalbumin-1 and apalbumin-2 on production of TNF-α is probably derived from their specific domains. It has been previously described (*17*) that these RJ-proteins possess 88% amino acids sequential identity. The human serum albumin, representing an albunoid protein was used as negative control, because it has no verifiable amino acids sequential homology to RJ- proteins. As can be seen (**Figure 3**, column 6), human serum albumin revealed the lowest stimulatory effect on TNF-α release (200 pg · mL^{-1}, SD ± 100 pg · mL^{-1}) in comparison with RJ- proteins. An interesting feature of these experiments is that the stimulatory effect of the oligomeric form (subunits with MW ca. 420 kDa) of apalbumin-1 (**Figure 3**, column 1) was rather low in comparison with the pronounced effect of its monomeric form (55 kDa) (**Figure 3**, column 2). It is possible that amino acids sequential motifs of the apalbumin-1 molecule, which are responsible for stimulation of TNF-α release are blocked in its oligomeric form by protein−protein interactions. The serine-valine-rich RJ-peptide apismin (*21*) was less effective as elicitor of TNF-α release by mouse macrophages (production 300 ± 80 pg · mL^{-1}) (**Figure 3**, column 3) than apalbumin-1 or apalbumin-2 (**Figure 3**, columns 2 and 4, respectively).

These results provide direct evidence that the release of TNF-α in monocytes (*12*) that was observed with 1% honey was stimulated by RJ-proteins present in honey (**Figure 1B**, lanes 3−7) while artificial honey failed to elicit TNF-α release in human monocytic cell line due to lack of these proteins. The amount of TNF-α produced by mouse macrophages induced by apalbumin-1 was 890 ± 210 pg · mL^{-1} (**Figure 3**, column 2), which is in agreement with the data on TNF-α release (average value 700 pg · mL^{-1}) in human monocytic cell line stimulated by honeys of different plant origin (*12*).

The release of TNF-α and probable other cytokines by mouse macrophages mediated by the apalbumin-1 could explain the broad therapeutic properties of honey. The similarities between the immune systems of insects and mammals (*33*−*35*) showed that TNF-α might play a role in cytokine-induced activation of genes important for immune response of honeybees and humans. In these processes, TNF-α could play a pivotal role as the factor participating on regulation of important cellular processes such as cell proliferation and inflammation.

New data on physiological properties of RJ and RJ-proteins, such as enhanced proliferation (*36*), suppression of allergic reactions (*37*, *38*), antifatigue effect (*39*), and antihypertensive activity (*40*), broaden their potential application in pharmacy and food industry. It is proposed that apalbumin-1 as the major protein component of honey could be used as potential marker for physiological evaluation of honey as a functional food by monitoring cytokines production in a suitable in vitro system. The ability of apalbumin-1 to stimulate production of cytokines release could by used as a tool for valuation of honey as a model for functional nutrigenomics.

Recently it has been found that apalbumin-1 is also the most abundant protein in both the Apis cerana RJ (*41*) and Africanized honeybees RJ (*42*). These results showed that apalbumin-1 was a fairly homogeneous protein among the RJs derived from different species of honeybees. It could be proposed that apalbumin-1 was present in honeys of the mentioned honeybee species. In light of these findings, it is possible to generalize our proposal for immunochemical testing of adulteration of all honeys as well as for evaluation of their physiological properties on the basis of presence of apalbumin-1 in honey.

ACKNOWLEDGMENT

The authors are grateful to Mrs. Kristina Radosovska for technical assistance.

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Received for review July 15, 2003. Revised manuscript received January 20, 2004. Accepted February 13, 2004. This work has been supported by VEGA grant Agency of Ministry of Education of Slovak Republic and Slovak Academy of Sciences Nr. 2/4059/04.

JF034777Y