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Major royal jelly proteins as markers of authenticity and quality of honey

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Until now, the properties of honey have been defined based exclusively on the content of plant components in the nectar of given plant. We showed that apalbumin1, the major royal jelly (RJ) protein, is an authentic and regular component of honey. Apalbumin1 and other RJ proteins and peptides are responsible for the immunostimulatory properties and antibiotic activity of honey. For the quantification of apalbumin1, an enzyme-linked immunosorbent assay (ELISA) was developed using polyclonal *anti*-apalbumin1 antibody. The method is suitable for honey authenticity determination; moreover it is useful for detection of the honey, honeybee pollen and RJ in products of medicine, pharmacy, cosmetics, and food industry, where presences of these honeybee products are declared. Results from the analysis for presence and amount of apalbumin1 in honeys will be used for high-throughput screening of honey samples over the world. On the basis of our experiments which show that royal jelly proteins are regular and physiologically active components of honey we propose to change the definition of honey (according to the EU Honey Directive 2001/110/EC) as follows: Honey is a natural sweet substance produced by honey bees from nectar of plants or from secretions of plants, or excretions of plant sucking insects, which honey bees collect, transform by combining with *major royal jelly proteins* and other specific substances of their own, deposit, dehydrate, store and leave in the honey comb to ripen and mature.

KEY WORDS: *royal jelly proteins; antimicrobial peptides; honey authenticity*

Honey is a mixture of various compounds of plant and honeybee origin such as sugars, proteins, enzymes, amino acids, vitamins, hormones, flavonoids, inorganic acids, and minerals. The properties and composition of honey can vary widely depending on the region, season, bee variety, plant source of nectar, storage time in honeycomb, mode of harvesting, and post-harvest storage (1). Honey is not only the basic food of honeybee colonies and a constitutive part of larval diet, but is also an important source of different antimicrobial compounds that play a vital role in the natural defence of all kinds of living organisms (2). Therefore, the quality and authenticity of honey is of major concern to both apiculturists and consumers.

The antibacterial activity of honey is defined as peroxide-related (3) and non-peroxide activity (4-8). The first one is due to the generation of hydrogen peroxide, an enzymatic product of gluconic acid formation from glucose, while the other is represented by antimicrobial compounds of floral origin, as well as honeybee proteins and peptides present in honey.

In Europe, there is an increasing demand for honeys of specific region, unifloral honeys, and niche markets such as organic honeys (9). The classic quality parameters such as water content, hydroxymethylfurfural (HMF) content,

and sensory analysis are no longer sufficient to test for these additional quality characteristics and to guarantee consumer satisfaction and safety (10, 11). Novel techniques include the analyses of plant derived volatiles (12), non-volatile typical plant derived compounds (13, 14), and DNA derived from a specific plant source of unifloral honeys (15). Various control methods have so far been used to assess honey authenticity including HPLC, HPTLC, GC-MS, IR-MS, IC, NMR, etc.

Nowadays, it is generally understood that the quality of honey and honeybee products should be defined in terms of physiological functions of their authentic components (5-8, 16-20). The most specific constituents of honey are proteins of honeybee origin. Honey proteins that have been identified and in the focus of attention until now are enzymes related to the carbohydrate metabolism such as invertase (21), glucose-6-oxidase (22), β-glucosidase (23), and diastase (24).

The most important authentic honeybee proteins in honey (5, 25) are royal jelly (RJ) proteins designated as apalbumins, which belong to a protein family consisting of nine members with M_r of 49-87 kDa. Apalbumin1 (apa1), apalbumin2 (apa2), and apalbumin3 (apa3) account for 90 % of the RJ protein content and have 72 % identical amino acid sequence (26-30). Minor RJ proteins are mainly the homologues of apalbumins (7), antimicrobial peptides (6, 31, 32), and enzymes (21-24). Recent RJ proteomic

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studies showed the presence of about 150 distinct proteins, one third of which were identified as apalbumins (33). RJ proteins display multifunctional features. For example, apa1 and apa2 stimulate tumour necrosis factor alpha (TNF-α) release (5), and apa3 also modulates immune response (34). A 57 kDa RJ protein enhanced proliferation of rat hepatocytes in primary culture (35). A honeybee defensin, royalisin, showed antimicrobial properties (6, 31, 36). Expression of antimicrobial peptides abaecin and defensin in honeybee larvae was enhanced following their infection with bacterial pathogen *Paenibacillus larvae* (37). Similarly, apa3 levels in the honeybee head increased after bacterial infection (38).

The activity of a honeybee product results from synergic effects of all its components. Several groups have demonstrated synergy between flavonoids, existing chemotherapeutics, and honeybee products (39-40). The research team of Kikuji Yamaguchi, Japan Royal Jelly Co. Ltd., has found that pharmacological actions of honeybee royal jelly on the nervous system are enhanced by nobiletin, a citrus flavonoid. Based on these experimental data, a drug containing royal jelly and nobiletin was developed to prevent or delay neurodegeneration in the brains of patients with Alzheimer's disease (41). Future studies may allow the development of a pharmacologically acceptable new class of agents based on described synergy effects.

The aim of this work is to show the importance of RJ proteins and peptides as physiologically active compounds of honeybee products. The major protein of RJ, apa1, is the main protein of honey as well. This protein is honeybee specific and cannot be replaced by other components or ingredients that may affect the method used for assessing the authenticity of honey. Therefore we suggest using apa1 as a marker for authenticity and quality of honey.

MATERIALS AND METHODS

Standards and antibodies

Apa1, as a standard protein, was prepared by ultracentrifugation of RJ (28). It was then freeze-dried and stored at -20 ºC.

Polyclonal antibody (pAb) against RJ proteins/peptides (*anti*-apa1, *anti*-apa2, *anti*-apisimin antibody) was prepared by immunisation of rabbits with an RJ protein/peptide (42, 43) and was stored at -20 ºC. Peroxidase-conjugated *anti*-rabbit IgG (SwAR Px) was obtained from the Institute of Sera and Vaccines (Prague, the Czech Republic).

Sample preparation

Royal jelly (RJ) of honeybee *Apis mellifera carnica*, Hontianka line, was collected 48 hrs after obtaining one day-old larvae and was stored at -20 °C. The RJ was diluted in water (1:10, w/v, MilliQ, Millipore, UK), stirred at room temperature for 30 min, and centrifuged at 15,000 *g* at 4 °C

for 20 min. For experiments, the water-soluble fraction of RJ proteins was used.

Honey samples. Polyfloral and/or honeys of different floral origin: acacia (*Robinia pseudoacacia*), linden (*Tillia spp.*), rapeseed (*Brassica napus*), dandelion (*Taraxacum officinale*), chestnut (*Castanea sativa*), and cherry (subgenus *Cerasus*) were prepared by vortexing honey with water (1:1, w/v, MilliQ, Millipore, UK) for 5 min at room temperature. Pollen-free honeys were prepared by filtration of diluted samples through a 0.8 µm membrane filter (Milipore Corporation, USA).

Sucrose syrup honey (SCCH). A honey chamber of bee colony was equipped with new empty combs and honeybees were fed with 1 litre of sucrose syrup (sucrose/water 1:1, w/v) for three days. The experimental hive was placed in green house to avoid the mixing of honey prepared by feeding bees with sucrose syrup and floral honey. The SCCH was then harvested from the new combs and stored at 18 °C in the dark.

Preparation of honey protein fraction. Honeys of different floral origin were vortexed for 5 min at 20 °C with milliQ water 1:1 (w/v). The samples were filtered using 0.8 µm MF-Milipore MCE Membrane and then dialysed against milliQ water for 24 h at 4 °C using dialysis tubing MWCO 3500 Da (Pirece, Rockford, IL, USA). The samples were then freeze-dried and stored at -20 °C. For testing purposes, the appropriate amount of protein was dissolved in water.

Determination of protein concentration

The protein content of samples was determined by microplate microassay according to Bradford (44). 100 µL of Quick Start Bradford reagent (BioRad, Laboratories, Inc., USA) was added to 100 µL of the sample or its dilution. The absorbance was measured at 595 nm. Bovine serum albumin (BSA, Sigma, USA) was used as a standard.

Sodium dodecyl sulphate-polyacrylamide electrophoresis (SDS-PAGE) and Western-blot analysis

Proteins were separated by SDS-PAGE in 10 or 12 % gels (45). Gels were either stained with Coomassie Brilliant Blue G-250 (Serva, USA) or electroblotted onto PVDF-membrane (ProBlott, Applied Biosystems, USA) following the manufacturer's instructions. RJ proteins were detected using specific *anti*-apalbumin pAb as was described previously (5, 42, 43). Immunoactive protein bands were visualised by incubating the blots in the chromogenic DAB/NiCl$_2$ solution.

Determination of apa1 in honeys by ELISA

Honey samples were analysed for apa1 as described previously in detail (46). The 96 well/flat-bottom microtiter plates (Brand, Germany) were coated with antigen - diluted honey samples at dilution of 0.05 and/or 0.001 % in milliQ water and/or standard solution of apa1 and incubated overnight at 4 °C. After washing with TBS buffer

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(100 mmol L^{-1} Tris and 150 mmol L^{-1} NaCl, pH 7.5) the plates were incubated with polyclonal rabbit *anti*-apa1 antibody in milk buffer (2 % non-fat milk in TBS) and then with peroxidase-conjugated *anti*-rabbit IgG in milk buffer for 1 h. 3 % ABTS (2,2'-azino-bis-(3-benzthiazoline-6-sulfonic acid, Southern Biotech, USA) was added to 50 mmol L^{-1} citrate buffer, pH 4.3, supplemented by hydrogen peroxide. The absorbance at 405 nm was read in a Microplate Spectrophotometer PowerWave™ XS (BioTek Instruments, INC, Winooski, Vermont, USA). Data processing and statistics were performed with Gen5 software (BioTek, USA).

Bacteria and media

Bacterial strain of *Bacillus subtilis* (*B. subtilis*) CCM 2217 (ATCC11774) was obtained from the Czech Collection of Microorganisms, Masaryk University, Brno, the Czech Republic, and *Paenibacillus larvae larvae* (*P. l. larvae*) ATCC2548 from the American Type Culture Collection, USA. The cultures were maintained as frozen stocks at -80 °C. The bacteria were grown in MYPGP medium (Difco, USA) supplemented with 0.1 mg mL^{-1} of thiamine hydrochloride (Sigma, USA).

Inhibition zone assay

Antibacterial diffusion test on agar plates was performed by spreading 8 mL of 0.5 % agar MYPGP medium mixed with spore suspension of *B. subtilis* or *P. l. larvae* (1x10^5 spores per mL) as described previously (6). 5 µL of the protein samples of known concentration was poured into the 2 mm diameter holes in the agar plates and incubated at 37 °C for 16-20 hours. Tetracyclin hydrochlorid and ampicilline (Serva, Heidelberg, Germany) at concentrations of 25 µg mL^{-1} and 50 µg mL^{-1} were used as controls of the inhibition effect.

Growth inhibition in liquid medium

The microplate method was used to test the antimicrobial properties of honey and RJ proteins. The bacteria mentioned above were grown in MYPGP medium supplemented with 0.1 mg mL^{-1} thiamine hydrochloride. The tested protein samples were added to the bacterial culture in exponential phase of growth at density of 10^5 CFU mL^{-1} and were incubated at 37 °C. Growth inhibition was monitored by measuring absorbance at 595 nm. Ampicilline and tetracycline hydrochloride were used as positive controls.

Monitoring TNF-α release

The ELISA kit (DUO Set, R&D systems, Abingdon, UK) was used as per manufacturer's instructions, as described previously, to monitor the TNF-alpha release from murine macrophages (5). Apa1 monomer (55 kDa), oligomeric form of apa1 (350 kDa), apa2, royal jelly peptide apisimin, 1 % (w/v) honey solution, proteins purified from

the honeys, and deproteinised honey samples prepared in complete RPMI 1640 medium were applied in the stimulation assay. Lipopolysaccharides of *Salmonella typhimurium* (LPS) were used as a positive control. The proteins were separated from honey by filtration using a Microsep device (Pall Life Sciences, Ann Arbor, MI, USA) with 3 kDa cut-off. Deproteinised honey solution [30 % (w/v)] was adjusted to 1 % (v/v) in complete RPMI 1640 medium. All prepared honey solutions were rendered sterile by membrane filtration (0.22 µm). The level of TNF-α was determined in cell culture supernatants collected after 3, 6, and 24 h of cultivation. A recombinant mouse TNF-α was used as a standard. The assay was repeated three times.

RESULTS AND DISCUSSION

Antimicrobial protein potential of honey

Our experimental data showed high variation of antimicrobial activity even between the honeys of same botanical origin. Growth inhibition of *P. larvae* was observed in protein fractions of acacia, cherry, rapeseed, and honeydew honeys (47). Further, we fractionated the acacia honey proteins by size exclusion chromatography (28) and obtained high molecular protein fraction (Mw over 30 kDa) with antimicrobial effect on honeybee pathogen, *P. larvae*, at concentrations of 2 and 5 mg mL^{-1} (Figure 1). These data indirectly show that high molecular antimicrobial proteins in honey could also be present in RJ.

The electrophoretic profile of different honeys (Figure 2) showed that major RJ proteins, mainly apa1, are the major proteins of honey as well. The presence of RJ proteins was confirmed by Western-blot analysis using specific pAbs. Minority homologues of RJ proteins were also detected (not shown).

Minority homologues of apalbumins were purified from supernatant fraction of water-soluble proteins of RJ by size exclusion chromatography (28). Middle molecular mass fraction (30-90 kDa) was used to follow purification of apa2a, the minority homologue of apa2, by DEAE cellulose ion exchange chromatography (7). As a first screening of the antimicrobial effect of some minority homologues of apalbumins we used diffusion test of growth inhibition of *B. subtilis* as a model organism (Figure 3). As it is shown in Figure 3A, two samples of RJ (RJ1, RJ2) slightly inhibited the growth of *B. subtilis*, while the inhibition zones of purified minority homologues of RJ proteins (Figure 3B, 1-5) and RJ peptide royalisin (Figure 3B, 6) were comparable with the inhibition effect of the commonly used antibiotics amplicillin and tetracycline at similar molar concentrations (Figure 3A, Figure 3B). An interesting observation was that apa2, the second most abundant protein of RJ did not show antibiotic properties (Figure 3A, apa2), while the antibiotic activity of its minority homologue, apa2a (apa2a) was significant (Figure 3B, 5).
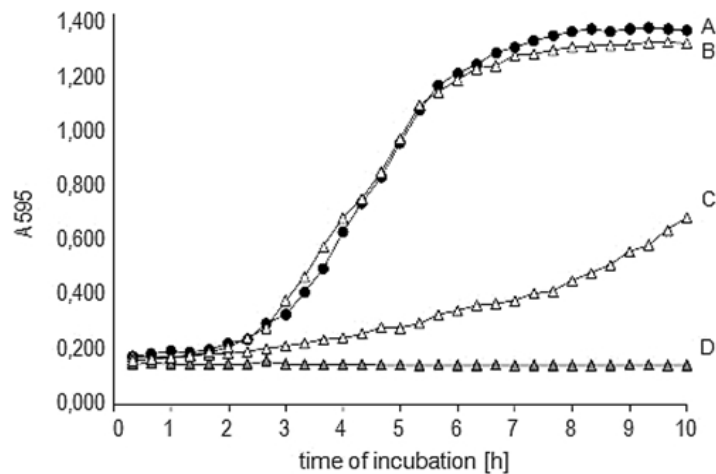
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Figure 1 *Antimicrobial effect of honey proteins. Growth inhibition of* P. larvae *(ATTC2547) by high molecular weight protein fraction of acacia honey at concentrations of: 1 mg mL⁻¹ (B), 2 mg mL⁻¹ (C), and 5 mg mL⁻¹ (D). Control (A), growth, tested microorganism without addition of antimicrobial agent; A595=absorbance at 595 nm*
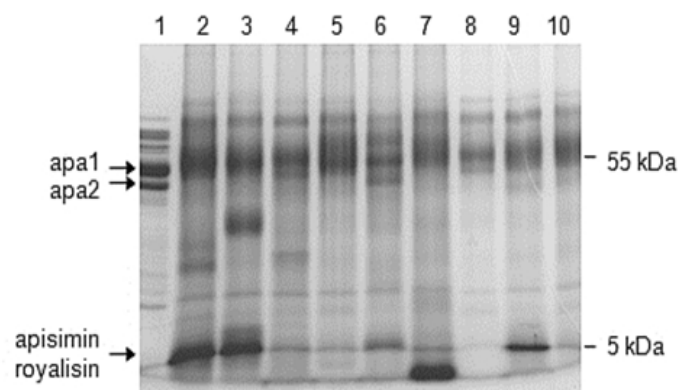

Figure 2 *Electrophoretic protein profile of honeys. 10 % SDS PAGE, Coomassie brilliant blue staining. Line 1 - royal jelly; line 2 - polyfloral honey 1; line 3 - polyfloral honey 2; line 4 - rapeseed honey; line 5 - chestnut honey; line 6 - cherry honey; line 7 - honeydew honey; line 8 - acacia honey; line 9 - linden honey; line 10 - honey made from sucrose syrup feeding*
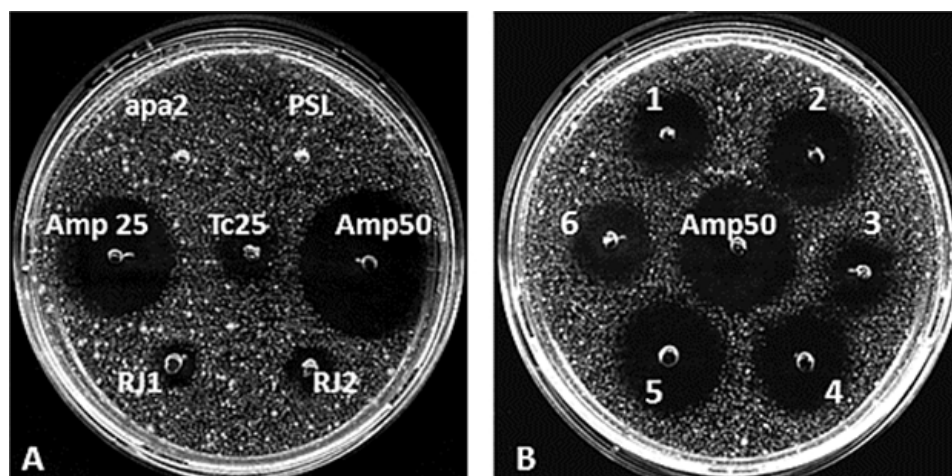

Figure 3 *Diffusion test of growth inhibition of* Bacillus subtilis *by royal jelly and minority homologues of RJ proteins. A: apa2 - apalbumin2 (2x10⁻⁴ mol L⁻¹); RJ1, RJ2 – royal jelly from different honeybee colonies; PSL - physiological solution as a negative control; Amp25 - ampicillin (7x10⁻⁵ mol L⁻¹); Amp50 - ampicillin (1.45x10⁻⁴ mol L⁻¹); Tc25 - tetracycline hydrochloride (7x10⁻⁵ mol L⁻¹). B: 1. - 4. minority homologues of apalbumins (1x10⁻⁵ mol L⁻¹) purified from royal jelly; 5. - apa2a (1.8x10⁻⁵ mol L⁻¹); 6. - royalisin (5.6x10⁻⁵ mol L⁻¹); Amp50 - ampicillin (1.45x10⁻⁴ mol L⁻¹)*

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Apa2a showed in SDS-PAGE a single band of Mw of 48 kDa, which was confirmed by MALDI-TOF-MS (7). The immunochemical analysis confirmed that apa2a is immunoactive to *anti*-apa2 antibody but Edman sequencing showed that N-terminus of apa2a differed to apa2. Molecular characterisation of apa2a by nanoLC-MALDI-TOF MS showed glycosilation sites of the protein different to the maternal apa2. Therefore, various minority homologues of apalbumins in RJ suggest their physiological functions differ from those of maternal apalbumins. This hypothesis was supported by the antimicrobial activity of apa2a, which inhibited the growth of honeybee pathogen *P. larvae* at concentration 1.8×10^{-4} mol L^{-1}. This corresponds to the effective inhibitory concentration (1×10^{-5} mol L^{-1}) of tetracycline but maternal apa2 did not show any antibiotic properties (7).

We have identified immunochemically in honey for the first time an antimicrobial and antifungal RJ peptide apisimin (32), using polyclonal *anti*-apisimin antibody. This peptide was detected in all tested samples including honey made from sucrose syrup feeding. Besides apisimin, we have also found in some honey samples antimicrobial RJ peptide royalisin (bee-defensin) (Figure 2). The inhibitory potential of royalisin and apisimin against *P. larvae* suggest their role as anti-foulbrood factor in honeybee colony (6, 32).

The presented data call attention to multifunctional properties of RJ and honey proteins with potential impact on fundamental research (i.e. the studies of novel mechanisms of action of antibacterial proteins) and in the field of drugs development (i.e. the application of RJ proteins as antibiotics in beekeeping).

Monitoring of physiological potential of honey by production of TNF-α

Tumour necrosis factor alpha (TNF-α) is a macrophage-derived peptide that is known to be an important mediator in various physiological and immunological events. TNF-α is a key cytokine involved in antiviral, antibacterial, and antiparasitic host defence mechanisms. We have studied the physiological potential of honey by monitoring TNF-α production by mouse macrophages after 3, 6, and 12 hours of incubation. We used acacia, linden, chestnut, and cherry honeys at concentration of 1 % (w/v) as well as the proteins purified from these honeys. The lipopolysacharid (LPS) from *Salmonella typhimurium* was used as a control stimulant. Honey samples were filtered through a 0.8 μm membrane to remove pollen before use. The filtration of honey had no significant influence on the protein concentration of honey determined by Bradford (Table 1) and the pollen present in honey seems to have no significant influence on the immunostimulating properties of honey. As it is shown in Figure 4, TNF-α release induced by honey proteins was slightly higher than in the case of honey samples, while in the presence of deproteinised honey no significant stimulation effect was observed (Table 2). The lowest values of TNF-α production were found in the presence of cherry honey (729.4 ng mL^{-1}) comparable to LPS (627.6 ng mL^{-1}) after 3 h of incubation. The highest stimulation effect was shown by the proteins of chestnut honey (1487.3 ng mL^{-1}) after 6 h of incubation, which corresponded to the highest average concentration of apa1 in chestnut honey (Table 3). This data confirmed our previous finding that RJ proteins, mainly apa1 and apa2, are responsible for the immunostimulatory potential of honey (5). The oligomeric form of apa-1 (420 kDa) has significantly lower immunostimulatory effect than the monomeric form (55 kDa) of the protein. The stimulating activity of apalbumins on production of cytokines is derived from their specific amino acid sequencing domains (5).

Quantification of apa1 in honey by ELISA

We found that honey and bee pollen contain RJ proteins of molecular mass from 3 to 90 kDa, which corresponds to the spectrum of proteins and peptides present in native RJ where apa1, the major protein of RJ, is predominant (Figure 2). Western-blot analysis using polyclonal *anti*-apa1 antibodies (not shown) confirmed our previous finding that the 55 kDa protein with N-terminal amino acid sequence N-I-L-R-G-E corresponded to apa1 (28, 46). With regard to abundance of apa1 in honey, we used apa1 as a protein standard for a developed enzyme-linked immunoassay (ELISA) (46). The limit of detection for apa1 was 2 ng mL^{-1} The concentration of apa1 in different honeys demonstrates variability mainly with respect to the botanical origin of samples (Table 3, Figure 5) and does not depend significantly on the geographical origin of honey or honeybee line.

The highest average content of apa1 was determined in chestnut honey (145.58 μg g^{-1}) in comparison with acacia (62.29 μg g^{-1}) and rapeseed (83.68 μg g^{-1}) honey. The amount of apa1 in polyfloral honeys varied in broader limits

Table 1 *Comparison of total protein content in non-filtered and filtered honeys. The concentrations are means of n samples of the same floral origin. Each value was calculated as a mean of five parallel analyses of the same sample*

Honey origin	n	Total protein of honey [μg g^{-1}]		Δ%[a]
		non-filtered	filtered	
Acacia	5	271.55	267.75	1.40
Chestnut	5	892.66	863.68	3.25
Rapeseed	5	572.96	532.57	7.05
Dandelion	5	685.06	644.67	5.89

[a] *percentage of differences in total protein content of non-filtered and filtered honeys*

Table 2 *Stimulation of TNF-α production by mouse macrophages after 2, 6, and 24 hours of incubation in the presence of 1 % honey samples in comparison with proteins of honeys. The lipopolysaccharides (LPS) of* Salmonella tryphimurium *were used as control*

Sample		TNF-α [pg ml⁻¹]		
		3 h	**6 h**	**24 h**
Acacia	*honey*	1279.0	1251.6	1231.9
	proteins	1081.7	1280.1	1383.6
Linden	*honey*	1195.0	1083.6	1201.2
	proteins	1027.9	1287.9	1426.4
Chestnut	*honey*	1106.1	909.4	1163.9
	proteins	1269.4	1487.3	1117.0
Cherry	*honey*	729.4	750.7	873.3
	proteins	1341.1	1241.1	1167.7
Deproteinised honey		29.5	31.0	35.4
LPS		627.6	964.1	791.2

Table 3 *The average content of apalbumin1 (apa1) in monofloral and polyfloral honeys. Each value of apalbumin1 was calculated as a mean of five parallel analyses of the same sample*

Honey origin	n	apa1 [μg g⁻¹ of honey]		
		min	**max**	**average**
Acacia	29	26.15	87.21	62.29
Linden	6	65.69	125.95	95.64
Chestnut	8	114.92	171.14	145.58
Rapeseed	7	34.00	103.35	83.68
Dandelion	6	98.39	157.69	132.20
Polyfloral	41	52.61	208.42	126.06
Sucrose syrup	2	7.61	7.72	7.66
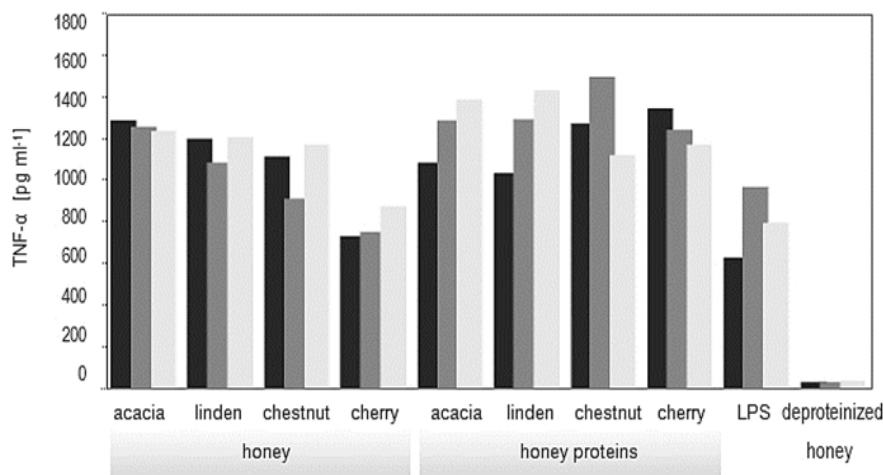
Figure 4 *TNF-α production by mouse macrophages in the presence of honey samples, proteins of honeys and deproteinised linden honey after 3, 6, and 24 hours of incubation. Lipopolysaccharides of* Salmonella typhimurium *(LPS) were used as a positive control*
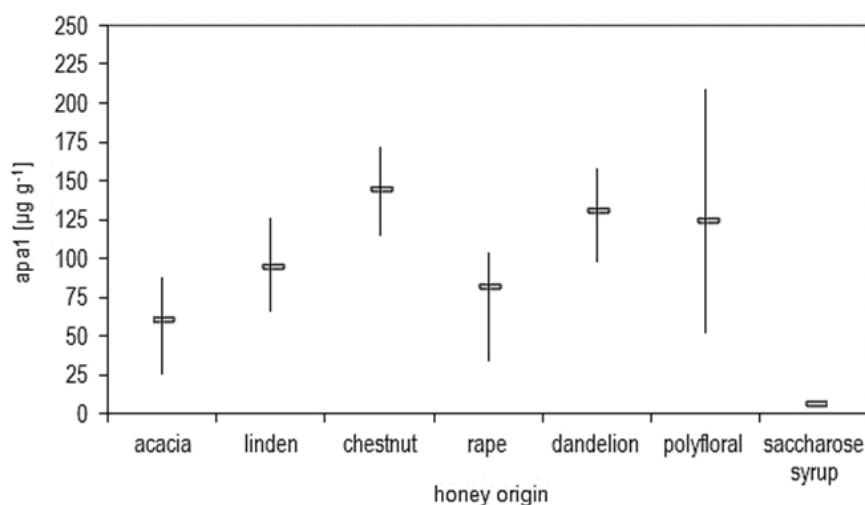
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Figure 5 *Sample distribution of apalbumin1 content in honey samples of different floral origin and in polyfloral honeys in comparison with honeys made from sucrose syrup feeding. (Apalbumin1 concentration in honey samples according to Table 3)*

than in monofloral honeys (from 52.61 µg g^{-1} to 208.42 µg g^{-1}). The average value of apa1 in polyfloral honeys (126.06 µg g^{-1}) was 15 times higher than in sucrose syrup honey (7.66 µg g^{-1}). Based on the large screening of polyfloral honeys (46), it can be supposed that the concentration of apa1 in honey below 50 µg g^{-1} would be indicative of the presence of industrial glucose syrups in honey or dilution of floral honey with the honey obtained by feeding the honeybee colony with sucrose syrup.

Honey authenticity based on determination of proteins and peptides secreted by honeybees to the honey presents a new approach to evaluation of honey as a functional food. It would be desirable for the honey sold commercially to include on the label information about the content of apa1 as a physiologically active component of honey.

CONCLUSION

Honeybee proteins are to become recognised as an important model for the study of antimicrobial defence, immunity, nutrigenomics, allergic reaction, development, mental health and longevity, as well as for the investigation of the role of RJ proteins and peptides in the defence system of honeybee colony and humans against pathogens. A systematic molecular-biological research of individual proteins and peptides of RJ has showed that they are multifunctional and biologically active compounds that underline the healing properties of honeybee products. Determination of the physiological capacity of honey based on authentic bee proteins and peptides provides a possibility for the exact characterisation of honey as a valuable nutrient.

Honey authenticity control based on determination of proteins secreted by bees in the processing of nectar to honey represents a new approach in honey adulteration detection. We have found that the most important physiologically active component of honey is the major protein of royal jelly - apa1, which cannot be replaced with other components or ingredients that may affect the method used for assessing the authenticity of honey. The presented ELISA method for immunochemical quantification of apa1 in honey can be used for: the first screening of honey authenticity and quality; detection of honey adulteration by low-cost industrial syrups (e.g. corn syrup, high-fructose corn syrup); quantification of apa1 in other honeybee products such as bee pollen and RJ; and determination of RJ content in various preparations used in food products, apitherapy, cosmetics, and pharmacy.

The evidence of objectivity of determination of apa1 in different honey samples from Europe was confirmed during the implementation of the project ''BEE SHOP'' (Bees in Europe and Sustainable Honey Production) 6. FP EU FOOD-CT-2006-022568. We continue to work on the high-throughput screening of honey samples over the world in cooperation with Slovak Association of Beekeepers, and with Japan Royal Jelly, Co., Ltd., Tokyo. The obtained data will serve as a tool for improvements of the European legislation governing honey.

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Glavni proteini matične mliječi kao markeri izvornosti i kakvoće meda

Do sada su svojstva meda bila definirana isključivo na temelju sadržaja komponenti nektara određene biljke. Mi smo pokazali da je apalbumin1, glavni protein matične mliječi, izvoran i uobičajeni sastojak meda. Apalbumin1, ostali proteini matične mliječi i peptidi odgovorni su za imunostimulatorna svojstva i antibiotsko djelovanje meda. Korištenjem poliklonalnog *anti*-apalbumin 1 protutijela osmišljen je imunoenzimski test (ELISA) za kvantifikaciju apalbumina 1. Metoda je ne samo prikladna za utvrđivanje izvornosti meda nego i korisna za detekciju meda, peluda i matične mliječi u medicinskim, farmaceutskim, kozmetičkim i prehrambenim proizvodima na kojima je naznačena prisutnost pčelinjih proizvoda. Rezultati analize prisutnosti i količine apalbumina 1 koristit će se za probir velike količine uzoraka meda diljem svijeta. Na temelju naših eksperimenata, koji pokazuju da su proteini matične mliječi uobičajene i fiziološki aktivne komponente meda, predlažemo izmjenu definicije meda (na temelju Direktive EU-a o medu 2001/110/EC): Med je prirodna slatka tvar koju pčele proizvode od nektara ili izlučevina biljaka ili izlučevina insekata koji sišu biljke. Nju pčele skupljaju, pretvaraju kombinacijom *glavnih proteina matične mliječi* i ostalih vlastitih specifičnih tvari, polažu, dehidriraju, pohranjuju i ostavljaju u saću da sazrije.

KLJUČNE RIJEČI: *antimikrobni peptidi; izvornost meda; proteini matične mliječi*