Proteomic identification and enzymatic activity of buckwheat (*Fagopyrum esculentum*) honey based on different assays

VERONIKA BORUTINSKAITE – GRAŽINA TREIGYTE – VIOLETA ČEKSTERYTE – BOGUMILA KURTINAITIENE – RUTA NAVAKAUSKIENE

Summary

Buckwheat (*Fagopyrum esculentum* Moench) blossom honey was analysed for enzymatic activity and protein content. Proteins were separated by one-dimensional denaturing electrophoresis (1DE) in sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) or resolved by two-dimensional denaturing gel electrophoresis (2DE). Using different mass spectrometry (MS) techniques, the main proteins of plant and honeybee *Apis mellifera carnica*, Pollmann origin were identified. Using direct MS analysis of honeybee proteome, a total of 87 proteins were identified in buckwheat honey. Proteins of honeybee origin included major royal jelly protein (MRJP), uncharacterized honeybee protein, high glutamic acid storage protein and enzymes α-glucosidase, α-amylase and glucosylceramidase. Separation of proteins by 1DE and 2DE revealed a smaller number of proteins, including 6 proteins of bee origin and 11 of yeast origin. In addition, catalase activity and glucose oxidase activity in buckwheat honey were estimated on the native PAGE. Our study demonstrates a useful approach to identification of honey proteins, which play an important role in human nutrition and immune defence system.

Keywords

buckwheat honey; royal jelly; proteins; separation; two-dimensional electrophoresis; mass spectrometry

Buckwheat (*Fagopyrum esculentum* Moench) blossom honey is characterized by a dark brown, almost black, colour and a sharp, sweet taste and molasses flavour [1, 2]. Higher content of phenols and flavonoids was determined in buckwheat honey compared to other types of honey. A study showed that *p*-hydroxybenzoic and *p*-coumaric acids could be treated as possible fingerprints of buckwheat honey [3]. Volatile compounds of buckwheat honey differ from those of other types of honey. Butanal and 2-methylbutanal have been reported as markers for this honey origin among the many volatile constituents found in buckwheat honey. The latter volatile compounds, as well as the presence of isovaleric acid, influence its aromatic and organoleptic properties [4]. The presence of *p*-coumaric and *p*-hydroxybenzoic acids is characteristic of buckwheat plants. *p*-Hydroxybenzoic acid was found to dominate in buckwheat grain with husks, accounting for 21 %, while *p*-coumaric for 9.8 % of the total phenolic acid content [5].

Enzymes in honey play an important catalytic function. Buckwheat honey exhibits high activity of the enzymes diastase (EC 3.2.1.1, α-amylase), up to 69.95 Gothe units per gram of honey (one Gothe unit will hydrolyse 10.0 mg of starch in one hour at 40 °C) and invertase (EC 3.2.1.26) 264.34 $U \cdot kg^{-1}$. Catalase (EC 1.11.1.6) activity in buckwheat honey reaches to 0.964 $\mu g \cdot g^{-1} \cdot min^{-1}$ and release of hydrogen peroxide is 65.84 $\mu g \cdot g^{-1} \cdot min^{-1}$ [6]. Catalase activity was detected in various kinds of honey, being the highest in heather honey but lower in oilseed rape and clover honey [7]. The

Veronika Borutinskaite, Institute of Biochemistry, Vilnius University, Sauletekio av. 7, LT-10257 Vilnius, Lithuania; Electronic Systems Department, Faculty of Electronics, Vilnius Gediminas Technical University, Naugarduko 41, LT-03227 Vilnius, Lithuania.

Gražina Treigyte, Bogumila Kurtinaitiene, Ruta Navakauskiene, Institute of Biochemistry, Vilnius University, Sauletekio av. 7, LT-10257 Vilnius, Lithuania.

Violeta Čeksteryte, Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, Instituto aleja 1, LT-58344 Akademija, Lithuania.

Correspondence author:
Violeta Čeksteryte, tel: (+370) 672 18175, e-mail: violeta@lzi.lt

enzyme catalase decomposes hydrogen peroxide and produces molecular oxygen and water. This enzyme protects cellular organelles and tissues from damage by peroxide, which is cytotoxic [8]. Glucose oxidase (GOD, EC 1.1.3.4) is needed to convert glucose to gluconic acid and hydrogen peroxide, which have an antimicrobial action. Activity of GOD in buckwheat honey was reported to be 1.13 $U \cdot g^{-1}$, while, in multifloral spring honey and oilseed rape honey, it was 0.55 $U \cdot g^{-1}$ and 1.08 $U \cdot g^{-1}$, respectively [9]. GOD oxidizes glucose in the unripe honey and produces hydrogen peroxide and gluconolactone. Hydrogen peroxide is associated with antibacterial properties of honey [10]. It is important to identify proteins that can be essential for antibacterial properties of honey. Enzymes are important indicators of honey freshness and purity [11].

The total content of nitrogen in nitrogenous substances in honey is very low and averages 0.43 mg per 1 g of honey. It is contributed to honey from bee gland secretion, plants nectar and yeasts [12]. Proteinaceous substances account for about 40–80 % of the total nitrogen content, while the other nitrogenous substances are free amino acids. Proteins in honey cause a lower surface tension and raise foam and air bubbles [13]. Generally, 100 g of honey contains 0.05–1.0 g of proteins and 0.03–0.07 g of free amino acids [14]. Besides the specific honey enzymes invertase, α-amylase and GOD, involved in carbohydrate metabolism, honey contains proteolytic enzymes. Proteolytic enzymes break the long chain molecules of proteins into shorter fragments, peptides, and subsequently into their components, amino acids. The recent studies on honey proteolytic enzymes revealed that honey proteases are able to degrade major royal jelly proteins (MRJP), and their proteolytic activity is associated with honey kind [15]. The authors identified 67 MRJP, α-glucosidase (EC 3.2.1.20), GOD and profilin in orange honey. It was stated that the most important are nine MRJP; however, those proteins can exhibit different action: MRJP1 stimulates liver regeneration [16], MRJP3 can provide immunoregulatory effects in vitro and in vivo [17], MRJP4 and MRJP5 are important sources of essential amino acids [18]. Nectar is mixed with protease enzymes introduced from the salivary glands of bees, which transform its chemical composition. It is believed that plant proteins can be digested by bee glands' proteases, therefore the specific protein of bee origin is detected in honey [19].

Lithuanian buckwheat honey was studied for diastase, catalase, invertase and GOD activities during storage and processing [6, 9]. In the current study, we collected Lithuanian buckwheat honey samples and investigated them for enzyme expression and protein composition. The findings in this area provided new knowledge on honey quality, which will help honey producers to find new marketing channels. The aim of this study was to evaluate catalytic activity and diversity of proteins in buckwheat honey by the means of different assays. The protein separation techniques used in this study provided new important information on the protein composition in buckwheat honey.

MATERIALS AND METHODS

Collection of honey samples

Monofloral buckwheat (*Fagopyrum esculentum* Moench) honey is specific to southern Lithuania. Buckwheat honey samples were collected by private beekeeper in an apiary located in Nemunas Loops Regional Park, Birštonas municipality (coordinates N 54° 35' 5", E 24° 2' 1"), Lithuania. The apiarist kept *Apis mellifera carnica* species in his farm. Honey samples were preserved in glass bottles in the dark and refrigerated (5 °C) until analysis. Botanical origin was determined by comparison of light microscopic images of pollen found in buckwheat honey to those of known pollen collected by hand [20]. The pollen content in the selected buckwheat honey samples was approximately 90 %.

Preparation of honey samples for enzyme activity analysis

The buckwheat honey samples were dissolved in distilled water (0.2 $g \cdot ml^{-1}$) and filtered through a Whatman glass fibre filter (diameter 21 mm) with a pore size of 1.5–5 μm (Merck, Darmstadt, Germany). Part of the solution was concentrated approximately five times in a Spectra/Por dialysis bag (cutoff 12–14 kDa; Serva, Heidelberg, Germany) kept on dry carboxymethyl cellulose (Sigma-Aldrich, St. Louis, Missouri, USA). The obtained solutions were used for native electrophoretic analysis.

Native electrophoresis

Native electrophoresis was performed in a vertical slab gel apparatus TV1000 (BiocomDirect, Bridge of Weir, United Kingdom). A separation gel containing 13 % or 15 % acrylamide, pH 8.6, cross-linked at 1 : 75 with *N, N*'-bismethylene acrylamide (Serva) was used. Samples of 10 μl or 20 μl of the earlier prepared honey solution were used. Protein molecular weight marker SM0671 (Thermo Scientific, Waltham, Massachusetts,

USA) was used for monitoring the progress of polyacrylamide gel electrophoresis. The electrophoresis buffer system was Tris-glycine (Serva), pH 8.9. The separation time was 3 h with a constant voltage of 300 V and a starting current of 120 mA. After electrophoresis, the gels were washed in distilled water for 30 min.

Detection of catalase activity

Catalase (EC 1.11.1.6) detection was performed using the method of PICHORNER et al. [21]. For gel electrophoresis, a 15% polyacrylamide gel (PAG) was prepared. After electrophoretic separation, the gel was soaked in a phosphate-buffered solution (0.06 mol·l^{-1}, pH 7.5) containing 0.1 mmol·l^{-1} *o*-dianisidine (Sigma-Aldrich) and 0.05 mmol·l^{-1} haemin (Sigma-Aldrich) dissolved in a minimal volume of 0.01 mol·l^{-1} sodium hydroxide, for 20 min (solution A). After this procedure, the gel was rinsed with distilled water and incubated in a phosphate-buffered solution (0.06 mol·l^{-1}, pH 7.5) containing 0.02 mmol·l^{-1} *o*-dianisidine and 0.1 mol·l^{-1} hydrogen peroxide for 2 min (solution B). The gel was again rinsed with distilled water immediately after the zones of catalase activity appeared as transparent bands on the brown background.

Detection of glucose oxidase

Localization of glucose oxidase (EC 1.1.3.4) in the gels was performed via its hydrogen peroxide production determined by peroxidase-catalysed *o*-dianisidine oxidation [22]. The reaction should take place inside the gel only to yield coloured zones. The peroxidase (molecular mass about 50 kDa) in the buffer solution must be enabled to diffuse into the gel. The swollen gels (13% PAG) were inserted into 50 ml of 0.1 mol·l^{-1} acetate buffer (pH 5.0), which contained 50 mg glucose, 2.5 mg horseradish peroxidase (173 U·mg^{-1}; Applichem, Darmstadt, Germany) and 5 mg *o*-dianisidine. Brown zones of enzyme activity on a colourless background were obtained.

Protein isolation from bee honey for protein separation

For the protein isolation from honey, 200 g of honey were diluted with 500 ml of bi-distilled water and proteins were precipitated by adding 4 volumes of cold 99.5% acetone and incubated overnight at –20 °C. Then, the precipitate was washed with 1 ml chilled 80% acetone several times and dried for 5 min in room temperature. The protein precipitate was suspended in isoelectric focusing (IEF) buffer containing 9 mol·l^{-1} urea, 2 mol·l^{-1} thiourea, 0.04 mol·l^{-1} dithiothreitol (DTT) and 4% [3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate] (CHAPS) with addition of 0.5% ampholyte-containing commercial buffer pH 3–10 (IPG) (GE Healthcare Life Sciences, Chicago, Illinois, USA). Proteins were extracted by vortexing for 1 h at room temperature. The mixture was centrifuged at 25 000 ×*g* for 20 min at +16 °C, the supernatant was collected and stored at –80 °C until analysis.

The proteins isolated from buckwheat honey were separated by one dimensional electrophoresis (1DE) in sodium dodecyl sulphate polyacrylamide gel (SDS-PAGE) system on gradient (7.5–15%) or resolved by two-dimensional gel electrophoresis (2DE). For 2DE in first dimension, isolated proteins were separated by isoelectric focusing using Immobiline DryStrip pH 3-11, 11 cm (GE Healthcare Life Sciences). In second dimension, ExcelGel SDS Gradient 8–18% precast gels (GE Healthcare Life Sciences) were used for SDS-PAGE performed according the manufacturer's instructions. Protein molecular weight marker SM1811 (Thermo Scientific) was used for monitoring the progress of SDS-polyacrylamide gel electrophoresis. For protein visualization, gels were stained with PageBlue protein staining solution, which is a colloidal Coomassie G-250 dye (CCB, Thermo Scientific). 2DE separation of honey proteins was carried out in three independent biological experiments.

In-gel digestion and mass spectrometry

Areas of interest were cut out from the 2DE gels and subjected to overnight in-gel tryptic digestion [23]. Briefly, the gel slices were dehydrated with 50% acetonitrile and then dried completely using a centrifugal evaporator (DNA Mini, Eppendorf, Hamburg, Germany). The gel slices were rehydrated in 30 μl of 25 mmol·l^{-1} ammonium bicarbonate (pH 8.3) containing 25 μg·ml^{-1} modified trypsin (Promega, Madison, Wisconsin, USA), and they were incubated overnight at 37 °C. Any solution remaining after the digestion was removed and placed in a fresh tube. Then, the gel slices were washed two times with 5% trifluoroacetic acid (Sigma-Aldrich) in 50% acetonitrile. The digestion and extract solutions were combined and evaporated to dryness. For matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) analysis, the peptides were dissolved in 3 μl of 30% acetonitrile and 0.01% trifluoracetic acid and were then prepared with a matrix (α-cyano-4-hydroxycinnamic acid) on the target plate. The analysis was performed in a 4800 MALDI TOF/TOF mass spectrometer (Applied Biosystems/MDS SCIEX, Ontario,

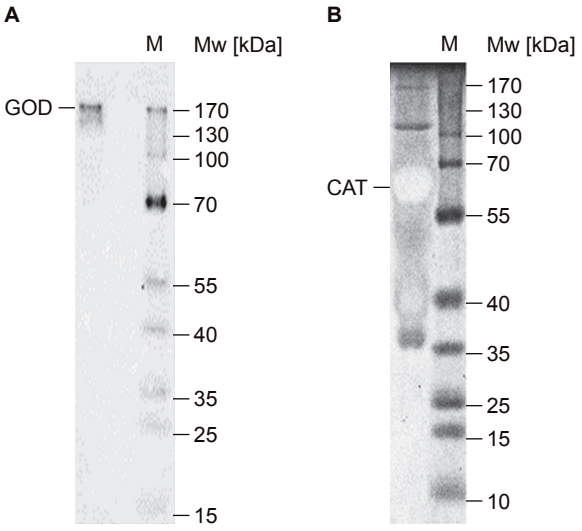

Fig. 1. Enzyme activity detection in buckwheat honey samples after separation by non-denaturing PAGE.

A – glucose oxidase, B – catalase.
GOD – glucose oxidase, CAT – catalase, M – molecular weight marker.

Canada), which was externally calibrated using synthetic peptides with known masses (Applied Biosystems). Mass spectrometry (MS) reflector mode setting was: *m/z* range 800–4 000, tandem mass spectrometry (MS/MS) mode settings were: collision energy 1 keV, collision-induced dissociation (CID) not used, fragment mass accuracy ± 0.1 Da. The mass information generated from the composite spectrum was submitted to a search performed with the free available UniProt database (UniProt Consortium, Hinxton, United Kingdom) [24].

Protein sample preparation for direct gel-free mass spectrometry analysis

For the direct gel-free mass spectrometry analysis, honey proteins were isolated as described previously, just being suspended in IEF buffer without ampholytes. Isolated honey proteins were concentrated by using Amicon Ultra-0.5 ml 30 kDa centrifugal filter unit (Sigma-Aldrich) and were denatured in 8 $mol \cdot l^{-1}$ urea, 100 $mmol \cdot l^{-1}$ DTT solution with continuous rotation at 13.3 Hz in a temperature-controlled shaker for 3 h at 37 °C. Trypsin digestion was done according to a modified protocol of Wisniewski et al. [25]. Briefly, proteins were washed in a column with a buffer containing 8 $mol \cdot l^{-1}$ urea. The proteins were alkylated using iodoacetamide. Buffer was exchanged by washing two times with 50 $mmol \cdot l^{-1}$ NH_4HCO_3 and proteins were digested overnight with TPCK Trypsin 20233 (Thermo Scientific). After overnight digestion, peptides were recovered by centrifugation and then two additional washes using 50% CH_3CN were combined, acidified, lyophilized, dissolved in 0.1% formic acid and then analysed by high definition mass spectrometry (HDMS) in a Synapt G2 mass spectrometer (Waters, Milford, Massachusetts, USA).

Data processing, searching and analysis

Raw data files were processed and searched using ProteinLynx Global SERVER (PLGS), version 2.5.2 (Waters). The following parameters were used to generate peak lists: (i) minimum intensity for precursors was set to 100 counts, (ii) minimum intensity for fragment ions was set to 30 counts and (iii) intensity was set to 500 counts. Processed data were analysed using trypsin as the cleavage protease, one missed cleavage was allowed and fixed modification was set to carbamidomethylation of cysteines, variable modification was set to oxidation of methionine. Minimum identification criteria included 2 fragment ions per peptide, 5 fragment ions per protein and minimum of 2 peptides per protein. The false discovery rate (*FDR*) for peptide and protein identification was determined based on the search of a reversed database, which was generated automatically using PLGS when global false discovery rate was set to 1 %. Functional protein association networks were constructed using free available AgBase, version 2.00 (Mississippi State University, Oxford, Mississippi, USA) [26].

RESULTS AND DISCUSSION

Detection of glucose oxidase activity

The described staining techniques for glucose oxidase activity on polyacrylamide gels are based on the fact that glucose oxidase reacts with one-electron scavengers in a reaction dependent on pH and inconstant oxygen concentration. The visible brown zone on the electrophoresis gel strip (Fig. 1A) indicated enzyme with molecular weight (Mw) of approximately 170 kDa. Mw of protein identified in present study was compatible with that previously observed for glucose oxidase composed of two subunits having Mw of 85 kDa each [27]. This indicated that our image showed a whole enzyme.

Detection of catalase activity

Catalase detection was also performed using native electrophoresis. Zones of catalase activity appeared as transparent bands on the brown background (Fig. 1B). The haemin-catalysed oxidation

of *o*-dianisidine in polyacrylamide gel resulted in a uniform brown staining of the gel within a few minutes. At positions where the decomposition of hydrogen peroxide was performed by catalase, sharp colourless bands appeared rapidly after insertion of the gel into incubation solution (*o*-dianisidine with hydrogen peroxide). As peroxidase is more effective in oxidizing *o*-dianisidine at the expense of hydrogen peroxide than haemin, zones of peroxidatic activity appeared as dark-brown strains. After a few minutes, maximum contrast between the colourless zones of catalase activity and the dark-brown zones of peroxidase activity on the amber background was obtained.

Visible band on the electrophoresis gel strip showed a protein of 60 kDa. Catalase is known to consist of 4 subunits of Mw of 60 kDa each [28]. Thus, our image showed individual enzyme subunits. Dark grey strips corresponded to proteins with peroxidase activity [21], Mw of which was approximately 35 kDa and of more than 100 kDa.

Catalase is an enzyme responsible for degradation of hydrogen peroxide. It is a protective enzyme present in nearly all animal cells. Catalase breaks peroxide excess of bees in food, thereby protecting it from the toxic effects [29]. Catalase has two enzymatic activities depending on the concentration of H_2O_2. If the concentration of H_2O_2 is high, catalase removes H_2O_2 by forming H_2O and O_2 (catalatic reaction). However, at a low concentration of H_2O_2 and in the presence of a suitable hydrogen donor, e.g., ethanol, methanol or phenol, catalase removes H_2O_2 while oxidizing the substrate (peroxidatic reaction) [28].

In this concern, important is the level of hydrogen peroxide in honey, which depends on the ratio of catalase and glucose oxidase activity. The higher the glucose oxidase level, the higher the peroxide level and, vice versa, the lower the catalase level, the higher the peroxide level. On the other hand, because catalase originates in plants, the level of catalase in honey will effectively determine the level of peroxide in honey. This will depend on how much pollen is collected by bees, the floral source of the pollen and also on the catalase activity of that pollen. Amylase and glucose oxidase were purified from the homogenate of the hypopharyngeal glands of the forager bee. Bands with Mw of 57 kDa and 85 kDa were detected when the active fractions for amylase and glucose oxidase were subjected to SDS-PAGE [27].

Identification of buckwheat honey proteins

The soluble proteins extracted from buckwheat blossom honey were analysed in three different ways. First, proteins were separated by SDS-PAGE, stained with CCB and then MS analysis was performed. Second, proteins separated by 2DE on immobilized pH gradient of 3–11 in first dimension (according to isoelectric point of

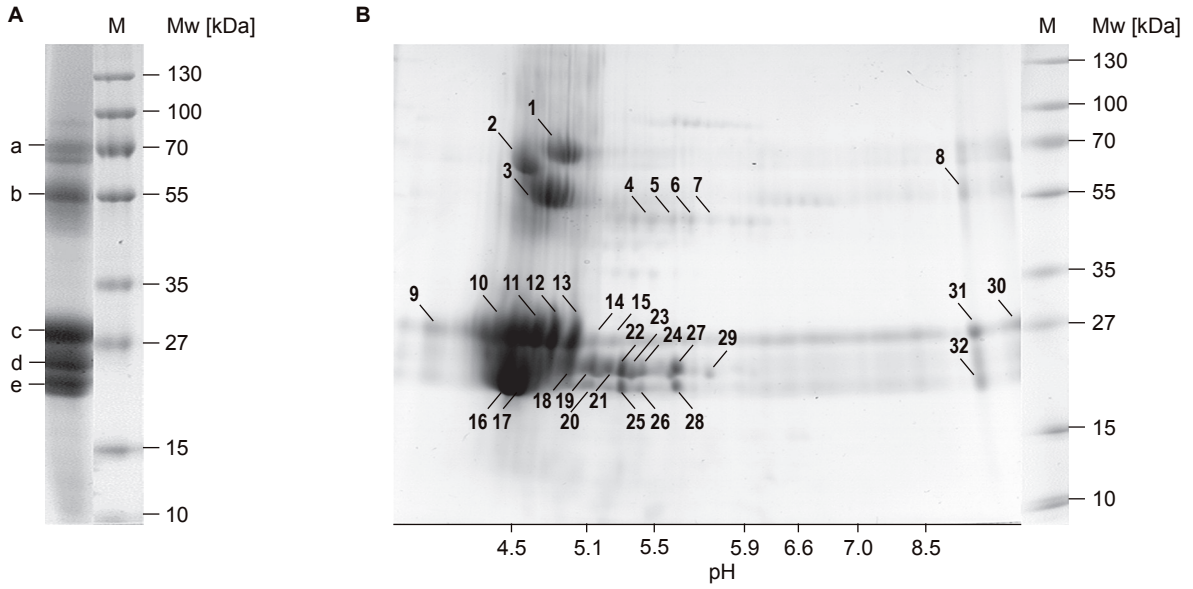

Fig. 2. Buckwheat honey proteins separated by electrophoresis.

A – sodium dodecyl sulphate polyacrylamide gel electrophoresis, B – wo-dimensional denaturing gel electrophoresis. M – molecular weight marker. Letters and numbers indicate the positions of proteins supplied for mass spectrometry analysis, identified and presented in Tab. 1.

Tab. 1. Proteins identified in buckwheat blossom honey by mass spectrometry analysis (MALDI-TOF MS).

Spot No.	Accession number	Protein	Organism species	Matching/ sequence coverage [%]	Number of peptides		Theoretical values		Experimental values	
					Theoretical	Experimental	Mw [kDa]	*pI*	Mw [kDa]	*pI*
a	Q17058	α-Glucosidase	*Apis mellifera*	39	57	14	65.523	5.06	67–71	
b	O18330	MRJP1	*Apis mellifera*	34	40	11	48.854	5.1	51–55	
c	Q8SW28	14-3-3 protein homolog	*Encephalitozoon cuniculi* (strain GB-M1)	17	35	6	29.622	5.11	24–29	
d	Q2UUH5	Methylthioribulose-1-phosphate dehydratase	*Aspergillus oryzae*	43	29	8	27.150	6.4	21–25	
e1	Q52FS9	Phosphatidylglycerol/phosphatidylinositol transfer protein	*Magnaporthe oryzae*	44	24	5	19.543	5.11	21–25	
e2	Q12322	Putative uncharacterized protein YOL114C	*Saccharomyces cerevisiae*	26	41	4	23.175	10.1	18–22	
1	Q17058	α-Glucosidase	*Apis mellifera*	48	57	16	65.523	5.06	68–70	4–5
2	P30655	Probable proteasome subunit beta type-5	*Schizosaccharomyces pombe*	22	26	6	29.967	6.44	59–68	4–5
3	Q17060	MRJP1	*Apis mellifera*	44	40	17	48.854	5.1	52–56	4–5
4	O77061	MRJP2	*Apis mellifera*	24	49	10	51.041	6.83	45–48	6–7
5	O77061	MRJP2	*Apis mellifera*	34	49	15	51.041	6.83	45–48	6–7
12	P87240	General negative regulator of transcription subunit 2	*Schizosaccharomyces pombe*	18	32	6	34.456	5.76	25–28	4–5
17	O74471	Cytochrome c oxidase subunit 6A, mitochondrial	*Schizosaccharomyces pombe*	19	21	4	15.189	8.89	20–25	4–5
21	O94498	Uncharacterized N-acetyltransferase C18E5.08	*Schizosaccharomyces pombe*	22	26	3	21.136	9.2	24–25	5–6
22	P0CX40	40S ribosomal protein S8-B	*Saccharomyces cerevisiae*	7	44	3	22.475	10.67	24–26	5–6
27	C8ZFP7	Mitochondrial inner membrane protease ATP23	*Saccharomyces cerevisiae*	8	37	3	26.903	7.57	22–26	6–7
32	Q9P383	Nuclear cap-binding protein subunit 2	*Schizosaccharomyces pombe*	9	28	3	20.785	8.87	18–22	10–11

Spots marked as a-e were identified by sodium dodecyl sulphate polyacrylamide gel electrophoresis. Spots 1–32 were identified by two-dimensional denaturing gel electrophoresis.
Mw – molecular weight, *pI* – isoelectric point, MRJP – major royal jelly protein.

proteins) and on SDS-PAGE in second dimension (according to molecular weight) and, after staining with CCB, MS analysis was performed. Third, soluble honey proteins were analysed by direct gel-free mass spectrometry analysis using HDMS Synapt G2 mass spectrometer.

Proteins from buckwheat blossom honey were separated by SDS-PAGE and approximately 4–5 protein bands were seen in SDS-PAGE gel (Fig. 2A). All bands were excised and subjected to in-gel tryptic digestion and identification by MS analysis (MALDI-TOF-MS) followed by plant/bee protein database search. 2DE map showed similar results in terms of protein groups as those obtained by SDS-PAGE (Fig. 2B). Thirty-two protein spots were cut out from 2DE gel and subjected to in-gel tryptic digestion. The positions of all proteins identified on 2D gels were within the expected range of their theoretical isoelectric points and Mw sizes. The following honeybee (*Apis mellifera*) proteins were identified: major royal jelly proteins MRJP1, MRJP2 and α-glucosidase (Tab. 1, spots b, 3–5). Also, some proteins of plant origin were identified, namely, methylthioribulose-1-phosphate dehydratase and phosphatidylglycerol/phosphatidylinositol transfer protein (Tab. 1, spot d, e1) *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* proteins were also identified by mass spectrometry in buckwheat honey (Tab. 1, spots e2, 12,17, 21, 22, 27, 32).

Results of MS analysis revealed the following proteins of *S. cerevisiae*: putative uncharacterized protein YOL114C OS (Tab. 1, spot e2, accession number Q12322) and 40S ribosomal protein S8-B OS (Tab. 1, spot 22, accession number P0CX40). Further, a protease was identified (Tab. 1, spot 27, accession number

C8ZFP7). The activity of protease in honey was first identified by ROSSANO et al. [15]. Protease enzymes produced by microorganisms are detected by means of an electrophoretic technique gel zymography [30, 31]. This technique provides information on molecular weights of hydrolytic enzymes, their forms and locations [32]. The activity of different forms of serine proteases and their effect on MRJP, specifically on MRJP1, in different kinds of honey were determined by bidimensional zymography (2-DZ) [15].

Among proteins associated with yeast *S. pombe*, the following were detected: probable proteasome subunit beta type-5 OS; general negative regulator of transcription subunit 2 OS; cytochrome c oxidase subunit 6A, mitochondrial OS; uncharacterized *N*-acetyltransferase C18E5.08 OS; nuclear cap-binding protein subunit 2 OS (accession numbers P30655, P87240, O74471, O94498, Q9P383). One protein, 14-3-3 protein homologue OS = *Encephalitozoon cuniculi* can be associated with fungi [33]. The proteasome proteins, like probable proteasome subunit beta type-5 OS and mitochondrial inner membrane protease ATP23 OS, can be characterized by ability to cleave peptides. Those proteins associated with above mentioned yeast were found in tested honey samples. Different species of genera *Saccharomyces* and *Schizosaccharomyces* are known to be present in honey [34, 35]. Other proteins belong to *S. cerevisiae* such as nuclear cap-binding protein subunit 2, Mitochondrial inner membrane protease ATP23 and others (Tab. 1). The presence of osmophilic yeasts (*Candida*, *Cryptococcus*, *Debaryomyces*, *Pichia*, *Rhodotorula*, *Torulaspora* or *Zygosaccharomyces*) in honey is an indication of the beginning of fermentation [36]. The presence of *S. cerevisiae* might be an indication of overfeeding bees with sugar paste prior to honey production. Either type of yeast is incompatible with high-quality honey [37].

Our previous study showed that total acidity of buckwheat honey tended to increase, diastase and catalase activities tended to decrease, and production of hydrogen peroxide tended to decrease during one-year storage at 5–8 °C [6]. The moisture content of Lithuanian buckwheat honey (18.4 %) is slightly higher than that of spring and rape honey, which is 17.2 % and 16.3 %, respectively [9]. Yeasts in honey do not multiply when moisture content is below 17.0 % [38]. However, the moisture content of honey stored in a refrigerator at 4 °C or at room temperature for 16 weeks increases by 0.4–1.2 % [39]. It can be concluded that buckwheat honey should be used within one year.

Antioxidant enzymes which were found in this assay, namely, GOD, cytochrome c oxidase and lactoylglutathione lyases (glyoxalases) can influence the level of antioxidant activity of buckwheat honey. Our previous investigation showed that processing buckwheat honey at 55–70 °C reduced GOD activity [9]. The antioxidant activity, evaluated by oxygen radical absorbance capacity (ORAC), of buckwheat honey stored for 6 weeks in a refrigerator at 4 °C showed a decrease from initial 12.68 mmol·kg^{-1} (expressed as Trolox equivalents) to 6.14–6.79 mmol·kg^{-1} and to 5.96–6.07 mmol·kg^{-1} when stored at room temperature. The decrease of ORAC in clover honey was lower in both storage conditions. This leads to the conclusion that the quality of buckwheat honey changes faster and more markedly than that of other types of honey [40].

It is known that royal jelly (RJ) is on average composed of 60–70 % water, 12–15 % crude protein, 10–16 % sugars, 3–6 % lipids and traces of vitamins, salts and free amino acid and some substances that contribute to the induction of bee-queen differentiation [41]. Today nine most abundant proteins of RJ are named major royal jelly protein (MRJP1 to MRJP9) and are encoded by genes (*mrjp1* to *mrjp9*) that have been identified in the honeybee genome. Interesting is that MRJP1 has a growth-factor-like activity, as the protein enhances DNA synthesis, maintains proliferation and suppresses apoptosis of rat hepatocytes [42, 43]. α-glucosidase breaks down starch and disaccharides and releases α-glucose [44]. In this study, we identified MRJP1 to MRJP9 RJ proteins in buckwheat blossom honey (Tab. 1, spots b, 3–5; Tab. 2, No. 12, 15, 18, 26, 29, 34, 39, 60, 66, 87).

In this study, using gel-free MS, we identified 87 proteins. We think that this method is much better for proteomic analysis than mass spectrometry after SDS-PAGE (which identified only 5 bands) or 2DE (which identified only 32 proteins). We identified transferrin with Mw of 78.63 kDa by gel-free MS (Tab. 2, No. 36). Transferrins belong to a family of iron-binding proteins that have been implicated in innate immunity and in vitellogenesis in insects [45]. Expression of transferrins is upregulated in response to infection in insects [46].

Protein glucosylceramidase was also identified in buckwheat honey using gel-free MS (Tab. 2, No. 63). It is known that glucosylceramidase (EC 3.2.1.45), (glucocerebrosidase, ceramide glucosidase and other names) deficit in insects leads to shorter lifespan, locomotor, memory and other behavioral deficits, neurodegeneration, and accumulation of insoluble protein aggregates that are

Tab. 2. Proteins isolated from buckwheat (*Fagopyrum esculentum*) blossom honey and identified by gel-free mass spectrometry analysis.

No.	Accession number	Protein	Organism species	GN	PE	SV	*Mw* [kDa]	*pI*	Number of peptides		Matching sequence [%]	*p*
									Theoretical	Experimental		
1	A0A078HEF2	BnaA03g11410D protein	*Brassica napus*	BnaA03g11410D	4	1	10.28	4.72	7	2	28.6	4.25×10^{-8}
2	A0A078J0K1	BnaCnng30450D protein	*Brassica napus*	BnaCnng30450D	4	1	85.15	7.76	76	17	22.4	3.16×10^{-6}
3	A0A078IJR4	BnaA06g23560D protein	*Brassica napus*	BnaA06g23560D	4	1	84.05	7.77	77	15	19.5	6.59×10^{-6}
4	A0A078G9V9	BnaC01g39620D protein	*Brassica napus*	BnaC01g39620D	3	1	38.08	7.42	39	13	33.3	1.02×10^{-5}
5	A0A078DHX7	BnaA03g08190D protein	*Brassica napus*	BnaA03g08190D	3	1	90.85	6.98	96	9	9.4	1.06×10^{-5}
6	A0A078CBH7	BnaA03g28400D protein	*Brassica napus*	BnaA03g28400D	3	1	84.92	6.17	89	17	19.1	1.54×10^{-5}
7	A0A078FW97	BnaA09g06900D protein	*Brassica napus*	BnaA09g06900D	4	1	84.06	7.19	75	18	24.0	2.20×10^{-5}
8	A0A078FSK2	BnaC08g40620D protein	*Brassica napus*	BnaC08g40620D	4	1	10.29	4.72	7	3	42.9	2.50×10^{-5}
9	A0A078CZ01	BnaC09g06400D protein	*Brassica napus*	BnaC09g06400D	4	1	83.74	7.17	74	18	24.3	2.72×10^{-5}
10	Q7Y1T6	Polygalacturonase	*Brassica napus*	Sta44G2	3	1	42.31	6.60	40	13	32.5	3.32×10^{-5}
11	Q17058	α-Glucosidase	*Apis mellifera*		1	1	65.56	5.06	57	63	110.5	3.44×10^{-5}
12	O97432	MRJP5	*Apis mellifera*	MRJP5	2	1	70.24	5.95	81	42	51.9	5.44×10^{-5}
13	A0A078I7E1	Non-specific lipid-transfer protein	*Brassica napus*	BnaA08g11810D	3	1	17.35	8.96	15	2	13.3	5.66×10^{-5}
14	Q25BT6	α-Glucosidase	*Apis mellifera*	hbg3	4	1	65.58	5.06	57	59	103.5	5.66×10^{-5}
15	D3JZ08	MRJP5	*Apis mellifera*		2	1	70.18	6.12	82	47	57.3	0.00012426
16	A0A078IL69	BnaC05g48040D protein	*Brassica napus*	BnaC05g48040D	3	1	83.72	6.19	88	15	17.0	0.00013056
17	A0A088A3F4	Uncharacterized protein (Fragment)	*Apis mellifera*	LOC727045	4	1	42.75	6.43	41	18	43.9	0.00020152
18	O77061	MRJP2	*Apis mellifera*	MRJP2	1	1	51.08	6.83	49	68	138.8	0.00021068
19	A0A078DNT9	BnaA08g18150D protein	*Brassica napus*	BnaA08g18150D	4	1	19.09	4.92	20	7	35.0	0.00021248
20	A0A078I9J4	BnaC02g43200D protein	*Brassica napus*	BnaC02g43200D	4	1	83.49	7.77	75	15	20.0	0.00023008
21	A0A088AU26	Uncharacterized protein	*Apis mellifera*	Ame.6033	4	1	53.80	6.60	52	68	130.8	0.00024871
22	A0A088A030	Uncharacterized protein	*Apis mellifera*	Gld2	3	1	69.35	5.58	54	8	14.8	0.00026388
23	A0A078CBK9	Non-specific lipid-transfer protein	*Brassica napus*	BnaC07g44200D	3	1	12.44	8.13	11	4	36.4	0.00026738
24	A0A087ZXA2	Uncharacterized protein	*Apis mellifera*	LOC551268	3	1	33.62	6.37	22	6	27.3	0.00030306
25	A0A078HUW9	BnaC05g43590D protein	*Brassica napus*	BnaC05g43590D	4	1	6.32	4.87	4	1	25.0	0.00034453
26	D3Y5T0	MRJP3	*Apis mellifera*	Mrjp3	2	1	61.65	6.65	61	44	72.1	0.00036884
27	A0A088AU22	Uncharacterized protein	*Apis mellifera*	Mrjp5	4	1	47.56	8.07	40	46	115.0	0.00042998
28	A0A078EC08	BnaC02g30660D protein	*Brassica napus*	BnaC02g30660D	4	1	10.98	7.74	10	1	10.0	0.00044339
29	Q6IMJ9	MRJP7	*Apis mellifera*	MRJP7	2	1	50.54	4.90	41	26	63.4	0.00063154
30	A0A088AU27	Uncharacterized protein	*Apis mellifera*	Mrjp7	4	1	50.85	4.91	42	25	59.5	0.00073238
31	A0A078DVE1	BnaA10g14580D protein	*Brassica napus*	BnaA10g14580D	3	1	97.78	9.12	113	8	7.1	0.00083876

Tab. 2. *continued*

No.	Accession number	Protein	Organism species	GN	PE	SV	*Mw* [kDa]	*pI*	Number of peptides		Matching sequence [%]	*p*
									Theoretical	Experimental		
32	A0A078C268	BnaC03g10390D protein	*Brassica napus*	BnaC03g10390D	3	1	90.70	6.98	95	8	8.4	0.00094543
33	A0A078EQS9	BnaC01g16500D protein	*Brassica napus*	BnaC01g16500D	4	1	19.74	5.29	19	4	21.1	0.00112676
34	O18330	MRJP1	*Apis mellifera*	MRJP1	1	1	48.89	5.10	40	67	167.5	0.00173373
35	A0A078G6I2	Lactoylglutathione lyase	*Brassica napus*	BnaA08g25110D	3	1	31.87	5.26	37	2	5.4	0.00199146
36	A0A088AFH7	Transferrin	*Apis mellifera*	Tsf1	3	1	78.63	6.77	90	7	7.8	0.00207476
37	A0A078EPU3	BnaC02g07610D protein	*Brassica napus*	BnaC02g07610D	3	1	84.43	5.98	87	20	23.0	0.00226977
38	A0A078GJY6	BnaC09g39920D protein	*Brassica napus*	BnaC09g39920D	3	1	84.47	6.05	88	20	22.7	0.00279593
39	Q6W3E3	MRJP6	*Apis mellifera*		2	1	49.79	5.89	39	33	84.6	0.00307086
40	A0A078E9S3	BnaA07g26180D protein	*Brassica napus*	BnaA07g26180D	4	1	109.66	5.28	80	3	3.8	0.00330839
41	A0A078GLG4	Malate dehydrogenase	*Brassica napus*	BnaC06g06220D	3	1	35.94	8.23	36	5	13.9	0.0035919
42	A0A078GIN3	BnaC05g34710D protein	*Brassica napus*	BnaC05g34710D	4	1	51.84	6.09	38	4	10.5	0.00567576
43	A0A078C1J3	BnaC03g08690D protein	*Brassica napus*	BnaC03g08690D	3	1	84.38	5.91	86	21	24.4	0.00762898
44	A0A078FUC7	BnaC09g36890D protein	*Brassica napus*	BnaC09g36890D	3	1	106.12	8.96	123	8	6.5	0.01442712
45	A0A078FHF1	BnaA02g12590D protein	*Brassica napus*	BnaA02g12590D	4	1	10.35	7.47	7	4	57.1	0.01453515
46	A0A088AU21	Uncharacterized protein	*Apis mellifera*	Mrjp6	4	1	49.88	5.95	39	37	94.9	0.01791861
47	A6YLP9	High Glx storage protein	*Apis mellifera*		2	1	112.19	6.43	58	4	6.9	0.02243202
48	A0A078FG82	BnaC03g14230D protein	*Brassica napus*	BnaC03g14230D	4	1	10.32	4.72	7	1	14.3	0.02387677
49	A0A078HZ31	Glyceraldehyde-3-phosphate dehydrogenase	*Brassica napus*	BnaC05g47450D	3	1	38.42	7.09	45	7	15.6	0.0271424
50	A0A088AJR6	Uncharacterized protein	*Apis mellifera*	LOC410149	4	1	52.95	5.26	41	2	4.9	0.02744587
51	A0A088AMK2	Uncharacterized protein	*Apis mellifera*	LOC413324	3	1	51.13	8.61	57	8	14.0	0.05258294
52	A0A087ZSJ1	Uncharacterized protein	*Apis mellifera*	LOC725484	4	1	10.16	8.76	10	4	40.0	0.09818074
53	A0A078DIA5	BnaA06g28250D protein	*Brassica napus*	BnaA06g28250D	4	1	20.53	8.28	29	1	3.4	0.1171258
54	A0A078GV11	BnaA02g25020D protein	*Brassica napus*	BnaA02g25020D	4	1	24.59	5.21	23	1	4.3	0.12289841
55	A0A078DXI5	BnaC03g32850D protein	*Brassica napus*	BnaC03g32850D	4	1	40.45	5.61	39	3	7.7	0.23367496
56	A0A078EKB9	BnaC07g35460D protein	*Brassica napus*	BnaC07g35460D	4	1	18.58	4.71	15	1	6.7	0.37390097
57	A0A078GVL8	BnaA06g25580D protein	*Brassica napus*	BnaA06g25580D	3	1	55.91	5.95	45	1	2.2	0.37523118
58	A0A078HSF0	BnaA04g13950D protein	*Brassica napus*	BnaA04g13950D	3	1	51.80	8.15	47	4	8.5	0.39565907
59	A0A078GAH2	BnaA06g37080D protein	*Brassica napus*	BnaA06g37080D	3	1	46.52	5.90	33	5	15.2	0.39949111
60	D3JXA7	MRJP4	*Apis mellifera*		2	1	53.01	5.89	43	21	48.8	0.42047707
61	A0A078EMV8	BnaC03g37770D protein	*Brassica napus*	BnaC03g37770D	4	1	28.40	5.63	19	2	10.5	0.42655874

Tab. 2. *continued*

No.	Accession number	Protein	Organism species	GN	PE	SV	*Mw* [kDa]	*pI*	Number of peptides		Matching sequence [%]	*p*
									Theoretical	Experimental		
62	A0A088AEG8	α-Amylase	*Apis mellifera*	LOC406114	3	1	55.81	7.22	53	26	49.1	0.43979619
63	A0A088APM4	Glucosylceramidase	*Apis mellifera*	LOC409708	3	1	59.31	5.34	49	7	14.3	0.45990225
64	A0A078DAX9	BnaA06g31170D protein	*Brassica napus*	BnaA06g31170D	4	1	19.39	4.98	19	2	10.5	0.50811129
65	A0A088AF74	Uncharacterized protein	*Apis mellifera*	LOC725114	4	1	10.07	5.70	7	1	14.3	0.50881543
66	Q6TGR0	MRJP8	*Apis mellifera*	Mrjp8	2	1	47.0	6.0	36	5	13.9	0.61681596
67	A0A087ZYX8	Uncharacterized protein	*Apis mellifera*	SP28	3	1	44.6	5.9	30	5	16.7	0.61883849
68	A0A087ZVX2	Uncharacterized protein	*Apis mellifera*	GMCOX14	3	1	73.1	5.9	69	25	36.2	0.78684466
69	A0A088AHL6	Uncharacterized protein	*Apis mellifera*	OC410219	4	1	58.7	5.1	84	3	3.6	0.81385278
70	Q9U8X5	α-Amylase	*Apis mellifera*		2	1	55.9	6.9	53	27	50.9	0.82207203
71	A0A088AU20	Uncharacterized protein	*Apis mellifera*	Mrjp4	4	1	52.9	5.9	43	19	44.2	0.92373436
72	A0A087ZSJ0	Uncharacterized protein	*Apis mellifera*	LOC100577210	4	1	11.6		9	7	77.8	0.97113635
73	A0A088ALP3	Uncharacterized protein	*Apis mellifera*		4	1	29.51	5.16	49	2	4.1	1.67E-05
74	A0A088AU24	Uncharacterized protein	*Apis mellifera*	LOC102654393	4	1	19.56	4.68	14	1	7.1	0.00038997
75	A0A078DVP2	BnaC02g26890D protein	*Brassica napus*	BnaC02g26890D	4	1	34.12	4.23	41	2	4.9	0.00139629
76	A0A078FLZ2	BnaC06g14290D protein	*Brassica napus*	BnaC06g14290D	4	1	37.00	4.51	61	2	3.3	0.00308425
77	A0A087ZXA0	Uncharacterized protein	*Apis mellifera*	LOC727193	3	1	34.13	6.30	26	11	42.3	0.02208167
78	A0A088A5D7	Uncharacterized protein	*Apis mellifera*	LOC552229	3	1	61.78	4.59	40	15	37.5	0.03023905
79	A0A078CED8	BnaA01g05100D protein	*Brassica napus*	BnaA01g05100D	4	1	20.24	5.25	19	1	5.3	0.03865292
80	A0A088AC16	Uncharacterized protein	*Apis mellifera*	LOC408608	4	1	19.43	6.60	21	9	42.9	0.04893423
81	A0A088ADM5	Uncharacterized protein	*Apis mellifera*	SP3	3	1	39.46	6.39	33	16	48.5	0.09315544
82	A0A088APM5	Glucosylceramidase	*Apis mellifera*	LOC409709	3	1	58.57	7.03	56	7	12.5	0.11554385
83	P00330	Alcohol dehydrogenase	*Saccharomyces cerevisiae**	ADH1	1	5	36.85	6.21	32	16	50.0	0.12648866
84	Q8N0N7	α-Amylase	*Apis mellifera*		3	1	56.01	7.22	54	28	51.9	0.1528523
85	A0A078CFF0	BnaA01g01570D protein	*Brassica napus*	BnaA01g01570D	4	1	30.93	4.56	15	4	26.7	0.21762234
86	A0A088A031	Uncharacterized protein	*Apis mellifera*	LOC406081	3	1	67.84	6.36	57	29	50.9	0.28834306
87	Q4ZJX1	MRJP9	*Apis mellifera*	MRJP9	2	1	48.7	8.7	43	9	20.9	0.73691881

Designations according to UniProt database (UniProt Consortium, Hinxton, United Kingdom): GN – GeneName, PE – ProteinExistence, SV – SequenceVersion.
Mw – molecular weight, *pI* – isoelectric point, *p* – significance value *(*ANOVA), MRJP – major royal jelly protein, * – *Saccharomyces cerevisiae* (strain ATCC 204508 / S288c).

normally degraded through an autophagy process [47].

In the present study we identified around 20 different plant proteins in the honey sample, e.g. Bna proteins, polygalacturonase (Q7Y1T6), non-specific lipid-transfer protein (A0A078CBK9), lactoylglutathione lyase (A0A078G6I2), malate dehydrogenase (A0A078GLG4), glyceraldehyde-3-phosphate dehydrogenase (A0A078HZ31) (Tab. 2). We should mention a big number of identified different Bna proteins, most of them uncharacterized, but some of them can be involved in methionine biosynthetic process (A0A078C268; A0A078FUC7; A0A078C1J3) or have polygalacturonase activity (A0A078HSF0; A0A078G9V9), hydrolase activity (A0A078I9J4; A0A078FW97), endopeptidase activity (A0A078GIN3). The other proteins detected by gel-free MS were non-specific lipid-transfer protein, transfer phospholipids and cross-membrane galactolipids (Tab. 2, No. 13, 23). They may play a role in wax or cutin deposition in the cell walls of expanding epidermal cells and certain secretory tissues. Polygalacturonase (pectin depolymerase, PG – Tab. 2, No. 10) degrades pectin and is important for the fruit ripening process, pollen tube elongation and abscission. Lactoylglutathione lyases (glyoxalases – Tab. 2, No. 35) are important in stress response and tolerance in plants [48]. It was shown that overexpression of the glyoxalase pathway in transgenic tobacco and rice plants has been found to control an increase of reactive oxygen species (ROS) and $Mg(^{2+})$ under stress conditions by maintaining glutathione homeostasis and antioxidant enzyme levels [49]. Malate dehydrogenase that was identified from gel-free MS analysis (Tab. 2, No. 41) plays a crucial role in the physiological processes of plant growth and development.

CONCLUSIONS

For the first time, we characterized protein content of buckwheat (*Fagopyrum esculentum* Moench) blossom honey using different techniques. Our results revealed that proteins found in honey fall into two main groups, being of plant origin and of bee origin. Proteins of plant origin can be attributed to the different functional groups according to their activity like non-specific lipid-transfer protein; involved in methionine biosynthetic process; antioxidant properties; in different plant development processes like abscission or growth. The main proteins of bee origin were associated with royal jelly proteins. By 2DE, three MRJP were identified, among which one protein of MRJP1 and two proteins of MRJP2. Using gel-free MS analysis, 9 major royal jelly proteins were identified (MRJP1–MRJP9). Among these, two MRJP5 proteins (accession numbers O97432 and D3JZ08) had the highest molecular weight (70.24 kDa and 70.18 kDa). Among the identified proteins, a storage protein High Glx of bee origin (accession number A6YLP9) had the highest molecular weight of all proteins identified in this study, 112.19 kDa.

Separation of proteins by 1DE and 2DE showed only two MRJP1 proteins with low expression differences. Likewise, low expression differences were noticed between two identified MRJP2 proteins and between two glucosidase enzymes of bee origin (accession number Q17058). Separation of proteins by 2DE coupled to mass spectrometry created an opportunity to reveal proteins associated with yeasts. The results provided also valuable information concerning honey storage. Data obtained by this study on protein diversity can be useful not only for researchers but also for beekeepers.

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