Proteomic studies of honeybee- and manually-collected pollen

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Abstract
In this study a new approach to quantitative and qualitative proteomic evaluation of pollen of different botanical origin was proposed. A monofloral mixture of plum (Prunus) – 86.83 ± 0.70% and willow (Salix spp.) – 13.7 ± 0.30% pollen, and monofloral plum (Prunus) pollen collected by bees was studied. Other pollen was collected manually from pear (Pyrus communis), apple tree (Malus sylvestris), cherry (Prunus/Cerasus) and wild cherry (Prunus avium). Samples were made during an early spring plant blossom. Proteins isolated from pollen samples were fractionated by a two-dimensional electrophoresis (2-DE) and about 50 proteins were identified using mass spectrometry analysis. A three-dimensional (3-D) visualization of proteins was performed. The intensities of protein spots differed in the orchard pollen samples more than ten times. The biological functions of the identified proteins differ, i.e. they are involved in transcription / translation, metabolic and other cellular processes.

The data revealed up-regulated process of the enzyme 5-methyltetrahydropteroyltriglutamate-homocysteine methyltransferase 1 in all pollen involved in methionine formation, which was the highest in monofloral orchard pollen collected by bees. This enzyme was identified in all the tested pollen. High level of expression of putative flavin-containing monooxygenase FMO GS-OX-like 11 protein was found in manually-collected pollen. This protein is important for plant protection against pathogens as well for plant hormone biosynthesis.

Key words: 2-D electrophoresis, 3-D visualization, mass spectrometry, pollen, proteins.

Introduction
Bee pollen has been recommended as a valuable dietary supplement, especially after surgery, to children with loss of appetite and to people working hard physically and mentally. Pollen has anti-inflammatory, detoxifying, antibiotic and sometimes even anti-allergic activity. All these properties are due to the unique composition of pollen (Komosinska-Vassev et al., 2015).

There are marked differences in the composition and content of carbohydrates between manually-collected pollen and pollen pellets (Conti et al., 2016). In the pollen collected from anthers of 15 studied plant species, the content of total carbohydrates varied in range from 1.22% to 5.76% and protein content – from 11.20% to 48.40% (Tidke, Nagarkar, 2015). Sugar content in pollen pellets ranged from 21.77% to 58.95% and protein content – from 13.8% to 30.4% (Conti et al., 2016).

Proteomic analysis is used in pollen germination research. It was found that carbohydrate / energy metabolism, wall metabolism, protein synthesis, degradation and signalling was overrepresented in the mature pollen of Orzya sativa L. ssp. japonica (Dai et al., 2006). In the latter research there were identified 322 unique proteins, 23% of them having more than one isoform. It can be supposed that carbohydrates are essential for energy metabolism in anther of plant pollen. Honeybee foragers collect pollen grains from flowers, moisten them with nectar and mouth secretions, and so transform to “bee pollen”. Addition of carbohydrates during the pollen collection process by bees changes pollen weight and influences pollen enzymatic modifications (Mauriello et al., 2017).

Protein content in pollen pellets is species-dependent, while sugar content is not species-specific. Foragers add between 4.3 and 44.4 μM sugar mg⁻¹ pollen load. The content of this component added to pollen grain is associated with pollen dimensions and weight as well as environmental factors (Leonhardt, Blüthgen, 2012). Pollen proteins are composed of eighteen amino acids, including essential amino acids vital for bee diet: methionine, tryptophan, arginine, lysine, histidine,
phenylalanine, isoleucine, threonine, leucine and valine (Negrao, Orsi, 2018). Studies show that nutritional needs of young bees for essential amino acids are greater compared to foragers (Paoli et al., 2014).

Kędžia (2008) has found nucleoproteins, such as histones, protamines, albumins and globulins, in pollen; the latter compounds are associated with nucleic acids. Pollen contains from 0.6% to 4.8% of nucleic acid. The author suggests that deoxyribonucleic acid (DNA) is concentrated in the nuclei of pollen cells of a generative nature, while ribonucleic acid (RNA) was identified in the nuclei of pollen cells of a vegetative nature.

There is little published data regarding proteomic studies of orchard pollen. Wang et al. (2018) have identified differentially expressed proteins among the pollen from the ‘Snowdrift’ crabapple, ‘Hongling’ crabapple, ‘Hongjin’ crabapple and ‘Gala’ apple trees. The authors have reported that differentially expressed proteins found in pollen are involved in these functions: metabolism related proteins, stress regulatory proteins and proteins involved in signal transduction. Proteomic analysis was used for the evaluation of changes in the apple fruit during different stages of ripening, maturation and senescence and the quality of apple (Shi et al., 2014).

Holmes-Davis et al. (2005) have identified 135 distinct proteins in Arabidopsis thaliana (Columbia ecotype) pollen that are involved in different processes like metabolism, energy generation or cell structure. Other studies quantified Arabidopsis pollen coat proteome and buckwheat (Fagopyrum esculentum) honey proteome (Mayfeld et al., 2001).

Honeybee-collected pollen from tea tree (Camellia sinensis), preserved at room temperature and at −20°C, was subjected to protein composition analysis (Li et al., 2008). Proteins in the pollen were separated by a two-dimensional electrophoresis (2-DE) in a sodium dodecyl sulphate-polyacrylamide gel (SDS-PAGE) system (2D-PAGE) and identified using a matrix-assisted laser desorption / ionization time of flight (MALDI-TOF) mass spectrometry (MS) analysis and other methods. The authors identified more proteins in frozen pollen compared to the pollen preserved at room temperature and stated that storage conditions affected the protein abundance in the tested pollen samples.

The variation of protein expression in pollen manually-collected from the red clover cultivar ‘Vycia’, berseem clover ‘Faron’ and white clover ‘Medanai’, analysed by a mass spectrometry previously was investigated (Treigytė et al., 2014). Pollen was stored at −80°C until analysis. Over 30 protein spots whose quantitative levels were most divergent in created and investigated clover pollen proteome map was detected. By means of MALDI-TOF mass spectrometry (MS/MS), analysis was performed in buckwheat honey. A total of 87 proteins were identified in this kind of honey. Using the native PAGE analysis method catalase and glucose oxidase activity in buckwheat honey was estimated (Borutinskaite et al., 2018).

The aim of present study was to develop a reference map of the bee- and manually-collected orchard pollen proteome using protein extraction technique followed by a two-dimensional electrophoresis (2-DE) and a mass spectrometry analysis and to compose a three-dimensional (3-D) visualization of protein spots, and characterize the potential functions of proteins and their expression.

Materials and methods

Honeybee-collected orchard pollen. All pollen samples were collected in the apiaries of Institute of Agriculture, Lithuanian Research Centre for Agriculture and Forestry, located in Kėdainiai district, Lithuania. Orchard pollen gathered by bees was collected in accordance with good beekeeping practices and did not interfere with normal colony growth (Gracham, 1992). Pollen gathered by honeybees was collected with a standard pollen trap purchased from the company “Wilara” (Lithuania). The trap was mounted on the hive entrance and pollen was collected in good weather during fruit tree blossoming. After removal from traps, the pollen was cleaned. One half of the bee-collected orchard pollen was marked as G1, and the other sample G2 was selected by colour and composed from G1. Botanical origin of G1 and G2 samples was confirmed microscopically. Botanical origin was determined by comparison of light microscopic images of pollen found in pollen samples to those of known pollen manually-collected (Čekstytė, 2012). The G1 sample consisted of 86.83 ± 0.70% plum (Prunus) and 13.7 ± 0.30% willows (Salix spp.) pollen, and G2 sample consisted of 100.0% plum pollen. All samples were kept in a refrigerator at −80°C in air-tight plastic bags until analysis.

Manually-collected pollen. Pollen samples were collected during early spring blossom of different fruit trees during 2013–2016. Pollen was collected from approximately 100–200 flowers picked from each plant. Samples of manually-collected pollen were composed from fruit plants: pear (Pyrus communis) (G3), apple tree (Malus sylvestris) (G4), cherry (Prunus / Cerasus) (G5), plum (Prunus) (G6) and wild cherry (Prunus avium) (G7). Pollen grains were manually-collected into Eppendorf tubes (Eppendorf AG, Germany) and immediately placed in a storage at −80°C until analysis. The amount of pollen collected was 50–70 mg per sample, each sampling was repeated three times.

Pollen expression results. About 400–500 pollen grains were counted in each sample. The frequency of pollen of each melliferous plant is expressed as percentage of the total pollen sum. Pollen considered as monofloral is mainly produced from one plant species or pollen content from one plant species is predominant (constituting more than 45.0%). The pollen content of other plant species is designated as follows: secondary pollen 16–45%; important minor pollen 3–15%; minor pollen. This methodology is applied for the determination of honey pollen botanical composition (Louveaux et al., 1978). The same methodology and range of pollen levels are used to evaluate botanical composition of bee-collected pollen and bee bread (Almeida-Muradiana et al., 2005; Čekstytė et al., 2016). Manually-collected pollen was of 100 % purity; therefore, the data from those samples were not subjected to statistical analysis.

Protein isolation from pollen. Proteins from mature pollen (approx. 20 mg) were isolated as described by Sheoran et al. (2007) with some modifications (Treigytė et al., 2014). Shortly, mature pollen was homogenized with acetone containing 10% trichloroacetic acid (TCA) and 1% dithiothreitol (DTT). The solution was centrifuged 20,000× g for 20 min at 4°C and pellet was washed two more times with acetone solution containing 1% DTT. The pellet was dried in vacuum and proteins were extracted with isoelectric focusing (IEF) lysis buffer containing 9 M urea, 2 M thiourea, 4% CHAPS (3-[3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), 1% DTT, 0.8% IPG (immobile pH gradient) buffer, pH 3–10. The solution was centrifuged 20,000× g for 20 min at 4°C and pellet was extracted again with IEF lysis buffer. After centrifugation, both extracts were combined, quantified (3–3.5 mg per 20 mg pollen) using the BioRad DC protein assay kit (Bio-Rad Laboratories, USA) and directly used for protein analysis or stored at −20°C until analysis. Three independent biological experiments were carried out.

Protein fractionation by two-dimensional electrophoresis (2-DE). Proteins isolated from bee- and manually-
collected pollen were resolved by 2-DE gel isoelectric point (pI) 3–11, gel gradient 7.5–20%. Areas of interest were cut out from the 2-DE gel and subjected to overnight in-gel tryptic digestion (Shevchenko et al., 1996; Treigytė et al., 2014).

**Mass spectrometry analysis.** 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 (a-cyano-4-hydroxycinnamic acid) on the target plate. The analysis was performed in a 4800 MALDI TOF/TOF analyser (Applied Biosystems/MDS SCIEX, Canada), which was externally calibrated using synthetic peptides with known masses. Mass spectrometry (MS) reflector mode setting was: m/z range 800–4000, 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 (The UniProt Consortium, UK).

**Image acquisition and data analysis.** Stained 2-DE gels were digitized on ImageScanner™ III scanner (GE Healthcare Bio-sciences, Germany) using software LabScan, version 6.0 (GE Healthcare Bio-sciences, Germany) application that is specialized for acquisition of 2-DE gel images. To ensure linearity of response and minimize software-induced variance in gel image analysis, calibration of the scanner was performed before scans using provided step tablet. Gels were scanned at 300 dpi resolution and saved in tiff format. Analysis of 2-DE gel images was performed using originally developed software prototype with new pre-processing, alignment, segmentation and subsequent analysis algorithms. All required tools were implemented using programming language MATLAB (The MathWorks, USA). Employed software tools allow: to crop gel images in order to keep only protein separation area; align images; automatically detect protein spots; manually edit protein spot area to eliminate false positives and false negatives if needed; quantify spots and estimate expression changes; visualize gel regions in 3-D or 2-DE. The process of 2-DE gel image analysis can be performed in two basic ways: spot detection and delineation are performed prior to image alignment, or in the reversed order (Dowsey et al., 2010). The following sequence of gel image analysis steps was used: image pre-processing, image alignment, spot detection with segmentation in registered images and differential analysis. Differential analysis provides ratios of normalized spot quantities that describe differences between experimental groups; changes of spot abundance between the gel groups (Treigytė et al., 2014).

**Results and discussion**

**Creation and characterization of two-dimensional electrophoresis (2-DE) protein gel maps.** In this study, the proteins in bee- and manually-collected pollen samples was determined. Proteins from pollen were separated on 2-DE systems: visualized and mass spectrometry analysis was performed. Results of comparative 2-DE gel (Fig. 1) image analysis with identified proteins are summarized in Table.

**Figure 1.** Protein isolation by two-dimensional electrophoresis (2-DE) gel (isoelectric point 3–11, gradient 5–18%)
<table>
<thead>
<tr>
<th>No.</th>
<th>Accession number</th>
<th>Description of protein</th>
<th>Theoretical MW, kDa</th>
<th>Experimental MW, kDa</th>
<th>G1:G2</th>
<th>G1:G3</th>
<th>G1:G4</th>
<th>G1:G5</th>
<th>G1:G6</th>
<th>G1:G7</th>
<th>G2:G6</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>18 Q9SSE5</td>
<td>Zinc finger protein CONSTANS-LIKE 9</td>
<td>40.7</td>
<td>5.8</td>
<td>40.6</td>
<td>8.4</td>
<td>−1.99</td>
<td>−1.67</td>
<td>1.14</td>
<td>−1.32</td>
<td>−1.52</td>
</tr>
<tr>
<td>2</td>
<td>18 Q9C7C3</td>
<td>Zinc finger CCCH domain-containing protein 36</td>
<td>25.9</td>
<td>9.5</td>
<td>12.7</td>
<td>7.8</td>
<td>−1.05</td>
<td>−1.39</td>
<td>−2.18</td>
<td>−1.60</td>
<td>−1.61</td>
</tr>
<tr>
<td>3</td>
<td>20 Q9MAT5</td>
<td>Protein arginine N-methyltransferase PRMT10</td>
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<td>5.1</td>
<td>16.9</td>
<td>4.7</td>
<td>−5.08</td>
<td>−2.97</td>
<td>−1.21</td>
<td>−2.53</td>
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<td>4</td>
<td>32 Q9LRK3</td>
<td>Mediator of RNA polymerase II transcription subunit 37a</td>
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<td>5.0</td>
<td>79.2</td>
<td>4.9</td>
<td>−1.10</td>
<td>1.58</td>
<td>−2.07</td>
<td>1.46</td>
<td>−1.04</td>
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<td>5</td>
<td>33 Q9SR07</td>
<td>Probable WRKY transcription factor 39</td>
<td>36.6</td>
<td>9.4</td>
<td>37.6</td>
<td>7.3</td>
<td>−1.12</td>
<td>−2.60</td>
<td>−1.63</td>
<td>−1.33</td>
<td>−2.05</td>
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<tr>
<td>6</td>
<td>34 P25209</td>
<td>Nuclear transcription factor Y subunit B</td>
<td>18.9</td>
<td>5.9</td>
<td>28.3</td>
<td>10</td>
<td>−1.21</td>
<td>−5.07</td>
<td>−1.61</td>
<td>−1.63</td>
<td>−2.27</td>
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<tr>
<td>7</td>
<td>47 P13911</td>
<td>DNA-directed RNA polymerase subunit alpha</td>
<td>38.9</td>
<td>7.2</td>
<td>36.9</td>
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<td>1.11</td>
<td>−1.73</td>
<td>−7.20</td>
<td>−1.10</td>
<td>−3.11</td>
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</table>

**Table.** Proteins identified from two-dimensional electrophoresis (2-DE) gel by mass spectrometry and their potential biological function.
### Table continued

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Accession No.</th>
<th>Function</th>
<th>Fold Change</th>
<th>MW (kDa)</th>
<th>PI</th>
<th>Fold Change</th>
<th>MW (kDa)</th>
<th>PI</th>
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<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>P01060</strong></td>
<td>Bowman-Birk type proteinase inhibitor 2</td>
<td>11.6 5.17 25.1 5.9</td>
<td>7.12</td>
<td>2.57</td>
<td>-3.94</td>
<td>-1.25</td>
<td>-8.55</td>
<td>1.13</td>
</tr>
<tr>
<td><strong>Q8LZ1</strong></td>
<td>CASP-like protein At1g15630</td>
<td>20.0 9.9 20.5 6.1</td>
<td>2.38</td>
<td>-1.92</td>
<td>-2.12</td>
<td>1.39</td>
<td>1.22</td>
<td>1.95</td>
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<tr>
<td><strong>Q952K5</strong></td>
<td>Zinc finger A20 and AN1 domain-containing stress-associated protein 6</td>
<td>17.5 8.9 11.8 11</td>
<td>4.00</td>
<td>1.78</td>
<td>-3.47</td>
<td>2.31</td>
<td>1.57</td>
<td>5.60</td>
</tr>
<tr>
<td><strong>1Q7T15</strong></td>
<td>Protein BREVIS RADIX</td>
<td>38.7 6.3 20.4 5.4</td>
<td>-1.15</td>
<td>-1.45</td>
<td>-4.36</td>
<td>-2.26</td>
<td>-1.35</td>
<td>-1.81</td>
</tr>
<tr>
<td><strong>P24924</strong></td>
<td>Trypsin inhibitor</td>
<td>19.5 6.2 12.6 3.1</td>
<td>-3.47</td>
<td>-11.3</td>
<td>-14.3</td>
<td>-6.41</td>
<td>-5.94</td>
<td>-4.65</td>
</tr>
<tr>
<td><strong>P01060</strong></td>
<td>Bowman-Birk type proteinase inhibitor 2</td>
<td>11.6 5.1 14.9 9.9</td>
<td>1.57</td>
<td>-2.08</td>
<td>-2.63</td>
<td>-1.15</td>
<td>1.08</td>
<td>1.25</td>
</tr>
<tr>
<td><strong>P38475</strong></td>
<td>Uncharacterized mitochondrial protein ymiE3</td>
<td>15.5 8.4 15 9.7</td>
<td>3.82</td>
<td>1.48</td>
<td>-1.09</td>
<td>1.31</td>
<td>1.08</td>
<td>-1.10</td>
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<tr>
<td><strong>Q9CTM1</strong></td>
<td>Putative FBD-associated F-box protein At1g55030</td>
<td>48.1 8.6 37.8 10</td>
<td>3.50</td>
<td>8.00</td>
<td>3.38</td>
<td>1.41</td>
<td>11.6</td>
<td>1.22</td>
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<tr>
<td><strong>P93208</strong></td>
<td>14-3-3 protein 2</td>
<td>28.8 4.7 25.4 4.2</td>
<td>-3.58</td>
<td>-1.08</td>
<td>1.11</td>
<td>-1.31</td>
<td>-1.17</td>
<td>-1.43</td>
</tr>
<tr>
<td><strong>Q6NPC9</strong></td>
<td>Probable RNA methyltransferase At5g51130</td>
<td>36.5 9.2 37.6 7.3</td>
<td>-1.12</td>
<td>-2.60</td>
<td>-1.63</td>
<td>-1.33</td>
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<td><strong>Q9TO43</strong></td>
<td>Putative F-box protein At4g58870</td>
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<td>2.76</td>
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<td>1.53</td>
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<tr>
<td><strong>Q9FHD5</strong></td>
<td>Cysteine-rich repeat secretory protein 5</td>
<td>31.8 5.5 37.2 5.8</td>
<td>-2.89</td>
<td>-4.13</td>
<td>-3.13</td>
<td>-2.72</td>
<td>-1.51</td>
<td>-1.05</td>
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</tbody>
</table>

### Notes
No. – spot number in 2-DE gel (G1–G7), numbers in column correspond to numbering of proteins in 2-DE gel; accession number in UniProt database (UniProt Consortium, 2015), MW – molecular weight, pl – isoelectric point; G1 – Prunus and Salix spp. mixture of monofloral orchard pollen collected by bees, G2 – monofloral Prunus pollen collected by bees, G3–G7 – manually-collected pollen: G3 – Pyrus, G4 – Malus sylvestris, G5 – Prunus / Cerasus, G6 – Prunus, G7 – Prunus avium. Changes in protein spot volumes are represented as ratios of averages of normalized spot volumes (Gx/Gy); an increase in spot abundance is represented with positive fold change and a decrease, with a negative fold change.

Protein spots in maps were overlapped and quantitative changes in protein levels were evaluated by computer assisted analysis (Table). Numbers (No.) in the 2-DE maps indicate the positions of proteins subjected to mass spectrometry analysis. Spot labels (No.) are the same as in Table. Representative images from one of the three experiments showing similar results are shown. A total of 48 proteins were identified by mass spectrometry. Their changes in various orchard pollen samples were designated and grouped according to cellular function (Table). Ratios of normalized spot quantities along with the experimental molecular weight (MW) and isoelectric point (pl) data were estimated. Identified proteins are marked with corresponding labels in Figure 2 and Table.

Isoelectric points of identified proteins were determined according to known pl values of the used pl standard 3–11. Molecular weights of protein spots were computed by comparing (using a standard curve) their relative mobilities to relative mobilities of the marker proteins (standards) whose molecular weight is known. Changes in protein spot volumes are represented as ratios of averages of normalized spot volumes, currently designing Gx/Gy. An increase in spot abundance is represented with positive fold change and a decrease, with a negative fold change. Table summarizes changes that were determined between protein maps of G1–G7 (Fig. 1). Twenty eight proteins were up- (increase) or down-regulated (decrease) in different pollen samples more than five times (Table).

**Protein three-dimensional (3-D) visualization and description of potential function of identified proteins in different pollen samples.** About 50 proteins in the pollen of different origin were indentified. Most (27) of the identified proteins are involved in metabolic and biosynthesis processes and molecule transport (Table, Fig. 2). A few proteins – subunits of V-type proton ATPase which play a role in metabolism was identified (Table, No. 5 Q39442 and No. 10 O23948). It was shown in literature that V-ATPase function is essential for Golgi organization and development of the male gametophyte (pollen), plant growth via V-ATPase-dependent endosomal trafficking (Zhou et al., 2016).
Other enzymes involved in metabolic process such NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (Table, No.7 Q1IWQ6 and No. 16 P26517), delta-1-pyrroline-5-carboxylate dehydrogenase (Table, No.12 Q8VZC3), monoxygenase (Table, Nos 17 and 19 Q95SH5), pyruvate dehydrogenase E1 (Table, No. 48 Q38799), probable aldehyde dehydrogenase (Table, No.12 Q8VZC3), were also found with changed expression levels between different pollen samples. Mostly four enzymes, such as catalase, superoxide dismutase, alcohol dehydrogenase and pyruvate decarboxylase, are believed to play a central role against oxidative stress in plants during photosynthesis and respiration (Alschner et al., 2002). Seven proteins (Q9SSE5, Q9C7C3, Q9MAT5, Q9LKR3, Q9SR07, P2S509 and P13911) are involved in transcription and five (Q8M9W2, P49397, A7M955, A1EAA43 and P29197) – in translation processes. The 11 proteins with unknown function was identified (Table, Fig.2).

**Figure 2.** The distribution of proteins, identified by mass spectrometry after two-dimensional electrophoresis (2-DE) gel, according to their biological function

Having assessed all the relationships Gx:Gy in protein spot volumes it was found that some quantities of particular protein type vary more than 10 times. The following proteins were selected from the highest to lowest protein spot volumes ratio for their spots: No.15 (arsenate reductase), No. 44–45 (5-methyltetrahydropteroylglutamate), No. 18 (UTP-glucose-1-phosphate uridylyltransferase), No. 17 (putative flavin-containing monoxygenase), No. 21 (trypsin inhibitor), No. 39 (translation initiation factor IF-1), No. 14 (profilin), No. 24 (putative F-box protein) and No. 43 (chaperonin CPN60). The automatic spot matching fails in such situations and the exact position of small protein spot was noticed only in 3-D view of the gel, followed by stereoscopic visualization. A 3-D visualization of matched and identified protein spots is shown in Figure 3.

Proteins identified in spots (14–18, 21, 24, 39 and 43–45) were overlapped in maps. Quantitative changes of proteins were evaluated by a computer-assisted analysis (Table 1) and the 3-D view of those protein spots was composed additionally by the mass spectrometry analysis. The identified protein fold change described above was ≥5.

Arsenate reductase (glutaredoxin) (Table, spot No. 15 Q10XS6) was overexpressed only in the sample from Malus sylvestris pollen. Arsenate reductase protein is required for arsenate resistance (Abbas et al., 2018). It was shown that absence of arsenic reductase in Arabidopsis results in accumulation of arsenic 10- to 16-fold more compared with wild-type plants and this can lead to toxic effects on pollen tube development (Dhankher et al, 2006). The Food Standards Agency’s final report provides data on the concentration of arsenic in fruit and vegetables. Arsenic was in low concentration (1–6.7 ng g⁻¹ fresh weight) in apple samples compared with other fruits and vegetables, which can be due to high arsenate reductase expression (Meharg et al., 2012).

Another protein, identified by mass spectrometry, whose protein spot volumes varies from 7.02×10⁻⁷ to 17.4 in different pollen samples, but predominates mostly in G1 sample, is 5-methyltetrahydropteroylglutamate synthase (Table, Fig. 3, spot No. 44 Q50008). This protein is involved in the pathway that synthesizes L-methionine – sulphur-containing amino acid required for protein synthesis (Ravanel et al., 2004). Methionine is the sulphur-containing amino acid that is essential for mammals and must therefore be derived entirely from the diet. Methionine can be found in pollen of different plants. UTP-glucose-1-phosphate uridylyltransferase (Table and Fig. 3, spot No. 18 Q9LKG7) was found in high expression levels in G4–G7 samples. In plant leaves, UTP-glucose-1-phosphate uridylyltransferase is a key part of the sucrose biosynthesis pathway. Also, this enzyme is essential for microgametophyte development during which the pollen reaches mature stage and desiccates (Wang et al., 2008).

Studies have revealed variation in expression of putative flavin-containing monoxygenase FMO GS-OX-like 11 protein (Table, spot No. 17): low expression in bee-collected samples from orchard and Prunus pollen (G1 and G2) and high expression in hand-collected samples from pollen of Pyrus communis pollen (G3) and Malus sylvestris (G4). It has been shown that its structure is similar to flavin-containing monoxygenase FMO from Arabidopsis. In literature, the data suggesting that over-expression of three FMO, a class of enzymes whose function in plants has been uncharacterized so far, resulted in resistance to pathogens can be found (Koch et al., 2006).

It was found fold changes in expression of proteins whose biological function in pollen is unknown: trypsin inhibitor (spot No. 21, P24924) and putative FBD-associated F-box protein At1g55030 (Table, Fig. 3, spot No. 24, Q9C7M1).

Translation initiation factor IF-1 protein – one of the essential components for the initiation of protein synthesis in chloroplasts – was more expressed in G1, G4 and G7 pollen samples than in G2–G3 and G5–G6 samples (Table, Fig. 3, spot No. 39 A1EA43). Somehow translation initiation factor IF-1 protein expression is higher and probably synthesis of pollen proteins is better in M. sylvestris and P. avium samples for unknown reasons.

The ratios of normalized spot volumes of profilin differ among the pollen of experimental groups (Table, Fig. 3, spot No. 14 O04725). It was found a small level of profilin protein in the sample of P. communis pollen collected by hand (G3) in comparison with all other samples. Arabidopsis profilins, low-Mᵋ actin monomer-binding proteins, were shown to play a role in cell elongation, cell shape maintenance, polarized growth of root hair and in determination of flowering time. Profilins are present in all eukaryotic cells and are identified as allergens in pollen (birch, grass, etc.), latex and plant foods (Ramachandran et al., 2000). So far only four proteins have been identified as Pyrus allergens: pathogenesis-related protein PR-10, nonspecific lipid-transfer protein 1, isoflavone reductase related protein and profilin. Actually, this genus is not commonly cited as a source of pollinosis. Only one study demonstrated that direct, prolonged contact with the pollen could cause eye allergy symptoms in a few individuals. Maybe it is the reason why in our samples, only in the Pyrus pollen sample was found very low amount of profilin (Yanagisawa et al., 1999).

One of the proteins, detected with some differences between the pollen samples, is chaperonin CPN60 (Table 1, Fig. 3, spot No. 43 P29197). It was found CPN60 in low amount in G4 (M. sylvestris) and...
with mass spectrometry. Kaupinis and Dr. Marija Ger for the excellent assistance (No. MIP-083/2015) from the Research Council of Lithuania.

The profilin of this pollen should not cause allergic symptoms for pollen consumers.

The data indicate that profilin expression level is very important for folding of Rubisco assembly, the key protein which is very important for enzyme responsible for respiration (Zhao, Liu, 2018). It was found data in literature that silencing CPN21 in grape seed led to grape seed abortion (Hanania et al., 2007).

In summary, 2-DE protein maps of pollen of different botanical origin collected by hand or by bees, evaluated changes in protein spot quantity, identified about 50 specific proteins and described their potential functions was compared.

Conclusions

1. In this study, for the first time, the reference maps of the bee- and manually-collected orchard pollen proteome by using protein extraction technique followed by a two-dimensional electrophoresis (2-DE) and mass spectrometry analysis was created. It was identified about 50 proteins from a total of ~200 protein spots.

2. The potential biological functions of these proteins vary from transcription, translation to metabolic cellular processes.

3. Different trends of the profilin expression were observed between bee- and manually-collected pollen. The data indicate that profilin expression level is not high in pollen mixture collected by bees from early blossom plants in the spring like plums, willows. Therefore, the profilin of this pollen should not cause allergic symptoms for pollen consumers.

4. All these data can be important for understanding the function and role of pollen protein which can lead to its possible application in food industry.

Acknowledgments

This research was funded by a grant (No. MIP-083/2015) from the Research Council of Lithuania. The authors thank Dr. Mindaugas Vailius, Dr. Algirdas Kaupinis and Dr. Marija Ger for the excellent assistance with mass spectrometry.

Received 12 06 2018
Accepted 03 03 2019

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Bičių ir rankomis surinktų žiedadulkų proteominė analizė

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Sody žiedadulkų proteomą (bendrojo baltymo) sudėties nera išsamiai aprašyta. Straišnyje pateikta skirtingos botaninės kilmės žiedadulkų kiekvieno ir koxybinė proteominė analizė. Tirtas surinktos baltymų svyruočių (Prunus) bei blizgūnių (Salix spp.) monoflorini močinio ir monočlorini močių (Prunus) žiedadulkės. Kitos žiedadulkių mėginių išskirti baltymai, vyšnių (Malus spectabilis), veislių (Trifolium pratense, T. alexandrinum) ir trešnių (Prunus avium) žiedadulkės. Nustatyta, kad identifikuotų baltymų biologinės funkcijos skiriasi ir yra susijusios su transkripcija / transliacija, metaboliniais ir kitais ląsteliniais procesais.

ISSN 1392-3196 / e-ISSN 2335-8947
DOI 10.13080/z-a.2019.106.019.024