Evaluation of Proteome Profiles of *Salix* spp. Pollen and Relationship Between Glucose Oxidase Activity and Pollen Content in Willow Honey

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Abstract

Proteins from hand- and bee-collected pollen were isolated and separated in sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and two-dimensional gel electrophoresis (2DE) system as well as by direct gel-free mass spectrometry (MS) analysis. Total thirty-six plant proteins with known functions and three uncharacterized were identified using mass spectrometry (MS) techniques from fourteen protein spots separated in 2D gel electrophoresis. Our study revealed two forms of profilin proteins with slightly different mass of 14.19 kDa and 14.09 kDa and beta actin fragment with 33.25 kDa both in hand- and bee-collected pollen as well proteins of thioredoxin family with molecular weight of 47.29 kDa. The antioxidant enzyme glucose oxidase (GOX) activity was investigated in twelve samples of monofloral willow honey, having *Salix* spp. pollen from 44.7 % to 80.2 %. Data of paired regression analysis between *Salix* spp. pollen content and GOX activity show moderate and negative correlation coefficient (r = -0.56).

Keywords: willow pollen; honey; protein expression; separation; two-dimensional electrophoresis; mass spectrometry

Introduction

Trees and shrubs, which belong to the family Salicaceae, include the genus *Salix* (willows), the genus *Populus* (poplars, aspens) (and the genus *Chosenia*, represented by the only species). The genus *Salix* (Salicaceae) comprises about 330-500 species, and around 65 species are growing in Europe (Argus 1997). *Salix* spp. has been cultivated as an energy plant for biomass production (Hanley and Karp 2013). The term biomass refers to non-fossilized and biodegradable organic material from plants and microorganisms (Demirbas 2010). Currently, in many countries high-density, short-rotation plantations of fast-growing tree species are grown on agricultural land or on fertile but degraded forest land for the production of woody biomass. Production from short-rotation plantations may replace wood from protected forest and contributes to conservation of valuable native forests (Christersson and Verma, 2006). *Salix* spp. cultivars for biomass and biofuel are grown on plantations in different districts in Lithuania (Konstantinavičienė et al. 2017, Bakšienė and Titova 2018) and *Salix viminalis* and poplar (*Populus nigra* L.) is grown in Western Lithuania. The average dry matter yield of *Salix viminalis* was 7720 t ha^{-1} on a naturally acidic moraine loam in Western Lithuania with pH $_{KCl}$ 4.31 – 4.64 (Jasinskas et al. 2017). Fertilization of short rotation plantations potentially lowers the price of stumpage of *Salix* spp., *Populus* spp., *Pinus taeda* L. However, intensive management of forest land has negative effects on ground water which is contaminated with herbicides and excess of nutrients. Therefore, sustainable management practices must be applied in order to maintain eco-balance in short rotation forests (Singh

2013). The main biofuel market trends are as follows: increased competition within forest industries and conventional agriculture, international trade flows, international debate about the sustainability of biofuel production (Demirbas 2010).

The germination of *Populus* species seeds is very limited (Braatne et al. 2007). The report of proteomic investigation in *Poplar* seed vigour change have been provided by Zhang et al. (2015). Authors stated that *Poplar* seed aging is directly associated with proteins involved in different metabolism processes, protein synthesis, destination, cell defence and rescue. Willow (*Salix caprea*) is one of the first flowering plants in the spring. Flowers of entomophilous *Salix* species are mostly pollinated by insects of Apoidea (*Apis mellifera*, *Bombus* spp.) (Lipiński 2010). Their catkins provide pollen and nectar, which are attractive food sources for bees, other insects and birds. *Salix caprea* is a melliferous plant species producing 150-200 kg/ha honey (Popovici et al. 2007, Ostaff et al. 2015). Inherent *Salix*-species growing in small areas and as solitary trees are important for the landscaping, therefore they should be protected. Tests with *Salix caprea* L. for biomass production were conducted in Sweden. A study showed that the total aboveground dry mass of *Salix caprea* L. averaged 95±50 t ha^{-1} (from 4 to 203 t ha^{-1}) and mean annual increment (MAI) amounted to 2.6 t ha^{-1} year (from 0.8 to 5.6 t ha^{-1}), (Anon 2010). Monofloral willow honey is harvested in many geographical areas in Lithuania (Čeksterytė et al. 2014). Minor components of honey determine its antioxidant properties or functionality associated with the protein contained in different kinds of honey (Gheldof et al. 2002). Stress proteins or heat shock proteins of 70 kilodalton (kDa) Hsp70 were detected in the nectaries of male and female flowers of *Salix gilgiana* the species of willow native to Japan and Korea (Futamura et al. 2000). Heat shock proteins (Hsp70) can protect cells from thermal or oxidative stress. Hsp70 are able to bind to hydrophobic residue proteins affected by stress and protect these partially denatured proteins from further aggregation and allow them to refold. The family of Hsp70 contains isoforms of similar proteins variable in size from 66 kDa to 78 kDa. The main function of Hsp70 is to protect and repair stress damaged proteins (Tavaria et al. 1996). These proteins are known to exist in all living organisms; however, Hsp70 was not identified in the pollen from white clover (*Trifolium repens* L.), red clover (*Trifolium pratense* L.) and berseem clover (Treigytė et al. 2014). Today, it is important to characterize protein content and find possible biomarkers in bee honey or pollen for further safe use in phytomedicine.

Flavonoids or some vitamins are not the only compounds which possess antioxidant properties. Nowadays, honey and pollen proteins and their interactions with flavonoids are receiving increased interest from researchers (Chua et al. 2013, Brudzynski and Maldonado-Alvarez 2015).

Our previous study showed that flavonoid hyperoside can serve as a marker for only one kind of spring honey that comes from willow. The total content of flavonoids in monofloral willow honey was lower compared to different kinds of summer honey (Čeksterytė et al. 2006). Volatile phenolic acids, including syringic acid (0.0-0.2%), *p*-coumaric acid (0.0-1.4%) and ferulic acid were identified in low concentrations in Croatian maple (*Acer* spp.) honey (Jerković et al. 2010). Lithuanian willow, oilseed rape, multifloral honey and beebread also contain *p*-coumaric acid (Baltrušaitytė et al. 2007). This acid is present in sporopollenin, the essential constituent of pollen cell walls and propolis. Propolis, also called "bee-glue" is collected from bud exudates of salicaceous plants like poplars (*Populus* spp.). Massively parallel RNA sequencing analysis revealed that *p*-coumaric acid can induce detoxification genes in honey bee (Mao et al. 2013). It has been found that kynurenic and salicylic acids are useful markers for willow honeydew honey (Tuberoso et al. 2011). Kynurenic acid (KYNA) is one of the kynurenine derivatives which can be used in treatment of brain disorders (Kostrzewa 2014, Erhardt et al. 2009). It is well known that salicylic acid is used as a medication for skin disorders. Together with hydroxy acids it is the main ingredient for skin care products (Madan, and Levitt 2014). Salicin structural composition (beta-D-Salicin 1 or 2-(hydroxymethyl) phenyl-O-beta-D-glucopyranoside) was the first phenolic glycoside discovered in nature. Salicin and its metabolite salicylic acid were investigated for their pharmacological properties (Mahdi 2014). Therefore, beneficial effects of combination of active components of beekeeping products might be obtained in their use for maintaining human health. Supplementation of honey with other bee products – beebread, propolis, and pollen – resulted in a significant increase in the total phenolic and flavonoid contents, antioxidant activity and reducing power, with the highest effect found for addition of beebread (Juszczak et al. 2016). The application of pollen for treatment is difficult due to its compositional diversity in the collected mixtures. Pollen diversity determines their properties, biological activity, and therapeutic effect (Denisow and Denisow-Pietrzyk 2016).

Most protein research are related to the study of their changes in the material being tested and differences were identified according to proteins spots intensity on gels used in experiments (Levander et al. 2007, Zhan et al. 2018).

The aim of the current study was to determine protein composition of monofloral willow (*Salix* spp.) pollen collected by hand and by bees and protein expres-

sion changes in hand- and bee-collected pollen; to estimate relationship between *Salix* spp. pollen composition and glucose oxidase (GOX) enzyme activity in willow honey.

Materials and Methods

Collection of pollen from willow (Salix caprea)

For the study of proteins, willow pollen grains were hand-collected from blooming willow (*Salix caprea*) catkins in April and were stored in Eppendorf tubes at "80 °C until analysis. Bee pollen collection was carried out in accordance with good beekeeping practice and did not interfere with colony growth (Grout 1992). Bee-collected pollen was sampled also in April from a standard pollen trap mounted on the hive entrance during abundant blossom of willow.

The samples were sorted and kept in a refrigerator at –80 °C in air-tight plastic bags until analysis. Manually and bee-collected pollen in experiment results marked as G1and G2.

Honey sampling areas

Samples of willow honey were collected from different districts in Lithuania: in Middle Lithuania, Kėdainiai district and North-west Lithuania, Telšiai district; Gomerta Landscape Reserve; Radviliškis District; Žemaitija National Park; Plunge district. We collected a total of fourteen monofloral willow honey samples of which nine were from Lithuania's protected landscape areas. The samples were preserved in dark glass bottles and refrigerated at 5 °C until analysis.

Microscopic analysis of honey pollen

Botanical origin of honey samples was determined according to (Louveaux et al. 1978). The pollen grain exine and shape on honey slides were visualized under a light microscope Nicon Eclipse E600. About 400–500 pollen grains were counted in each sample. The content of pollen of each melliferous plant is expressed as percentage of total pollen sum. Monofloral honey is mainly produced from one plant species and pollen from one plant species is predominant, i.e. accounts for more than 45.0%. The pollen content of other plant species is designated as follows: secondary pollen 16–45%; important minor pollen 3–15% and minor pollen < 3.0%.

Botanical origin was determined by comparison of light microscopic images of pollen found in willow honey to those of known pollen collected by hand (Čeksterytė 2012).

Protein isolation from pollen

Proteins from mature pollen (approx. 20 mg) were isolated as described by (Sheoran et al. 2009). 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 more than twice 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% [3-(3-cholamidopropyl) dimethylammonio-1-propanesulfonate] (CHAPS), 1% DTT, 0.8% IPG buffer, pH 3–10 (GE Healthcare Life Sciences, Chicago, Illinois, USA). The solution was centrifuged 20.000 × g for 20 min at 4°C and pellet was extracted again with the lysis buffer. After centrifugation, both extracts were combined and directly used for protein analysis or stored at –20°C until analysis.

Protein fractionation by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS/PAGE) and two-dimensional gel electrophoresis (2-DE)

The proteins isolated from hand- and bee-collected pollen were fractionated on gradient (7.5 – 15%) polyacrylamide gel and resolved by 2-DE. For 2-DE, an Immobiline DryStrip kit, pH range 3–11, and Excel gel SDS, gradient 8–18%, were used. It was carried out according the manufacturer's instructions (Immobiline DryStrip kit for 2DE with Immobiline DryStrip and Excel gel SDS, Pharmacia Biotech, Sweden). For protein visualization, gels were stained with Colloidal Coomassie G-250 (Bio-Rad Laboratories, USA). For 2-DE fractionation of pollen proteins, three independent biological experiments were carried out.

In-gel digestion and matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS)

Areas of interest were cut out from the 2-DE gels. Proteins were analysed by direct gel-free mass spectrometry analysis. For this, isolated proteins were applied on Amicon Ultra-0.5 mL 30 kDa centrifugal filter unit (Sigma-Aldrich, USA). Trypsin digestion was done according to a modified FASP protocol (Wisniewski, Zougman, Nagaraj and Mann 2009). Briefly, proteins were washed with buffer containing 8 M urea. The proteins were alkylated using iodoacetamide. Buffer was exchanged by washing twice with 50 mM NH_4HCO_3 and proteins digested overnight with TPCK Trypsin 20233 (Thermo Scientific, USA). 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) Synapt G2 mass spectrometer (Waters Corporation, UK).

Data processing, searching and analysis

Raw data files were processed and searched using ProteinLynx Global SERVER (PLGS) version 2.5.2 (Waters

Corporation, UK). 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, (iii) intensity was set to 500 counts. Processed data was 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 (Borutinskaitė et al. 2017). 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 ProteinLynx Global Server (PLGS, Waters) when global false discovery rate was set to 1 %.

Image acquisition and data analysis

Visualized 2-DE gels were digitized using ImageScanner™ III imager (GE Healthcare Biosciences, Germany) together with LabScan 6.0 software application. Prior to gel acquisition, scanner calibration was performed using provided step tablet. Gels were scanned at 300 dpi resolution, 16-bit pixel depth, and exported as TIFF™ format graphic files. Analysis of 2DE gel images was performed using custom pre-processing, alignment, segmentation, spot pairing and subsequent data analysis and visualization tools. The software toolset was implemented using MatLab™ programming language (The MathWorks, USA). Basic steps of the gel image analysis workflow that was adopted here are the following: image pre-processing, image alignment, spot detection-segmentation in aligned images, and differential analysis. For an extended description of the developed and applied 2DE gel image analysis algorithms used in the current experiments, please refer to Treigyte et al. (2014). Differential analysis provides changes in protein spot volumes (PSV) that describe differences between experimental groups (changes of spot abundance between the gel groups), and are computed as ratios of averages of normalized spot volumes. The data required for differential analysis (protein spot region, spot correspondences between gel images) has been collected during image alignment and segmentation steps. Changes in PSV are marked as G1:G2. An increase in spot abundance is represented with positive fold change and a decrease with a negative fold change (-).

Determination of glucose oxidase (GOX) activity

The GOX activity was measured by oxygen consumption rate, based on the glucose oxidation reaction, by the modified method (Kretavičius et al. 2010). The enzymatic oxygen consumption was detected amperometrically using Clark-type membrane oxygen electrode. The reaction was performed in the 1.4 mL PMMA cell, thermostated at 37 °C. Analytical grade, previously desiccated D-glucose (Lach-Ner, Czech) solutions were prepared one day before analysis for mutarotation reaction to be complete. The reaction mixture consisted of 3 mol/L glucose in the sodium acetate buffer (0.1 mol/L, pH=6.0). Honey samples were dissolved in distilled water, in ratio 1g/2 mL solution. Oxygen uptake rate was recorded after addition of 0.05 mL of the assay solution to the reaction mixture. The controller was used to apply a voltage to the Pt electrode that is –0.6 V vs. Ag/AgCl reference electrode. The resultant current proportional to the oxygen concentration in the air-saturated solution was displayed and saved with a data logger. The GOX activity is expressed as micromoles of oxygen consumed in 1 min by the enzyme contained in 1g of honey (U/g).

Statistical analysis

The analyses were carried out in triplicate. The results of GOX activity were analysed using the ANOVA programme. Estimated values are expressed as means ± standard deviations (SD). Correlation coefficients were calculated by the MS Excel software using the multiple regression statistic type of analyses.

Results

Identification of proteins in hand- and bee- collected willow (Salix caprea) pollen

Proteins from hand- and bee-collected pollen were isolated and separated in SDS/PAGE and visualized by Colloidal Coomassie Blue (CCB) staining (Figure 1a).

The bee- and hand-collected proteins were separated in 2DE system on pH 3-11 IPG strips and stained with CCB for visualization (Figure 1b). Proteins of interest (14 spots) were cut off from gels and were analysed by direct gel-free mass spectrometry. Characteristics (Mw, pI, Score and Coverage) of identified proteins from hand- and bee-collected pollen are presented in Table 1. More detailed data on identified proteins are presented in supplement (S1). Two forms of profilin proteins with slightly different mass 14.198 Da and 14.090 Da (accession numbers are B9RKF5 and A9PBI2) were identified in bee- and hand-collected pollen (Figure 1, spot No 1). It is known that profilin controls actin polymerization and binds to membrane phospholipids (Valenta et al. 1992). Many actin binding proteins maintain in an active form by binding to membrane phospholipids. The activation of phospholipids and enzymes of lipid kinases, phosphotases, phopholipases can release profilin and re-model actin-binding proteins (Pareek et al. 2010). Profilins are present in all eukaryotic cells and are identified as allergens in pollen (birch, grass, etc.), latex and plant foods (Salazar et al. 2014). Profilins are one of the

Table 1. Proteins separated on 2DE and identified by mass spectrometry from hand- and bee-collected willow (*Salix caprea*) pollen

	SwissProt Accession number	Name of protein	Mw (Da)	pI (pH)	PLGS Score	Coverage (%)
1	B9RKF5	Profilin	14198	4.37	5533.242	17.5
	A9PBI2	Profilin	14090	4.51	1388.565	32.0
	A9PAR7	60s acidic ribosomal family protein	11423	4.19	753.7521	38.5
2	B9N635	Pollen Ole e 1 allergen and extensin family	17746	4.58	3404.224	27.3
3	-					
4	P00330	Alcohol dehydrogenase 1	36825	6.21	5800.421	49.4
	B9SX15	Peptidyl prolyl cis trans isomerase	18125	8.74	2289.276	19.5
5	B9GEM9	Elongation factor 1B alpha subunit 2 family protein	24066	4.27	19394.47	34.8
	A9PA21	Uncharacterized protein	22445	4.12	18671.62	59.5
	A9P8Q7	14 3 3 like family protein	28709	4.55	3242.619	41.3
6	B9HLA3	Glyceraldehyde 3 phosphate dehydrogenase	37062	7.39	32257.73	24.6
	A9PJ53	Putative uncharacterized protein	38360	7.13	22880.42	66.2
	L0AT09	Sinapyl alcohol dehydrogenase	39423	6.37	14943.67	30.6
	B9SRH4	Fructose bisphosphate aldolase	38613	6.62	9141.485	23.1
	P00330	Alcohol dehydrogenase 1	36825	6.21	6224.062	49.4
	B9N0N1	Pectinacetylesterase family protein	42776	6.59	525.438	8.6
	T1WWB9	Cinnamyl alcohol dehydrogenase 5	39035	6.27	498.847	11.1
7	F8WL60	Beta actin Fragment	33252	5.11	73688.57	62.9
	A0A088ARP9	Uncharacterized protein	41769	5.14	53880.95	40.4
	B9HXH1	S adenosylmethionine synthase	43154	5.40	3764.452	24.5
	Q9MBB8	Polygalacturonase	41565	5.96	488.2868	7.6
	B9HY30	Phosphoglycerate kinase	42652	5.59	431.676	11.4
	B9RH03	Vacuolar ATP synthase subunit C putative	42590	5.29	221.9318	6.3
8	B9RFQ1	S adenosylmethionine synthase	44233	5.41	9197.645	39.9
	A9P851	Translation initiation factor eIF 4A family protein	46840	5.22	4530.067	49.6
	B9IAL5	Thioredoxin family protein	47290	4.98	1148.288	29.9
9	U5GC14	Uncharacterized protein	52611	4.76	2260.943	15.6
	B9RFQ1	S adenosylmethionine synthase	44233	5.417	2004.635	21.3
10	A9PAR0	Elongation factor 1 alpha	49448	9.34	22372.68	43.4
	A0A0A6ZAK1	Elongation factor 1 alpha	49245	9.40	15595.15	30.6
	A9PG38	Elongation factor 1 alpha	49402	9.42	13954.1	32.7
11	B9HMG7	Heat shock protein 70 cognate	71129	4.90	7512.242	25.6
	B9RYP6	Heat shock protein putative	73235	4.97	2791.835	26.0
12	Q6S3D6	Cytosolic phosphoglucomutase	63219	5.35	13211.21	51.3
	B9HW41	Cytosolic phosphoglucomutase family protein	63048	5.08	10886.97	42.6
	B9HK35	Vacuolar ATP synthase catalytic subunit A family protein	68575	5.24	1680.983	28.5
13	B9HK35	Vacuolar ATP synthase catalytic subunit A family protein	68575	5.24	21812.82	60.3
	Q6S3D6	Cytosolic phosphoglucomutase	63219	5.35	4375.066	38.3
14	B9HV14	Vacuolar ATP synthase catalytic subunit A family protein	68656	5.24	11382.97	46.5
	B9IGD0	Pyruvate decarboxylase family protein	65028	5.40	291.2979	3.8

Note: Mw – molecular weight; pI – isoelectric point; PLGS Score is calculated by the Protein Lynx Global Server v2.3 software to analyse all available mass spectrometry data and is a statistical measure of accuracy of identification. A higher score implies greater confidence of protein identity. Coverage (%) – sequence coverage

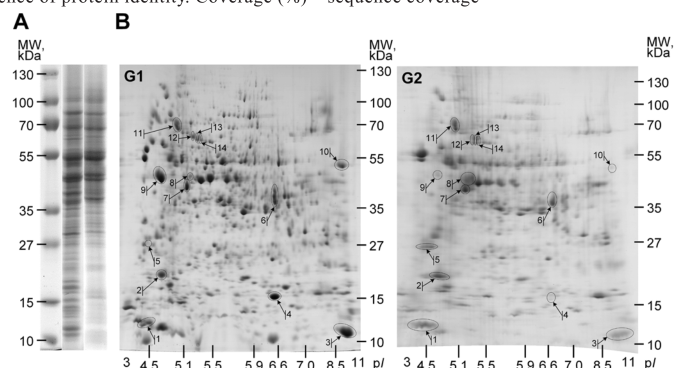

Figure 1. Willow pollen proteins separated by electrophoresis. (**A**) – SDS-polyacrylamide gel electrophoresis (SDS/PAGE) of proteins extracted from hand (G1) - and bee (G2) -collected willow pollen. (**B**) – two-dimensional gel electrophoresis (2DE) of proteins extracted from hand (G1) and bee (G2)-collected willow pollen. Equal amount of proteins (10 µg/lane) was loaded for SDS-PAGE, and proteins (200 µg/gel) were separated by two-dimensional gel electrophoresis and stainded by PageBlue. Lane M – molecular weight marker (kDa), pI – isoelectric points

most common pan allergens to be studied because they are responsible for a large number of sensitisations and are clearly related to cross-reactivity and co-sensitisation (Landa-Pineda et al. 2016). It is known that *Arabidopsis thaliana* has five isoforms of profilins: three profilins, which are mainly in all plant tissues and the egg cell, two isoforms found only in pollen and different forms of profilin can be associated with its interaction with actin isoforms (Meagher 1991, Kraft and Sehon 1993, Müssar et al. 2015). Our previous study revealed four forms of profilins and six forms of actin with molecular weights of 14.2 – 14.4 kDa and 41.6 – 41.8 kDa, respectively in the pollen collected from different clover species (Treigytė et al. 2014). Proteins involved in actin reorganization processes were also found in willow pollen: beta-actin fragment (F8WL60, spot No 7) and vacuolar ATP synthase (spot Nos. 7, 12, 13, 14). The mass of beta actin fragment was 33.25 kDa.

Also, in spot No. 2 we identified another protein, Pollen Ole e 1 allergen and extensin family protein (B9N635). Ole e 1 is a well-characterized allergenic protein from olive pollen and is a polymorphic protein found in glycosylated (80% to 85% of the total allergen) and non-glycosylated forms.

Also, we characterized proteins involved in protein biosynthesis process: B9GEM9 (Elongation factor 1B alpha subunit 2 family protein, spot No 5), A9PAR0 (Elongation factor 1 alpha, spot No 10) and A9P851 (Translation initiation factor eIF 4A family protein, spot No. 8). In tobacco, eIF-4A is encoded by a gene family with one isoform, eIF-4A8, being exclusively expressed in pollen and its phosphorylation is required during pollen germination (Op den Camp and Kuhlemeier 1998). The protein A9PAR7 (60s acidic ribosomal family protein, spot No 1) is the component of large ribosomal complex and is important for translation process. 14-3-3 like family protein (A9P8Q7, spot No. 5) belongs to 14-3-3 family of proteins which are DNA regulatory elements found in plant genes. B9HLA3 (glyceraldehyde 3 phosphate dehydrogenase, spot No 6) protein can help to maintain photosynthetic efficiency and plant development under saline conditions (Chang et al. 2015). Cytosolic phosphoglucomutase (Q6S3D6, spot No. 12) was identified mostly in bee-collected pollen. The lack of both cytosolic and plastidial PGM isoforms activities in *Arabidopsis* resulted in dwarf growth, premature die off, and inability to develop a functional inflorescence (Malinova et al. 2014). Heat shock proteins (HSP) were also found in bee- collected pollen, a conserved molecular chaperone with important contribution to plant's survival under environmental stresses (B9HMG7, spot No 11).

Comparing protein expression level between hand and bee collected pollen

Using computational analysis, we detected that the expression of identified proteins differed between hand- and bee-collected pollen (Table 2). Differences were noted in protein spot volumes (PSV) in all their groups (1 – 14). Data in Table 2 summarizes changes of protein spot volumes (PSV) that were determined between protein maps of G1 and G2 (Figure 1). An increase in spot abundance is represented with positive fold change and a decrease, with a negative (–) fold change.

Table 2. The changes in expression of hand- and bee-collected willow (*Salix caprea*) pollen proteins separated on 2DE

Spot No.	Fold change G1:G2	Exp. Mw, kDa	Exp. pI
1	-1.23	12.2	4.3
2	-1.65	18.2	4.6
3	1.15	11.2	9.0
4	5.41	15.1	6.4
5	-6.09	24.1	4.3
6	-1.07	36.1	6.5
7	-1.66	41.9	5.0
8	-8.72	46.2	5.1
9	11.01	45.6	4.6
10	6.07	51.9	8.9
11	-1.48	80.2	4.9
12	-1.54	70.6	5.2
13	1.29	68.8	5.2
14	-2.09	68.4	5.3

Note: G1 – hand-collected pollen; G2 – bee-collected pollen; Exp. Mw – experimental molecular weight; Exp. pI – experimental isoelectric point

We found that some proteins were expressed more (fold change G1:G2>0) in hand-collected pollen (spots Nos. 3, 4, 9, 10, 13) while others were expressed more in bee-collected pollen (fold change G1:G2<0, spots Nos. 1, 2, 5-8, 11, 12, 14). Alcohol dehydrogenase 1 (ADH; P00330) and peptidyl prolyl cis trans isomerase (B9SX15) were identified in spot No 4 with positive fold change G1/G2=5.41 (Figure 1a, Table 1). Activity of ADH is essential for the survival of plants during anaerobic condition (Johnson et al. 1994). PPIase (Peptidyl prolyl cis trans isomerase) belongs to the cyclophilin-type PPIase family. Physiological role of these proteins in plants is still a matter of speculation. They may play important roles in protein folding, mRNA processing, protein degradation, and signal transduction and thus may be crucial during both development and stress responsiveness (Romano et al. 2004). The highest negative spot volumes (PSV) ratio (–8.72) was determined between proteins of hand-collected and bee-collected pollen samples (G1:G2) in protein group at spot No. 8, while the highest positive one (11.01) at spot No. 9. These data indicate that proteins of bee- collected pollen in spot No. 8 overexpressed over hand collected pollen and vice versa in spot No. 9. Protein S adenosylmethionine synthase was identified both in spots Nos. 8 and 9, however its confidence identity was greater in spot No. 8 com-

pared to No. 9 and were 9197.645 and 2004.635, respectively. The low proteins overexpression of hand-collected pollen was found in proteins groups in spots Nos. 3, 13, and bee- collected pollen in spots Nos. 1, 2, 6, 7 where the ratio of PSV were 1.15, 1.29 and -1.23, -1.65, -1.07, -1.66, respectively. We can maintain that protein profilin which has been identified in the pollen is two isoforms only slightly overexpressed in bee-collected pollen.

Glucose oxidase (GOX) content in willow honey

GOX is a protein secreted into honey, pollen or beebread from honeybee hypopharyngeal glands (White et al. 1963). GOX activity was measured in twelve honey samples of monofloral willow honey, in which willow pollen accounted for 44.7 % – 80.2 % (Figure 2). *Salix* spp. pollen predominated in honey (H1) and (H4) and accounted for 80.2 % and 77.8 %, respectively, and GOX activity was 0.51 U/g and 0.57 U/g, respectively. *Brassica napus* pollen was not found in H1- H5 and H7 samples. in which GOX activity was low 0.1 – 0.51 U/g. H6, H11 and H12 honey samples containing 39.2%, 18.2 % and 37.0 % of *Brassica napus* pollen, respectively, were distinguished by the highest GOX activity (1.03 – 1.56 U/g). The relationship between *Salix* spp. pollen composition and GOX activity in honey is presented in Figure 3. The correlation coefficient was moderate negative (r= -0.56) and determination coefficient (R^2) was not strong 0.316. GOX activity for all tested samples varied in range from 0.10 to 1.56 U/g. *Salix* spp. pollen accounted for 69.8 % and *Malus domestica* for 14.2% in H5 honey; however, GOX activity was low 0.35 U/g in this sample. *Acer* spp. was found in all willow honey samples and varied in range from 0.8 % to 11.0 %. GOX activity was

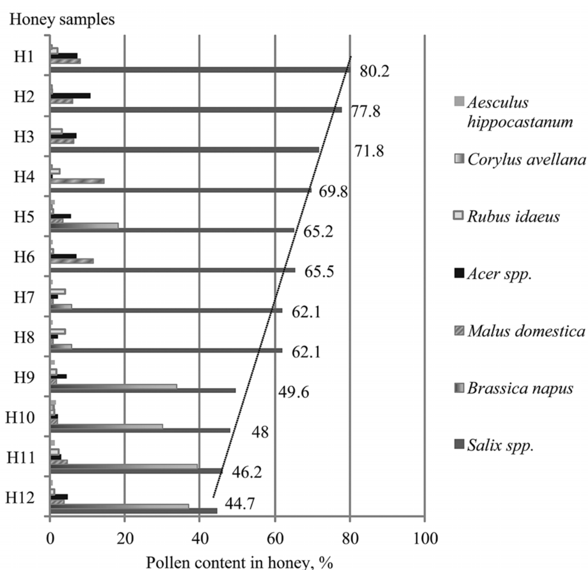

Figure 2. Pollen content in willow honey

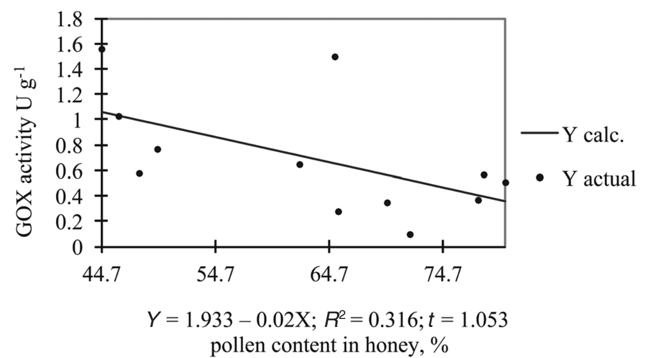

Figure 3. Relationship between glucose oxidase activity and *Salix* spp. pollen content in willow honey

low (0.37 U/g) in the sample (H3) containing high *Acer* spp. and *Salix* spp., percentage 11.0% and 77.8%, respectively, but no *Brassica napus* pollen.

Our data confirmed the variability of GOX activity in the studied monofloral willow honey samples. In our previous study, very weak GOX activity 0.87 ± 0.01 U/g was found in fresh monofloral bee-collected willow pollen compared with that in oilseed rape (*Brassica napus* L.) pollen, 7.44 ± 0.54 U/g. GOX activity in the samples of monofloral beebread, composed of 81.65 % oilseed rape pollen and 72.4 % willow pollen was as high as 21.6 ± 3.4 U/g and 10.1 ± 0.3 U/g, respectively (Čeksterytė et al. 2014). The colour of willow honey ranges from white to pale yellow (Wieczorek et al. 2014). Evaluation of honey antioxidant activity confirmed that light honey possesses lower free radical scavenging activity compared to dark honey. Light colour of honey indicates a low total phenolic (TPC) content (Wilczyńska 2010).

The twelve forms of enzyme family of oxidoreductases uridine diphosphate (UDP)-glucose 6-dehydrogenase) with molecular weight 52.8 – 53.1 kDa were found in our previous proteomic study of red clover (*Trifolium pratense* L.) and white clover (*Trifolium repens* L.) pollen (Treigytė et al. 2014). However, identification of proteins of *Salix* spp. pollen did not reveal enzymes attributed to oxidoreductases. Oxidoreductases act at the reducing end of glucose on the first hydroxyl group. These enzymes are divided into two groups, glucose oxidases (GOXs) and glucose dehydrogenases (GDHs). In reactions, glucose oxidases use oxygen and produce hydrogen peroxide. Glucose dehydrogenases act as electron transfers to various natural and artificial electron acceptors (Ferri et al. 2011).

Discussion

It has been reported that *Salix caprea* L. flowers are about 50% insect-pollinated (Vroege and Stelleman 1990). While *Salix alba*, *Salix elaeagnos*, *Salix*

daphnoides and *Salix triandra* are pollinated mainly by insects, but wind pollination also takes place. Seed set in inflorescences of the above-mentioned wind-pollinated *Salix* species is also possible (Karrenberg et al. 2002). Compared to much anemophilous tree pollen, *Salix* pollen is less allergenic (Ribeiro et al. 2009). Protein expression in the group, containing profilins as pan allergens as well as pollen Ole e 1 allergen and extensin family was not high compared to all expression levels determined in our study.

In our study, computer-assisted method was adjusted for evaluation of increase/decrease of the protein expression level in the analysed hand- and bee-collected willow pollen protein maps and mass spectrometry method was employed for identification of unknown proteins. Large molecular weight proteins and their abundance interfere with low-molecular weight proteins (LMW) in the tested material and can complicate the identification of proteins (Chen et al. 2015). The use of 2-DE proteins separation with immobilized pH gradients (IPGs) combined with protein identification by mass spectrometry (MS) is being complemented by the rapid development of high sensitivity mass spectrometers. 2-DE with immobilized gradients pH 2.5-12 makes it possible to identify alkaline proteins. Protein study using 2-DE with IPGs methodology create opportunities to separate proteins from complex mixtures according to their isoelectric point (pI), molecular weight (MW), relative abundance and creates a map of proteins. Proteins separated by this technology, reflect changes in protein expression level and the protein isoforms (Görg et al. 2005). The equipment of matrix-assisted laser desorption ionization (MALDI)-time of flight (TOF) mass spectrometry, is often used to perform peptide mass fingerprinting (PMF) (Nordhoff et al. 2001). In our experiment low-molecular weight proteins (LMW, ≤ 30kDa): two profilins and 60s acidic ribosomal family protein was found in one spot No.1 of tested *Salix* pollen. Two proteins with a different MW were identified at one spot No. 4. The latter are proteins peptidyl prolyl cis trans isomerase having LMW and alcohol dehydrogenase 1, which MW is as much higher, 36.825 kDa. Another LMW proteins group was identified from spot No. 5. The molecular weight of the proteins identified in this group varies within the range from 22.445 to 28.709 kDa. The proteins above 30 kDa was found with MW (33.252 – 73.235 kDa) at spots No 6 – 14. Among which spot No. 7 contain six different proteins, and their MW varied in range from 33.352 to 42.652 kDa. Spot No. 10 contains three isoforms of the alpha subunit of the elongation factor-1 complex, with similar MW 49.402, 49.448 kDa, while spot No. 5 contained one isoform of an elongation factor 1B alpha subunit 2 family protein, 24.066 kDa. Protein studies are very important in medicine. Zhan et al. 2018 have identified 42 and 63 proteins/spot using Orbitrap-based mass spectrometers [Liquid chromatography (LC)-Orbitrap Velos MS)] in cases of human cancers. More than twenty years ago the protein analysis techniques, determined only one protein per electrophoretic 2-D spot (Rabilloud 2013).

During honey harvesting process, mandibular and hypopharyngeal glands of bees' secret proteases to nectar, therefore original nectar proteins can be digested and changed and detected as specific proteins of bee origin. The secretion of honeybee thoracic salivary glands is important for honey maturation and pollen and wax moistening (Maurizio 1975, Simpson 1963). Proteins found in honey and pollen are of bee and plant origin and reflect ecological lifestyle of insects as well as environmental ecological situation on molecular level (Fujita et al. 2010).

Using the proteomics test method, twelve forms of UDP-glucose 6-dehydrogenase with a theoretical molecular weight (Mw) of 52.8 to 53.1 kDa in clover pollen were found (Treigytė et al. 2014). Recently, we have investigated the protein content of oilseed rape pollen (hand- and bee-collected) and rape honey using the following methods: SDS-PAGE, 2D-E separation and gel-free analysis. Proteins were subjected to mass spectrometry (MS) analysis (Borutinskaitė et al. 2017). In this assay we identified seven ribosomal protein isoforms and one heat shock protein of oilseed rape pollen using 2-DE separation. Gel-free analysis method was informative in determining the composition of plant proteins in oilseed rape honey. Nineteen oilseed rape honey proteins were determined using gel-free protein approaches, among which alcohol dehydrogenase 1, glyceraldehyde 3 phosphate dehydrogenase and polygalacturonase were common to oilseed rape honey and willow pollen. Protein composition of chestnut, acacia, sunflower, eucalyptus and orange honey was investigated by Girolamo et al. (2012) who used the SDS-PAGE method and subsequently identified proteins by mass spectrometry. However, in this assay there were determined only major royal jelly protein MRJPs (1-5), α-glucosidase and defensin - 1, but the attempts to identify plant proteins were unsuccessful. Only one plant enzyme glyceraldehide-3-phophate dehydrogenase was found in acacia (*Robinia pseudoacacia*) honey. Simuth et al. (2004) has analysed royal jelly protein in honeybee products corresponding to 55 kDa proteins by Western-blot analysis and N-terminal amino acid sequence. The sequence was identical to that of apalbumin-1, which is the most abundant protein in royal jelly. This protein was not identified in our experiments because mass spectrometry analysis of proteins of hand- and bee-collected willow pollen was conducted after 2DE fractionation, and the molecular weight of most of the proteins was lower or higher than 55 kDa.

The techniques applied enabled us to simultaneously identify MRJPs and other bee and plant origin proteins involved in many biological processes (Treigytė et al. 2014, Čeksterytė et al. 2016, Borutinskaitė et al. 2017). Using gel-free MS analysis, a total of 87 proteins were identified, including MRJPs (1–9) in Lithuanian buckwheat honey (Borutinskaitė, et al. 2018). Our study shows that we mastered the methods for protein separation in plant pollen by SDS-PAGE or 2DE and gel-free analysis and protein identification by MS.

Abscisic acid (ABA) and salicylic acid (SA), which belong to hormones, as well as proteins Hsp70 are present in *Salix* spp. plants as well as in willow honey (Nambara and Marion-Poll 2005, Futamura et al. 2000, Rivas-San Vicente and Plasencia 2011, Jerković et al. 2014). Low antioxidant activity and abscisic acid (ABA) were determined in Polish willow honey, which possesses numerous volatile compounds (Jerković et al. 2014). Previously we investigated radical scavenging activity in assays with 1,1-diphenyl-2-picrylhydrazyl radical (DPPH), and 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulphonic acid (ABTS) as well as identified phenolics compounds such as kaempferol, chrysin, apigenin and determined pollen amount in Lithuanian willow and other honeys (Baltrušaitytė et al. 2007, Čeksterytė et al. 2013). In this study we identified proteins of thioredoxin family with a molecular weight of 47.29 kDa in *Salix* pollen. Plant proteins such as redoxins (thioredoxins, glutaredoxins) act as antioxidants (Meyer et al. 2008). Plants have antioxidant system which reduces the effect of reactive oxygen species (ROS) among which are molecules of H_2O_2, O_2 as well as different ions. Some of those antioxidant enzymes, including thioredoxin, are able to combine with ROS (Rao et al. 2014). Thioredoxins (TRXs) are abundant in plants and participate in a large number of vital processes in plant cells. The TRXs system is responsible for plant photosynthesis and acclimatization to a variety of environmental conditions (Thormählen et al. 2016).

Monofloral willow honey is harvested in New Zealand, Italy, Spain, Estonia, Lithuania and Poland (Tan et al. 1990, Persano Oddo, L. and Piro, R. 2004, De la Fuente et al. 2007, Kirs et al, 2011, Čeksterytė et al. 2013, Jerković et al. 2014). Lithuanian willow honey shows variable GOX activity. In previous studies we found that GOX content in buckwheat, spring, and oilseed rape honey was as follows: 1.13; 1.08; 0.55 U/g, respectively (Kretavičius et al. 2010). In the current study, willow honey with the highest *Salix* spp. pollen content ranging from 71.8 % to 80.2 % had low GOX activity ranging from 0.10 to 0.509 U/g. GOX is a natural component of honey. Its expression is variable and increases in the hypopharyngeal glands with the age of worker bees. Honeybee nutrition or genetic factors may be essential for GOX expression level. GOX content was found to vary in relation to honey origin, as well as within the same floral source from different geographical locations (Bucekova et al. 2014). It has been reported that polyfloral diets enhance immune function of bees and GOX activity (Alaux et al. 2010).

Conclusions

Comparison of protein expression in hand- and bee-collected pollen makes it possible to evaluate their valuable functional properties and their changes.

A total of thirty-six plant proteins with known functions and three uncharacterized functions were identified from fourteen protein spots separated in 2D gel electrophoresis using mass spectrometry (MS) techniques

All proteins identified in this study are important for pollen development and stress responsiveness of plants. Expression of proteins with one protein cytosolic phosphoglucomutase was higher in hand-collected pollen, compared with other protein group having two forms of those proteins, in which expression was higher in bee-collected pollen. Computed data show the same tendency of opposite protein expression level where PSV were 1.29 and –1.54, respectively.

The influence of *Salix* spp. pollen found in honey on antioxidant activity was not observed. GOX activity showed moderate and negative correlation coefficient ($r = -0.56$).

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Supplementary material (see **Appendix**)

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Appendix

Table S1. List of proteins separated on 2DE and identified by mass spectrometry from hand- and bee-collected willow (*Salix caprea*) pollen (xlsx 17 kb)

	Accession	Entry	Description
1	B9RKF5	B9RKF5_RICCO	Profilin OS Ricinus communis GN RCOM 1048920 PE 3 SV 1
	A9PBI2	A9PBI2_POPTR	Profilin OS Populus trichocarpa GN POPTR 0003s04610g PE 2 SV 1
	A9PAR7	A9PAR7_POPTR	60s acidic ribosomal family protein OS Populus trichocarpa GN POPTR 0004s19680g PE 2 SV 1
2	B9N635	B9N635_POPTR	Pollen Ole e 1 allergen and extensin family protein OS Populus trichocarpa GN POPTR 0001s40290g PE 4
3	-		
4	P00330	ADH1_YEAST	Alcohol dehydrogenase 1 OS Saccharomyces cerevisiae strain ATCC 204508 S288c GN ADH1 PE 1 SV 5
	B9SX15	B9SX15_RICCO	Peptidyl prolyl cis trans isomerase OS Ricinus communis GN RCOM 1259280 PE 3 SV 1
5	B9GEM9	B9GEM9_POPTR	Elongation factor 1B alpha subunit 2 family protein OS Populus trichocarpa GN POPTR 0001s23190g PE 3
	A9PA21	A9PA21_POPTR	Uncharacterized protein OS Populus trichocarpa GN POPTR 0003s18990g PE 2 SV 1
	A9P8Q7	A9P8Q7_POPTR	14 3 3 like family protein OS Populus trichocarpa GN POPTR 0005s17740g PE 2 SV 1
6	B9HLA3	B9HLA3_POPTR	Glyceraldehyde 3 phosphate dehydrogenase OS Populus trichocarpa GN POPTR 0008s17940g PE 3 SV 2
	A9PJ53	A9PJ53_9ROSI	Putative uncharacterized protein OS Populus trichocarpa x Populus deltoides PE 2 SV 1
	L0AT09	L0AT09_POPTO	Sinapyl alcohol dehydrogenase OS Populus tomentosa PE 3 SV 1
	B9SRH4	B9SRH4_RICCO	Fructose bisphosphate aldolase OS Ricinus communis GN RCOM 0383870 PE 3 SV 1
	P00330	ADH1_YEAST	Alcohol dehydrogenase 1 OS Saccharomyces cerevisiae strain ATCC 204508 S288c GN ADH1 PE 1 SV 5
	B9N0N1	B9N0N1_POPTR	Pectinacetylesterase family protein OS Populus trichocarpa GN POPTR 0004s24210g PE 4 SV 1
	T1WWB9	T1WWB9_POPTO	Cinnamyl alcohol dehydrogenase 5 OS Populus tomentosa GN cad5 PE 2 SV 1
7	F8WL60	F8WL60_RHISY	Beta actin Fragment OS Rhizophora stylosa GN RsAct1 PE 2 SV 1
	A0A088ARP9	A0A088ARP9_APIME	Uncharacterized protein OS Apis mellifera GN LOC102653846 PE 3 SV 1
	B9HXH1	B9HXH1_POPTR	S adenosylmethionine synthase OS Populus trichocarpa GN POPTR 0010s16330g PE 3 SV 1
	Q9MBB8	Q9MBB8_SALGI	Polygalacturonase OS Salix gilgiana GN SgPG3 PE 2 SV 1
	B9HY30	B9HY30_POPTR	Phosphoglycerate kinase OS Populus trichocarpa GN POPTR 0010s17870g PE 3 SV 1
	B9RH03	B9RH03_RICCO	Vacuolar ATP synthase subunit C putative OS Ricinus communis GN RCOM 1445580 PE 4 SV 1
8	B9RFQ1	B9RFQ1_RICCO	S adenosylmethionine synthase OS Ricinus communis GN RCOM 1436370 PE 3 SV 1
	A9P851	A9P851_POPTR	Translation initiation factor eIF 4A family protein OS Populus trichocarpa GN POPTR 0018s07650g PE 2
	B9IAL5	B9IAL5_POPTR	Thioredoxin family protein OS Populus trichocarpa GN POPTR 0014s15820g PE 3 SV 2
9	U5GC14	U5GC14_POPTR	Uncharacterized protein OS Populus trichocarpa GN POPTR 0005s12410g PE 4 SV 1
	B9RFQ1	B9RFQ1_RICCO	S adenosylmethionine synthase OS Ricinus communis GN RCOM 1436370 PE 3 SV 1
10	A9PAR0	A9PAR0_POPTR	Elongation factor 1 alpha OS Populus trichocarpa GN POPTR 0008s04230g PE 2 SV 1
	A0A0A6ZAK1	A0A0A6ZAK1_9ROSI	Elongation factor 1 alpha OS Euphorbia lathyris PE 2 SV 1
	A9PG38	A9PG38_POPTR	Elongation factor 1 alpha OS Populus trichocarpa GN POPTR 0010s22570g PE 2 SV 1
11	B9HMG7	B9HMG7_POPTR	Heat shock protein 70 cognate OS Populus trichocarpa GN POPTR 0008s05470g PE 3 SV 1
	B9RYP6	B9RYP6_RICCO	Heat shock protein putative OS Ricinus communis GN RCOM 1312280 PE 3 SV 1
12	Q6S3D6	Q6S3D6_POPTO	Cytosolic phosphoglucomutase OS Populus tomentosa PE 2 SV 1
	B9HW41	B9HW41_POPTR	Cytosolic phosphoglucomutase family protein OS Populus trichocarpa GN POPTR 0010s11970g PE 3 SV 2
	B9HK35	B9HK35_POPTR	Vacuolar ATP synthase catalytic subunit A family protein OS Populus trichocarpa GN POPTR 0008s00560g
13	B9HK35	B9HK35_POPTR	Vacuolar ATP synthase catalytic subunit A family protein OS Populus trichocarpa GN POPTR 0008s00560g
	Q6S3D6	Q6S3D6_POPTO	Cytosolic phosphoglucomutase OS Populus tomentosa PE 2 SV 1
14	B9HV14	B9HV14_POPTR	Vacuolar ATP synthase catalytic subunit A family protein OS Populus trichocarpa GN POPTR 0010s26000g
	B9IGD0	B9IGD0_POPTR	Pyruvate decarboxylase family protein OS Populus trichocarpa GN POPTR 0016s12760g PE 3 SV 1

Appendix (*Continued*)

mW (Da)	pI (pH)	PLGS Scor	Peptides	Theoretic	Coverage	Precursor	Products	Digest Pe	Modified	Products F	Products I	Amount (f	Amount (	Protein ID
14198	4,3799	5533,242	5	5	17,5573	2,8121	66	2	0	6,9872	0,009303	89,6502	1,2738	18326
14090	4,5161	1388,565	4	8	32,0611	1,8097	45	3	0	7,0637	0,01152	17,0105	0,2399	2194
11423	4,1968	753,7521	2	11	38,5965	1,1074	15	2	0	11,2255	0,015471	1,6167	0,0185	1508
17746	4,5835	3404,224	8	15	27,3292	1,237	97	7	0	6,5387	0,007762	60,4477	1,0734	118587
36825	6,2168	5800,421	18	25	49,4253	1,9677	214	16	0	8,2401	0,012468	50	1,8425	139780
18125	8,7495	2289,276	4	12	19,5122	2,9042	41	4	0	5,494	0,009452	23,4662	0,4256	28585
24066	4,27	19394,47	11	18	34,8214	1,8878	177	7	0	6,4201	0,007242	315,5023	7,5976	120766
22445	4,1294	18671,62	9	13	59,5122	2,3448	142	8	0	8,467	0,008543	16,4948	0,3705	3090
28709	4,5586	3242,619	11	24	41,3386	3,1007	74	11	0	9,6876	0,010066	121,9063	3,5021	1989
37062	7,3916	32257,73	10	26	24,6334	1,5115	142	9	0	7,7351	0,008958	276,1528	10,2413	91064
38360	7,1367	22880,42	32	27	66,2011	2,1211	579	25	0	7,6497	0,010333	626,0129	24,0295	58648
39423	6,3721	14943,67	14	31	30,663	1,5358	195	13	0	6,3111	0,010937	143,3757	5,6561	136724
38613	6,6284	9141,485	11	27	23,1844	3,4628	217	10	0	7,1662	0,008362	48,7873	1,885	15887
36825	6,2168	6224,062	20	25	49,4253	1,7779	210	16	0	7,9983	0,011959	50	1,8425	139780
42776	6,5962	525,438	4	24	8,6294	0,992	37	4	0	8,1399	0,009716	11,537	0,4938	105949
39035	6,2754	498,847	5	23	11,1111	2,8018	49	7	0	10	0,008792	11,2037	0,4376	136781
33252	5,1138	73688,57	25	26	62,963	5,7292	459	18	1	7,3209	0,016652	825,4976	27,4679	136965
41769	5,1431	53880,95	11	34	40,4255	3,9517	220	11	0	7,8142	0,021967	57,8564	2,4182	142273
43154	5,4009	3764,452	10	33	24,557	2,9272	93	8	0	8,7907	0,010647	49,4389	2,1349	94029
41565	5,9663	488,2868	3	27	7,6336	2,5283	41	6	0	10,333	0,011121	17,273	0,7184	53521
42652	5,5972	431,676	3	32	11,4713	1,679	52	7	0	9,9705	0,01036	9,5214	0,4064	121438
42590	5,2983	221,9318	3	30	6,383	11,5859	34	6	1	10,1415	0,0122	2,5367	0,1081	32211
44233	5,417	9197,645	17	33	39,9504	8,8081	208	14	0	8,2652	0,008915	147,2215	6,5162	43608
46840	5,2236	4530,067	19	42	49,6368	3,1365	216	18	3	7,9544	0,012733	59,5054	2,7891	3878
47290	4,9819	1148,288	12	35	29,9539	7,1535	103	11	0	9,2562	0,010455	41,0296	1,9415	96614
52611	4,7651	2260,943	18	58	15,6904	3,477	185	14	1	8,4879	0,009728	935,21	49,2345	99540
44233	5,417	2004,635	7	33	21,34	1,3296	53	7	0	7,775	0,009966	24,4632	1,0828	43608
49448	9,3442	22372,68	42	45	43,4298	2,5209	508	23	1	6,1957	0,012387	1097,465	54,3029	1933
49245	9,4087	15595,15	36	45	30,6488	3,5231	377	19	0	6,2815	0,009445	710,642	35,0184	52075
49402	9,4248	13954,1	30	45	32,7394	2,6587	311	16	0	5,9788	0,009504	72,4521	3,5816	2610
71129	4,9028	7512,242	22	52	25,6173	5,2581	292	19	0	7,6367	0,009803	113,2766	8,0623	96458
73235	4,9775	2791,835	28	63	26,0542	2,2158	291	24	0	9,1266	0,013145	115,5401	8,4669	38500
63219	5,3599	13211,21	33	54	51,3746	3,2245	512	28	1	7,0681	0,00874	206,404	13,0568	136935
63048	5,083	10886,97	27	53	42,6117	5,2421	420	21	2	7,159	0,009225	132,8999	8,3844	103297
68575	5,2412	1680,983	17	61	28,5714	5,2937	158	18	2	9,3387	0,009241	17,6768	1,213	122994
68575	5,2412	21812,82	42	61	60,3531	1,9703	581	33	3	7,2654	0,008794	136,9437	9,397	122994
63219	5,3599	4375,066	25	54	38,3162	6,3865	220	24	0	7,8183	0,009939	24,5871	1,5553	136935
68656	5,2471	11382,97	31	62	46,549	2,2639	454	28	2	6,9993	0,009042	122,3598	8,4062	93968
65028	5,4067	291,2979	2	38	3,8017	1,7962	28	5	0	8,0017	0,009394	9,1521	0,5955	91327