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APPLICATION OF TWO-DIMENSIONAL ELECTROPHORESIS AND MASS SPECTROMETRY FOR THE DETECTION OF ALLERGENS IN SELECTED VARIETIES OF WHEAT, OATS AND BUCKWHEAT

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ABSTRACT

The number of people suffering from food allergies and intolerances has been increasing in recent years and cereal proteins are the most common food allergens. Pseudocereals represent perspective alternative in nutrition with a positive effect on the human body. The aim of the work was to analyze the proteome of selected varieties of wheat (*Triticum aestivum* L.), oats (*Avena sativa*) and buckwheat (*Fagopyrum esculentum* Moench.) using two-dimensional electrophoresis (2DE) and mass spectrometry in order to detect the presence of potentially allergenic proteins. Using the PDQuest program, 221 protein spots ranging from 4.13 to 9.89 µl with experimental molecular weights from 12.42 kDa to 140 kDa were quantified in 2DE gels of wheat. In the oat sample, 168 protein spots were quantified in the range pI of 4.02 to 9.93 and an experimental molecular weight of 14.81 kDa to 67.96 kDa. Buckwheat proteins were separated on a 2DE gel into 208 protein spots in the 3 to 9.83 pI region with an experimental molecular weight of 10.10 kDa to 115 kDa. By comparing the data with the Allergome database, allergens Tri and 26, Tri and 33, Tri and 36, Tri and alpha Gliadin, Tri and 20 were detected in wheat, Ave s 11S allergens in oats and Fag e 1 allergen in buckwheat. 2DE together with mass spectrometry have been shown to be suitable and sensitive methods for the detection of allergens in food crops.

Keywords: allergens, buckwheat, mass spectrometry, oat, two-dimensional electrophoresis, wheat

INTRODUCTION

Cereals and pseudocereals are important agricultural crops and are an integral part of human nutrition. The grain contains a significant amount of proteins, carbohydrates, fats, fiber, vitamins and minerals. Cereals are a natural source of energy, nutrients, but also various anti-nutritional substances, such as allergens. In recent years, the number of people suffering from civilizational diseases, such as food allergies or various intolerances, has been increasing. Some foods can cause an allergic reaction or problems caused by food intolerance in people with sensitivity to a specific allergen. The most common food-induced diseases include celiac disease, baker's asthma, wheat-dependent exercise-induced anaphylaxis (WDEIA) and other food allergies **(Mickowska *et al.*, 2012; Chňapek *et al.*, 2021; Žiarovská and Urbanová, 2022).**

Adverse food reactions can be divided into food allergies (hypersensitivity) and food intolerances, depending on the reactions of the immunological system. Food allergy is classified as an immune-mediated adverse reaction, while food intolerance is not an immune-mediated reaction **(Mills and Shewry, 2004).** A food allergy is an adverse immune response to certain types of food. Any food can cause an allergic reaction. Chicken eggs, cow's milk, wheat, shellfish, fruit and buckwheat have been proven to account for up to 75% of all food allergies **(Sancho and Mills, 2010; Matsuo *et al.*, 2015**).

Many of the allergic diseases have a rapid onset of unwanted symptoms after exposure to the allergen, which greatly facilitates the identification of the cause of their occurrence. On the other hand, there are many allergic diseases where the onset of symptoms is longer and the symptoms are not clearly attributed to the relevant allergen exposure **(Woodfolk *et al.*, 2015).**

Food allergens are antigenic molecules that induce an immune response. Allergen-specific immunoglobulin E (IgE) antibodies play a key role in case of food allergy. Food allergies are divided into IgE-mediated immune and non-IgE-mediated immune reactions. IgE-mediated allergic reactions to ingested food can manifest on one or more organs such as the skin, digestive tract, respiratory system, cardiovascular system. Clinical manifestations that are associated with immediate food allergic reactions are anaphylaxis, urticaria, atopic dermatitis, asthma **(Mills and Shewry, 2004; Nakamura and Teshima, 2013).**

Most plant food allergens belong to several protein families, suggesting that protein structure and biological activity play an important role in determining their allergenic properties. Three groups of food allergenic proteins in plants have been identified, namely the prolamin supergroup, the cupin supergroup, and the "Bet v1" group **(Breiteneder and Radauer, 2004).**

The prolamin supergroup includes three major groups of plant food allergens, namely 2S albumins, nonspecific lipid transfer proteins (nsLTPs), and cereal α-amylase/trypsin inhibitors. All these low-molecular-weight proteins are rich in cysteine, have a similar three-dimensional structure rich in α-helices, and are resistant to thermal processes and proteolysis. 2S albumins are the main group of storage proteins of dicotyledonous plants and include allergens of nuts, sesame, and mustard. Allergens nsLTPs are found in fruits, nuts and vegetables. Cereal α-amylase and protease inhibitors include wheat, barley, rice, and corn allergens **(Breiteneder, 2006).**

Cupins are a functionally distinct group of ß-structured proteins. They represent the main globulin storage proteins of legumes and nuts. Globulins are divided into 7S vicilin-like globulins and 11S legumin-like globulins. Globulins are allergens found in peanuts, soy, lentils, walnuts, hazelnuts and sesame **(Breiteneder and Radauer, 2004).** "Bet v1" group mainly contains allergens of fruits of the Rosaceae family (apple, cherry, apricot, pear) and vegetables of the Apiaceae family (celery, carrot). Allergens of the "Bet v1" group are not resistant to heating and digestion **(Breiteneder and Mills, 2005).** Some research has shown that low molecular weight (LMW) glutenins, α- and γ-gliadins are the major allergens of wheat-allergic patients and have identified the QQQPP motif as an IgE-binding epitope **(Matsuo *et al.*, 2004).**

Wheat (*Triticum aestivum* L.) is an important source of allergens that induce many types of food allergies, such as wheat allergy, baker's asthma, wheat dermatitis or WDEIA. Wheat profilin (Tri and 12), non-specific LTP (Tri and 14), α-amylase inhibitors (AAI, Tri and 15, Tri and 28–30), wheat germ agglutinins (Tri and 18), thioredoxin (Tri and 25), thiol reductase homolog (Tri and 27), triose phosphate isomerase (Tri and 31), 1-cys-peroxiredoxin (Tri and 32), serpin (Tri and 33), glyceraldehyde-3-phosphate dehydrogenase (Tri and 34), dehydrin (Tri and 35), α-purothionine (Tri and 37), serine proteinase inhibitor (Tri and 39), peroxidase and glutathione S-transferase in soluble fractions were identified as allergens of baker's asthma, wheat food allergy and wheat urticaria **(Matsuo *et al.*, 2015).**

It is important to distinguish celiac disease from a food allergy to wheat and wheat dust. Typical symptoms of allergy are skin reactions, asthma and anaphylactic shock. During an allergic reaction, there is an increase in IgE antibodies, which means that epitopes in gluten allergy are not identical to celiac epitopes **(Socha *et al.*, 2011).**

The latest advances in molecular biology methods allow efficient analysis of food allergens from various plant sources, which is very important for predisposed individuals who should have a sufficient assortment of safe foods available. Allergen studies have shown that allergen specific IgE antibodies and peptide epitopes are suitable indicators for identifying patients with food allergy, predicting clinical severity, and detecting changes in food tolerance **(Matsuo *et al.*, 2015).**

Various methods of proteomic research are used for the detection of plant allergens, including electrophoretic, immunochemical methods and various types of chromatography and mass spectrometry. The most frequently used method is enzyme-linked immunosorbent assays (ELISA), which can be used to detect the concentration of antigen or antibodies in a biological sample. It is highly sensitive, reproducible and enables rapid determination of residue limits within the industrial processing of food sources. Proteomic techniques in combination with Western blot analysis enable the identification of allergens in the total sample extract. To a significant extent, they contribute to the acquisition of new knowledge that serves to develop diagnostic methods by detecting the binding of IgE antibodies to specific allergens. In the current period, it is possible to use modern methods of molecular biology for the analysis of allergens, such as two-dimensional electrophoresis in combination with immunoblotting and mass spectrometry, which enables the identification of allergens in very small quantities and the sequential processing of unknown sample extracts **(Sancho and Mills, 2010; Baumert, 2014).**

The aim of present study was to analyze the proteome of wheat, oat and buckwheat grains by modern molecular methods (two-dimensional electrophoresis and mass spectrometry) with an emphasis on the detection of proteins that cause allergic reactions in people with a genetic predisposition.

MATERIAL AND METHODS

Plant material

Grains of wheat (*Triticum aestivum* L.) variety Elinor, oats (*Avena sativa* L.) variety Zvolen and buckwheat (*Fagopyrum esculentum* Moench) variety Špačinská 1 were analyzed. The selected varieties are the most cultivated in agricultural practice in Slovakia and are used for the preparation of food products. The samples were obtained from the collection of plant genetic resources stored in the Gene Bank of the Slovak Republic (National Agriculture and Food Centre, Research Institute of Plant Production, Piešťany, Slovak Republic).

Protein Extraction and Two-Dimensional Electrophoresis (2-DE)

Extraction of storage proteins

Dry, ripe grains were mechanically homogenized with liquid nitrogen. Protein extraction was carried out at 4°C according to the modified methodology of **Schägger (2006).**

The extracted proteins were precipitated 5 times with 0.1 mol.L^{-1} ice-cold ammonium acetate in 100% methanol and stored overnight at -20°C. In order to obtain the sediment, the samples were centrifuged at 4 °C and 15,000 rpm for 20 minutes on the second day, and the precipitate was washed twice with ice-cold 80% acetone and then centrifuged for 10 minutes at 4 °C and 10,000 rpm^{-1}. Finally, the precipitate was washed with 70% ice-cold ethanol and centrifuged for 20 minutes at 4°C and 10,000 rpm. The protein precipitate was dissolved in IEF buffer [8 mol.L^{-1} urea, 2% (w/v) CHAPS, 0.5 mol.L^{-1} DTT, 0.2% (v/v) ampholyte Pharmalyte 3 -10 for IEF, redistilled water] and protein concentration was determined by the protocol of **Bradford (1976).**

Isoelectric focusing (SERVA IPG BlueStrips manual in modification)

Based on the determined concentration of proteins in the sample, an adequate amount was taken (according to the selected gel strip), which was supplemented with IEF buffer solution to a volume of 125 µL. In the focusing vessel, the sample was incubated with a 7 cm gel strip, pH 3-10 (SERVA IPG BlueStrips in modification) for 60 minutes at room temperature. The gel strip was overlaid with mineral oil and placed in a Bio-Rad PROTEAN i12 IEF CellUnit. Active rehydration took place for 12 hours at 50 V and was followed by isoelectric focusing under the following conditions: 150 V, 150 Vh; 500 V, 500 Vh; 4000 V, 15,000 Vh and terminated with a constant voltage of 500 V.

Electrophoretic separation of grain storage proteins (SERVA IPG BlueStrips manual in modification)

After isoelectric focusing, the gel strips were incubated for 15 minutes in an equilibration solution [6 mol.L^{-1} urea, 1.5 mol.L^{-1} Tris-HCl (pH 8.8), 5% (w/v) SDS, 30 % (v/v) glycerol, redistilled water] containing 2% (w/v) DTT followed by 2.5% (w/v) IAA. The gel strip was placed on a 10% polyacrylamide gel [11.43 ml mol.L^{-1} Tris –HCl (pH 8.8), 17.48 ml AA-BIS solution (54.49 g acrylamide and 0.72 g N, of N'-methylenebisacrylamide in a volume of 250 ml), 0.3 ml of 10% (w/v) SDS solution, 0.76 ml of 4% (w/v) ammonium persulfate solution, 0.06 ml of TEMED], then the protein marker Spectra Multicolor Broad Range Protein Ladder (Thermo Scientific) and 0.5% agarose containing bromophenol blue were applied. Electrophoresis was carried out in a Bio-Rad Mini-PROTEAN Tetra System in an electrode buffer environment (28.2 g glycine, 6 g Tris-HCl, 2 g SDS to a volume of 2000 ml with distilled water) at 10 mA and 80 V approximately 3.5 hours.

Protein staining, visualization and evaluation

Gels were stained overnight in Coomassie Brilliant Blue solution containing 20% (v/v) ethanol, 1.6% (v/v) phosphoric acid, 8% (w/v) ammonium sulfate, 0.08% (w/v) Coomassie Brilliant Blue G-250 and then washed in distilled water to remove excess dye. The electrophoretic profiles of individual samples were scanned with a Bio-Rad GS-800 Calibrated Densitometer scanner, then adjusted with the Quantity One program and evaluated using the PDQuest™ 2-D Analysis Software program from Bio-Rad.

Protein identification by Mass Spectrometry (MS)

In-gel digestion **(Shevchenko *et al.*, 2006** in modification**)**

Protein spots were excised from gels and de-stained with 600 µl of 0.05 mol.L^{-1} ammonium bicarbonate (NH_4HCO_3) containing 50% acetonitrile (ACN). The samples were shaken on a shaker for 15 minutes at 1000 rpm and then the supernatant was removed. To dehydrate the gel, 400 µL of ACN was added to the samples, shaken for 5 min on a shaker, and the supernatant was removed. To reduce the samples, 100 µL of 0.01 mol.L^{-1} DTT in 0.1 mol.L^{-1} NH_4HCO_3 was added, the solution was incubated for 30 min at 50 °C at 500 rpm, and the supernatant was removed. After cooling, 400 µL of ACN was added to the sample, mixed and the supernatant was removed. For alkylation, 100 µL of freshly prepared 0.05 mol.L^{-1} indole-3-acetic acid (IAA) in 0.1 mol.L^{-1} NH_4HCO_3 was added to the samples, the sample was incubated for 30 minutes at room temperature in the dark at 500 rpm and then the supernatant was removed. Samples were dehydrated by adding 400 µL of ACN, shaken for 5 min, and the supernatant was removed. Again, 600 µL of 0.05 mol.L^{-1} NH_4HCO_3 containing 50% ACN was added, incubated for 15 min at 1000 rpm and the supernatant removed. To dehydrate the gel, 400 µL of ACN was added to the samples, shaken for 5 min, and the supernatant was removed. The samples were prepared for digestion with trypsin.

Subsequently, 10 ng.μL^{-1} trypsin in 0.01 mol.L^{-1} NH_4HCO_3 containing 10% ACN was added to the samples, in such an amount that the gel pieces were immersed in the solution. The samples were incubated for 120 min at 4°C to allow the solution to soak into the gel. The supernatant was removed and an amount of 0.01 mol.L^{-1} NH_4HCO_3 containing 10% ACN was added to the gel so that the gel was immersed in the solution. Protein cleavage took place at 37°C in a thermostat with air circulation for 6 to 12 hours. The individual sample tubes were centrifuged and 40 µL of a solution containing 70% ACN and 1% trifluoroacetic acid was added to each tube. The samples were incubated for 15 minutes at 37°C and 1000 rpm in a shaker. The supernatant was collected in a new tube, then the extraction was repeated and both supernatants were combined. The extracts were concentrated to 10 µL in a vacuum centrifugal evaporator and stored at -20 °C.

Identification of proteins using LC-MS/MS analysis

The cleaved proteins were diluted in 100 µl of 3% ACN solution. The sample was separated by liquid chromatography on a chip with a column with a volume of 40 nL. The separation cartridge was 300SB-C18-ZX. The volume of the injected sample was 1 µL and peptides were separated and concentrated in the pre-column of the chip with a flow rate of 3 µL per minute using a mobile phase of 3% aqueous ACN solution. Peptides were washed into the chamber of the ion source, through the analytical column, by the gradient of the mobile phase by switching the chip to the analytical mode. The analytical pump gradient setting for 45 minutes was as follows: water concentration was from 97% to 5% and ACN from 3% to 95%, while at the 50th minute the water concentration was again 97% and ACN 3%. The peptides were successively ionized in the Agilent Chip Cube Interface using a modified ESI method. Next, the ions were analyzed by an Agilent 6530 Accurate-Mass Q-TOF mass spectrometer, which worked in Auto MS/MS mode. In the MS mode, the m/z range was set from 300 to 2000 and the scanning speed was 8 spectra per second, in the MS/MS mode from 100 to 3000 m/z with the scanning speed to 3 spectra per second. Peptides were used as an isotopic model in the experiment. The setup of the device allowed the selection of precursor ions according to the charge and subsequently according to the abundance, while the selected values of the peptide charges were 2, 3 and more.

Processing of the obtained data and identification of the basic components was carried out in the program Agilent MassHunter Workstation Software Qualitative analysis. The analysis of already processed data describing the individual

components of the analyte was performed with the Agilent SpectrumMill program. The data processed in this way were compared with the UniProt, NCBIprot and Allergome proteomic databases.

RESULTS AND DISCUSSION

In recent years, strategies used for the detection and quantification of allergens found in food have been significantly developed. The introduction of new modern mass spectrometry techniques, the improvement of bioinformatics and the expansion of genomic databases have enabled the emergence of a new proteomic scientific field, allergenomics. Allergenomics has become a powerful tool for the characterization and quantification of food protein allergens that are associated with IgE. **(Di Girolamo *et al.*, 2015).**

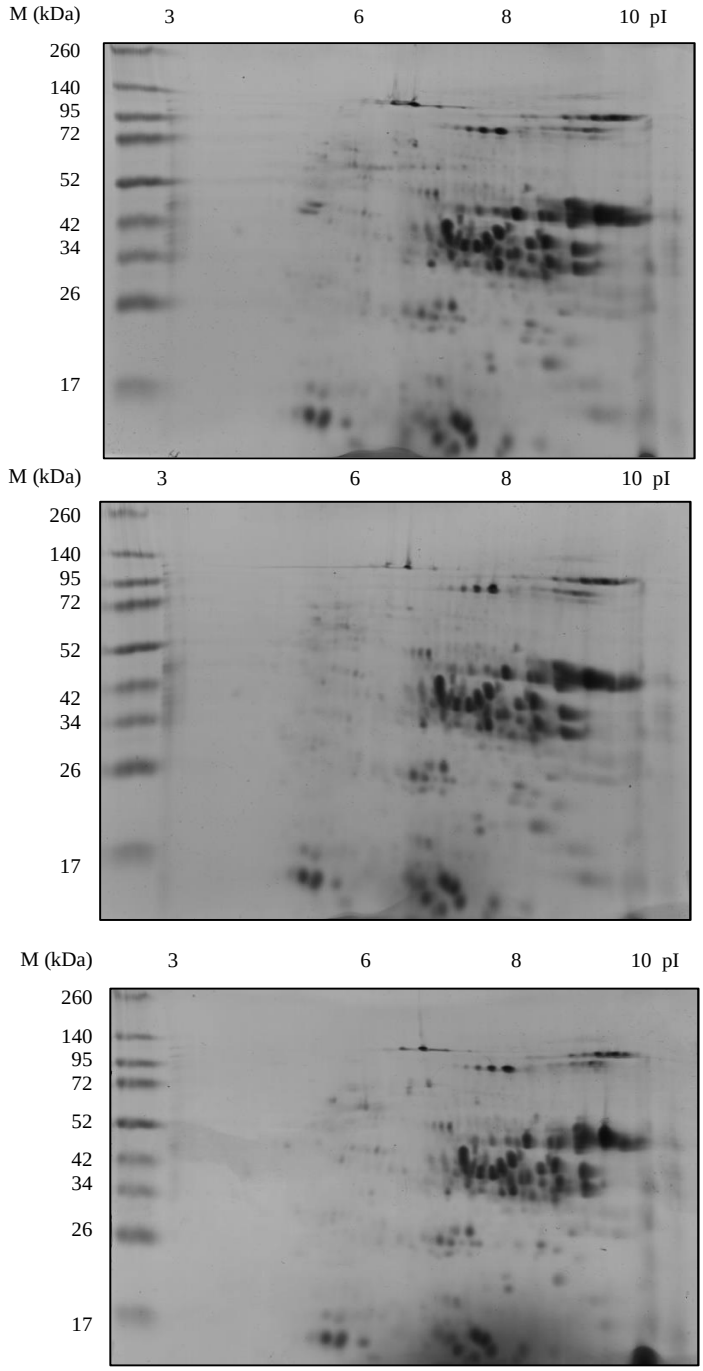


Figure 1 Biological triplicate of 2DE gels of the wheat variety Elinor. M – molecular weight marker (in kDa)

The most frequently used methods in allergenomics are mass spectrometry (MALDI-TOF, MS/MS), PCR, ELISA, various types of electrophoresis (SDS-PAGE, 2-DE), isoelectric focusing (IEF) and high-performance liquid chromatography (HPLC) **(Picariello *et al.*, 2011).** Studying the fractional composition of grain proteins allows estimating the amount of protein components, but the highest resolution of individual polypeptides is achieved by two-dimensional gel electrophoresis **(Skylas *et al.*, 2000).** Two-dimensional polyacrylamide gel electrophoresis (2-DE) including isoelectric focusing (IEF) and polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS-PAGE) is the most effective method for the separation of a large number of proteins **(Nalęcz *et al.*, 2009).** Many other proteomic techniques are used for protein identification, such as liquid chromatography coupled to mass spectrometry (LC-MS/MS), which is among the most promising non-immunological methods for the identification, quantification and differentiation of proteins based on the precise molecular weight of peptide biomarkers **(Alves *et al.*, 2017).**

The extracted wheat, oat and buckwheat proteins were separated by 2DE gel electrophoresis using 7 cm gel strips with an immobilized pH gradient in the range of 3-10 (Fig. 1, 4, 7). From the 2DE gel of wheat (Fig. 3), oats (Fig. 6) and buckwheat (Fig. 9) selected protein spots were cut out based on the results of Western blot, which were obtained from the previous research activity of the authors and published in the paper of **Chňapek *et al.* (2021)**. The samples were digested with trypsin and subsequently these proteins were identified by mass spectrometry. The obtained data were processed in the program Agilent MassHunter Workstation Software Qualitative analysis, where individual components were identified. The analysis of the processed data took place in the Agilent SpectrumMill program and the data were subsequently compared with the UniProt, NCBIprot and Allergome proteomic databases.

Analysis of 2DE wheat gels quantified 221 protein spots that were found in biological triplicates. Proteins were separated (Fig. 2) in the range from 4.13 to 9.89 pI with experimental molecular weight from 12.42 kDa to 140 kDa. 10 protein spots were cut from the 2DE gel of wheat (Fig. 3), which were identified using mass spectrometry (Tab. 1).

By comparing the obtained data with proteomic databases, it was found that three protein spots (904, 1290, 1866) were identified as HMW-GS with a theoretical molecular weight of 87.22 kDa to 127.36 kDa with pI from 6.04 to 8.23. The wheat HMW-GS identified were Dx5 (904), Dy10 (1290) and 12 (1866). These results agree with the results obtained by SDS-PAGE published in **Chňapek *et al*. (2022),** where the presence of the 5+10 subunit pair was detected in the Elinor wheat sample. LMW-GS were identified in two protein spots (3185, 2568) with theoretical molecular weights from 37.34 kDa to 37.52 kDa in the pI region of 9.69 to 9.81. Two protein spots (2478, 1573) were identified as gliadin/avenin-like protein with a pI of 6.21 and a molecular weight of 22.74 kDa.

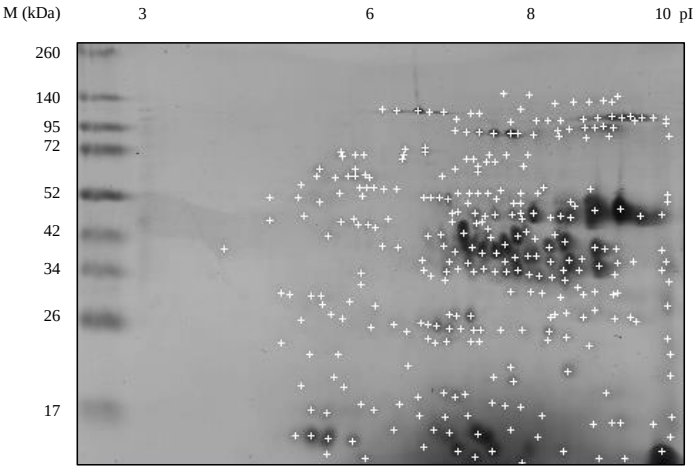


Figure 2 2DE gel of the wheat variety Elinor evaluated in the PDQuest program. White marks – quantified protein spots, M – molecular weight marker (in kDa)

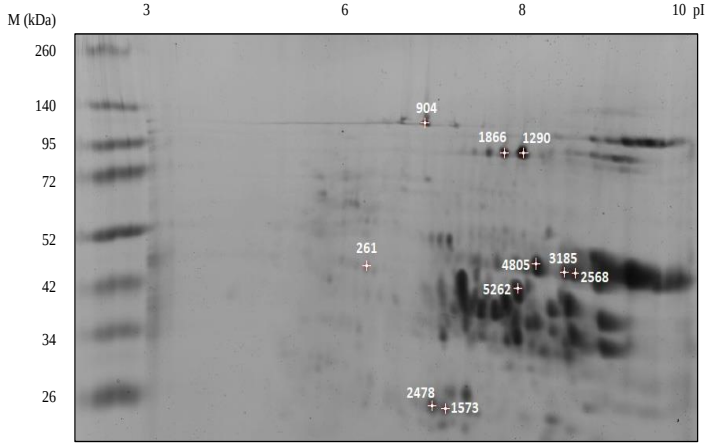


Figure 3 Protein map of the wheat variety Elinor. Protein spots are identified in Table 1, M – molecular weight marker (in kDa)

Protein spot 5262 contained α-gliadin Gli2-LM2-12 with a pI of 8.11 with a theoretical molecular weight of 36.37 kDa and protein spot 4805 was identified as γ-gliadin D2, D4 with a pI of 9.98 and a molecular weight of 37.61 kDa. Protein spot 261 was identified as serpin Z1B with a molecular weight of 36.84 kDa and a pI of 5.34. According to the UniProt database, all identified proteins belong to the storage proteins of wheat and are located in the endosperm of the grain. Serpin is a protease inhibitor and is localized in the extracellular space. By comparing the data obtained by mass spectrometry with the Allergome allergen database, it was found that out of 10 identified proteins, 7 were allergens (Tab. 1). Among the wheat allergens, the Tri and 26 allergen identified in three HMW-GS protein spots (904, 1866, 1290) was the most frequently occurring. Other allergens identified were Tri and 33 in protein spot 261 (serpin Z1B), Tri and 36 in protein spot 2568 (LMW-GS), Tri and alpha Gliadin in protein spot 5262 (α-gliadin Gli2-LM2-12) and Tri and 20 in protein spot 4805 (γ-gliadin D2, D4).

Table 1 Wheat proteins identified by LC-MS/MS

Wheat								
Number on the gel	**Identified protein**	**Number in the database**	**Score**	**Function**	**Localization in the cell**	**Number in the database Allergome**	**Theor. Mr [kDa]**	**Theor. pI**
904	DX5, HMW-GS	P10388	19.10	storage protein	endosperm	2898 Tri a 26	127.36	6.04
1866	12, HMW-GS	P08488	9.61	storage protein	endosperm	2898 Tri a 26	87.45	8.08
1290	DY10, HMW-GS	P10387	17.38	storage protein	endosperm	2898 Tri a 26	87.22	8.23
261	Serpin Z1B	P93693	23.91	protease inhibitor	extracellular space	5724 Tri a 33	36.84	5.34
3185	LMW-GS	R9XT62	21.76	storage protein	endosperm	-	37.34	9.69
2478	Gliadin/avenin-like protein	D2KFG9	22.62	storage protein	-	-	22.74	6.21
1573	Gliadin/avenin-like protein	D2KFG9	7.16	storage protein	-	-	22.74	6.21
2568	LMW-GS	Q571Q5	15.64	storage protein	endosperm	2674 Tri a 36	37.52	9.81
5262	α-gliadin Gli2-LM2-12	Q1WA40	8.49	storage protein	endosperm	3682 Tri and alpha_Gliadin	36.37	8.11
4805	γ-gliadin (D2, D4)	M9TGF7 Q94G92	15.75	storage protein	endosperm	3678 Tri a 20	37.61	9.98

Legend: Theor. Mr – theoretical molecular weight, Teor. pI – theoretical value of pI

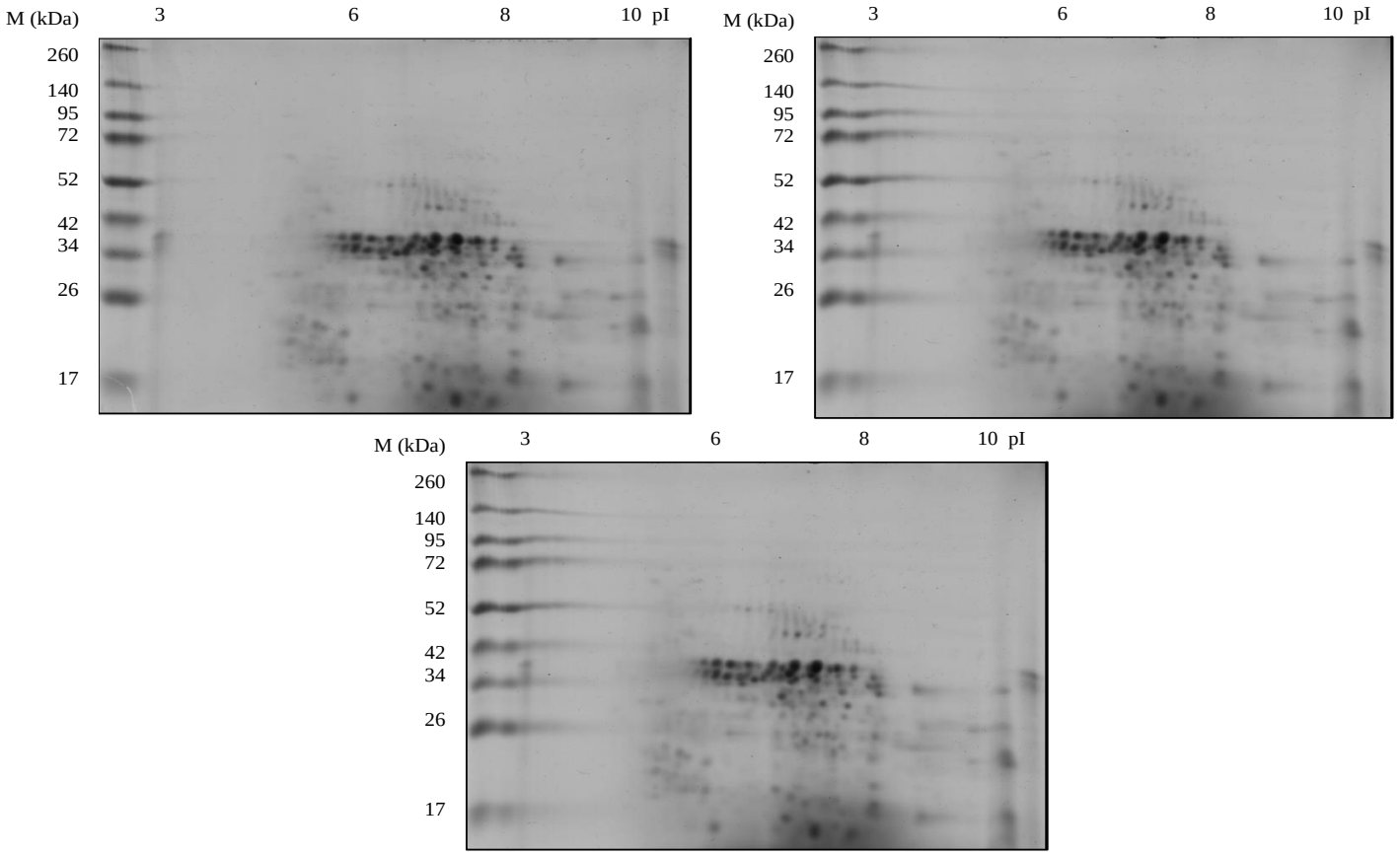


Figure 4 Biological triplicate of 2DE gels of the oat variety Zvolen. M – molecular weight marker (in kDa)

The obtained results correspond with the results of other authors who have published works in this area. According to **Tatham and Shewry (2012),** wheat gluten is divided into monomeric gliadins, which are soluble in alcohol solutions and are formed by ω5-, ω1,2-, α/β- and γ-gliadins, and polymeric glutenins, which are divided into HMW- GS and LMW-GS. Each wheat variety contains from three to five HMW-GS, which are further divided into x- and y-type. All hexaploid wheats contain 1Bx, 1Dx and 1Dy subunits, the presence of which determines the quality of wheat flour. Varieties with the *Glu-D1d* allele consisting of genes 1Dx5-1Dy10 produce stronger dough than those with the *Glu-D1a* allele consisting of genes 1Dx2-1Dy12. Celiac-active and toxic peptides are found in all gluten-forming proteins, but the relative proportion of gluten varies depending on genetic factors such as wheat type and variety in combination with environmental factors such as agro-climatic conditions, soil type, fertilization and agro-techniques **(Hajas *et al*., 2018; Noma *et al*., 2019).**

The wheat epitopes most commonly associated with reactions in celiac patients are from α-gliadins and some from γ-gliadins, ω-gliadins, HMW-GS and LMW-GS. The main allergens associated with WDEIA are ω-gliadins and HMW-GS. LMW-GS can also trigger WDEIA because they share epitopes with the major wheat allergen known as ω5-gliadin. Metabolic proteins from soluble fractions such as α-amylase, β-amylase, peroxidases and serpins also induce IgE-mediated allergies associated with wheat consumption. Although soluble proteins are the main allergen of wheat allergies, especially baker's asthma, gliadins are also a presumed cause of respiratory allergies **(Alves *et al*., 2018; Burkhardt *et al*., 2018; Yokooji *et al*., 2019).**

According to **Wu *et al.* (2012)** serpins are proteins, several of which regulate proteolysis. The molecular weight of most serpins is 40 kDa to 50 kDa. Serpins have been found in a number of crops such as barley, rye, oats and wheat. According to **Li *et al.* (2018)** serpin accumulation in maturing wheat grain suggests its potential role in flour functionality. The glutamine-rich serpin region is similar to glutamine-rich storage proteins that affect dough properties. According to **Larré *et al.* (2011)** serpins are potential allergens of baker's asthma and food allergies. According to **Matsuo *et al.* (2015)** Tri and 33 and Tri and 20 were identified as allergens in patients with baker's asthma and wheat food allergy. LMW-GS (Tri and 36) has been identified as the allergen that causes food allergy to wheat and WDEIA in patients living in European countries. HMW-GS (Tri and 26) was identified as the allergen responsible for WDEIA in Japanese patients.

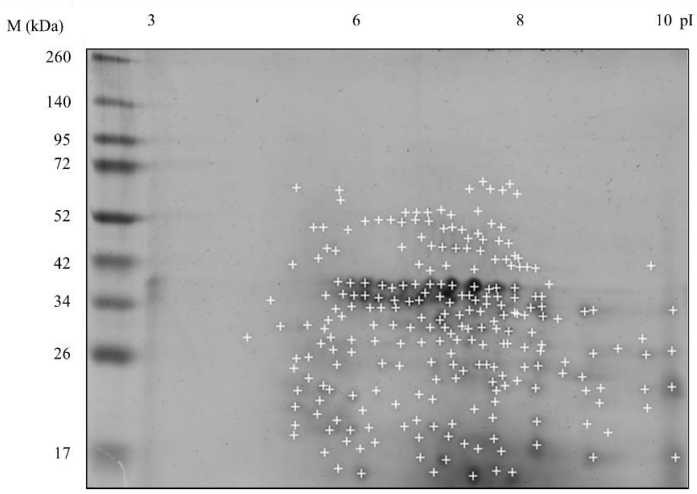


Figure 5 2DE gel of the oat variety Chosen evaluated in the PDQuest program. White marks – quantified protein spots, M – molecular weight marker (in kDa)

In biological triplicates of 2DE gels from oats of the Zvolen variety, 168 protein spots were quantified (Fig. 5), which were separated in the pI region from 4.02 to 9.93 with experimental molecular weights from 14.81 kDa to 67.96 kDa. For protein identification using mass spectrometry, five protein spots were cut (Fig. 6) and by comparing the data with databases, it was found that all identified oat proteins (Tab. 2) were storage proteins located in the endosperm of the grain.

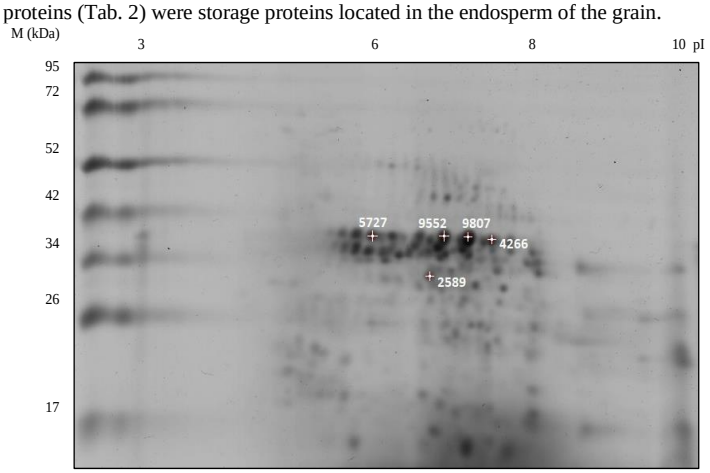


Figure 6 Protein map of the oat Zvolen variety. Protein stains are identified in Table 2, M – molecular weight marker (in kDa)

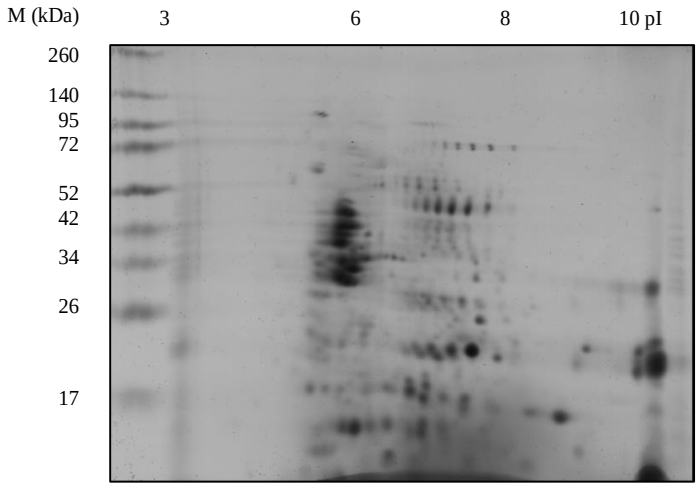
Three protein spots (9552, 9807, 5727) were identified as 12S globulin with a pI from 7.89 to 9.67 and a theoretical molecular weight from 53.77 kDa to 59.07 kDa. Protein spot 4266 was identified as an 11S globulin with a pI of 9.55 and a theoretical molecular weight of 62.26 kDa. A gliadin-like avenin with a pI of 6.72 and a theoretical molecular weight was detected in protein spot 2589. Of the five protein spots, four (9552, 9807, 5727, 4266) were identified as allergenic proteins, all of which were identified as Ave s 11S oat allergen according to the Allergome database.

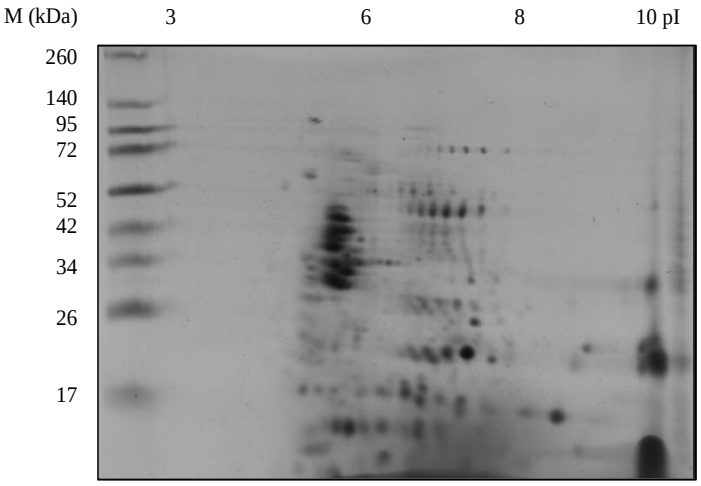
Table 2 Oat proteins identified by LC-MS/MS

Oat								
Number on the gel	**Identified protein**	**Number in the database**	**Score**	**Function**	**Localization in the cell**	**Number in the database Allergome**	**Theor. Mr [kDa]**	**Theor. pI**
9552	12s globulin	O49258	14.66	storage protein	endosperm	10849 Ave s 11S	58.57	8.8
9807	12s globulin	O49257	14.31	storage protein	endosperm	10849 Ave s 11S	53.77	9.67
5727	12s grain storage globulin 2	P14812	7.90	storage protein	endosperm	10849 Ave s 11S	59.07	7.89
2589	Gliadin-like avenin	L0L6J0	24.62	storage protein	endosperm	-	33.24	6.72
4266	11s globulin	Q38779	3.92	storage protein	endosperm	10849 Ave s 11S	62.26	9.55

Legend: Theor. Mr – theoretical molecular weight, Teor. pI – theoretical value of pI

According to **Esfandi *et al.* (2019)** oats have the highest protein content among cereals. Protein consist of globulins (50-80%), albumins (1-12%), prolamins (4-15%) and glutenins (<10%). Oats are the only cereal whose storage protein is globulins formed by 12S, 7S and 3S fractions (**Chang *et al.*, 2011**). The main fraction of oat proteins is 12S globulin, structurally similar to legume 11S globulin, which consists of two subunits with a molecular weight of 32 kDa (A-subunit) and 22 kDa (B-subunit).





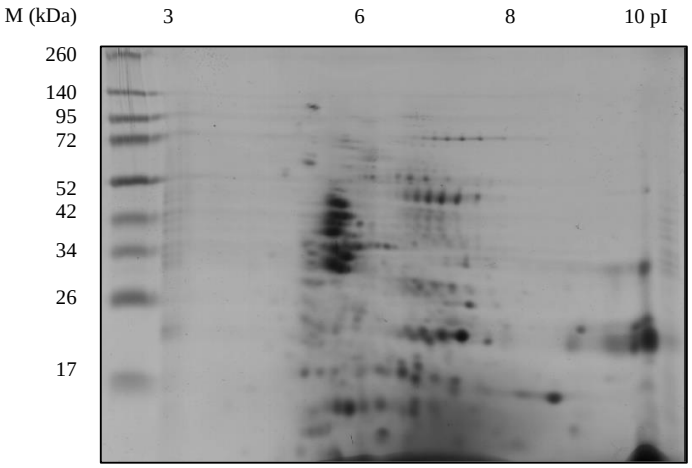


Figure 7 Biological triplicate of 2DE gels of the buckwheat Špačinská 1 variety. M – molecular weight marker (in kDa)

The A-subunit is made up of an acidic polypeptide, while the B-subunit is basic, with both polypeptides connected by disulfide bonds. Minor fractions are 7S and 3S globulins **(Nieto *et al.*, 2014). Esfandi *et al.* (2019)** found in the set of oat varieties, that the majority of peptides were represented by 12S globulin, followed by 12S grain storage globulin and 11S globulin. **Comino *et al.* (2016)** identified oat proteins by mass spectrometry. Protein spots were excised from 2DE gels based on their intensity after immunoblotting. All proteins were identified as avenins. These proteins belonged to α- and γ-gliadin-like fractions, some of which reacted with the anti-33-mer antibody. This research proved the existence of new potentially toxic peptides for celiac patients. According to **Klose *et al*. (2009),** oat gluten-forming proteins are likely to be in the range of 36 kDa to 55 kDa and pI 5-8. The mentioned researches correspond with the achieved results of the detection of allergens in oats.

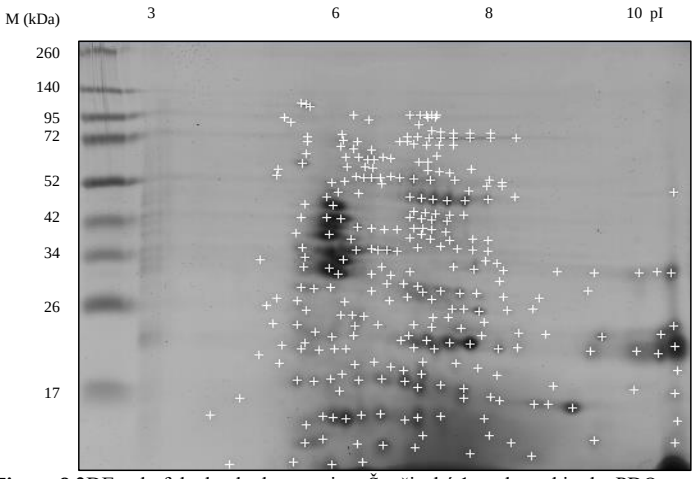


Figure 8 2DE gel of the buckwheat variety Špačinská 1 evaluated in the PDQuest program. White marks – quantified protein spots, M – molecular weight marker (in kDa)

A total of 208 protein spots were quantified in biological triplicate 2DE gels from buckwheat (Fig. 7). Protein spots of buckwheat Špačinská 1 were separated in the region from 3 to 9.83 pI with an experimental molecular weight from 10.10 kDa to 115 kDa. Six protein spots were excised from the gel (Fig. 9), of which three (5244, 1108, 1268) were identified as 13S globulins with a pI between 5.39 and 9.34 with a theoretical molecular weight of 21.56 kDa to 64.86 kDa. All 13S globulins are grain storage proteins according to the UniProt database. Protein spot 1420 with a pI of 5.89 and a theoretical molecular weight of 24.44 kDa was identified as 1-cys peroxiredoxin, which has peroxiredoxin activity in the cell.
An aspartate protease whose function is an aspartate-type endopeptidase activity was detected in protein spot 525 with a theoretical molecular weight of 49.36 kDa and a pI of 9.26. Protein spot 504 was identified as a flavonoid 3'-hydroxylase with a pI of 6.08 and a theoretical molecular weight of 50.45 kDa, which is responsible for iron ion binding and oxidoreductase activity (Tab. 3).
Two proteins were identified in protein spot 1268 by mass spectrometry. The first 13S grain storage globulin protein was identified by the Allergome database as the Fag e 1 allergen. The second 13S globulin/legume-like protein was 100% identical to the major buckwheat allergen storage protein listed in the Allergome database as Fag e 1 buckwheat allergen according to the UniProt database.

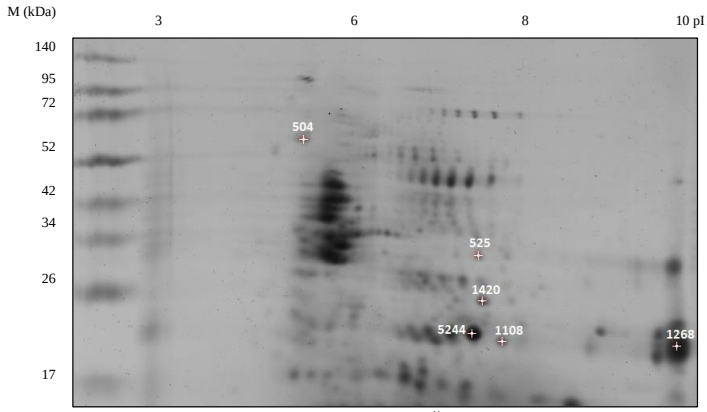


Figure 9 Protein map of the buckwheat variety Špačinská 1. Protein spots are identified in Table 3, M – molecular weight marker (in kDa)

Table 3 Buckwheat proteins identified by LC-MS/MS

Buckwheat								
Number on the gel	**Identified protein**	**Number in the database**	**Score**	**Function**	**Localization in the cell**	**Number in the database Allergome**	**Theor. Mr [kDa]**	**Theor. pI**
5244	13S globulin	W6JNG6	140.76	storage protein	endosperm	-	50.74	5.39
1108	13S globulin	Q84MJ4	25.74	storage protein	endosperm	-	50.73	5.51
1420	1-Cys peroxiredoxin	Q9SP12	47.22	Peroxiredoxin activity	cell	-	24.44	5.89
525	Aspartate protease	Q6QJL5	6.17	Aspartate-type endopeptidase activity	-	-	49.36	9.26
504	Flavonoid 3'-hydroxylase	R4H461	4.47	binding of iron ions, oxidoreductase activity	-	-	50.45	6.08
1268	13S grain storage globulin 1	O23878	26.25	storage protein	-	698 Fag e 1	64.86	5.68
	13S globulin/Legumin-like protein	D2JWR2	131.16	storage protein	-	100% match with the main allergenic storage protein K4PY05 698 Fag e 1	21.56	9.34

Legend: Theor. Mr – theoretical molecular weight, Teor. pI – theoretical value of pI

These findings support the results obtained by other authors. **Sano *et al.* (2014)** reported that the protein composition of buckwheat is different compared to wheat. 13S globulin makes up 43% of all grain proteins and belongs to the group of legume-like proteins that are part of the 11S globulin group. The main allergens of buckwheat grain are proteins with molecular weights of 9, 16, 19 and 24 kDa. Other identified allergens are proteins with a molecular weight of 30, 43 and 67 kDa. One of the most dominant allergens is the β-polypeptide of the main grain storage protein 13S globulin with a molecular weight of 24 kDa, Fag e 1 **(Sano *et al.*, 2014).**

Buckwheat is one of the five main allergenic foods, as it contains several different allergens that can cause anaphylactic reactions in patients with buckwheat allergy. Proteins with a molecular weight of 8 kDa to 100 kDa have been identified as the main buckwheat allergens.

The identified allergen is a protein with a molecular weight of 56 kDa, which is similar to the main allergen storage protein and legume-like 13S globulin **(Zheng *et al.*, 2018).** Allergens with a molecular weight of 22 kDa and 15 kDa are designated as Fag e 1 and Fag e 2 **(Morita *et al.*, 2006).**

From the results of the proteome analysis of selected varieties of wheat, oat and buckwheat genotypes using the 2DE method and LC-MS analysis, it can be concluded that interspecies differences in the representation of individual protein subfractions were found. Allergens that are specific to a particular crop have been shown to occur in all analyzed crop species. On the other hand, it is very important to analyze also the individual varieties of the food-important crop. It was proven using Elisa method **(Chňapek *et al.*, 2021)** that the wheat varieties showed a gluten content in a wide range (28 899 – 43 278 $mg.kg^{-1}$), which greatly exceeds the set limit according to Codex Standard 118-1979 (20 $mg.kg^{-1}$) for patients with celiac disease. In the oat samples, the gluten content varied from 10,57 $mg.kg^{-1}$ to 57,37 $mg.kg^{-1}$, while the representation of celiac active polypeptides varied considerably in individual oat varieties. In selected buckwheat samples, a very low gluten content was determined with an average value of 2.03 $mg.kg^{-1}$, they meet the limit for the label "gluten-free" and can be used for the production of foods suitable for patients suffering from celiac disease. Elisa results were also confirmed by Western blot analysis.

Gálová *et al*. (2012) determined the content of gluten proteins in a set of different crops (wheat, barley, rye, oats, spelt, buckwheat, chickpea) using the RIDASCREEN ® ELISA test, and found that wheat, barley and rye varieties exceeded the limit of gluten protein content, while in oat samples, the gluten content ranged from 60 $mg.kg^{-1}$ to 21 300 $mg.kg^{-1}$, depending on the variety. Some oat varieties met the limit for gluten-free food and some exceeded the limit, so oats are still a risky crop for people with celiac disease.

It follows from the above that it is very important to know the presence of allergens in individual food plant sources, to detect them using appropriate methods and then to produce food intended for consumption by predisposed individuals.

CONCLUSION

In recent years, the demand for the production of food suitable for at-risk population groups suffering from food allergy or intolerance to any of the protein components of cereal grains has come to the fore. The initial screening of the proteome of an agriculturally important crop is based on the detection of the required qualitative protein components. Using two-dimensional electrophoresis, proteins are separated in the first dimension by isoelectric focusing based on their isoelectric point, and further separation in SDS-PAGE also takes into account their molecular weight. Proteins separated in this way can be reliably detected and characterized by mass spectrometry. By subsequently comparing the obtained sequences of polypeptide chains with the Allergome database, it is possible to reliably detect the relevant allergen in the given crop. The obtained results deepened and expanded current knowledge and the possibilities of allergen detection in cereals and pseudocereals by modern molecular methods. Allergens were confirmed to be present in all types of analyzed crops. Based on the above, it is necessary to consider the selection of the raw material base for the production of foods intended for the nutrition of predisposed individuals.

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