

Identification of oat (*Avena sativa*) and buckwheat (*Fagopyrum esculentum*) proteins and their prolamin fractions using two-dimensional polyacrylamide gel electrophoresis

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Abstract Total (non-fractionated) kernel proteins and the prolamin fraction (soluble in 75% ethanol) were extracted from oat (*Avena sativa*) var. Flämingstern kernel and from buckwheat (*Fagopyrum esculentum*) var. Kora kernel. As for buckwheat, extraction was effective only after kernel dehulling which allowed the removal of tannins and phenolic compounds that form complexes with proteins during extraction. The extracted proteins were analyzed using two-dimensional polyacrylamide gel electrophoresis (2D-PAGE). Gels of the prolamin fractions of oat and buckwheat were used as reference gels in order to detect prolamins on gels of total kernel proteins. The occurrence of 26 and 29 spots corresponding to prolamins was found on gels of total oat proteins and on gels of total buckwheat proteins, respectively. The electrophoretic images of oat and buckwheat prolamins revealed organized subregions containing spots with similar isoelectric points (pI) and various molecular weights (MW), mostly on oat prolamin gels and spots of similar molecular weights with various isoelectric points, mostly on buckwheat prolamin gels. Such organized subregions can be used as identifiers for the occurrence of prolamin fractions in total proteins (particularly as regards buckwheat proteins).

Keywords Oat and buckwheat proteins · Prolamins · Proteomics · 2D-PAGE · Electrophoretic image analysis

Introduction

Prolamins are the main storage proteins of the majority of cereals (including wheat, barley and rye), where they account for 30–50% of total protein content. In cereals such as oat and buckwheat, they are found in much lower amounts. The average prolamin content of oat (*Avena sativa*) and buckwheat (*Fagopyrum esculentum*) is 10–15% and only 1–4%, respectively, in relation to the total protein content [1]. Buckwheat is considered to be potentially well tolerated by patients with celiac disease, and is recommended for wholesome food production [2].

Proteomic examinations are becoming increasingly important in food science for the identification of prolamin fractions [3–6]. The increasing importance of proteomic examinations in this direction results from the increasingly frequent application of electrophoretic, chromatographic, spectrometric, immunological and bioinformatic techniques to:

- distinguish cereal varieties,
- distinguish protein fractions within a certain protein,
- compare the molecular similarity of proteins in wheat, oats and buckwheat, among others,
- confirm the safety of, e.g., oats or buckwheat proteins in a gluten-free diet.

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) involving isoelectric focusing (IEF) and electrophoresis in polyacrylamide gel in the presence of sodium dodecyl sulphate (SDS-PAGE), proposed originally by O'Farrell [7], is currently the most effective method of separating a large number (hundreds or even thousands) of proteins. Recent progress towards the development and applications of this method has been discussed by Garfin [8] and Görg et al. [9] in their review papers. A qualitative

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analysis aimed at detecting specific proteins involves both 2D electrophoresis as an independent research method, and electrophoresis with mass spectrometry [10].

2D electrophoresis has proven useful in research concerning the identification of cereal varieties [11–14]. Proteomic examinations of cereals using 2D electrophoresis currently focus on wheat proteins [15] and rice proteins [16], while little research has been done on oat or buckwheat proteins. Using 2D gels, Jacobsen et al. [11] classified prolamins of different wheat varieties which were indistinguishable using matrix-assisted laser desorption/ionization-time of flight-mass spectrometry (MALDI-TOF-MS) combined with an artificial neural network (ANN). MALDI-TOF mass spectra of prolamins of these varieties were similar to such an extent that it was impossible to distinguish them with the use of ANN (this was mainly due to the same molecular weight of the major proteins and lack of detectable differences in their relative content). However, the 2D-PAGE produced spots of minor proteins characteristic of each of the varieties because the analyzed extracts contained proteins of similar molecular weights but different isoelectric points. Studies aimed at improving this method involved, among others, the determination of the effect of changing spot location in gels on the repeatability of analyses [17]. A controversial issue is the possibility that spots originating from various proteins may overlap. This can be an important consideration for detecting proteins or groups of proteins. Some authors [18–20] emphasize the significance of this issue, while others [21] question its importance. Research has also been undertaken to determine the repeatability of results obtained using 2D electrophoresis [22, 23]. Another area of intensive study is the search for efficient methods for protein fractionation prior to 2D-PAGE analysis [24, 25].

DiCagno et al. [26] used 2D-PAGE to detect proteins of oat and buckwheat in flour obtained from the mixture of grain originating from various plants and also applied this method to monitor the proteolysis of cereal proteins. These authors studied prolamins mixtures of different plant species (oats and buckwheat, among others), fermented by lactic acid bacteria and those applied in the production of sourdough bread. In their paper, 2D images of the prolamins protein fractions and their polypeptides of different doughs made of a mixture of wheat, millet, oat and buckwheat were presented. Yano et al. [6] used 2D electrophoresis, followed by the proteolysis and sequencing of peptides, to identify the allergenic proteins of buckwheat. The allergenic proteins they found did not belong to the group of prolamins.

The aim of this study was identification and characterization of total oat and buckwheat kernel proteins and their prolamins fractions using 2D-PAGE image analysis. This is a preliminary study, whose main objective was an attempt to find identifiers for the occurrence of prolamins fractions

in total proteins. Gels of the prolamins fractions of oat and buckwheat were used as reference gels in order to detect prolamins on gels of total kernel proteins. In order to compare spots on the reference gels and on gels of total proteins of oat and buckwheat, the characteristic spots of similar shape and intensity (anchoring spots) and characteristic groups of spots were searched for the purpose of identification.

Materials and methods

Materials

The experimental material consisted of the kernel of oat var. Flämingstern variety and buckwheat var. Kora. Oat kernel was harvested in 2005 by the farming company Przedsiębiorstwo Hodowlane Lochow-Petkus Polska Sp. z o.o. (Prusy-Kondratowice, Poland) and buckwheat kernel was harvested in the same year at the Plant Growing Station Palikije (Wojciechów Lubelski, Poland). The material was stored at 5 °C in hermetically sealed plastic containers.

Ethanol, methanol, acetic acid and o-phosphoric acid 85% were purchased from Polish Chemical Reagents (Poland). All other chemicals were purchased from Sigma-Aldrich (Germany).

Methods

Flour sample preparation and protein extraction

Refined kernel was ground in an IKA A10 laboratory mill (Labortechnik, Germany) and sieved through a 250- μ m mesh sieve. Buckwheat kernel was dehulled before grinding.

Extraction of oat and buckwheat prolamins

Oat prolamins were extracted in several stages of sequential extraction, using a modified Simpson's method [27]. The first stage of the extraction involved adding 4 ml of Tris(hydroxymethyl)aminomethane (Tris)-HCl solution (50 mM, pH 8.8, 1% (w/v) polyvinylpyrrolidone (PVPP), 14% (v/v) phenylmethylsulfonyl fluoride (PMSF)) to 1 g of flour. Extraction was conducted for 1 h at 4 °C, shaking every 15 min. Then, the obtained extract was centrifuged (20,000 $\times$ g, 20 min, 15 °C), and the supernatant containing albumins and globulins was rejected. The procedure of extraction and centrifugation was subsequently repeated. The pellet was washed with 5 ml of deionized water, by mixing for 2–3 min, and centrifuged (under the conditions specified above). Prolamins were extracted twice, by pouring 4 ml 75% (v/v) ethanol over the pellet, shaking and storing samples for 2 h at 25 °C [27, 28].

After centrifugation, the obtained supernatant containing proteins was vaporized in a needle concentrator (Technique, UK) to 1/4 of the initial volume of the extract, lyophilized, and was stored afterwards at -80°C . The extraction of buckwheat prolamins was carried out in the same way, using a fivefold amount of flour.

The oats' prolamin extraction procedure was performed twice which resulted in a subsequent series of three and four repetitions of the oats' prolamin extract (seven repetitions in total). They originated from two different flour samples from the same lot of oat kernels. The buckwheat prolamin extracts originated from one series of extraction (three repetitions).

Extraction of total oat and buckwheat kernel proteins

5 ml of extraction buffer [6 M urea 1% (w/v) Triton X-100, 0.5% (w/v) DL-dithiothreitol (DTT), 0.5% (v/v) immobilized pH gradient (IPG) buffer pH 3–10, 1% (w/v) PVPP, 14% (v/v) PMSF] was added to 500 mg of flour and carefully mixed. Extraction was conducted for 1 h at room temperature, shaking every 15 min. After this time, the extracts were centrifuged ($40,000\times g$, 1 h, 15°C). The obtained supernatant (containing proteins) was stored at -80°C [27, 28].

Three repetitions for each kind of extract of the total oat and buckwheat kernel proteins were prepared. The extraction procedure for the total oat kernel proteins was repeated.

As a result of the protein extraction, four kinds of protein extracts were obtained, which were named: (1) oat prolamins, (2) buckwheat prolamins, (3) total oat kernel proteins, and (4) total buckwheat kernel proteins. The lyophilisates of these extracts from all repetitions were subjected farther to 2D-PAGE analysis.

2D electrophoresis of prolamin fractions and of total oat and buckwheat kernel proteins IEF separation [29]

After the measurement of protein concentration according to Bradford [30], 2.5 mg of oat prolamin lyophilisate and 5 mg of buckwheat prolamin lyophilisate was dissolved in 450 μl of buffer for rehydration (8 M urea 0.4% (w/v) DTT, 2% (w/v) Triton X-100, 0.5% (v/v) IPG buffer pH 3–10, bromophenol blue). Samples were subjected to 10 min of ultrasound treatment and stored for a subsequent 50 min at room temperature. 450 and 500 μl of the prepared oat or buckwheat solution, respectively, were taken for IEF analysis, and the solution was placed on gel strips, IPG Dry Strips with linear pH gradient (18 cm, pH 3–10, Bio-Rad Polska Sp. z o.o.). In the case of total oat and buckwheat kernel proteins, 420 μl of rehydration buffer was added to 180 μl of the extract, collecting 500 μl of the protein solution for IEF analysis. The rehydration of gel strips was conducted for 13 h at 30 V. IEF separation was conducted

in the first dimension apparatus Ettan™ IPGphor™ (Amersham Biosciences, Uppsala, Sweden), under the following conditions: 1 h at 100 V, 1 h at 300 V, 1 h at 600 V, 0.5 h at 1,000 V, 0.5 h gradient up to 8,000 V and 8 h at 8,000 V.

SDS-PAGE separation [31]

Proteins separated in the first dimension were equilibrated for 15 min in an equilibration buffer [6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulphate (SDS), 50 mM Tris–HCl buffer of pH 8.8, bromophenol blue] with the addition of 1% (w/v) DTT, and then for another 15 min in an equilibration buffer of the same composition, with the addition of 2.5% (w/v) iodoacetamide (IAA) instead of DTT. After equilibration, IPG strips were placed in the second dimension apparatus Ettan™ DALTsix (Amersham Biosciences, Uppsala, Sweden). Protein separations were conducted in 12.5% polyacrylamide gels (dimensions: $215 \times 276 \times 1$ mm), as follows: 2 h at 5 mA/gel and 18 h at 15 mA/gel. A concentrated electrode buffer pH 8.3 [96 mM Trizma base (Tris), 0.5 M glycine, 0.4% (w/v) SDS] was diluted with deionized water at a 1:2 ratio in the upper chamber and at a 1:4 ratio in the lower chamber. Strips were placed onto gels, and a 0.5% solution of agarose in an electrode buffer was poured over their surface.

Gel staining and destaining [32]

After separation in the second dimension, gels were stained by the “Coomassie Blue” method using a modified Neuheff's method. The colloidal Coomassie Brilliant Blue G-250 (Sigma–Aldrich, Germany) solution was used for staining (0.12% (w/v) Coomassie Brilliant Blue G-250, 10% (w/v) orthophosphoric acid, 10% (w/v) ammonium sulphate, 20% (v/v) methanol). Before staining, gels were fixed for 1 h (40% (v/v) ethanol, 10% (v/v) acetic acid) and rinsed twice with deionised water for 10 min. After staining (12 h), gels were destained in 1% (v/v) acetic acid, until a destained background was obtained.

Computer-aided image analysis of gels

Destained gels were scanned and subjected to an image analysis with the use of ImageMaster 2D software (Amersham Biosciences, Uppsala, Sweden). Isoelectric points and molecular weights were calculated for all spots. Averaged gels of prolamin fractions were used as reference gels (seven gels for oat proteins and three gels for buckwheat proteins). The different amounts of gel for oats and buckwheat resulted from the fact that, in the case of oats, the authors accounted for gel images obtained from 2D electrophoretic separation, preceded by an initial experiment aiming at the optimization of prolamin protein extraction.

Prolamin proteins were extracted according to a modified method proposed by Simpson [27] for sequencing wheat proteins containing significantly greater amounts (percentage content) of prolamins than oat or buckwheat. Oat was meant to serve as a model protein. Moreover, oat protein was chosen to select an optimum protein concentration for further 2D electrophoretic analyses. To study many varied protein concentrations based on oats, the number of gels for oats was greater than for buckwheat.

In order to compare spots on the reference gels and on gels of total kernel proteins of both plants (six gels for total oat kernel proteins and three gels for total buckwheat kernel proteins), characteristic spots of similar shape and intensity (anchoring spots) and groups of spots were found whose relative shifts were similar on prolamin fraction gels and on total kernel protein gels. The above spots were identified by the operator. Spots neighboring to the selected spots were identified automatically. Such a strategy of gel comparison is recommended by Kjærsgaard et al. [33]. Due to the fact that certain spots can be invisible on some gels [13], the following criteria were adopted with respect to spots considered characteristic of the prolamin fraction. While creating averaged prolamin gels, spots occurring on at least six out of seven oat gels, and two out of three buckwheat gels, were taken into account. Spots found on total kernel protein gels were also considered, provided that they were visible on reference gels and on at least two out of three gels.

Results and discussion

Specificity of the isolation and purification of oat and buckwheat proteins

Research concerning prolamins is particularly difficult because of their heterogeneity and insolubility in most solvents. Studies on prolamins are hindered by the specific chemical constitution of these proteins, since they make up a complex of many polypeptides differing in electrical charge, molecular weight, sequence and isoelectric point. The identification of more or less allergenic fractions requires previous separation and purification of specific proteins forming the complex [3].

In the case of some plants and cereals, such as buckwheat, the process of protein isolation can be additionally hampered by the presence of anti-nutritional factors, including phenolic compounds such as tannins and flavonoids [34]. It is a well-known fact that tannins and proteins form insoluble complexes that are stabilized by hydrogen bonds or covalent bonds that may disturb or even prevent the isolation of proteins from plant extracts. It is therefore necessary to remove tannins through the addition of substances containing groups similar to peptide bonds, e.g., PVPP [35].

In view of the above problems with both the extraction and isolation of prolamins, new and improved methods for prolamin isolation and structural analysis are being developed [36, 37]. According to Michaud and Asselin [38], the choice of the appropriate extraction procedure, extractant type, concentration and reducing agents is determined by the specific properties of proteins and has a crucial effect on the subsequent quantity and quality analyses, the composition of protein fractions and on the quality of separation. In many cases, in order to prevent protein modification during extraction it is recommended to add compounds such as SDS, trichloroacetic acid (TCA), ethylenediaminetetraacetic acid (EDTA) and proteases inhibitors, or to prevent protein-polyphenols interactions, using PVPP or reducers [35, 38]. In the current study, the extraction method was improved through the addition of 1% (w/v) PVPP and 14% (v/v) PMSF to the extraction buffer, which was used both in the extraction of prolamins and total proteins of oat and buckwheat. It was not a true replica of the method recommended by Simpson [27], but rather a modification. The attempt in this study to remove tannins by adding PVPP, as recommended in literature, proved unsuccessful with respect to dehulled buckwheat. The dehulling of buckwheat kernels partially solved the problem of their separation, particularly with regard to prolamin proteins (found in trace amounts in dehulled buckwheat). Apart from that, the prolamin fraction of buckwheat required lyophilization prior to electrophoretic separation. This was confirmed by the much better separability of dehulled buckwheat kernels, reported for good-quality lyophilisates. More prolamin spots were observed and the images were less blurred, which probably resulted from the presence of phenolic compounds. “Pure” (not containing any detectable spots) images of polyacrylamide gels were obtained for unde-hulled buckwheat (data not shown). The hull of a kernel contains a relatively large amount of phenolic compounds that form complexes with proteins, which impedes the total extraction of proteins and, subsequently, their electrophoretic separation. The extraction buffer was supplemented with PVPP, to remove the phenolic compounds and with PMSF, to eliminate the process of proteolysis by native proteases which can be found in the extraction buffer after extraction. Such a procedure allowed the authors to obtain even better quality of lyophilisates and electrophoretic images with no greater smudges and the spots on the gels were easier to detect and identify.

Characteristics of 2D electrophorograms of prolamins and total kernel proteins from oat and buckwheat

The gel images of oat prolamins and total oat kernel proteins are presented in Figs. 1 and 2, respectively. The gel images of buckwheat prolamins and total buckwheat kernel

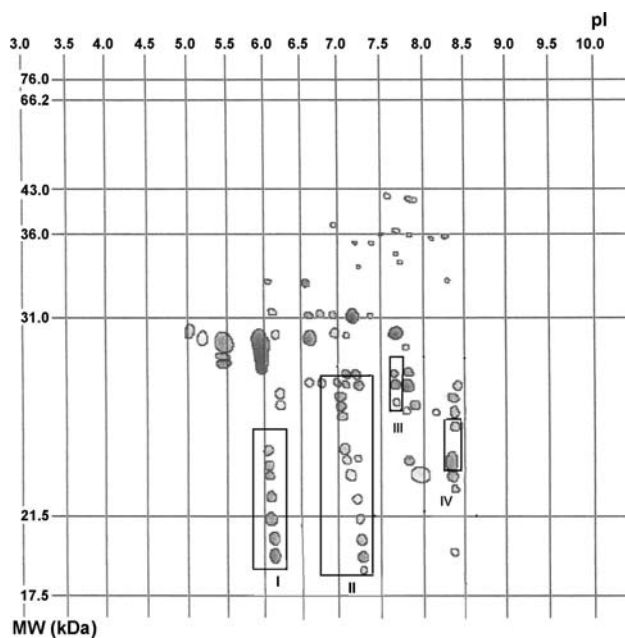


Fig. 1 2D-electrophoretic image of oat prolamins (var. Flämingstern) with marked organized subregions

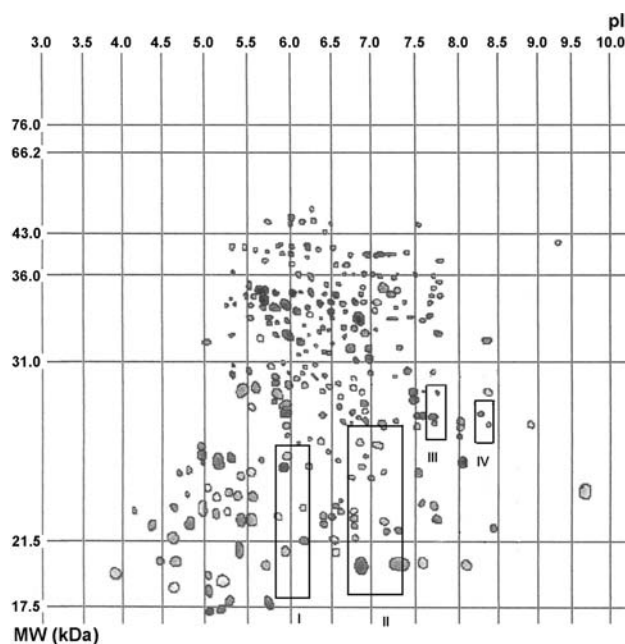


Fig. 2 2D-electrophoretic image of total oat kernel proteins (var. Flämingstern) with marked subregions corresponding to the organized subregions in the image of oat prolamins (Fig. 1)

proteins are presented in Figs. 3 and 4, respectively. The lists of spots assigned to the prolamin fractions of oat and buckwheat are presented in Tables 1 and 2, respectively.

The gel images of oat prolamin fractions contain four characteristic series of spots with similar isoelectric points, differing in molecular weight (Fig. 1). They cover the subregions of isoelectric points of about 5.9–6.2 and molecular

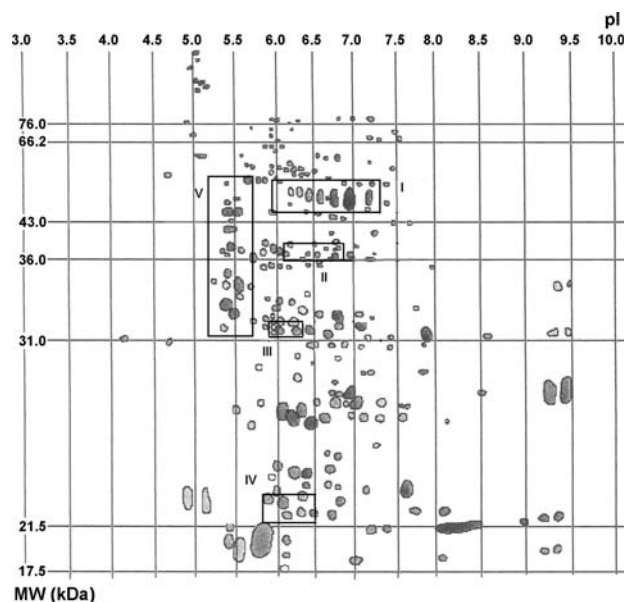


Fig. 3 2D-electrophoretic image of buckwheat prolamins (var. Kora) with marked organized subregions

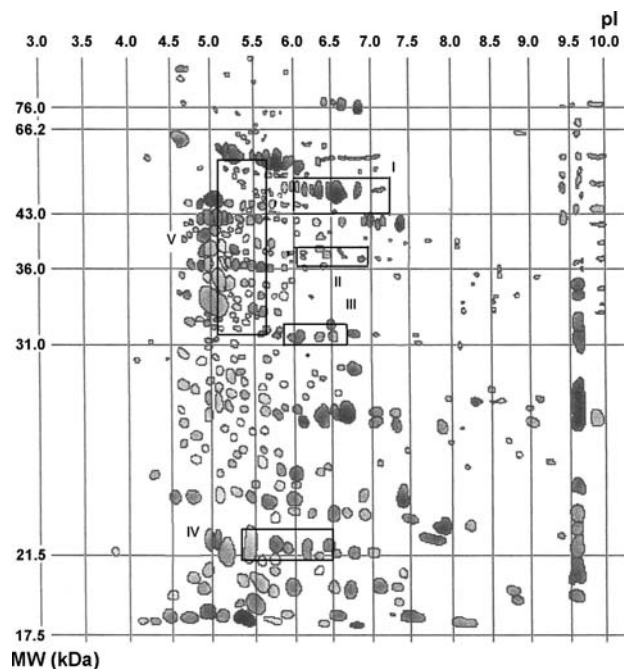


Fig. 4 2D-electrophoretic image of total buckwheat kernel proteins (var. Kora) with marked subregions corresponding to the organized subregions in the image of buckwheat prolamins (Fig. 3)

weights ranging from 20 to 23.9 kDa (subregion I, 7 spots), isoelectric points of 6.6–7.4 and molecular weights ranging from 19.9 to 25.1 kDa (subregion II, 14 spots), isoelectric points of about 7.7 and molecular weights ranging from 24.8 to 25.7 kDa (subregion III, 3 spots), and isoelectric points of 8.0–8.4 and molecular weights ranging from 23.9 to 24.8 kDa (subregion IV, 2 spots). The organized

Table 1 List of oat prolamins spots assigned to the prolamins fraction (Fig. 1) present in the gel images of total oat kernel proteins (Fig. 2)

Subregion	Number	Observed pI value	Observed MW value [kDa]
		Average value $\pm$ SEM	Average value $\pm$ SEM
I	1	6.14 $\pm$ 0.06	23.85 $\pm$ 0.09
	2	6.04 $\pm$ 0.06	23.01 $\pm$ 0.09
	3	6.10 $\pm$ 0.06	22.55 $\pm$ 0.09
	4	5.99 $\pm$ 0.06	20.91 $\pm$ 0.09
	5	6.00 $\pm$ 0.06	20.53 $\pm$ 0.09
	6	6.07 $\pm$ 0.06	20.16 $\pm$ 0.07
	7	6.05 $\pm$ 0.06	19.99 $\pm$ 0.07
II	8	6.79 $\pm$ 0.13	25.06 $\pm$ 1.00
	9	6.97 $\pm$ 0.07	24.76 $\pm$ 0.74
	10	7.37 $\pm$ 0.07	24.65 $\pm$ 0.10
	11	6.84 $\pm$ 0.07	24.44 $\pm$ 0.98
	12	7.31 $\pm$ 0.07	24.08 $\pm$ 0.10
	13	6.82 $\pm$ 0.14	23.74 $\pm$ 0.71
	14	6.59 $\pm$ 0.07	23.57 $\pm$ 0.09
	15	7.02 $\pm$ 0.02	23.41 $\pm$ 0.09
	16	7.38 $\pm$ 0.02	22.83 $\pm$ 0.09
	17	6.62 $\pm$ 0.07	22.64 $\pm$ 0.09
	18	6.64 $\pm$ 0.07	22.18 $\pm$ 0.09
	19	6.62 $\pm$ 0.01	21.85 $\pm$ 0.09
	20	7.12 $\pm$ 0.07	21.72 $\pm$ 0.22
	21	7.28 $\pm$ 0.07	19.87 $\pm$ 0.07
III	22	7.73 $\pm$ 0.08	25.69 $\pm$ 0.09
	23	7.71 $\pm$ 0.08	25.10 $\pm$ 0.09
	24	7.75 $\pm$ 0.08	24.81 $\pm$ 0.09
IV	25	8.05 $\pm$ 0.02	24.81 $\pm$ 0.09
	26	8.36 $\pm$ 0.02	23.88 $\pm$ 0.09

Spots present on the reference gel (averaged oat prolamins gel) and on at least two out of six total oat kernel protein gels. Spots present on the averaged gel are found on at least six out of seven oat prolamins gels

SEM standard error of the mean

subregions of spots can be useful identifiers for the occurrence of a given group of proteins [19, 39]. The above groups of spots are difficult to distinguish on the electrophorograms of total oat kernel proteins (Fig. 2). The differences between the same subregions in Figs. 1 and 2 refer to different spot location in the particular subregions, especially in subregions I and II. In subregion I (Fig. 1), the spots are located one above the other, whereas the spots in the same subregion in Fig. 2 are dispersed. In subregion II (Fig. 1), clear individual spots are visible and in Fig. 2 some prolamins appear as large, blending spots, difficult to distinguish while performing a computer analysis of the image.

As shown in Table 1, only a small number of oat protein spots can be assigned to the prolamins fraction. In gel

Table 2 List of buckwheat prolamins spots assigned to the prolamins fraction (Fig. 3) present in the gel images of total buckwheat kernel proteins (Fig. 4)

Subregion	Number	Observed pI value	Observed MW value [kDa]
		Average value $\pm$ SEM	Average value $\pm$ SEM
I	1	6.03 $\pm$ 0.78	56.83 $\pm$ 0.57
	2	6.17 $\pm$ 0.43	50.14 $\pm$ 0.50
	3	7.25 $\pm$ 0.36	49.93 $\pm$ 0.49
	4	6.33 $\pm$ 0.13	49.91 $\pm$ 0.40
	5	6.99 $\pm$ 0.35	49.71 $\pm$ 0.39
	6	6.70 $\pm$ 0.54	49.25 $\pm$ 0.49
	7	6.50 $\pm$ 0.13	49.24 $\pm$ 0.41
	8	6.20 $\pm$ 0.31	46.54 $\pm$ 0.47
	9	6.05 $\pm$ 0.18	46.44 $\pm$ 0.46
II	10	6.58 $\pm$ 0.13	39.68 $\pm$ 0.79
	11	6.53 $\pm$ 0.59	38.21 $\pm$ 0.76
	12	6.24 $\pm$ 0.81	38.07 $\pm$ 1.14
	13	6.36 $\pm$ 0.83	38.04 $\pm$ 0.76
	14	6.62 $\pm$ 0.60	37.79 $\pm$ 0.38
III	15	6.84 $\pm$ 0.41	37.46 $\pm$ 0.37
	16	5.86 $\pm$ 0.70	32.32 $\pm$ 0.65
IV	17	6.22 $\pm$ 0.06	22.27 $\pm$ 0.19
	18	5.89 $\pm$ 0.47	22.22 $\pm$ 0.44
	19	6.60 $\pm$ 0.40	22.10 $\pm$ 0.22
V	20	5.65 $\pm$ 1.07	58.78 $\pm$ 1.18
	21	5.55 $\pm$ 0.44	40.50 $\pm$ 0.41
	22	5.36 $\pm$ 0.53	37.63 $\pm$ 0.75
	23	5.24 $\pm$ 0.68	37.40 $\pm$ 0.75
	24	5.72 $\pm$ 1.03	36.60 $\pm$ 1.09
	25	5.46 $\pm$ 0.60	36.01 $\pm$ 0.36
	26	5.17 $\pm$ 0.88	34.56 $\pm$ 0.69
	27	5.67 $\pm$ 0.23	33.27 $\pm$ 0.67
	28	5.51 $\pm$ 0.61	32.54 $\pm$ 0.65
	29	5.38 $\pm$ 0.11	31.44 $\pm$ 0.31

Spots present on the reference gel (averaged buckwheat prolamins gel) and on at least two out of three total buckwheat kernel protein gels. Spots present on the averaged gel are found on two out of three buckwheat prolamins gels

SEM standard error of the mean

images of total oat kernel proteins (Fig. 2), groups of spots were found in place of single spots. A reduction in the amount of protein in the sample of the prolamins fraction resulted in the disappearance of several low-intensity spots.

In the buckwheat prolamins fractions (Fig. 3), five characteristic subregions can be distinguished, clearly visible also on the total buckwheat kernel protein gel (Fig. 4). In four of these subregions, proteins have similar molecular weights, while their isoelectric points are different (Table 2). These subregions cover:

- the range of isoelectric points of 6.0–7.3 and molecular weights of 46.4–56.8 kDa (subregion I, 9 spots),
- the range of isoelectric points of 6.2–6.8 and molecular weights of 37.5–39.7 kDa (subregion II, 6 spots),
- the range of isoelectric points of 5.9 and molecular weight of 32.3 kDa (subregion III, 1 spot)
- the range of isoelectric points of 5.9–6.6 and molecular weight of about 22 kDa (subregion IV, 3 spots).

In the fifth organized subregion, proteins have similar values of isoelectric points, but differ in molecular weight (Table 2). This subregion covers the range of isoelectric points of 5.2–5.7 and observed molecular weights of 31.4–58.8 kDa (subregion V, 10 spots). Spots in this subregion demonstrate higher intensity on total buckwheat kernel protein gels (Fig. 4) than on buckwheat prolamin gels (Fig. 3). This may indicate accidental overlapping of prolamin spots with the spots of other proteins. Preliminary fractionation of proteins increases the intensity of spots [24, 25], i.e., produces an effect opposite to that observed in the present experiment. Pietrogrande et al. [18, 19] and Campostrini et al. [20] showed that the phenomenon of spot overlapping is commonly found. Experimental research [19, 20] indicates that about 70% of spots contain single proteins (singlets). The theoretical calculations of Pietrogrande et al. [18] suggest that single proteins should account for a much lower percentage of spots. This discrepancy was explained by Hunsucker and Duncan [21], who claim that a significant number of spots have one dominant protein, and that contaminating proteins are undetectable since they occur in insufficient amounts.

The standard error of isoelectric points was higher than the standard error of molecular weights [17]. This was probably caused by geometric disturbances in gels. As for molecular weights, the effect of disturbances could be adjusted by applying the pattern of molecular weight on each gel. There is no such possibility in the case of isoelectric point. Geometric disturbances are not an impediment to matching spots on gels, as currently applied techniques permit their adjustment [17]. The method of placing spots used in this study solved this problem. In order to compare spots from various gel images of proteins of both plants, characteristic spots of similar shape and intensity (anchoring spots) as well as groups of spots with a similar mutual shift were found. These spots were identified by the operator while spots neighbouring the selected spots were identified automatically. Such a strategy of gel comparison is recommended by Kjærsgaard et al. [33].

The higher number of spots on buckwheat protein gels in comparison with oat protein gels (Tables 1 and 2) could suggest a higher heterogeneity of the former. The difference concerns both prolamins and total kernel proteins.

The location of spots corresponding to oats and buckwheat prolamins in 2DE images in the paper by Di Cagno et al. [26] differs from the location of the analogous spots on the electrophorograms presented here. Contrary to the earlier work, linear strips and the MW range shifted to lower masses were used in the present study. The observed discrepancies are more significant for buckwheat prolamins because considerably more spots attributed to the prolamin fraction were found. Similarly, more spots were detected and identified for oats prolamins than in the paper by Di Cagno et al. [26]. The oat prolamin images in the current study, however, indicate a certain similarity to the prolamin image of the above-mentioned authors. Both images show a spot series of similar pI and different molecular weight (visible four subregions of similar spots).

2D electrophoresis is useful for detecting buckwheat prolamins. The lack of sequences of those proteins in databases makes it impossible to identify them directly with the use of the classical strategy of “peptide mass fingerprinting”, commonly applied in proteomic examinations [4, 10]. The characteristic, organized subregions containing spots with similar molecular weights and different isoelectric points or with similar isoelectric points and different molecular weights (called “spot trains”) can be recommended as identifiers for the presence of prolamins. Buckwheat proteins can be identified by sequencing fragments and searching local sequence similarity with other prolamins, e.g., with the use of the strategy described by Shevchenko et al. [40].

Some progress in the proteomic characteristics of buckwheat proteins could be achieved by the application of proteolysis and sequencing of peptides obtained with the use of a tandem of mass spectrometry with ionization through electrospraying (ESI–MS/MS), followed by a comparison of the sequences of peptides obtained with known sequences of prolamins of other cereals. A similar strategy was applied by Yano et al. [6] while searching for allergenic buckwheat proteins, although the proteins they identified did not belong to the group of prolamins.

Conclusions

The identification of the proteins of oat and buckwheat kernels is possible using 2D polyacrylamide gel electrophoresis. Buckwheat proteins (prolamin fractions as total protein) are more heterogenic than oat proteins (more spots were observed on the gel images of buckwheat proteins than on the electrophoretic images of oat proteins). The organized subregions containing spots with similar isoelectric points and various molecular weights or with similar molecular weights and various isoelectric points can play a key role as

identifiers for prolamin fraction occurrence in total proteins. They were particularly well-visible on the gel images of buckwheat (prolamin fraction as total protein).

The proper preparation of samples, particularly the removal of tannins and phenolic compounds which form complexes with proteins and disturb 2D-PAGE, is of profound importance for prolamin analysis.

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