

Proteomic analysis of *Lupinus angustifolius* (var. Zeus and Bojar) and *Lupinus luteus* (var. Lord and Parys) seed proteins and their hydrolysates

Running title: Lupin seed proteins and their hydrolysates

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Abstract

BACKGROUND: Proteins enzymatic digestion is a very complex process, during which some components are degraded, while others remain in an unchanged form. Moreover, the enzymatic hydrolysis is one of the most popular methods used to reduce the allergenicity of food proteins. In this study, the efficiency of enzymatic hydrolysis of lupin seeds proteins was assessed by proteomic analysis performed by two-dimensional gel electrophoresis (2-DE) coupled with mass spectrometry identification. Two digestion systems were used, namely, oriented digestion carried out by trypsin and the model *in vitro* digestion mimicking conditions present in the gastrointestinal tract.

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RESULTS: The comparisons of 2-DE maps of proteins isolated from different lupin seeds species revealed that the differences in proteins expression were observed mainly in the central parts of gels, i.e., in the molecular weight range from 20 to 70 kDa, and the pH range 5-7. A total number of 27 differentially expressed proteins spots were successfully identified by mass spectrometry analysis. An important reduction in the number of proteins spots on 2-DE maps was observed when trypsin, and the *in vitro* digestion model were applied. The protein spot insensitive to digestion in both hydrolysis systems was identified as β -conglutin.

CONCLUSIONS: The results provide insight into the nature of the digestion process that may take place after lupin seed proteins intake and highlight the important fact that some of the proteins are insensitive to digestive enzymes activity. Moreover, evaluation of digestion activity of trypsin towards lupin seed proteins may be used for the development of specific processes of hypoallergenic food production.

Keywords: lupin seeds, storage proteins, two-dimensional electrophoresis, *in vitro* digestion, proteins hydrolysis

INTRODUCTION

Lupin seeds are already widely used as an ingredient in food products. Their average annual world production already exceeds a million tons. Within countries in which lupin is widely used by the food industry, Australia is the most important seeds producer, followed by Poland, Chile, Ukraine, and Belarus.¹ The seeds attractiveness is mainly related to a high protein content (33-46%), characterised by a preferred amino acid composition, and the desired technology properties. Furthermore, lupin seeds also contain compounds, such as oligosaccharides, phenolic compounds, and tocopherols, which possess documented health benefits.²

Until now, it has been confirmed that the health promoting properties of lupin seeds are due to the presence of the protein fraction - γ -conglutin, that can be used effectively in the treatment of diabetes.³⁻⁵ Based on the exposure of human hepatocyte cell line (HepG2) to γ -conglutin Lovati *et al.*,³ found that the mechanism of action of this protein is similar to that of insulin. Moreover, the study of these authors has also shown that the effect of γ -conglutin is comparable with metformin - recognised antidiabetic medicine. According to Capraro *et al.*,⁶ this protein fraction is able to penetrate into HepG2 cells, and this process is based on the pinocytosis phenomenon. Furthermore, γ -conglutin undergoes the multiple phosphorylations after introduction into HepG2 cells. Studies conducted in animals have shown that the addition of the lupin protein extract to the diet also causes a reduction in blood cholesterol levels.⁷ The potential mechanism responsible for these health-promoting properties of lupin seed proteins is associated with an increase in LDL receptor activity.⁷

Currently the clear trend of utilisation of enzyme technology in food sciences can be observed.⁸ The particular interest is associated with obtaining biologically and functionally active peptides, which can be obtained by hydrolysis of proteins of animal, as well as plant origin. However, enzymatic hydrolysis can be also utilised in order to reduce allergenicity of

food products. In this efforts, the strategy applying specific and strictly targeted proteolytic enzymes is used in order to obtain the repetitive hydrolysates characterised by defined properties.⁸ All of the above-mentioned features make lupin seed proteins a very attractive and desirable ingredient in food production. Unfortunately, with the increase in food utilisation of lupin seeds, its potential allergenic properties were revealed.⁹ This phenomenon is associated with the fact that after complex digestion processes, some lupin proteins undergo hydrolysis, while others remain unchanged.

Therefore, the aim of the presented study was to investigate processes that can take place when lupin seed proteins are digested after intake. The material consists of two lupin seeds species, i.e., *Lupinus angustifolius* (economically most important lupin species), and *Lupinus luteus* (so far negligible analysed). Moreover, particular attention was devoted to assessing the possibility of using the specific protease to carry oriented hydrolysis. Best to our knowledge, it is the first work where the proteomic approach was used in order to characterise lupin seed proteins hydrolysates.

MATERIALS AND METHODS

Reagents

Pepsin from the porcine gastric mucosa (P7000, activity ≥ 250 units/mg protein), pancreatin from the porcine pancreas (P1750, activity equivalent to 4 $\times$ U.S.P. specifications), trypsin from the porcine pancreas, which was treated by N-tosyl-L-phenylalanyl chloromethyl ketone (TPCK) in order to prevent any chymotrypsin activity (T1426, $\geq 10,000$ units/mg protein), bile (B8381), Tris(hydroxymethyl) aminomethane, sodium dodecyl sulfate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), trifluoroacetic acid (TFA), glycine, acrylamide, and Coomassie Brilliant Blue G -250 were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals used in this study were of analytical grade.

Materials

Narrow-leafed (*L. angustifolius*, var. Zeus and Bojar) and yellow (*L. luteus*, var. Lord and Parys) low-alkaloid lupin seeds were obtained from the Plant Breeding Station Smolice, Przebedowo branch, Poland (harvested in 2012). All samples were milled using an IKA M20 universal laboratory mill (IKA, Staufen, Germany), sieved to obtain a fraction below 0.6 mm and stored in closed polyethylene bags at -18°C. Prior analyses all samples were defatted for 2 h using an automatic Soxhlet Bühi Extraction System B-811 (Bühi Labortechnik, Flawil, Switzerland) with *n*-hexane as a solvent.

Trypsin digestion of lupin seed proteins

Isolation and digestion of lupin seed proteins were carried out applying the method described previously.¹⁰⁻¹¹ Briefly, lupin seed proteins were resuspended in water to the final concentration of 1 mg mL⁻¹, adjusted to pH 7.5 with 0.1 mol L⁻¹ NaHCO₃ and the enzyme solution (1 mg mL⁻¹ in water) was added in the ratio of 1:30 enzyme/lupin seed proteins (v/v). The sample was incubated for 1 h at 37°C under gentle stirring. The reaction was stopped by changing pH to 2 by adding 0.1 mol L⁻¹ HCl.

Enzymatic digestion of lupin seed proteins in the model system

The digestion in *in vitro* gastrointestinal track model used steps corresponding to digestion in the stomach and the small intestine. The colon stage of the digestive track was omitted. This type of *in vitro* gastrointestinal track, despite the simplicity, provides valuable information about protein digestion susceptibility and absorption of the hydrolysis products due to the fact that these processes take place mostly in the central part of the gastrointestinal track.

The *in vitro* digestion model was developed on the basis of method describing digestion of various food products.^{10,12-13} Lupin seed proteins were isolated from 10 g of defatted lupin flour, which roughly corresponds to two slices of bread made with 5% addition of lupin seeds. The proteins isolation procedure was in accordance with previously used.¹⁰⁻¹¹

Digestion of lupin seed proteins in the stomach was simulated by the addition of pepsin in an amount of 0.288 g. The enzyme was dissolved in 6 mL of 0.1 mol L⁻¹ HCl. Before the enzyme application pH of lupin seed proteins, samples were adjusted to 2.0 by the addition of the appropriate dose of 1 mol L⁻¹ HCl. After 2 hours, the pH of the sample was increased to 6.0 by the addition of 1 mol L⁻¹ NaHCO₃ and followed by 10 mL of pancreatic tract extract (0.06 g of pancreatin with the addition of 0.36 g of bile salts dissolved in 0.1 mol L⁻¹ NaHCO₃) addition. After 30 minutes from the introduction of pancreatic extract, the pH of the sample was raised to 7.4 by the addition of an appropriate amount of 1 mol L⁻¹ NaHCO₃. Digestion was carried out at this stage for next 2 hours at 37°C and then the digested samples were purified by 0.45 µm PVDF syringe filters. Finally, the samples were concentrated and desalted by using Amicon filters with a 3 kDa cutoff (Millipore, Bedford, MA, USA). All samples were stored frozen till the analyses.

Samples preparation for two-dimensional electrophoresis

Lupin seed proteins were isolated by shaking 500 mg of defatted lupin flour with 1 mL of buffer consisting 7 mol L⁻¹ urea, 2 mol L⁻¹ thiourea, 20 g L⁻¹ CHAPS, 64 mmol L⁻¹ dithiothreitol (DTT), 20 mL L⁻¹ carrier ampholyte and a set of protease inhibitors for 2 h. The samples were then centrifuged for 30 min (20,000 g at 4°C) and supernatants containing proteins were transferred directly for future analyses.

Lupin seed proteins digestion products before 2-DE separation were precipitated, concentrated and transferred into the appropriate buffer by using Ready Prep 2D cleaning kit (Bio-Rad, Irvine, CA, USA) accordingly to the manufacturer recommendations.

The proteins concentration was determined using a 2D Quant Kit[®] (GE Healthcare, Uppsala, Sweden), following the procedure supplied by the manufacturer.

Two-dimensional electrophoresis

Separations of the lupin seed proteins in a first dimension were done on Immobiline DryStrip[®] strips with a length of 24 cm and a pH gradient of 3 to 10 (GE Healthcare, Uppsala, Sweden). The strips were subjected to the rehydration for 12 h in a solution containing 800 µg (for analytical gels) or 1200 µg (for preparative gels designed for mass spectrometry identification) of the lupin seed proteins. The strips were then subjected to focusing at a temperature of 20°C with stepwise voltage increase up to 8000 V reaching a total value of 90,000 Vhs. Isoelectric focusing was performed utilizing the Ettan IPGphor[®] 3 unit (GE Healthcare, Uppsala, Sweden).

Before separations in the second dimension, the strips were reduced and alkylated using 375 mmol L⁻¹ Tris-HCl pH 8.8 buffer containing 6 mol L⁻¹ urea, 20 g L⁻¹ sodium dodecyl sulfate (SDS), 200 mL L⁻¹ glycerol, 0.02 g L⁻¹ bromophenol blue, successively containing 65 mmol L⁻¹ DTT (reducing disulfide bridges) and 243 mmol L⁻¹ iodoacetamide (alkylation of reduced disulfide bridges), for 15 min, respectively.

The proteins were separated in a second direction on a 120 g L⁻¹ polyacrylamide gels with 200 × 250 × 1 mm dimensions in Ettan DaltSix[®] system (GE Healthcare, Uppsala, Sweden). Separations were conducted for 17 h at 10°C at a constant current of 15 mA per gel. The reference molecular weight marker (Precision Plus Protein; Bio-Rad, Irvine, CA, USA) was added in parallel with the tests samples. Following separations, gels were stained in a

colloidal solution of Coomassie Brilliant Blue G-250 in accordance with the method suggested by Ulrike *et al.*,¹⁴ and documented using ImageScanner III scanner (GE Healthcare, Uppsala, Sweden). Comparative protein expression and the spot analyses were performed by using ImageMaster 2D Platinum 7.0 software (GE Healthcare, Uppsala, Sweden).

Three replications of 2-DE gels were done for each of the lupin seed proteins extracts, and the products obtained by lupin seed globulin digestions.

Mass spectrometry protein identification

Selected protein spots separated by 2-DE were identified using mass spectrometry accordingly to previously described procedure.¹⁵ Analyses were performed in the Environmental Mass Spectrometry Laboratory (Institute of Biochemistry and Biophysics, Academy of Sciences, Warsaw). Briefly, this identification included the separation of the peptides using nano-liquid chromatography (Waters, Milford, MA, USA) followed by detection in an Orbitrap Velos tandem mass spectrometer analyser (Thermo Scientific, Waltham, MA, USA). Prior to analyses, the samples were subjected to the standard gel digestion. Before the digestion, the proteins were reduced with 100 mmol L⁻¹ DTT (30 minutes at 56°C) and blocked with 0.5 mol L⁻¹ iodoacetamide (45 min at room temperature). Next, prepared samples were subjected to digestion with trypsin (V5111; Promega, Madison, WI, USA) at 37°C for 12 h. After digestion, the samples were acidified by using 10 g L⁻¹ TFA. The obtained peptide mixtures were analysed on nanoAcquity® BEH C18 1.7 µm, 100 µm × 100 mm column (186003546; Waters, Milford, MA, USA). Separations were carried out in gradient mode using a solution A (water containing 10 g L⁻¹ formic acid) and B (acetonitrile containing 10 g L⁻¹ formic acid). The flow rate was 250 nL min⁻¹, gradient profile consisted of 5-35% B from 0 to 180 min. Separated peptides were immediately directed into the ion source of the mass spectrometer, operating in MS and MS/MS mode. In order to avoid

contamination between tested samples, each analysis was preceded by equilibration of the column in accordance with the gradient profile described previously, but without injection. The results were analysed in Mascot Distiller program (Matrix Science, Boston, MA, USA) and then screened for sequence identity using the software Mascot search engine (Matrix Science, Boston, MA, USA) with the defined trypsin specificity. Parameters set up for the search of sequence identity in the NCBI database were as follows: the parent ion mass tolerance of 20 ppm and MS/MS mass accuracy of 0.2 Da. The identification included the possibility of optional modifications: oxidation of methionine, N-glycosylation, methylation, and carboxymethylation of a cysteine.

RESULTS AND DISCUSSION

Proteomic analysis of lupin seed proteins

The expression of proteins isolated from studied lupin seed species was compared using 2-DE followed by mass spectrometry identification of individual spots. Representative proteins profiles (2-DE maps) are shown in Fig. 1. The obtained electrophoretic profiles, within each of the tested varieties, were highly similar with an average score of 80% coverage of protein spots. This fact confirms repeatability of isolation, and separation of the analysed samples. Image analyses of 2-DE maps revealed the presence of an average of 544 (*L. angustifolius* var. Zeus), 389 (*L. angustifolius* var. Bojar), 555 (*L. luteus* var. Lord) and 492 (*L. luteus* var. Parys) protein spots, characterised by wide molecular weight range (from 10 kDa to 250 kDa) and isoelectric points from 3 to 10. Interspecies differences in protein expression were observed mainly in the central parts of the gels (the pH range 5-7). Within this part of gels, protein spots characterised by the same molecular weight but the different isoelectric point were present, e.g., spot number 6 (Fig. 1a). This is a conserved feature of lupin 2-DE maps, in which presence of rows of spots with a similar molecular weight but

varying pI values are observed.¹ This observation is associated with the presence of different isoforms of the same protein with different degrees of post-translational modifications.¹ Furthermore, these rows of spots are placed forming clusters of rows of closely spaced molecular weight values, reflecting a differential proteolytic cleavage from common proteins (e.g., α -conglutin fraction of *L. luteus*, Fig. 1c).

During a comparative analysis of 2-DE maps, a total of 27 protein spots that differentiate the two studied species were selected for the mass spectrometry identification. Based on the results they were classified into α -, β - and γ -conglutins fractions (Table 1; detailed peptides matched are provided in Supporting information Table 1). The proteins identification using the MASCOT algorithm was done based on the result above score 60, and above 10% of an amino acid sequence coverage. The presence of only three conglutin fractions can be explained by the fact that lupin seeds mostly consist of storage proteins, while the concentration of catalytic, structural, regulatory and defense proteins are less abundant. The obtained results are in accordance with available interspecific comparative analyses of lupin seed proteins, which also revealed presence of only conglutin families (α , β , γ) on 2-DE maps.¹⁶⁻¹⁷ However, most of the relevant studies have been focused on *L. albus* (white lupin seeds) and *L. angustifolius* (narrow-leafed lupin seeds), and only a few have been conducted on the other economically important lupin species, such as *L. luteus* and *L. mutabilis*.¹ Due to the lack of protein sequences derived from *L. luteus* α -, β - and γ -conglutin fractions available in NCBI database, the protein spots isolated from *L. luteus* 2-DE maps were assigned to the corresponding fraction of *L. angustifolius* (Table 1). This fact shows a high homology within conglutin fractions derived from seeds of various lupin species.

In most cases, the theoretical molecular weights and isoelectric points of 27 identified proteins using mass spectrometry were different from the values which were experimentally determined based on 2-DE (Table 1). These differences result from the fact that the sequences

available in the databases, and used during identification mostly refer to the precursor proteins, whereas the lupin seed proteins are subjected to complex post-translational proteolysis processes (mainly post-translational cleavage and glycosylations). Thus, obtained gels present the final products of genes expression whose molecular weights and isoelectric points differ from their precursors (Fig. 1, Table 1).

So far, detailed data concerning polypeptide chains cleavage sites during lupin seed proteins maturation have been limited. Only γ -conglutin maturation has been well characterised in our previous study.¹⁵ This protein fraction consists of two disulphide linked subunits (Cys190-Cys408) and during maturation is N-glycosylated at Asn98.¹⁵ On the basis of this knowledge, the protein spot number 27, which was originally identified as the γ -conglutin precursor (MW 45364 Da, pI 7.72), finally was qualified as the smaller subunit of γ -conglutin (MW 16696/16900, pI 6.02/6.81; Table 1).

On average of 326 and 407, the same proteins spots were identified in the case of the analysed varieties of *L. angustifolius* and *L. luteus*, respectively. Even the slight difference in the value of isoelectric point of spots 7 and 14, which was found in the case of *L. angustifolius* var. Bojar and Zeus (Fig. 1a, b) proved to be the same protein fraction (β -conglutin) during mass spectrometry identification (Table 1). These results indicate a very conserve proteins profile within analysed lupin seed varieties.

Islam *et al.*,¹⁸ studied proteome of seed storage proteins among four *L. angustifolius* cultivars. Reported results indicated that main proteins differentially expressed in studied seeds were identified as β -conglutin, while most of α -, δ - and γ -conglutins showed similar expression pattern among the cultivars.¹⁸ The lupin seeds proteomic studies typically involved seeds other than those of the *L. luteus*. Recently, Ogura *et al.*,¹⁹ have carried out detailed proteomic characterisation of *L. luteus* seeds proteins. These authors identified 737 proteins in 341 spots based on classical proteomic approach (2-DE combined with mass spectrometry

identification). The results allowed them to create a catalogue of proteins involved in different pathways. Although the high number of identified proteins present even in small quantities was reported by these authors, the main spots present on the 2-DE map corresponded to storage proteins fractions. Moreover, β -conglutin was identified in 205 spots while α -conglutin in 65 spots.¹⁹ This fact additionally confirmed that lupin seeds proteome contains mainly storage proteins.

Characterisation of lupin seed proteins hydrolysates

Enzymatic hydrolysis is one of the most recognised method used to reduce the allergenicity of food proteins.⁸ A detailed analysis of proteins which were not sensitive to trypsin hydrolysis was done using 2-DE. The Fig. 2 shows representative gels of proteins isolated from studied lupin seeds species (Fig. 2a, b), and their tryptic hydrolysates counterparts (Fig. 2c,d). The obtained results highlighted considerable differences in 2-DE maps that took place during hydrolysis of proteins of the studied species of lupin seeds. The main lupin seed proteins, characterised by the molecular weights of 10 to 250 kDa and isoelectric point in the range of 5-7 (Fig. 2a, b), were hydrolysed effectively during trypsin activity (Fig. 2c,d). Not hydrolysed proteins were defined by a narrow molecular weight ranged from 10 to 75 kDa and 4-6 isoelectric point (Fig. 2c,d).

The results obtained during 2-DE separation of lupin seed proteins hydrolysates provided additional confirmation about γ -conglutin insensitivity to trypsin digestion (Fig. 2 c,d). In all of the analysed trypsin hydrolysates, the protein spots characterised by the molecular weights of 16.10/16.90 kDa and the isoelectric points 6.68/6.81, were detected (Fig. 2 c,d; Table 1). These results correspond to the theoretical molecular weight of small subunit of γ -conglutin (theoretical molecular weight 16.54 kDa and isoelectric point 6.02), and were confirmed during mass spectrometric identification of selected spots of undigested lupin seeds samples (spot numbers 13 and 27; Table 1). Moreover, also characteristic spots

with molecular weight of 27.50 kDa and isoelectric point ranging between 8-9, were detected in 2-DE maps of lupin seed proteins samples digested by trypsin (Fig. 2 c,d). These spots can correspond to the theoretical molecular weight of large subunit of γ -conglutin (theoretical molecular weight 28.85 kDa and isoelectric point 8.58), however, additional mass spectrometry identification is required.

Different models simulating the conditions of a gastrointestinal tract are used to assess the digestibility and the bioavailability of food components.²⁰ Based on the literature review provided by Hur *et al.*,²⁰ by 2011 more than 80 different kinds of *in vitro* digestion models were developed. Depending on the type of a food product and complexity of a matrix, models differ significantly from each other. These differences mainly concern the number of steps included in digestion (digestion taking place in mouth, stomach and small intestine) and composition of digestive fluids (enzymes, salts, buffers, compounds lowering surface tension).²⁰ Very recently, a new, unified, and harmonised *in vitro* digestion model (INFOGEST) was proposed.²¹ However, because of the complexity of faithful reproduction of the physicochemical and physiological phenomena occurring in a gastrointestinal tract, the results obtained in *in vitro* models might differ from those that actually take place *in vivo*. Nevertheless, due to the fact that such models, i.e. *in vitro*, are easy to apply, they are widely used for the initial analyses of novel food.

The in-depth analysis of proteins remaining after the model *in vitro* digestion was possible with the use of 2-DE (Fig. 2e, f). These results also confirmed that the majority of protein spots of main storage lupin proteins were hydrolysed (Fig. 2e, f). The proteins which remain after the model digestion were characterised by a molecular weight of 10 and 80 kDa and isoelectric point in a range of 4-6. In the case of *L. angustifolius* majority of proteins that were not hydrolysed were identified as α -conglutin (Fig. 2a, e; Table 1). On the other hand, in the *L. luteus* hydrolysates proteins resistant to proteolytic enzymes during the digestion

belong to β -conglutin fraction (Fig. 2b, f; Table 1). Unlike in the case of samples that were digested only with trypsin, the *in vitro* hydrolysates were characterised by absence of the protein spots corresponding to γ -conglutin fraction. This fact is consistent with our previous studies describing γ -conglutin digestion susceptibility to different digestive enzymes. These results indicated that pepsin, as well as chymotrypsin lead to hydrolysis of this particular lupin protein fraction.⁹⁻¹¹ During the model *in vitro* digestion pepsin is used firstly in order to mimic digestion taking place in stomach. Subsequently, the pancreatin extract mimicking small intestine digestion part of digestive tract contains chymotrypsin. The presence of these enzymes during the *in vitro* digestion explains the absence of γ -conglyutin spots on 2-DE maps (Fig. 2e, f). Characteristic protein spots were present in native lupin seed proteins 2-DE maps and samples hydrolysed by trypsin as well as in the samples after the model *in vitro* digestion. These proteins spots corresponds to β -conglutin (spots number 5 and 20; gi|328684567; Fig. 1; Table 1), and its presence is particularly important due to the fact that literature data indicate that proteins from this fraction exhibit allergic properties, e.g. *Lup an 1* allergen.¹⁷

Various processing conditions might affect lupin seeds proteins profile. Sirtori *et al.*,²² studied effect of various thermal and mechanical processing on a *L. angustifolius* protein isolate. The authors evaluated processing conditions by separation of samples with the use of 2-DE. The results indicate that, even after harsh industrial processing, some of the proteins from α -, β - and δ -conglutin fractions remain in a stabile form.²² However, the authors did not carry out mass spectrometry identified of protein spots that were not degraded after the processing.

So far, digestion susceptibility of lupin seed proteins has been studied mainly by utilising one-dimensional SDS-PAGE electrophoresis. Main enzymes used in these studies utilised pepsin, pancreatin, trypsin, chymotrypsin, and the double digestion model (pepsin

followed by pancreatin digestion).⁹⁻¹¹ SDS-PAGE electrophoresis analysis of crude lupin seeds proteins revealed the great similarity of protein profiles within varieties of the same species. *L. angustifolius* seed proteins are mostly characterised by molecular weights in the range of 68 to 50 kDa while *L. luteus* seeds contained mostly proteins with molecular weights ranging from 66 to 48 kDa.⁹⁻¹¹ All of these proteins belong to the α - and β -conglutin fractions, which act as storage proteins in lupin seeds.²³ Lupin seed proteins susceptibility to pepsin hydrolysis, and the double digestion system was evidenced by the absence of protein bands on gels after SDS-PAGE.⁹⁻¹¹ When these enzymes were used, proteins from α - and β -conglutin fractions were effectively digested. The conserved feature of hydrolysates obtained after pancreatin activity is the presence of the characteristic protein bands corresponding to γ -conglutin fraction, i.e., 46 kDa – γ -conglutin monomer, 31.4 kDa – α -subunit of monomer, and 16.5 kDa – β -subunit of monomer.^{9-11,24}

The proposed hydrolysis system with specific protease (trypsin) may be an initial idea for setting up the production process of lupin hypoallergenic foods/low allergenicity foods. Moreover, utilising this hydrolysis system provides additional profits over the initial reduction of allergenicity - after this specific hydrolysis the important lupin seed protein fraction remains undigested – γ -conglutin. γ -Conglutin is characterised by a number of unusual features, among which its unique hypoglycaemic in mammals is most important.³⁻⁴ There are some reports that provide information that γ -conglutin might have antigenic properties, however, those reports indicate that rather these properties are related to the fact that this protein fraction is glycosylated. It is a known fact that glycans attached to proteins might costs non-specific binding of IgE antibodies.²⁵

CONCLUSIONS

Immunoreactive properties of food ingredients may change in a random or oriented manner. These changes can be a result of physical, chemical, and biotechnological actions. The

molecular basis associated with these changes results from inactivation or destruction of allergenic epitopes, and formation of new epitopes (e.g. increased access to hidden epitopes). The presented results provided a description of the changes and the nature of the processes taking place as a result of proteolysis of lupin seed proteins. Information about β -conglutin digestion insensitivity to the various proteolytic enzymes is of particular interest due to the fact that this protein fraction might cause allergic reaction. Moreover, the described oriented hydrolysis system can contribute to the development of production processes of food products utilising lupin seeds that will be characterised by reduced allergenicity, and at the same time having the unique health-promoting properties (presence of γ -conglutin fraction). The fact that after hydrolysis some of the proteins might remain in unaffected form require future studies aimed to assess if those proteins have immunoreactive properties.

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Figures captions

Figure 1. The two-dimensional electrophoresis maps of the lupin seed proteins isolated from *L. angustifolius* (a, b) and *L. luteus* (c, d). The first dimension was done on a 3-10 linear strip, and the second dimension was run on 12% gel. The protein spots for which mass spectrometry identification was made are numbered. Rectangles enclose α -conglutin fraction (solid line), and β -conglutin fraction (dashed line); the solid ellipses enclose the small subunit of γ -conglutin.

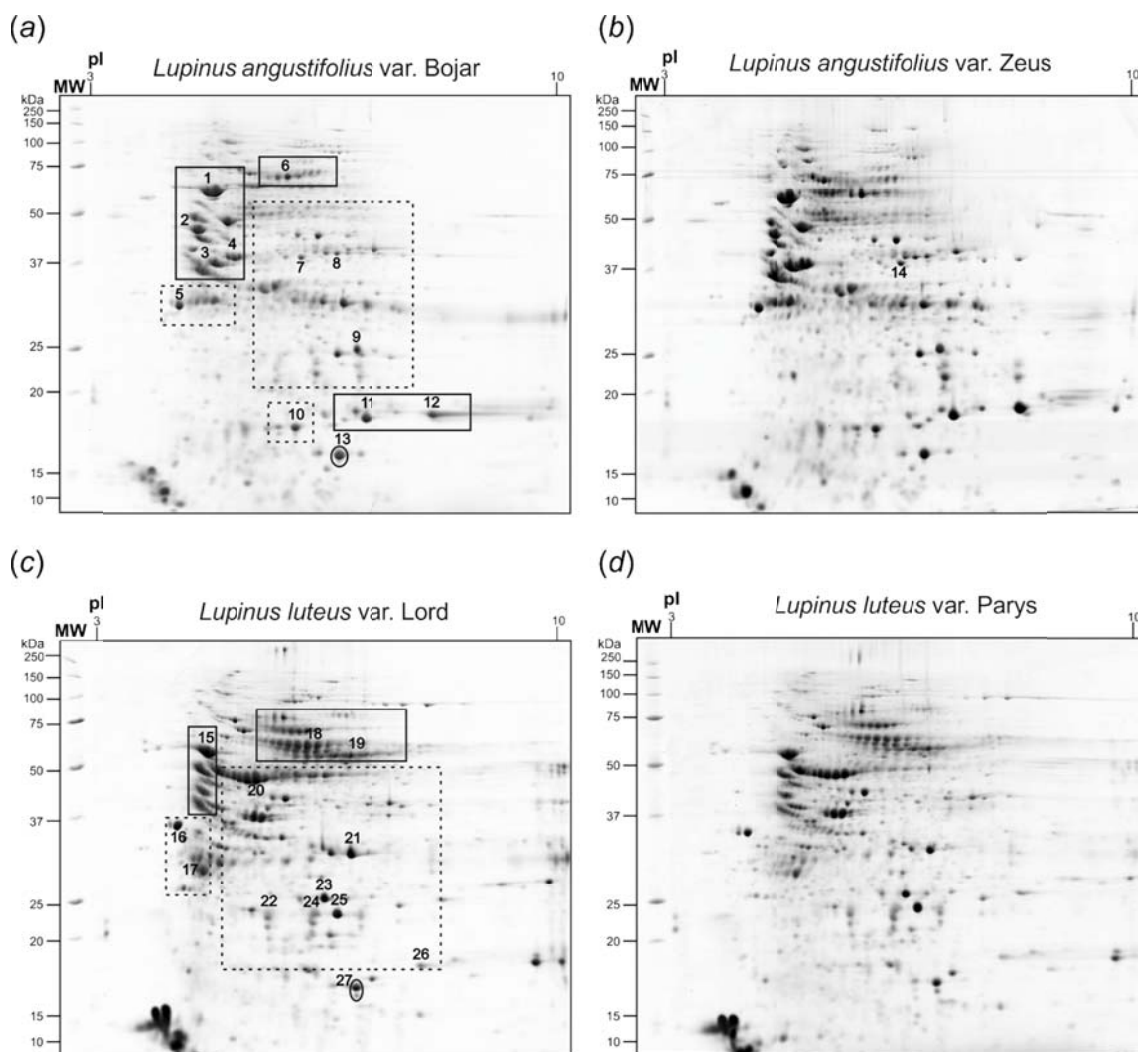
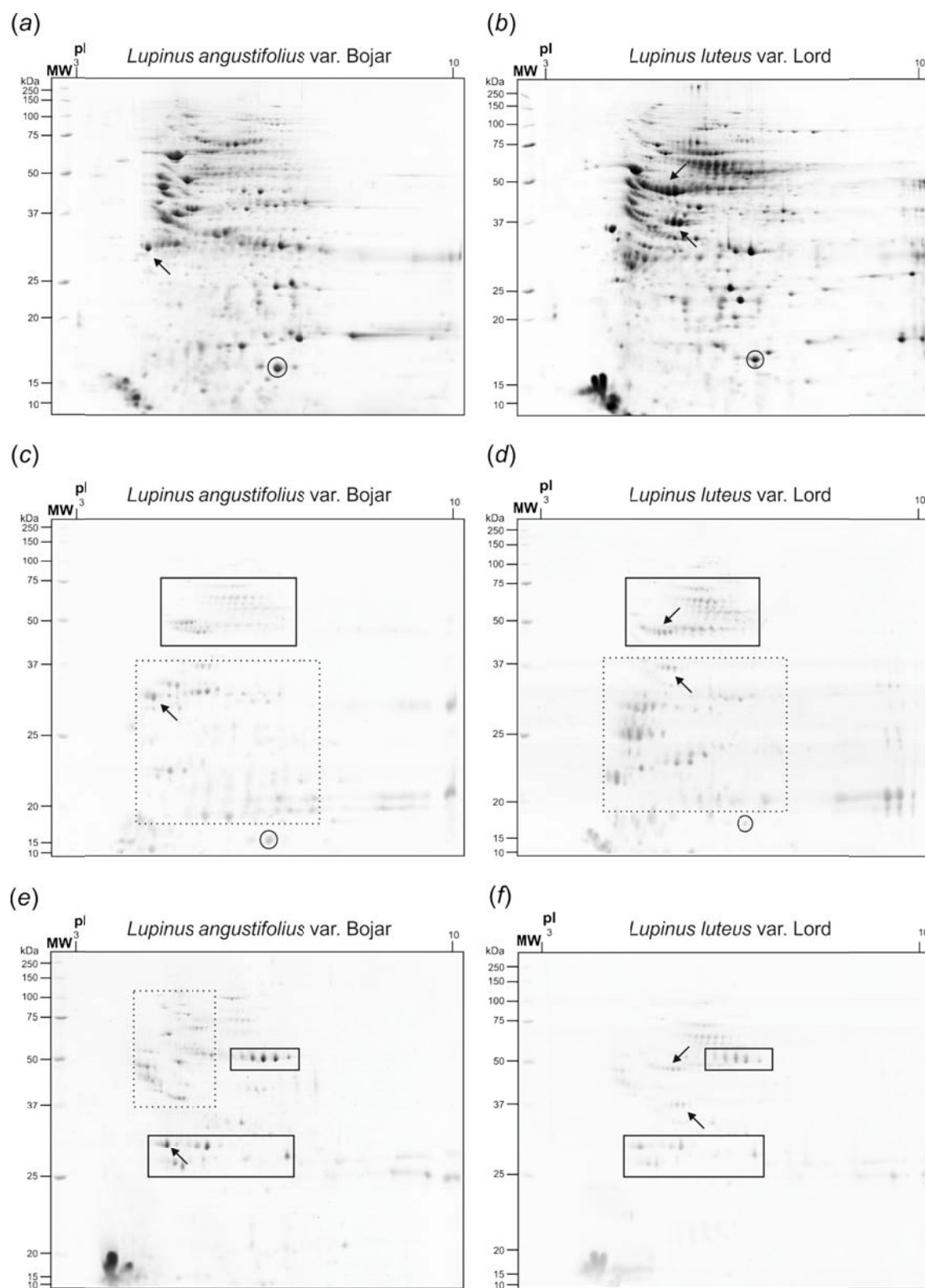


Figure 2. The representative two-dimensional electrophoresis maps of the lupin seed proteins and their hydrolysis products. The proteins used in the analysis were isolated from *L. angustifolius* var. Bojar (*a, c, e*) and *L. luteus* var. Lord (*b, d, f*). Panels (*a*) and (*b*) show crude lupin seed proteins maps. The hydrolysis was conducted by trypsin (*c, d*), as well as in the *in vitro* model system (*e, f*). The rectangles enclose proteins fractions that remain after digestion and were similar (solid line) or different (dashed line) in studied lupin seeds species. The solid ellipses enclose the small subunit of γ -conglutin. The arrows indicate protein spots present in the crude lupin seed proteins extracts, and remained after the digestions.



Tables

Table 1. Mass spectrometry identification of the lupin seed proteins separated by means of 2-DE.

Spot Nr ^a	Identified protein ^b	NCBI accession number ^c	Matched peptides ^d	Sequence coverage (%) ^e	Score ^f	Theoretical pI/MW (Da) ^g	Experimental pI/MW (Da) ^h
1	α -conglutin (<i>L. angustifolius</i>)	gi 328684561	35	60	37030	5.12/74606	4.84/60800
2	α -conglutin (<i>L. angustifolius</i>)	gi 328684561	29	55	18025	5.12/74606	4.62/46200
3	α -conglutin (<i>L. angustifolius</i>)	gi 328684559	17	38	10524	5.26/58157	4.60/36500
4	α -conglutin (<i>L. angustifolius</i>)	gi 328684559	21	51	10331	5.26/58157	4.99/38600
5	β -conglutin (<i>L. angustifolius</i>) ^{**}	gi 328684567	12	23	1168	5.64/70658	4.38/31400
6	α -conglutin (<i>L. angustifolius</i>)	gi 328684561	14	34	1028	5.12/74606	5.92/64300
7	β -conglutin (<i>L. angustifolius</i>)	gi 169950562	22	42	2296	5.82/71853	6.08/38300
8	β -conglutin (<i>L. angustifolius</i>)	gi 169950562	29	43	8797	5.82/71853	8.73/40100
9	β -conglutin (<i>L. angustifolius</i>)	gi 169950562	22	38	3492	5.82/71853	7.09/25900
10	β -conglutin (<i>L. angustifolius</i>)	gi 169950562	28	48	35203	5.82/71853	6.13/17800
11	α -conglutin (<i>L. angustifolius</i>)	gi 328684563	12	22	15528	5.31/67683	7.29/18600
12	α -conglutin (<i>L. angustifolius</i>)	gi 328684561	16	26	16648	5.12/74606	8.27/19000
13	γ -conglutin (<i>L. angustifolius</i>) [*]	gi 662366	12	26	23634	7.66/49570	6.68/16100
14	β -conglutin (<i>L. angustifolius</i>)	gi 169950562	23	41	1819	5.82/71853	6.49/38300
15	α -conglutin (<i>L. angustifolius</i>)	gi 328684561	7	11	6499	5.12/74606	4.52/58000
16	β -conglutin (<i>L. angustifolius</i>)	gi 328684571	8	16	602	5.96/69366	4.08/36000
17	β -conglutin (<i>L. angustifolius</i>)	gi 149208401	14	28	6631	5.34/61490	4.59/31000

18	β -conglutin (<i>L. angustifolius</i>)	gi 149208401	22	41	8460	5.34/61490	6.08/62600
19	β -conglutin (<i>L. angustifolius</i>)	gi 149208401	23	41	7086	5.34/61490	6.34/47900
20	β -conglutin (<i>L. angustifolius</i>) ^{**}	gi 328684567	14	28	3433	5.64/70658	5.29/47100
21	β -conglutin (<i>L. angustifolius</i>)	gi 149208401	13	26	1569	5.34/61490	6.72/31600
22	α -conglutin (<i>L. angustifolius</i>)	gi 328684559	12	25	17378	5.26/58157	5.49/23200
23	β -conglutin (<i>L. angustifolius</i>)	gi 149208401	9	19	890	5.34/61490	6.33/25600
24	α -conglutin (<i>L. angustifolius</i>)	gi 328684559	11	22	8861	5.26/58157	6.15/20500
25	β -conglutin (<i>L. angustifolius</i>)	gi 149208401	10	18	1313	5.34/61490	6.51/23700
26	β -conglutin (<i>L. angustifolius</i>)	gi 328684567	12	22	1738	5.64/70658	7.85/18200
27	γ -conglutin (<i>L. angustifolius</i>) [*]	gi 223005	3	16	1366	6.02/16696	6.81/16900

^a Protein spot corresponding to identified protein on the gels (Figure 1).

^b Identified homologs protein and the organism from which it proceeds.

^c Database accession number according to NCBI.

^d Number of peptides matched with *MASCOT* search algorithm (www.matrixscience.com).

^e Amino acid sequence coverage for the identified protein.

^f *MASCOT* search probability based on the Mowse score.

^g Theoretical isoelectric point and molecular weight (Da) of identified protein retrieved from the protein database (NCBI).

^h Experimental isoelectric point and molecular weight (Da) of identified protein separated by means of 2-DE.

^{*} The protein spot present in the samples after trypsin digestion.

^{**} The protein spot present in the samples hydrolysed by trypsin as well as in the samples after the model *in vitro* digestion.