



Characterisation of different digestion susceptibility of lupin seed globulins



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ABSTRACT

This study describes *in vitro* digestion of lupin seed globulins by pancreatin, trypsin and chymotrypsin. Lupin seed globulins turned out to be almost totally susceptible to chymotrypsin digestion. When pancreatin or trypsin were used for digestion of lupin seed globulins, γ -conglutin appeared to be resistant to proteolysis. Different fluorescence spectroscopic methods such as fluorescence anisotropy, fluorescence lifetimes and fluorescence quenching measurements were used for detailed characterisation of this phenomenon. A potential reason for γ -conglutin insensitivity to digestion may be related to the fact that lysine, as well as arginine, are positively charged at cell physiological pH. Simultaneously, flavonoids at this pH are partially ionised, which may lead to the occurrence of ionic interactions between these molecules at pH 7.5. The confirmation of this explanation may be the fact that γ -conglutin and vitexin form a static complex, which was observed using fluorescence quenching measurements.

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1. Introduction

Lupin is a leguminous plant which can be successfully employed in food production as a source of high quality protein and it can be a good substitute for soybean (Duranti, 2006; Lampart-Szczapa et al., 2003). Albumins and globulins are two major classes of protein present in lupin seeds. Lupin seed globulin consists of α -, β -, δ - and γ -conglutin. During digestion of those proteins in the model system it was reported that γ -conglutin is resistant to pancreatin digestion (Capraro, Magni, Scarafoni, & Duranti, 2009; Czubinski et al., 2012). This conglutin fraction exhibits a close sequence and structural similarity with soybean basic 7S globulin (Yoshizawa et al., 2011). The structure of lupin γ -conglutin strongly depends on environmental pH therefore this protein can form tetramer at pH 7 and exists as a monomer under acidic pH (4.0) (Capraro, Spotti, Magni, Scarafoni, & Duranti, 2010).

Phenolic compounds are secondary plant metabolites that are classified as bioactive food components (Lampart-Szczapa et al., 2003). Lupin seeds revealed the presence of phenolic acids, mainly *trans*-ferulic (Sosulski & Dabrowski, 1984) or hydroxybenzoic acids (Dueñas, Hernandez, Estrella, & Fernandez, 2009). Kamel (2003) detected apigenin-7-*O*- β -apiofuranosyl-6,8-di-*C*- β -glucoyranoside in *Lupinus hartwegii*. In the seeds of white, yellow and narrow-leaf lupin grown in Europe it was found that flavonoid glycosides

(apigenin-6,8-di-*C*- β -glucoyranoside and apigenin-7-*O*- β -apiofuranosyl-6,8-di-*C*- β -glucoyranoside) are two predominant phenolic compounds (Siger et al., 2012). Apigenin is present in the human diet in the form of glycosides, where the tricyclic core structure is linked to a sugar moiety through hydroxyl groups (*O*-glycosides) or directly to carbon (*C*-glycosides). It is believed that hydrolysis is the first step of flavonoid metabolism. After hydrolysis of flavonoids glucuronidation, methylation, sulphuration or hydroxylation take place. Those conjugates are present in the human body in the blood stream and the lymphatic system (Murota & Terao, 2005). Apigenin, which is present in lupin seeds, shows strong antioxidant and anti-inflammatory activity in the human body. This compound, known as a free radical scavenger (Bors & Saran, 1987), inhibits lipid peroxidase-catalysed oxidation and LDL oxidation, as well as prevents oxidation of endogenous antioxidants such as vitamin E and C and glutathione (Chan, Galatti, & O'Brien, 1999; Joranic, Steenken, Tosic, Marjanovic, & Simic, 1994). Apigenin also shows anticancer activity, inhibits the activity of tyrosine kinase and topoisomerase, and protects DNA from oxidative damage (Boege, Straub, Kehr, Boesenberg, & Christiansen, 1996). According to Lefort and Blay (2013), the epithelium of the gastrointestinal tract is exposed to a higher concentration of apigenin, thus this flavonoid also influences the gastrointestinal cancer development. It inhibits growth and proliferation of cancer cells, stimulates apoptosis and inhibits the growth of blood vessels that cause tumour growth. Apigenin is able to reduce cancer cell glucose uptake, it inhibits remodelling of the extracellular matrix,

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inhibits cell adhesion molecules that participate in cancer progression, and opposes chemokine signaling pathways that direct the course of metastasis into other locations (Lefort & Blay, 2013). Apigenin glycosides may bind to proteins within plant sources and be released as those proteins are broken down as part of the digestive process (Czubinski et al., 2012).

Interactions between the different compounds present in foods are common and can simultaneously influence nutritional and functional properties of food products. Interactions of proteins and phenolic compounds are very complex reactions that cause biological and chemical modification, e.g. change in taste, the formation of haze in beverages or reduction of protein digestibility (Haslam, 1989). It is suggested that there are two different mechanisms of interaction of proteins with phenolic compounds. The first mechanism involves two steps: creating protein–phenolic complexes and subsequently the precipitation of the complex (Haslam, 1989). The second mechanism involves the reaction of cross-linking (Emmambaux, 2004). There is limited information concerning the molecular basis that determines formation of the flavonoid–protein complexes. In essence, non-covalent interactions seem to play a key role in this phenomenon. Complexation of phenolic compound with proteins can be considered in several aspects. Those complexes might strongly affect the nutritional potential of polyphenols by masking their antioxidant capacity, and on the other hand, causes protein precipitation or lack of protein digestion susceptibility (Arts et al., 2002; Czubinski et al., 2012). The food matrix in which phenolic compounds occur can significantly promote the formation of flavonoid–protein complexes. Changes in the nature of phenolic compounds at different environmental pH values may affect their stability, solubility, as well as leading to the formation of complexes with proteins (Friedman & Jürgens, 2000; Stojadinovic et al., 2013; Tungjai et al., 2008). Information about the pH of aqueous solution may influence the possible formation of phenolic compound–protein interaction that is especially valuable, because during the digestion process phenolic compounds have to deal both with the strongly acidic (during digestion in the stomach by pepsin), as well as alkaline environments (during digestion in the duodenum involving pancreatin). The previous study has shown, based on the example of lupin seed globulins, that γ -conglutinin can form complexes with flavonoids released from other proteins during pancreatin digestion at pH 7.5 (Czubinski et al., 2012).

The use of different enzymes is required in order to explain processes that may occur during the digestion of lupin seeds globulins at the stage of the duodenum. The main proteolytic enzyme present in pancreatin is trypsin. This enzyme shows specificity to hydrolyse the peptide bond only between positively charged amino acids, such as arginine or lysine. However, within pancreatin chymotrypsin is also present. Chymotrypsin can hydrolyse the peptide bond between more hydrophobic amino acids, such as phenylalanine, tyrosine or tryptophan. Lupin seeds globulin digestion susceptibility can be studied based on the difference in the specificity of the above mentioned enzymes. This study describes lupin seed globulins digestion by pancreatin, trypsin and chymotrypsin. A potential interaction between lupin native flavonoids and γ -conglutinin at pH 7.5 is also presented.

2. Materials and methods

2.1. Reagents

Vitexin (5,7,4'-trihydroxyflavone-8-C-glucoside), pancreatin from the porcine pancreas (P1750, activity equivalent to 4× U.S.P. specifications), trypsin from the porcine pancreas, TPCK treated in order to prevent any chymotrypsin activity (T1426,

$\geq 10,000$ units/mg protein), chymotrypsin from the porcine pancreas (C4129, ≥ 40 units/mg protein), tris(hydroxymethyl) aminomethane, sodium dodecyl sulphate (SDS), N,N,N',N'-tetramethylethylenediamine (TEMED), glycine, acrylamide and Coomassie Brilliant Blue R-250 were purchased from Sigma–Aldrich (St. Louis, MO, USA). All other solvents and chemicals used in this study were of analytical grade. Redistilled water was used in the resin-based column chromatography, while ultrapure water was purified via the Milli-Q system (Millipore, Bedford, MA, USA).

2.2. Materials

Narrow-leaf lupin seeds (*L. angustifolius*, cv. Zeus) were obtained from the Plant Breeding Station Smolice, Przebędowo branch. The seeds were milled with an IKA M20 universal laboratory mill (IKA-Werke GmbH&Co, Staufen, Germany), sieved to obtain a fraction below 1.6 mm and defatted using the automatic Soxhlet Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland). Extraction with *n*-hexane was carried out for two hours. Defatted lupin flour was stored in closed polyethylene bags at -18°C until the analysis.

2.3. Protein extractions and γ -conglutinin purification

Protein extraction was carried out according to the procedure described by Czubinski et al. (2012). Briefly, albumins were removed from defatted lupin flour using extraction with water pH 8.0 containing 10 mM CaCl_2 , 10 mM MgCl_2 (34 ml per 1 g of lupin flour) for 4 h. Then samples were centrifuged for 1 h at 20,000g (model 6K15, Sigma, Osterode am Harz, Germany) and the pellet devoid of albumin was used for further extraction. Globulins extraction was performed by 4-h elution using 0.1 M Tris–HCl pH 7.5 buffer containing 10% NaCl (w/v), Na_3N 0.05% (w/v) (34 ml per 1 g of lupin flour). Then samples were centrifuged for 1 h at 20,000g and proteins were precipitated from the supernatant by adding ammonium sulphate (561 g/l). Salting was carried out for 30 min and then samples were centrifuged for 20 min at 20,000g. The precipitate containing salted globulins was resuspended in 50 mM Tris–HCl pH 7.5 buffer (5.7 ml per 1 g of lupin flour). All stages of protein extraction from defatted lupin flour were performed at 4°C with constant mechanical stirring. The obtained lupin globulin extract was filtered through 0.45 μm PVDF syringe filter (Millipore, Bedford, MA, USA) and desalted using an automatic FPLC system with the HiTrap Desalting column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl buffer at pH 7.5. Desalted proteins were collected in 2 ml fractions and immediately used for analysis.

γ -Conglutinin was purified from desalted globulin by combination of anion and cation exchange chromatography. Briefly, desalted globulins (5 ml) were subjected on HiTrap Q column (Pharmacia, Uppsala, Sweden) equilibrated with 50 mM Tris–HCl pH 7.5 buffer. Under these conditions γ -conglutinin was not adsorbed on the column. Fractions containing γ -conglutinin were collected. γ -Conglutinin containing fractions obtained in five anion exchange chromatography runs were concentrated on 30 kDa molecular weight cutoff Amicon centrifugal filters (Millipore, Bedford, MA, USA) up to 5 ml. Cation exchange purification was run by subjecting concentrated γ -conglutinin fractions (5 ml) on HiTrap SP column (Pharmacia, Uppsala, Sweden) and separation in linear gradient of NaCl from 0 M to 1 M was carried out.

All protein fractions were collected in 2 ml fractions and analysed by SDS–PAGE electrophoresis under non-reducing condition. The fraction containing γ -conglutinin was concentrated to the required concentration by 30 kDa molecular weight cutoff Amicon centrifugal filters (Millipore, Bedford, MA, USA).

2.4. Protein concentration measurements

Protein content was assayed according to Bradford (Bradford, 1976) using BSA as a standard. Analyses were carried out at wavelength $\lambda = 595$ nm (UV–Vis spectrophotometer SP 8001, Metertech Inc. Taipei, Taiwan).

2.5. Enzymatic digestion of lupin globulins in model system

Digestion was carried out by applying the method described in our earlier study for enzymatic treatments of lupin seed globulins (Czubinski et al., 2012). Three different proteolytic enzymes were used, i.e. pancreatin, trypsin as well as chymotrypsin. Briefly, for all enzyme treatments lupin globulins were resuspended in water to a final concentration of 1 mg protein per 1 ml, adjusted to the pH 7.5 with 0.1 M NaHCO_3 and enzyme solution (1 mg/ml in water) was added at a ratio of 1:30 enzyme/lupin globulins (w/w). 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 M HCl.

2.6. Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS–PAGE)

SDS–PAGE electrophoresis of desalted lupin globulins, as well as products of their enzymatic hydrolysis was carried out according to the method proposed by Laemmli (1970) using 12.5% polyacrylamide gel in a mini-Protean electrophoresis system (Bio-Rad, Hercules, CA, USA). Polypeptide bands were stained in Coomassie Brilliant Blue G-250 for 12 h. Relative molecular masses of protein were determined by a comparison to molecular weight markers (Fermentas, Lithuania).

2.7. Methanol extracts of phenolic compounds

To obtain phenolic compounds from seed of narrow-leaf lupin seeds (*L. angustifolius*, cv. Zeus), defatted seeds were extracted three times with 80% methanol. In brief, 5 g of sample were extracted with 50 ml methanol three separate stages over for 30 min at 50 °C. After centrifugation (10 min at 5000g) (model 6K15, Sigma, Osterode am Harz, Germany) the precipitate was again re-extracted twice following the same steps. The three supernatants were combined and evaporated under reduced pressure using an R-215 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland) to a volume of 50 ml.

2.8. Column chromatography purification of flavonoids

Purification of lupin seed methanol extracts was carried out according to the method proposed by Rybarczyk and Amarowicz (2007). For this purpose a column was filled with silica gel (Merck, Darmstadt, Germany). The filled column was washed with three volumes of methanol and one volume of the mobile phase consisting of chloroform–methanol–water (35:65:10; v/v/v). Five millilitres of extract were applied on the column. The eluate was collected in 4 ml fractions. Chromatographic separation was monitored at 335 nm. On the basis of the designated chromatogram the flavonoid fraction was combined and evaporated under reduced pressure using an R-215 rotary evaporator (Büchi Labortechnik AG, Flawil, Switzerland). Identification and quantification of flavonoids was carried out by combining identification characteristics of MS fragmentation patterns as well as quantitative UV–DAD analyses utilising vitexin as an equivalent, as described previously (Siger et al., 2012).

2.9. UV–Vis spectra of flavonoids in different pH

McIlvaine buffer at different pH values was used in spectroscopic studies of flavonoid behaviour. The absorption spectra were measured in the range 250–600 nm with a UV–Vis SP 8001 spectrophotometer (Metertech Inc. Taipei, Taiwan) in a 1×1 cm quartz cuvette. Experiments were conducted by mixing 2 ml of buffer of each pH values buffer with 20 μL of flavonoids separated by column chromatography. Final flavonoids content in cuvette was 0.158 $\mu\text{g}/\text{ml}$ (expressed as vitexin equivalents).

2.10. Fluorescence anisotropy measurements

Lupin globulins for fluorescence studies were diluted to a concentration of 48 $\mu\text{g}/\text{ml}$ with 50 mM Tris–HCl pH 7.5 buffer. The emission anisotropy was measured at 422 nm, with the excitation wavelength of 305 nm, using an appropriate instrumental procedure for anisotropy calculation applying a Perkin–Elmer LS-55 fluorimeter (Perkin Elmer, Waltham, MA, USA). The final anisotropy was the average of at least five consecutive measurements of fresh samples. All spectroscopic measurements were performed in a 1×1 cm quartz cuvette.

2.11. Fluorescence lifetime measurements

Fluorescence lifetimes of digested lupin seed globulins were measured with a TimeHarp 100 PC-board (PicoQuant) for time correlated single photon counting with 72 ps per channel resolution. A 5000F coaxial subnanosecond flashlamp (IBH) filled with nitrogen with maximum emission centered at 337 nm and pulse FWHM of 1.3 ns was used as excitation source. A 337 nm XL30 interference filter from Laser Components was used as the excitation window to avoid leaking from the nitrogen spectrum.

For γ -conglutinin fluorescence lifetime study the excitation source was a sub-nanosecond pulsed UV LED 290 with the maximum emission centered at 290 nm and a 0.7 ns wide pulse with full width at half maximum (FWHM), powered by a PDL 800-D driver (all the instruments from PicoQuant, Berlin, Germany). The emission was measured with a PMA 182 detector head (PicoQuant).

The data were analysed by an exponential reconvolution method using a nonlinear least-squares fitting program. The goodness of fit was estimated by using χ^2 value.

2.12. Fluorescence quenching studies

Lupin globulins for fluorescence quenching studies were diluted to a concentration of 50 $\mu\text{g}/\text{ml}$ with 50 mM Tris–HCl pH 7.5 buffer and during titration 1 mM vitexin solution was used. The fluorescence emission was measured in the range of 280–420 nm, with excitation wavelength of 240 nm, using a Shimadzu 5001 PC spectrofluorometer (Shimadzu, Kyoto, Japan). The excitation wavelength was selected on the basis of the excitation spectrum. The final fluorescence intensity was the average of at least three consecutive measurements of fresh samples. All the spectroscopic measurements were performed in a 1×1 cm quartz cuvette. The Stern–Volmer plots were prepared using fluorescence intensities collected at 343 nm.

2.13. Bioinformatic analysis

The γ -conglutinin model was predicted by homology modelling using the Swiss-Model program (Kiefer, Arnold, Künzli, Bordoli, & Schwede, 2009; Peitsch, 1995; Wallner & Elofsson, 2003). The soybean basic 7S globulin X-ray structure deposited in the Protein Data Bank (PDB) was used as a template (PDB accession number:

3AUP-A). The model quality assessment was checked using the ProQ program (Wallner & Elofsson, 2003). Figures were prepared with PyMOL (<http://www.pymol.org/>).

2.14. Statistical analysis

Results are presented as means $\pm$ standard deviation from three replicates of each experiment. Differences between mean values were determined by the analysis of variance (ANOVA). The post-hoc analysis was performed by the Tukey test. All tests were considered significant at $p < 0.05$. Statistical analysis was performed using Statistica 10.0 (StatSoft, Inc., Tulsa, OK, USA) software.

3. Results and discussion

3.1. Digestion susceptibility of lupin globulins

Different susceptibility of lupin seed globulin to pancreatin, trypsin and chymotrypsin digestion was monitored by SDS–PAGE electrophoresis under non-reducing condition (Fig. 1). Our previous research reported that pepsin, as well as pancreatin followed by pepsin digestion, leads to total digestion of lupin seed globulins. At present, susceptibility of lupin globulins to digestion by trypsin and chymotrypsin was studied. Presence of γ -conglutin was confirmed by the band at about 45 kDa on separated gel. In the case of pancreatin digestion it was confirmed that γ -conglutin turned out to be totally resistant. Presence of the γ -conglutin characteristic band at about 45 kDa was observed not only in a sample after pancreatin treatment, but also in trypsin-treated lupin globulins (Fig. 1). Pancreatin is a mixture of several digestion enzymes where, among proteases, trypsin is the main enzyme, so this fact can confirm similar results obtained in SDS–PAGE separation of those samples. Trypsin cleaves peptide chains mainly at the carboxyl side of lysine or arginine, while chymotrypsin, the other protease present in pancreatin, preferentially cleaves peptide chains on the carboxyl side of tyrosine, tryptophan or phenylalanine. In

the case where trypsin is used for digestion while analysing the γ -conglutin sequence (Q42369), there are 25 cleavages possible. Duranti, Di Cataldo, Sessa, Scarafoni, and Ceciliani (2002) studied trypsin proteolytic resistance of denatured γ -conglutin and re-folded protein. When γ -conglutin was subjected to acid treatment before digestion with trypsin, the protein was totally digested. Those authors found that divalent metal ions, mainly Zn^{2+} and Ni^{2+} , promote refolding of γ -conglutin, which leads to restoration of resistance to trypsin proteolysis.

The phenomenon of γ -conglutin resistance to pancreatin as well as trypsin digestion can be explained not only by the fact that there are relatively few peptide bond cleavage sites. One of the potential explanations of low γ -conglutin susceptibility to digestion by those enzymes can be related to the potential blocking of amino acids, between which the peptide bond should be cleaved. This fact can be identified by the formation of covalent addition products with phenolic compounds. Schilling, Sigolotto, Carle, and Schieber (2008) studied the formation of complexes between amino acid derivatives and chlorogenic acid quinone at pH 7.0. Those authors found that covalent bonds were formed between quinone and lysine derivatives. Quinones are products of phenolic compound oxidation. Oxidation of lupin seed polyphenols is possible in the presence of native lipoxygenase (Czubinski, Dwiecki, Siger, Lampart-Szczapa, & Nogala-Kalucka, 2012). Another potential reason for γ -conglutin ineffective digestion is related with the fact that both lysine and arginine at cell physiologically pH are positively ionised. Simultaneously, flavonoids at this pH are partially ionised, which may lead to the occurrence of ionic interactions between those molecules. However, further studies are necessary to establish this phenomenon.

Digestion of almost all lupin seed globulins was observed in case of chymotrypsin (Fig. 1). Obtained results were very similar to those reported in the case of pepsin digestion (Czubinski et al., 2012). This phenomenon can be related to the similar specificity of pepsin and chymotrypsin. When analysing the γ -conglutin sequence (Q42369) by enzyme specificity, 97 and 124 potential numbers cleavages are possible in the case of chymotrypsin and pepsin,

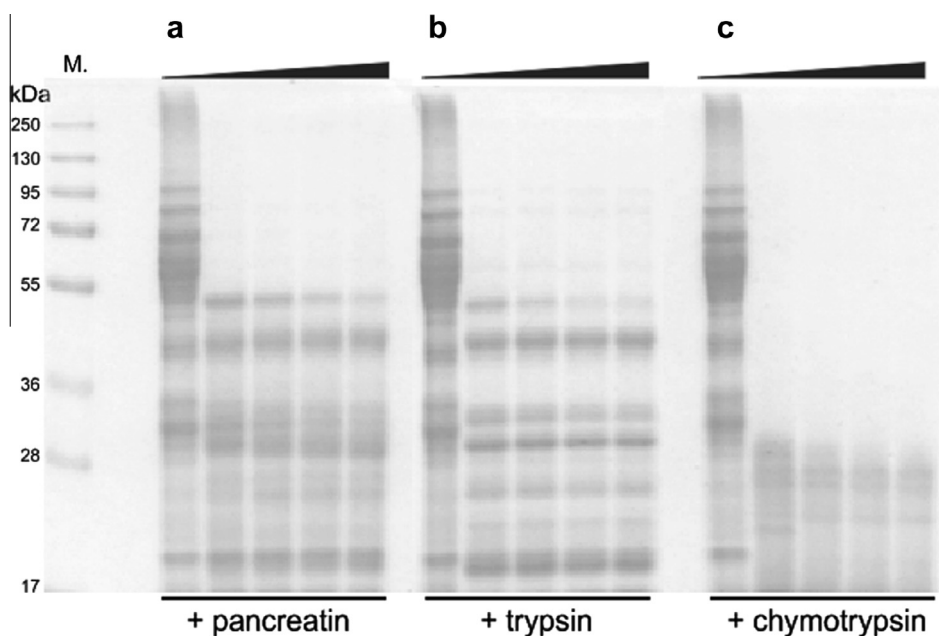


Fig. 1. SDS–PAGE patterns under non-reducing conditions of lupin globulins digested in different ways. Lupin globulins were incubated with pancreatin (a), trypsin (b), as well as chymotrypsin (c) [30:1 (w/w), pH 7.5, 37 °C] and digestion susceptibility was checked at 15-min time intervals up to 60 min. For comparison, the crude SDS–PAGE patterns of undigested lupin seed globulins are also shown. When lupin seed globulins were digested with pancreatin or trypsin, the obtained SDS–PAGE patterns were the same. Simultaneously, digestion time in the case of both pancreatin and trypsin digestion does not affect γ -conglutin hydrolysis susceptibility. Results obtained from chymotrypsin digestion of lupin seed globulins indicate that this enzyme successfully performs hydrolysis. M, molecular weight marker.

respectively (Gasteiger et al., 2005). A majority of cleavage position sites during digestion by pepsin and chymotrypsin are also similar. However, those enzymes are present in two different conditions, as γ -conglutin can occur as a tetramer (pH 7.0 at which chymotrypsin is active) or a monomer (pH 2.0 at which pepsin is active) (Capraro et al., 2010). In both environmental conditions γ -conglutin is totally digestible by pepsin and chymotrypsin, thus it may be concluded that oligomerisation of this protein is not related to digestion susceptibility by those enzymes. Capraro et al. (2009) studied pepsin digestion susceptibility of γ -conglutin at different pH. They noted that γ -conglutin is totally digested at pH 2.0 and 3.0 by pepsin but at pH 4.0, i.e. when this enzyme is minimally active the protein was unaffected. They recorded similar results when bromelain was used for digestion of γ -conglutin. When bromelain was incubated with γ -conglutin at pH 4.0 the protein was digested, while an increase in pH value caused resistance of γ -conglutin to bromelain digestion. Based on this fact Capraro et al. (2009) proposed a theory that γ -conglutin in the native conformation at neutral pH is resistant to pepsin proteolytic digestion.

Differences in lupin globulins digestion susceptibility were also measured, based on fluorescence parameters (anisotropy and fluorescence lifetime). 7-O- β -apiofuranosyl-6,8-di-C- β -glucopyranoside is the dominant flavonoid present in lupin seed globulin after digestion with various enzymes as well as in lupin seed phenolic extract (Czubinski et al., 2012; Siger et al., 2012). It was proven that flavonoids liberated from other lupin globulin proteins, as a result of pancreatin-catalysed digestion, are bounded by γ -conglutin (Czubinski et al., 2012). This phenomenon was observed by differences in fluorescence anisotropy and lifetime value of the free flavonoid and flavonoid-protein complex. Anisotropy measurements were performed at 305 nm excitation and 422 nm emission wavelength, which are related to tracing of microenvironment nature of flavonoids. The formed flavonoid-protein complex causes a higher anisotropy r value than in the case when proteins are not complexed with flavonoids. The fluorescence anisotropy r values of lupin seed globulin digestion performed by pancreatin, as well as trypsin, were 0.189 and 0.195, respectively (Table 1). In the case when lupin seed globulins were digested with chymotrypsin the r value decreased to 0.138 (Table 1). The decrease of the anisotropy value was observed as a result of an increase of motional freedom of flavonoid when lupin seeds globulin were totally digested. A similar reduction in r value to that observed in pepsin and pepsin followed by pancreatin digestion can correspond to the complete digestion of lupin seed proteins (including γ -conglutin) (Czubinski et al., 2012).

Changes in lupin seed globulins digestion susceptibility and the related phenomenon of the release of flavonoids manifest themselves also in the change of fluorescence lifetime. After lupin seed globulins were totally digested the fluorescence lifetime was longer than in the case when some proteins remained undigested and could capture flavonoids. The fluorescence lifetime of lupin seed globulins digested by pancreatin and trypsin was 2.0 and 2.2 ns, respectively (Table 1). When these two enzymes were used, γ -conglutin remained undigested (Fig. 1). Simultaneously, flavonoids liberated from other proteins can bind then to γ -conglutin and it was suggested that a locally concentrated flavonoid on the γ -con-

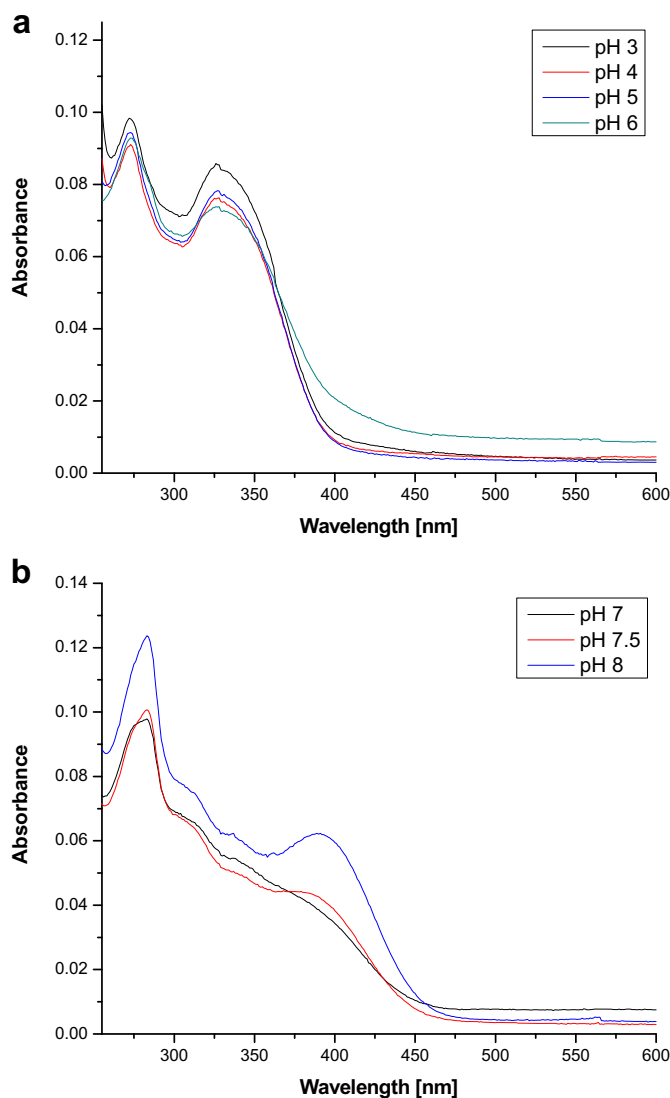


Fig. 2. Absorption spectra of lupin seeds flavonoids in buffer at different pH. The effect of pH (3–8) effect on absorption spectra was verified by diluting lupin seed flavonoids in an appropriate buffer. The McIlvaine buffer was used in order to obtain an appropriate pH value. When lupin seed flavonoid were diluted in a buffer with pH within the range from 3 to 6 (a) the absorption maximum was 335 nm, while a change of pH to 7–8 (b) causes a shift in the absorption spectrum (maximum at 390 nm). The change in the absorption maximum due to changes in pH value is associated with flavonoid ionisation.

glutin surface resulted in lifetime shortening by dynamic quenching (Lakowicz, 2006). An increase in lifetime was observed in chymotrypsin digested lupin seed globulins (3.9 ns) (Table 1). A relatively longer fluorescence lifetime, which was observed in chymotrypsin digestion, is caused by the disappearance of local concentration sites and finally the release of flavonoids from the protein–flavonoid complex. Similar results where lupin seed globulins were almost completely digested was observed in our previous study in

Table 1

Steady-state fluorescence anisotropy (r) and fluorescence lifetimes of lupin globulins digested with different enzymes in 50 mM Tris–HCl, pH 7.5, buffer.

Sample	Anisotropy (r)	Lifetime (ns)
Lupin globulins digested with pancreatin (50 μ g/mL)	0.189 $\pm$ 0.003 ^b	2.0
Lupin globulins digested with trypsin (50 μ g/mL)	0.195 $\pm$ 0.018 ^b	2.2
Lupin globulins digested with chymotrypsin (50 μ g/mL)	0.138 $\pm$ 0.006 ^a	3.9

Letters in a column shows statistically significant differences at $P < 0.05$. The estimated lifetime resolution is $\Delta\tau = 0.05$ ns.

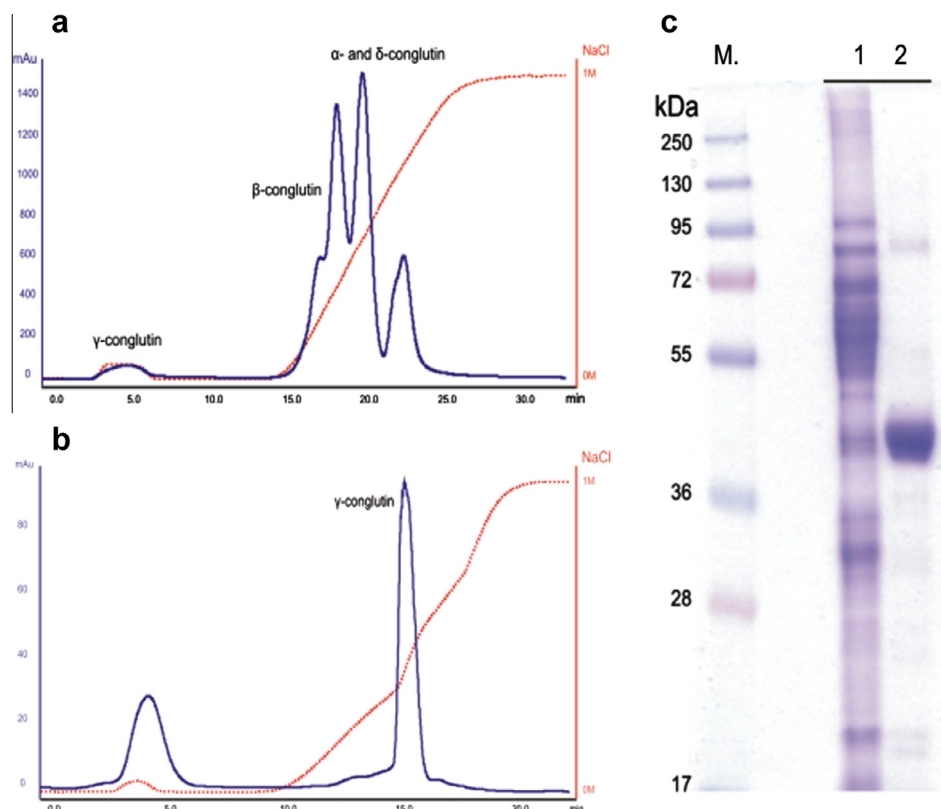


Fig. 3. Chromatogram of lupin seed globulins separated by ion exchange chromatography. Separation was performed in 50 mM Tris–HCl buffer at pH 7.5 in an anion exchange column in the 0–1 M NaCl gradient (a). Under these separation conditions γ -conglutin has a positive charge, therefore this fraction leaves the column first (at 0 M NaCl). In the next stage, collected γ -conglutin was separated at a cation exchange column in the 0–1 M NaCl gradient (b). Purity of obtained γ -conglutin was monitored by SDS–PAGE under non-reducing conditions (c). Based on SDS–PAGE separation purity of γ -conglutin (2) was estimated at 98%. For comparison, the crude lupin seeds globulin SDS–PAGE patterns are also shown (1). M, molecular weight marker.

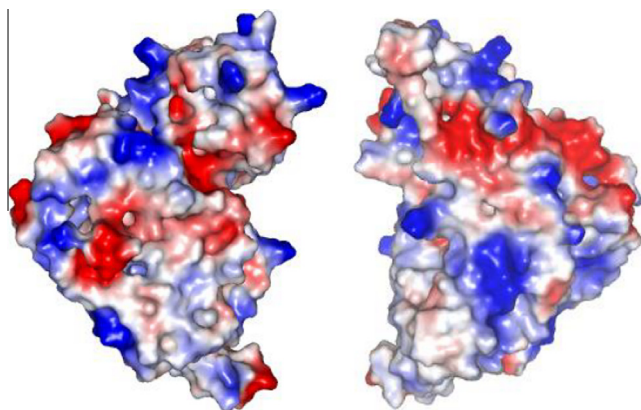


Fig. 4. Surface electrostatic charge distribution of γ -conglutin. Red regions of the surface correspond to negative charges; blue regions of the surface correspond to positive charges.

the case of pepsin followed by panceratin digestion (Czubinski et al., 2012).

3.2. Characterisation of lupin seed flavonoids

The presence of phenolic compounds in lupin seeds has been reported in many studies. Two dominant flavonoid compounds present in methanol extracts from lupin seeds were identified as apigenin-6,8-di-C- β -glucopyranoside and apigenin-7-O- β -apiofuranosyl-6,8-di-C- β -glucopyranoside (Siger et al., 2012). Simulta-

neously, none of these compounds are present in leaves and roots of 3-week-old plants, which may indicate the potential conversion of apigenin glycosides in course of germination and plant development (Muth, Marsden-Edwards, Kachlicki, & Stobiecki, 2008). It is a known fact that during protein recovery from legumes alkali buffers are usually used. A flavonoid fraction was extracted from defatted lupin seed by 80% methanol and further purified in a silica gel filed column, where mobile phase consists of chloroform–methanol–water (35:65:10, v/v/v). This method facilitates separation of phenolic acids, which are present as minor phenolic compounds in lupin seeds from apigenin derivatives. The qualitative and quantitative composition of the flavonoid fraction confirmed the presence of both apigenin C-glycosides in the sample (Supplementary data). Different pH of the aqueous environment, in which flavonoids are found can influence their solubility. For example, hesperidin and naringenin solubility increases 4-folds when pH 1.5 is changed to 8 (Benavente-García, Castillo, Del Baño, & Lorente, 2011). Another important phenomenon strongly associated with environmental pH changes is flavonoid ionisation. It was reported that an increase in the pH value might promote formation of phenolic compound–protein complexes (Czubinski et al., 2012; Stojadinovic et al., 2013). Flavonoids are good chromophores that absorb light between 200–400 nm. This fact can be utilised to trace changes in ionisation at different pH. Apigenin derivatives purified from lupin seeds were dissolved in different McIlvaine buffers (pH 3–8). UV–Vis spectra were recorded in at 200–600 nm (Fig. 2). At different pH buffers in which flavonoids were dissolved, shifts in the maximum of absorbance were observed. A shift in band II can be observed when analysing the UV–Vis spectrum of lupin seed flavonoids at pH below 6 and at pH 7 or above. In the case of apigenin derivatives at pH below 6 the maximum in

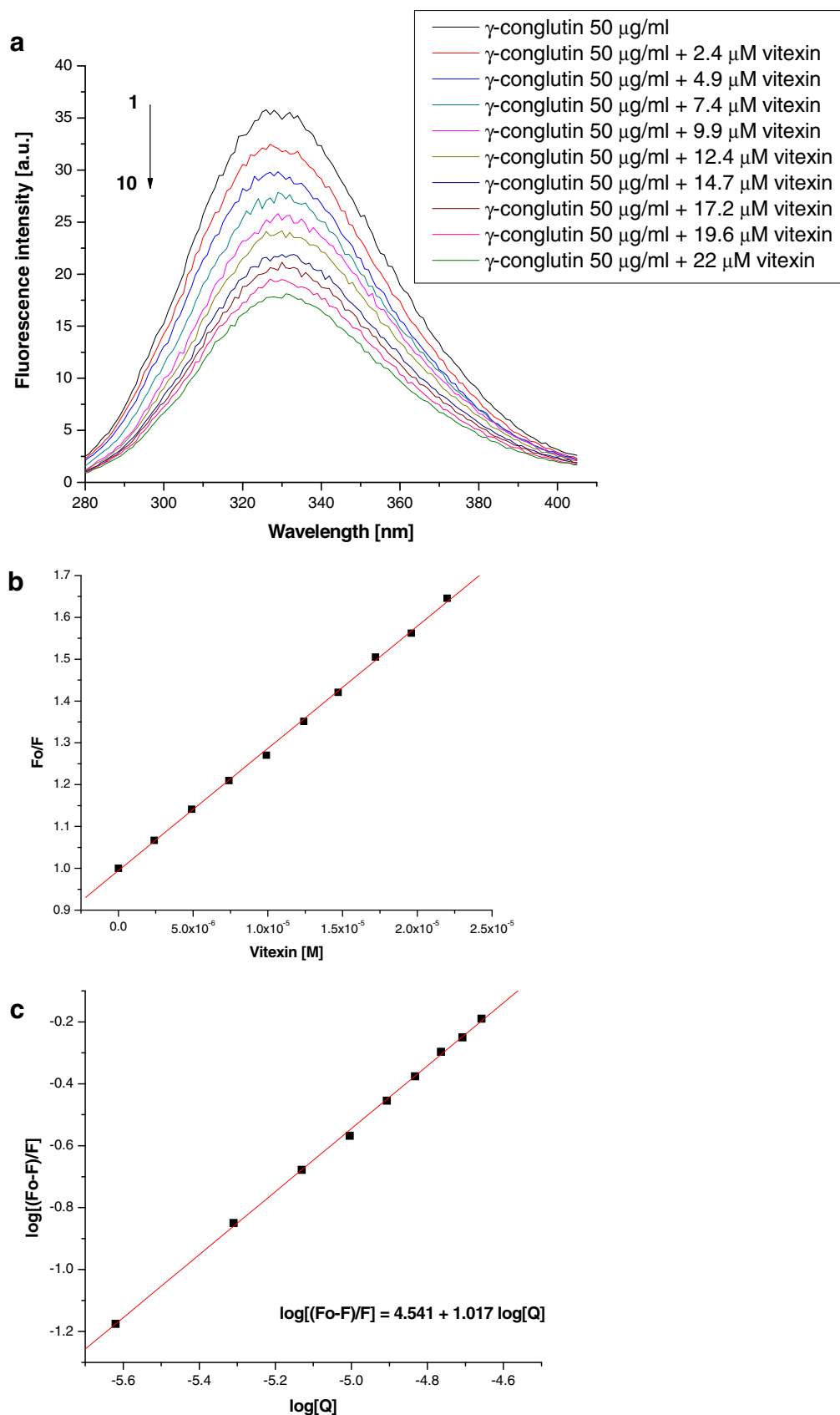


Fig. 5. Fluorescence spectra of γ -conglutinin (50 $\mu\text{g/ml}$) titrated with vitexin in 50 mM Tris–HCl buffer pH 7.5 at room temperature (a). The Stern–Volmer plots of F_0/F at 343 nm at room temperature as a function of concentration of quencher (vitexin) in γ -conglutinin (50 $\mu\text{g/ml}$) solution in 50 mM Tris–HCl buffer pH 7.5 (b). A double logarithmic Stern–Volmer plot at 343 nm for γ -conglutinin (50 $\mu\text{g/ml}$) – vitexin system (Q – vitexin concentration) in 50 mM Tris–HCl buffer pH 7.5 at room temperature (c).

the UV–Vis spectrum was at 335 nm, while at pH 7 or above the maximum differed significantly (390 nm). The change in the maximum of absorbance from 335 nm to 390 nm can be explained by the change in flavonoid ionisation. Tungjai et al. (2008) studied the effect of pH on UV–Vis spectra of apigenin and observed that maximum absorbance at 335 nm corresponded to the neutral form of this compound, while the ionic form had maximum absorbance at 390 nm. Those authors estimated pKa of C7-OH of apigenin at 7.86. Flavonoids present in lupin seeds are apigenin glycosides and therefore those compound can slightly differ in the pKa value; nevertheless, it can be concluded that in the protein extraction buffer (pH 7.5) those compounds are partially ionised.

3.3. Interaction between γ -conglutin and vitexin (fluorescence quenching studies)

An interaction between γ -conglutin and vitexin was studied based on fluorescence quenching effects. The measurement of fluorescence parameters is a useful technique providing information on the molecule microenvironment and is often used in investigations of polyphenol–protein interactions and in binding affinity studies (Dwiecki, Górnaś, Wilk, Nogala-Kałucka, & Polewski, 2007; Dwiecki, Neunert, Polewski, & Polewski, 2009; Kroll, Rawel, & Rohn, 2003; Liu, Qi, & Li, 2010; Rawel, Czajka, Rohn, & Kroll, 2002; Stojadinovic et al., 2013). According to the literature data excitation at 240–280 nm of proteins induced the emission of fluorescence in the range of 340–350 nm as a result of the presence of aromatic amino acids and a decrease in this fluorescence emission range can be related to ligand binding (Liu et al., 2010; Rawel, Rohn, Kruse, & Kroll, 2002; Rawel et al., 2002). In the study vitexin (apigenin-8-C-glucopyranoside) was used as a flavonoid quencher, because this compound is structurally closely related to the analysed flavonoids from lupin seeds. γ -Conglutin was purified using anion and cation exchange chromatography. Purity of γ -conglutin was confirmed by SDS–PAGE separation at non-reducing conditions (Fig. 3). Quenching fluorescence measurements were performed in Tris–HCl pH 7.5 buffer, where lupin seeds flavonoids are negatively ionised (Fig. 2), while γ -conglutin at this pH is positively ionised (Fig. 4). When the γ -conglutin solution (50 μ g/ml) was titrated with vitexin (2.4–22 μ M), fluorescence quenching at about 343 nm was observed (Fig. 5a). Fluorescence quenching is described by the Stern–Volmer Eq. (1) (Stojadinovic et al., 2013):

$$F_0/F = 1 + K_{sv} [Q] \quad (1)$$

where F_0 and F are fluorescence intensities before and after addition of the quencher, K_{sv} is the Stern–Volmer quenching constant and $[Q]$ is the concentration of the quencher. A linear Stern–Volmer plot indicates that one mechanism of quenching appears, either static (complex formation) or dynamic (collision of fluorophore with the quencher). In the case of γ -conglutin quenching in the presence of vitexin the obtained Stern–Volmer plot was linear, with the Stern–Volmer quenching constant (K_{sv}) equal to 29,215 [M^{-1}] (Fig. 5b). The fluorescence quenching rate constant (k_q) was calculated from Eq. (2), where τ_0 is a fluorescence lifetime of fluorophore without a quencher (Stojadinovic et al., 2013):

$$K_{sv} = k_q \tau_0 \quad (2)$$

The γ -conglutin fluorescence decay is double exponential at pH 7.5 and the observed values were 2.5 ns and 9.5 ns (data not shown). Taking into account those values, the fluorescence quenching rate constant k_q does not exceed 3.08×10^{12} [$M^{-1}s^{-1}$]. The maximum value possible for diffusion-limited quenching in water is about 10^{10} [$M^{-1}s^{-1}$] (Stojadinovic et al., 2013). The calculated bimolecular quenching rate constant is more than two orders of magnitude higher than the maximum diffusion controlled limit. It means that there is a complex formation between γ -conglutin

and vitexin, corresponding to the static mechanism of fluorophore quenching. Then a double logarithmic Stern–Volmer Eq. (3) for the static mechanism of quenching can be applied and calculation of binding constant (K_a) and the number of binding sites (n) in the γ -conglutin molecule may be calculated (Q is a quencher concentration) (Stojadinovic et al., 2013):

$$\log[(F_0 - F)/F] = \log K_a + n \log [Q] \quad (3)$$

A double logarithmic Stern–Volmer plot for the γ -conglutin – vitexin system is presented in Fig. 5c and the calculated binding constant (K_a) and the number of binding sites (n) are $3.47 \times 10^4 M^{-1}$ and 1.017, respectively. The calculated K_a is in the range observed for various dietary polyphenols bound to another globulin – β -lactoglobulin: e.g. resveratrol (10^4 – $10^6 M^{-1}$) (Liang, Tajmir-Riahi, & Subirade, 2007) and epigallocatechin-3-gallate (10^4 – $10^5 M^{-1}$) (Kanakakis et al., 2011; Shpigelman, Israeli, & Livney, 2010), at neutral pH. At pH 7.2 the K_a value observed for polyphenols from black tea, green tea, coffee and cocoa bound to β -lactoglobulin ranged from: $5.85 \times 10^3 M^{-1}$ to $1.46 \times 10^5 M^{-1}$. The value $n = 1.017$ means that one molecule of vitexin is able to bind to one molecule of γ -conglutin (the 1:1 complex). A similar stoichiometric ratio was observed at pH 7.2 in the case of β -lactoglobulin complexes with polyphenols from black tea ($n = 0.75$), green tea ($n = 0.88$), coffee ($n = 0.84$) and cocoa ($n = 0.99$) (Stojadinovic et al., 2013).

4. Conclusions

To conclude, presented results provide the first information concerning susceptibility of γ -conglutin to chymotrypsin digestion. The unique flavonoid binding phenomenon of γ -conglutin is another important property of this protein that to date has not been reported in literature. Such a phenomenon may be possible due to the electrostatic attraction between the positively charged γ -conglutin and flavonoids partially ionised at pH 7.5. γ -Conglutin – vitexin complex formation with a stoichiometric ratio of 1:1 was observed using fluorescence quenching measurements. Analysis of γ -conglutin binding ability requires future analysis, especially because both flavonoid composition changes during germination and the content of this unique protein fraction remain the same as in seeds.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.foodchem.2013.08.015>.

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HPLC chromatogram (recorded at $\lambda=335$ nm) of lupin seed flavonoids extract purified by column chromatography (for details please see Material and Methods section). Presence of two domain peaks [apigenin-6,8-di-C- β -glucoyranoside (a) and apigenin-7-O- β -apiofuranosyl-6,8-di-C- β -glucoyranoside (b)] of apigenin derivatives were confirmed. Percentage content of those compounds was almost equal.

