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Release of Flavonoids from Lupin Globulin Proteins during Digestion in a Model System

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ABSTRACT: Lupin seed globulin proteins form complexes with flavonoids, predominantly apigenin *C*-glycosides. Enzymes typical for the gastrointestinal tract were used to hydrolyze lupin seed globulins. Release of native flavonoids as a result of the proteolysis reaction was observed. Different analytical methods such as size exclusion chromatography, HPLC-MS, and fluorescence spectroscopy (steady-state fluorescence, fluorescence anisotropy, fluorescence lifetimes) were used for a detailed characterization of this phenomenon. Flavonoids liberated from lupin globulin proteins as a result of pancreatin-catalyzed digestion were bound by γ-conglutin resistant to this enzyme. Two possible mechanisms of this interaction may be suggested: hydrogen bonding between oligosaccharide chains of glycoproteins and the sugar moieties of the flavonoid glycosides or electrostatic attraction between positively charged γ-conglutin and flavonoids partially ionized at pH 7.5.

KEYWORDS: *lupin, flavonoids, apigenin C-glycosides, γ-conglutin, flavonoid−protein complexes*

INTRODUCTION

The key nutritional role of grain legumes is unquestioned, due to the massive presence of macro- and micronutrients. Among these nutrients, proteins play a relevant role due to their amino acid composition, which can easily be balanced in the diet.[1,2] Lupin seeds contain two main classes of protein, albumins and globulins. Globulins are typical storage proteins that constitute about 90% of all proteins present in seeds, and they mainly consist of α-, β-, δ-, and γ-conglutins.[3,4] Several suggestions for the role of legumes in the prevention of relevant diseases are available and represent the basis for the health claims on legume seeds. White lupin (*Lupinus albus*) γ-conglutin has structural similarity to xyloglucan-specific endo-β-1,4-glucanase inhibitor proteins (XEGIPs) and *Triticum aestivum* xylanase inhibitor (TAXI-1). γ-Conglutin also exhibits the ability to bind to the hormone insulin and to the insulin-like growth factors IGF-1 and IGF-II, and its pharmaceutical role similar to that of the hypoglycemic drug metformin has been suggested.[5] In addition to these findings, other biological activities have been attributed to lupin seed proteins, including plasma cholesterol and triglyceride lowering effects,[6] antihypertensive properties,[7] and angiotensin converting enzyme (ACE) inhibitory activity.[2,8] Bread enriched with narrow-leaf lupin protein (NLL protein) and fiber may help reduce blood pressure and the risk of cardiovascular disease.[9]

Lupin seeds, similarly to other legumes, are a rich source of phenolic compounds. Polyphenols, which provide by definition more than one phenolic hydroxyl group, represent one of the most ubiquitous and numerous groups of plant metabolites. Due to their reducing properties as electron-doning agents, polyphenols can act as antioxidants by means of free radical scavengers.[10] In the case of lupin seeds, the presence mainly of phenolic acids and flavonoids was recorded.[11−14] Sosulski and Dabrowski[11] reported that defatted flours of 10 legumes contained only soluble esters of *trans*-ferulic, *trans*-*p*-coumaric, and syringic acids. Lampart-Szczapa et al.[13] showed the presence of acids such as protocatechuic, *p*-hydroxybenzoic, chlorogenic, vanillic, *p*-coumaric, and ferulic in lupin seeds. The effect of narrow-leaf lupin (*Lupinus angustifolius* L.) seed germination on the content of phenolic compounds was also studied.[14]

There is a lot of information concerning interactions between phenolic compounds and proteins. Current knowledge provides information that flavonoid−protein interaction may impair flavonoid bioavailability or even in some cases mask the antioxidant activity of these compounds.[15] However, details of the nature of the mechanisms responsible for these phenomena have not been explained yet. Interactions based mainly on noncovalent mechanisms are suggested to occur, but some authors provide information about covalent reactions that may also take place.[16−18]

There are several aspects of flavonoid−protein interaction for which a precise description is especially demanded. One aspect is changes of these complexes' digestibility with enzymes typical for the gastrointestinal tract. Such an analysis may provide valuable information about the release of phenolic compounds from their natural connections after the consumption of food and feed containing lupin seeds. This study describes the interaction between flavonoids native to lupin seeds (predominantly apigenin *C*-glycosides) with globulin proteins.

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MATERIALS AND METHODS

Reagents. Vitexin (5,7,4′-trihydroxyflavone-8-*C*-glucoside), pepsin (from porcine stomach mucosa), pancreatin (from porcine pancreas), tris(hydroxymethyl)aminomethane, sodium dodecyl sulfate (SDS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), glycine, acrylamide, and Coomassie Brilliant Blue R-250 were purchased from Sigma-Aldrich (St. Louis, MO). Acetonitrile, methanol, and formic acid (all of HPLC grade) were obtained from Merck (Darmstadt, Germany). All other solvents and chemicals used in this study were of analytical grade. Redistilled water was used in the resin-based column chromatography, whereas ultrapure water purified via a Milli-Q system (Millipore, Bedford, MA) was used during the HPLC analysis.

Materials. Narrow-leaf lupin seeds (*L. angustifolius* cv. Zeus) were obtained from the Plant Breeding Station Smolice, Przebędowo branch. All samples 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 stored in closed polyethylene bags at −18 °C until the analysis.

Protein Extractions. All samples were defatted using an automatic Soxhlet Büchi Extraction System B-811 (Büchi Labortechnik AG, Flawil, Switzerland). The extraction with *n*-hexane was carried out for 2 h. Extraction of lupin seed globulins was carried out using a method of Melo et al.[19] and Dooper et al.[20] with some modifications. Briefly, albumins were removed from defatted lupin flour using extraction with water, pH 8.0, containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ (34 mL/g of lupin flour) for 4 h. Then samples were centrifuged for 1 h at 20000*g*, and the pellet devoid of albumin was used for further extraction. Globulin extraction was performed by 4 h of elution using 0.1 M Tris-HCl, pH 7.5, buffer containing 10% NaCl (w/v) and NaN_3 0.05% (w/v) (34 mL/g of lupin flour). Then samples were centrifuged for 1 h at 20000*g*, and proteins were precipitated from the supernatant by adding ammonium sulfate (561 g/L). Salting was carried out for 30 min, and then samples were centrifuged for 20 min at 20000*g*. The precipitate containing salted globulins was resuspended in 50 mM Tris-HCl, pH 7.5, buffer (5.7 mL/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 a 0.45 μm PVDF syringe filter (Millipore) 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 stored at −18 °C until future analysis.

Protein Concentration Measurements. Protein concentration was determined by colorimetric Bradford method.[21] Analyses were carried out at wavelength λ = 595 nm (UV−Vis spectrophotometer SP 8001, Metertech Inc., Taipei, Taiwan). To determine protein concentration a standard curve based on bovine serum albumin (BSA) has been made.

Enzymatic Digestion of Lupin Globulins in Model System. Digestion of lupin globulins was carried out by applying the method proposed by Capraro et al.[22] for enzymatic treatments of γ-conglutin. Three different combinations of proteolytic enzymes were used: pepsin, pancreatin, and a combined treatment with pepsin and pancreatin in consecutive steps. Briefly, for pepsin digestion lupin globulins were resuspended in water to the final concentration of 1 mg/mL and adjusted to pH 2 with 0.1 M HCl, and the enzyme solution (1 mg/mL in water) was added in the ratio of 1:30 enzyme/lupin globulins (w/w). The sample was incubated for 1 h at 37 °C under gentle stirring. Reaction was stopped by changing the pH to 7.5 by adding 0.1 M $NaHCO_3$. For pancreatin treatment lupin globulins were resuspended in water to a final concentration of 1 mg of protein/mL and adjusted to pH 7.5 with 0.1 M $NaHCO_3$, and enzyme solution (1 mg/mL in water) was added in the ratio of 1:30 enzyme/lupin globulins (w/w). The sample was incubated for 1 h at 37 °C under gentle stirring. Reaction was stopped by changing the pH to 2 by adding 0.1 M HCl. Combined pepsin and pancreatin digestion was performed in such a way that first pepsin treatment sample was brought to pH 7.5 and then pancreatin was added.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Eletrophoresis (SDS-PAGE). SDS-PAGE of lupin globulins as well as products of their enzymatic hydrolysis was carried out according to the method proposed by Laemmli[23] using 12.5% polyacrylamide gel. Polypeptide bands were stained in Coomassie Brilliant Blue G-250 for 12 h. Relative molecular masses of protein were determined by comparison to molecular weight markers (Fermentas, Vilnius, Lithuania).

Size Exclusion Chromatography (SEC). SEC analyses of lupin globulins treated with pepsin and pancreatin as well as pepsin followed by pancreatin were carried out using high-performance liquid chromatography (HPLC; Waters 600 pump, Milford, MA) on a Shodex Protein KW-803 (8.0 × 300 mm) (Showa Denko, Japan) column, in isocratic conditions. The column was equilibrated and eluted with 50 mM Tris-HCl, pH 7.5, buffer. The flow rate was 1.5 mL/min. The injection volume was 25 μL, and the column temperature was maintained at 25 °C. Signal was monitored at 200−600 nm with the diode array detector (Waters 2998 PDA).

Liquid Chromatography−Mass Spectrometry Analyses (LC-ESI/MS^n). Sample for LC-ESI/MS^n analyses was prepared by separation of pepsin digested lupin globulins on reversed-phase (RP) HPLC (Waters 600 pump) on an XBridge BEH300 C18 (4.6 × 250 mm, 5 μm) (Waters) column, equilibrated with solvent A (H_2O containing 0.5% formic acid, v/v). Gradient elution was performed by adding solvent B (acetonitrile) up to 75% in 65 min. The flow rate was 1.0 mL/min. The injection volume was 25 μL, whereas the column temperature was maintained at 25 °C. Signal was monitored at 200−600 nm with the diode array detector (Waters 2998 PDA). A characteristic peak eluting at about 24 min was collected. This procedure was carried out five times, and obtained fractions were combined and lyophilized. For further LC-ESI/MS^n analyses the obtained lyophilisate was resuspended in methanol.

LC-ESI/MS^n analyses were performed using an Agilent 1100 HPLC instrument (Palo Alto, CA) combined with an ion trap (IT) mass spectrometer, model Esquire 3000 (Bruker Daltonics, Bremen, Germany) using an XBridge C18 column (2 × 150 mm, 3.5 μm) (Waters), at a 0.2 mL/min flow rate; the samples were injected through a 10 μL injection loop. The elution from the LC column was carried out using mixtures of two solvents: A (H_2O containing 0.5% formic acid, v/v) and B (acetonitrile). Gradient steps were as follows: 0−2 min isocratic at 0.5% B, 2−35 min linear gradient to 30% B, 35−50 min linear gradient up to 98% B, and 50−60 min isocratic at 98% B, followed by a return to the starting conditions and column equilibration steps. Flavonoid glycosides were identified by their mass and UV spectra and/or by their retention times compared with the data obtained previously.

The mass spectrometer was equipped with an electrospray ionization (ESI) source. The source parameters were as follows: ESI source voltage of 4 kV, nebulization with nitrogen at 25 psi, dry gas flow of 7.0 L/min at a temperature of 310 °C, skimmer 1 voltage of 12.4, collision energy set to 1 V and ramped within 40−200% of this value. Helium was used as collision gas. The instrument operated under EsquireControl version 5.1, and data were analyzed using the Data Analysis version 3.1 package delivered by Bruker.

Steady-State Fluorescence Measurements. Lupin globulins for fluorescence studies ware diluted to a concentration of 48 μg/mL with 50 mM Tris-HCl, pH 7.5, buffer. Vitexin was dissolved in the same buffer. The steady-state fluorescence of the samples was measured in the range from 425 to 590 nm with excitation wavelengths of 305 nm. The measurements were performed with a Shimadzu 5001 PC spectrofluorometer. The emission anisotropy was measured at 422 nm, with excitation wavelength of 305 nm, using appropriate instrument procedure for anisotropy calculation applying a Perkin-Elmer LS-55 fluorometer. The final anisotropy was the average of at least five consecutive measurements of fresh samples. All of the spectroscopic measurements were performed in a 1 × 1 cm quartz cuvette at 22 °C.

Fluorescence Lifetime Measurements. Fluorescence lifetimes 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

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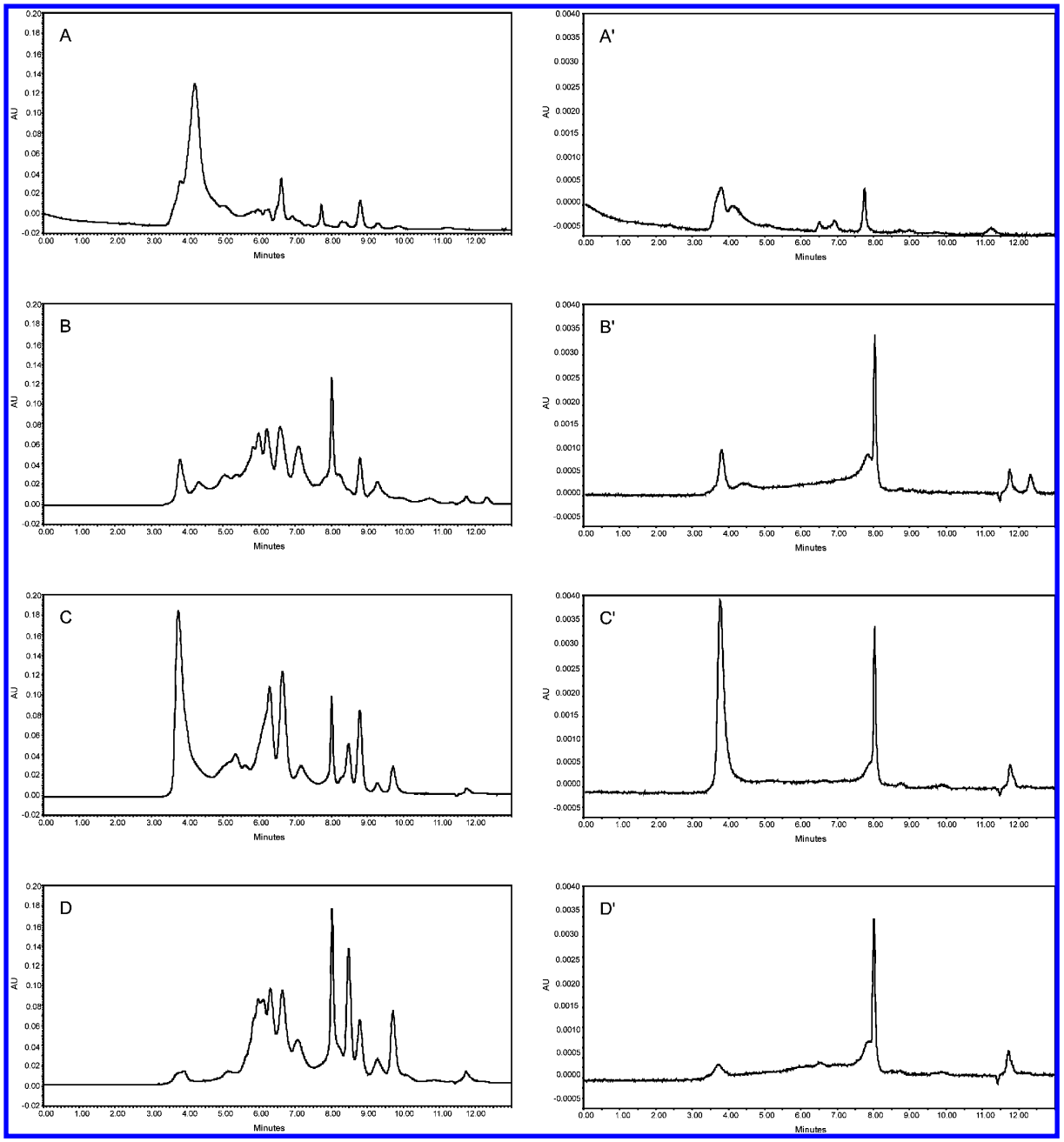

Figure 1. SEC chromatograms of lupin globulin undigested (A, detection wavelength 214 nm; A′, detection wavelength 335 nm) and digested with pepsin (B, detection wavelength 214 nm; B′, detection wavelength 335 nm), pancreatin (C, detection wavelength 214 nm; C′, detection wavelength 335 nm) as well as pepsin followed by pancreatin (D, detection wavelength 214 nm; D′, detection wavelength 335 nm).

ns was used as excitation source. The emission was measured with a PMA 182 detector head (PicoQuant). A 337 nm XL30 interference filter from Laser Components was used as excitation window to avoid leaking from the nitrogen spectrum. The data were analyzed by an exponential reconvolution method using a nonlinear least-squares fitting program. The goodness of fit was estimated by using χ^2 values.

Statistical Analysis. Results are presented as the mean ± standard deviation from three replicates of each experiment. A P value of <0.05 was used to denote significant differences between mean values determined by the analysis of variance (ANOVA) with the assistance of Statistica 8.0 (StatSoft, Inc., Tulsa, OK) software. The homogeneity of variance was checked by Levene's test. Homogeneous groups were determined by Tukey's post-hoc test. Analysis was performed with the assistance of Statistica 8.0 (StatSoft, Inc.) software.

RESULTS AND DISCUSSION

Characteristic of Lupin Globulins Digested in Model System. Results obtained during the SEC separation of native lupin seed globulins, as well as proteins digested with pepsin and pancreatin, are shown on Figure 1. Chromatograms were recorded at two different wavelengths: 214 nm as typical for detection of peptide bonds[24] and 335 nm as typical for detection of flavones (e.g., apigenin derivatives).[25,26]

The chromatogram of undigested lupin globulins was dominated by a peak of coeluting proteins characterized by high molecular weights (Figure 1A). Coelution due to a limited resolution of the chromatographic column caused the appearance of α-, β-, and γ-conglutins in this peak. In native form these proteins have molecular weights from 143 kDa for

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β-conglutin to 430 kDa for α-conglutin.[2] A peak characterized by the same retention time as observed for proteins with high molecular weights dominated the chromatogram (Figure 1A′) recorded at a wavelength typical for phenolic compounds (335 nm). This suggested the presence of flavonoids that might be connected with these proteins.

Chromatograms obtained for pepsin-treated lupin globulins provided information about the appearance of low molecular weight polypeptides being products of the digestion of seed proteins susceptible to this enzyme (Figure 1B). Digestion of proteins with pepsin caused a marked increase of the area of the peak observed at retention time (RT) close to 8 min on chromatograms recorded at λ = 335 nm (Figure 1B′; Table 1).

Table 1. Peak Area of the Samples Separated by SEC with Detection at a Wavelength of 335 nm[a]

sample	peak area	
	RT 3.7 min	RT 8.0 min
undigested lupin globulins	35070 ± 570 c	6277 ± 179 a
lupin globulins digested with pepsin	9693 ± 287 b	26908 ± 362 d
lupin globulins digested with pancreatin	52429 ± 793 d	18889 ± 258 b
lupin globulins digested with pepsin followed by pancreatin	4029 ± 264 a	25855 ± 353 c

[a]Retention time 3.7 min corresponds to undigested proteins; retention time 8.0 min corresponds to free flavonoids. Letters in a column show statistically significant differences at $P < 0.05$.

A simultaneous decrease of the peak area at RT about 3.7 min, corresponding to flavonoids bound to the globulins, was observed in this chromatogram (Table 1). These data allowed suggesting the release of flavonoids during the digestion of lupin globulins by pepsin.

A characteristic peak eluting at 3.7 min may be observed on chromatograms of pancreatin-digested lupin globulins (Figure 1C,C′). The peak remaining after digestion at RT 3.7 min may suggest the presence of γ-conglutin, which exhibits resistance to the digestion with pancreatin.[22] Native γ-conglutin is a glycosylated tetramer protein with a molecular weight of about 200 kDa.[2] An intensive peak recorded at λ = 335 nm (Figure 1C′) was observed at the same retention time as recorded for γ-conglutin in chromatogram C (Figure 1). The area of this peak was nearly twice as large as the area recorded for the undigested lupin globulins on chromatogram A′ (Figure 1; Table 1). Such a phenomenon suggests that γ-conglutin resistant to pancreatin may capture flavonoids released during the digestion of the other lupin globulins by this enzyme.

Chromatogram D (Figure 1) provides information that almost all globulins were digested by pepsin followed by pancreatin. Only one intense peak typical for released free flavonoids and eluting at about 8 min may be observed after this treatment (Figure 1D′; Table 1).

Information obtained from the SEC analyses concerning the susceptibility of lupin globulins to the proteolytic enzymes activities was confirmed by SDS-PAGE. Generally, lupin globulin proteins exposed to pepsin, as well as pepsin followed by pancreatin treatment, were totally digested and no generated peptides could be seen (lines B and D on Figure 2). Treatment of lupin globulins (Figure 2A) with pancreatin caused the hydrolysis of many proteins (Figure 2C). However, γ-conglutin turned out to be totally resistant to this enzyme activity, and two main bands of 29 and 18 kDa were observed, which

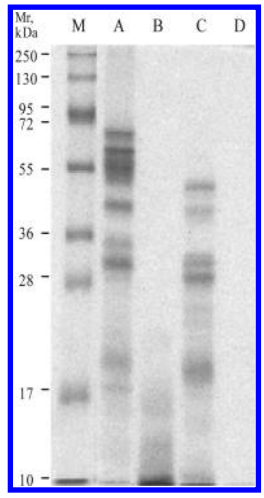

Figure 2. SDS-PAGE patterns of lupin globulins digested in different ways (A, undigested lupin globulins; B, lupin globulins digested with pepsin; C, lupin globulins digested with pancreatin; D, lupin globulins digested with pepsin followed by pancreatin). M, molecular weight marker.

corresponded to γ-conglutin large and small subunits, respectively. These results confirm the observation recorded by Capraro et al.,[22] concerning γ-conglutin resistance to pancreatin attack.

Liquid Chromatography−Mass Spectrometry Identification of Flavonoids. Digestion products obtained after treatment of lupin seed globulins with pepsin were submitted to RP-HPLC with the DAD at wavelengths of 214 and 335 nm corresponding to absorption of peptide bonds and polyphenolic compounds, respectively (Figure 3). The peak eluting at RT about 24 min had the UV spectrum shown in Figure 3 with absorbance maxima at λ = 216, 270, and 336 nm characteristic for *C*-glucosides of apigenin.[26] HPLC−mass spectrometry experiments (LC-ESI/MS^n) were performed to confirm the identity of the compound present in this peak. Samples for LC-ESI/MS^n were obtained by combining the studied peak from five consecutive RP-HPLC runs because the amount of compound was too small to obtain a good-quality multiple MS step spectrum. A single chromatographic peak was obtained as a result of LC-ESI/MS^n rechromatography of such an obtained sample. The MS spectrum of the compound present in this peak corresponded to the fragmentation pattern (Figure 4) of apigenin 7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside.[27]

Siger et al.[27] reported that apigenin glycosides are the main phenolic compounds that occur in lupin seeds (in detail 68.74 mg/100 g dm). One of these compounds, apigenin 7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside, has been identified in the current study as the main compound that is released during digestion of lupin seed proteins. Simultaneously, this compound occurs in lupin seeds at a concentration of approximately 40 mg/100 g dm.[27] The other compounds that appear in the chromatogram recorded at a 335 nm wavelength obtained by separation of pepsin digestion product of lupin seed proteins (Figure 3) can correspond to free apigenin aglycone. In the course of lupin development quantitative and qualitative changes in the composition of phenolic compounds can take place.[28] The presence of apigenin 7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside, which is able to bind lupin seed proteins, was not found after the germination of lupin seeds.[29]

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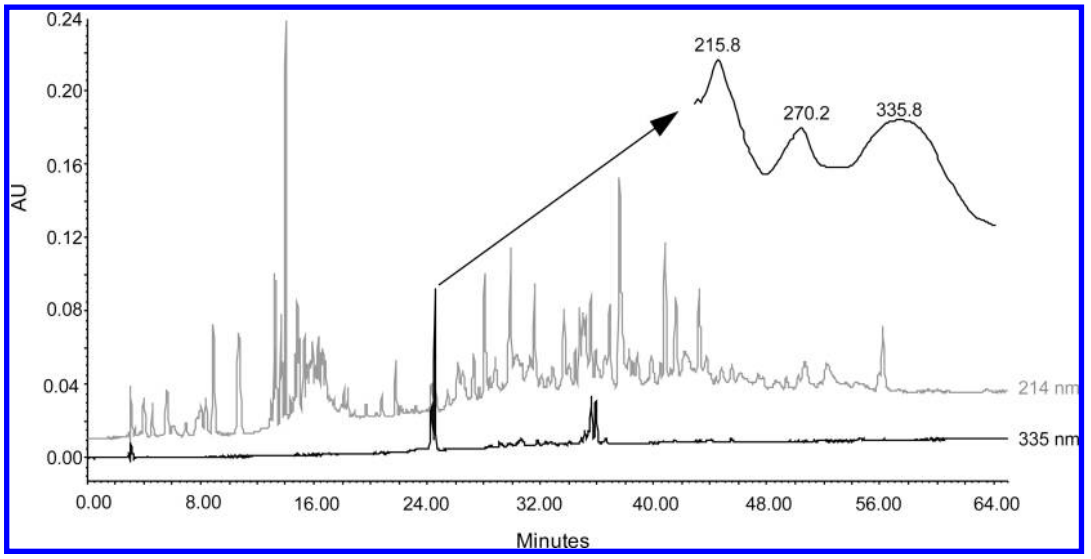

Figure 3. RP-HPLC chromatogram of lupin globulin digested by pepsin with detection at two wavelengths (214 nm, characteristic for peptide bond; 335 nm, characteristic for flavonoids) and characteristic UV spectrum of peak eluting about 24 min.

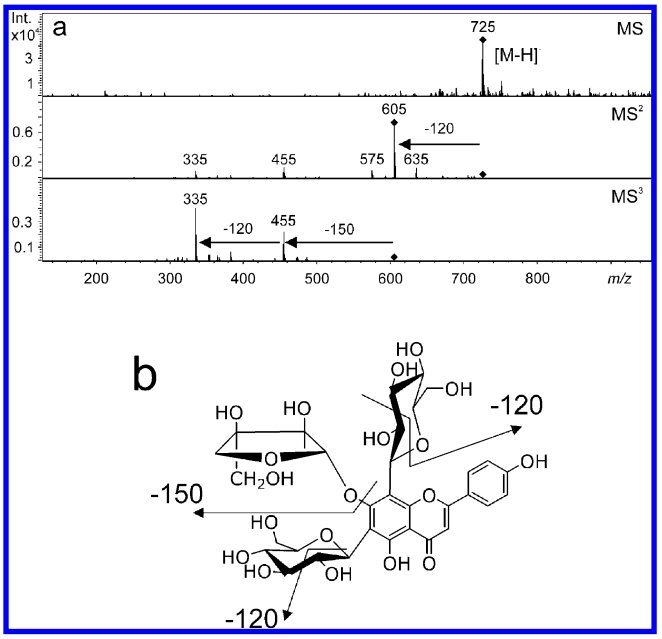

Figure 4. Mass spectrum (a) and fragmentation pathways (b) of apigenin 7-*O*-*β*-apiofuranosyl-6,8-di-*C*-*β*-glucopyranoside identified as the major phenolic compound released from lupin seed globulins during proteolysis with pepsin.

Phenolic acids, the presence of which in lupin seeds is widely demonstrated in the literature, were not detected during the digestion of lupin seed globulins. This may be due to the relatively low content of phenolic acids in lupin seeds, the average content of which does not exceed 10 mg/100 g.[13,27]

Flavonoid−Protein Interactions: Fluorescence Study. Fluorescence parameters (emission, lifetime, anisotropy) measurement is a useful technique for providing information on the molecular microenvironment and is often used in investigations of polyphenol−protein interactions.[16,30−33] Dominant flavonoids present in lupin seeds are apigenin glucosides: apigenin-6,8-di-*C*-*β*-glucopyranoside and apigenin 7-*O*-*β*-apiofuranosyl-6,8-di-*C*-*β*-glucopyranoside.[27] Those compounds are structurally closely related to vitexin (apigenin-8-*C*-*β*-glucopyranoside). All three substances mentioned above are glycosides of apigenin. The emission fluorescence spectra of vitexin (3−15 μM) excited at 305 nm in 50 mM Tris-HCl, pH 7.5, buffer are presented in Figure 5. Vitexin exhibited a weak

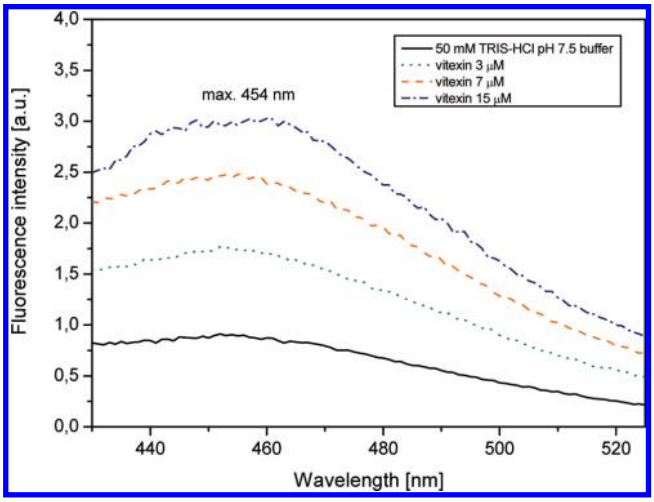

Figure 5. Fluorescence emission spectra of vitexin in 50 mM Tris-HCl, pH 7.5, buffer, excited at 305 nm.

fluorescence emission with maximum at about 454 nm (Figure 5). As the glucopyranose is a nonfluorescent compound, the observed fluorescence is caused by the phenolic moiety present in apigenin glycosides. Excitation at 305 nm of a sample containing undigested lupin globulins generated the fluorescence emission with maximum at about 438 nm (Figure 6). According to the literature data, excitation at 280 nm of a model protein (BSA) induced the emission of fluorescence in the range of 340−350 nm resulting from the presence of aromatic amino acids.[33,34] Therefore, emission observed at 438 nm in the case of lupin globulins presently investigated (Figure 6) came most probably from flavonoids bound to the protein. A slight red shift (from 438 to 444 nm) of the fluorescence emission maximum was observed (Figure 6) when the analyzed samples were digested with different proteolytic enzymes and the bound flavonoids were released. This effect probably came

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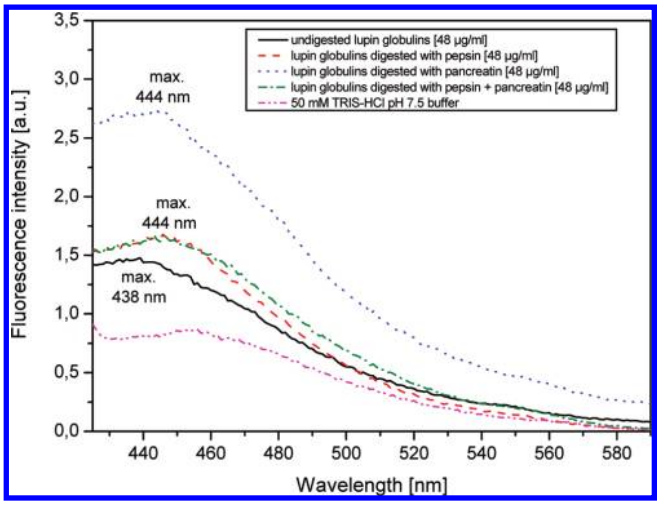

Figure 6. Fluorescence emission spectra of undigested and digested lupin globulins in 50 mM Tris-HCl, pH 7.5, buffer, excited at 305 nm.

from a change of microenvironment of flavonoids after this treatment. The reduced mobility of the flavonoid bound to the protein molecule usually resulted in enhanced fluorescence intensity or blue shift of fluorescence emission maximum. Such phenomena were observed by Sengupta and Sengupta,[35] when increased concentration of human serum albumin was added to the quercetin solution. In our study release of polyphenols from digested proteins resulted probably in increased mobility of the flavonoid molecule and change of the microenvironment. Those changes caused a blue shift of flavonoid emission maximum.

The measurement of steady-state fluorescence anisotropy (r) is a useful tool to study binding of flavonoids with proteins,[35] and it may provide more information about lupin globulin–flavonoid interaction during digestion. The fluorescence anisotropy value (r) of the undigested protein was 0.245 (Table 2). When the lupin globulins were digested with pepsin,

Table 2. Steady-State Fluorescence Anisotropy (r) and Fluorescence Lifetimes of Vitexin and Lupin Globulins Undigested/Digested with Different Enzymes in 50 mM Tris-HCl, pH 7.5, Buffer[a]

sample	anisotropy (r)	lifetime (ns)
vitexin (15 μM)	0.129 ± 0.025 a	
undigested lupin globulins (48 μg/mL)	0.245 ± 0.043 b	1.30
lupin globulins digested with pepsin (48 μg/mL)	0.185 ± 0.042 c	2.20
lupin globulins digested with pancreatin (48 μg/mL)	0.287 ± 0.070 d	1.20
lupin globulins digested with pepsin followed by pancreatin (48 μg/mL)	0.136 ± 0.020 a	3.65

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

the r value decreased to 0.185. The decrease of the anisotropy value was a result of an increase of motional freedom of flavonoids as a consequence their release during protein digestion. The anisotropy decreased to 0.136 when globulins were digested with pepsin and pancreatin (Table 2). This value was close to the anisotropy measured for vitexin standard in 50 mM Tris-HCl, pH 7.5, buffer (r = 0.129). Our measurements suggested that flavonoids were totally liberated after digestion of lupin globulins with both enzymes. Surprisingly, the highest anisotropy value was recorded in the samples of globulin digested with pancreatin (r = 0.287). Lupin glycoprotein γ-conglutin resistant to pancreatin[22] was the main protein present in these samples, which was confirmed by the SDS-PAGE. We suppose that flavonoids liberated from other proteins as a result of pancreatin-catalyzed digestion were bound by γ-conglutin during this process, which would explain the increase of the anisotropy value of proteins digested with pancreatin (0.287) above the level registered for the undigested lupin globulin (0.245; Table 2). Such phenomena may be possible due to electrostatic attraction between positively charged γ-conglutin[36] and partially ionized flavonoids (at pH 7.5). Another type of protein–flavonoid interaction may be hydrogen bonding between oligosaccharide chains of glycoproteins and the sugar moiety of flavonoid glycosides. However, determination of mechanisms of interaction between the γ-conglutin and native lupin polyphenols requires further research.

The release of flavonoids from lupin globulins during digestion manifests itself also in the changes of the lifetime of the polyphenol fluorescence. The fluorescence lifetime of flavonoids in undigested protein amounted to 1.30 ns (Table 2), and it increased after digestion of protein in the presence of pepsin as well as pepsin followed by pancreatin to 2.20 and 3.65 ns, respectively. A slight decrease of the emission lifetime (to 1.2 ns) was observed only in the case of samples digested with pancreatin without pepsin. Relatively short lifetimes observed in the undigested total globulins as well as in samples containing undigested lupin γ-conglutin suggested that flavonoids bound on these proteins surface were probably locally concentrated, which resulted in lifetime shortening by dynamic quenching.[37] Digestion of lupin protein by pepsin or pepsin and pancreatin caused the disappearance of these local concentration sites and the release of flavonoids. This phenomenon resulted in the loss of fluorescence dynamic quenching and emission lifetime extension.

To summarize, lupin seed globulins bind phenolic compounds present in seeds. Digestion of these proteins with pepsin and pepsin followed by pancreatin causes release of native flavonoids, which may be concluded on the basis of the HPLC-MS identification as well as fluorescence measurements. The most abundant compounds released from the digested proteins are apigenin glycosides (mainly 7-*O*-β-apiofuranosyl-6,8-di-*C*-β-glucopyranoside), which are also the major phenolic compounds present in seeds of different lupin species. The resistance of lupin glycoprotein γ-conglutin to digestion with pancreatin has been confirmed. We suppose that flavonoids liberated from other proteins as a result of pancreatin-catalyzed digestion are bound by γ-conglutin. Such a phenomenon may be possible due to electrostatic attraction between positively charged γ-conglutin and flavonoids partially ionized at pH 7.5. Another type of protein–flavonoid interactions may be hydrogen bonding between oligosaccharide chains of glycoproteins and the sugar moieties of the flavonoid glycosides. However, to establish the detailed mechanism of flavonoid–lupin globulin interactions, further studies are necessary. The results of the fluorescence lifetime measurements suggest that flavonoids bound to native lupin globulins as well as absorbed onto γ-conglutin surface during the pancreatin treatment of the sample form locally concentrated clusters.

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