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Differential Recovery of Lupin Proteins from the Gluten Matrix in Lupin−Wheat Bread As Revealed by Mass Spectrometry and Two-Dimensional Electrophoresis

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Supporting Information

ABSTRACT: Bread made from a mixture of wheat and lupin flour possesses a number of health benefits. The addition of lupin flour to wheat flour during breadmaking has major effects on bread properties. The present study investigated the lupin and wheat flour protein interactions during the breadmaking process including dough formation and baking by using proteomics research technologies including MS/MS to identify the proteins. Results revealed that qualitatively most proteins from both lupin and wheat flour remained unchanged after baking as per electrophoretic behavior, whereas some were incorporated into the bread gluten matrix and became unextractable. Most of the lupin α-conglutins could be readily extracted from the lupin−wheat bread even at low salt and nonreducing/nondenaturing extraction conditions. In contrast, most of the β-conglutins lost extractability, suggesting that they were trapped in the bread gluten matrix. The higher thermal stability of α-conglutins compared to β-conglutins is speculated to account for this difference.

KEYWORDS: lupin, wheat, bread, protein, interaction, extractability

INTRODUCTION

Use of plant-derived proteins is widely accepted as a way of meeting the demand for health requirements in food products. Lupin (*Lupinus* spp.) is a legume crop well adapted for a wide range of climates and soils and has been investigated for its health benefits in relation to its high protein and fiber contents.[1,2] It represents a relatively new health-food source, and its consumer awareness and acceptance are still low. One efficient way to bring lupin-associated health benefits to consumers is to add lupin flour to a widely consumed food product such as bread.

The main attribute of lupin is its high protein and dietary fiber as well as negligible amounts of starch content. It has been proven that bread enriched by lupin flour has the potential to provide health benefits, such as increased satiety and reduced energy intake,[2] decrease blood pressure,[1] and decreased blood glucose level.[3] The mixing of flour from different sources such as lupin and wheat has major effects on the properties of the final product.[4] Wheat gluten is required for breadmaking because it provides the basis for forming a network that has viscoelasticity and gas retention properties as dough is formed.[5,6] When wheat flour is supplemented with other flours rich in proteins, the added protein molecules interact with the wheat protein (gluten) network in both direct and indirect ways: the direct way is an interaction between added proteins and gluten proteins by cross-linking, and the indirect way is related to competition for water availability required by wheat gluten proteins.[7−9] During dough preparation and baking, different ingredients such as oxidizing agents, salts, and water that promote and affect the formation of protein cross-links[10] could also be affected by lupin flour addition. The interactions between soybean and wheat proteins in bread baking have been studied.[6,9] Results demonstrated that the addition of soybean flours to wheat flour at relatively low levels (approximately 5% by weight) has negative effects on bread quality attributes such as extensibility properties and gas retention, thus decreasing consumer acceptability.[6] Similarly, the addition of lupin flour to wheat flour breaks the connectivity of the wheat gluten matrix,[11] resulting in reduced consumer-level quality.[12] Some of these negative effects can be overcome by the addition of extra gluten during processing.[2]

It has been reported that adding soybean flour to wheat flour in breadmaking decreases the solubility of soybean protein as measured by its decreased extraction from soy−wheat bread.[7,9] The possible reason for this solubility change is that covalent cross-links (different disulfide bonds) are formed during baking. Some soybean proteins were also suggested to be bound into the bread matrix by noncovalent bonds. In a study on wheat protein changes during breadmaking, the decrease of protein extractability in the bread has been suggested to be due to protein cross-linking and/or aggregation.[13] It also suggested that disulfide bonds were mainly responsible for protein insolubility as a result of formation of very high molecular weight protein matrix in bread.

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The aim of the current study was to monitor the lupin and wheat flour protein complexes formed in breadmaking by using a combination of protein analysis techniques including intact protein analysis by MALDI-TOF and 2-D protein separation followed by MS/MS protein identification. A detailed analysis of protein from lupin−wheat bread was carried out to define the extractability of a wide range of proteins. The relationships of these properties to possible health attributes are discussed.

MATERIALS AND METHODS

Flour and Bread Samples. Lupin−wheat bread was prepared by substituting 40% of the wheat flour present in the normal wheat bread with lupin kernel flour. Extra gluten was added to the lupin−wheat bread to match the amount found in wheat flour.[2] Breads were baked as a single batch at Bodhi's Bakery, Fremantle, Western Australia, following the procedure used to prepare breads for studying health attributes of lupin-enriched bread.[2] The same batch of lupin and wheat flour samples was used in this study. The lupin flour was from a single cultivar, 'Kalya', of *Lupinus angustifolious*, and the wheat flour was a commercially available mix generally used to prepare normal wheat bread.

Protein Extraction. Under nonreducing and nondenaturing conditions, protein was extracted by using 0.5 M NaCl (pH 7.0) based on the techniques outlined in refs 14 and 15 During the extraction process, both the bread and flour samples were defatted by hexane at a 20:1 ratio.[16] The extraction buffer (0.5 M NaCl) was added to the flour and bread sample at the ratio of 15 mL/g, and the protein was extracted by stirring at 4 °C for 4 h. The extraction buffer for protein under reducing and denaturing conditions contained 8 M urea, 4% CHAPS, 60 mM DTT, and 2% (v/v) IPG buffer. The extraction buffer was added to the defatted flour and bread sample at the ratio of 20 mL/g, and protein was extracted at room temperature for 3 h.[17] In both cases the protein extract (supernatant) was collected by centrifugation at 12000*g* for 30 min.

For 2-D electrophoresis, all protein extracts were precipitated by incubation with ice-cold acetone at −20 °C for 16 h followed by centrifugation. The protein pellet was then washed with 10% ethanol and then with acetone containing β-mercaptoethanol (0.07%) to remove the additional salts. Ten milligrams of dried protein was dissolved in rehydration buffer containing 7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, and 2% IPG buffer for 5−6 h at room temperature. Protein concentration was determined by using an RC DC protein assay kit (Bio-Rad, Hercules, CA) and a Lambda 25 UV−vis spectrometer (PerkinElmer). For each sample, 1100 μg of protein was loaded onto IPG strips (Bio-Rad).

Intact Protein Analysis by MALDI-TOF. An overall survey of proteins from lupin−wheat bread, normal wheat bread, and lupin and wheat flours was conducted by using matrix-assisted laser desorption ionization time-of-flight mass spectrometry.[18] Protein extracted by 0.5 M NaCl was directly mixed with matrix (sinapic acid dissolved in 0.05% trifluoroacetic acid and 50% acetonitrile) at a 9:1 ratio. A 1 μL sample from the mixture was spotted on a MALDI-TOF plate and left at room temperature to dry. Spotting on the plate was repeated once, and the analysis was carried out on a Voyager DE PRO Biospectrometry workstation (PerSpective Biosystem), operated in linear mode. Each mass spectrum was the result of 500 laser shots on 10 random positions of the spot. The mass accuracy for linear mode was 1.01 ppm. The mass range was set to 5000−80000 Da. The machine was calibrated by using a Sequazyme Peptide Mass Standards Kit (Applied Biosystem) following sinapic acid matrix calibration mixture 3. The results from MALDI-TOF were analyzed using the Voyager machine companion software, Data Explorer, to produce the protein spectrum profiles.

Two-Dimensional Gel Electrophoresis. Isoelectric focusing (IEF) was conducted on 17 cm IPG strips with pH 3−10. The strips were rehydrated with buffer (7 M urea, 2 M thiourea, 2% CHAPS, 65 mM DTT, and 2% IPG buffer) containing 1100 μg of protein for 12 h. Strips were focused at 60000 Vh, with a maximum of 10000 V, at 20 °C using a Protein IEF cell (Bio-Rad). Before running SDS-PAGE, the strips were equilibrated with 50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% bromophenol blue containing 65 mM DTT for 15 min and for another 10 min by substituting DTT with 135 mM iodoacetamide in the same buffer.

Protein separation was carried out on 12% acrylamide/bis (31.5:1) gels, using a Protean II Xi cell (Bio-Rad). The running buffer consisted of 2.5 mM Tris-Base, 19.2 mM glycine, and 0.01% SDS. The gels were stained by Coomassie Brilliant Blue (CBB). Protein standards (Bio-Rad) were used to estimate the molecular size of the proteins. To minimize experimental variability, all samples were run three times with individual extraction and IEF.

The gels were analyzed by a 2-D Proteomic Imaging System (PerkinElmer) using ProScan 4.0 software. The digital gel maps of different samples were analyzed and compared by using Progenesis Same Spots software (Nonlinear Dynamics). Master gels were generated for each sample by matching all of the available gels. Normalization was carried out by determining the gain factor for each sample, which can be modeled as $y_i/y'_i = 1/\alpha_k$, where y_i is the measured abundance of feature i on sample k, $1/\alpha_k$ is the gain factor for sample k, and y'_i is the normalized abundance of feature i on sample k.[19]

Protein Identification by MS/MS. Protein spots of interest were excised from CBB-stained 2-D gels and were identified by mass spectrometric peptide sequencing. To avoid the overlapping parts of closely related spots, the center portion of each spot was sampled. The spots were analyzed by Proteomics International Ltd. Pty, UWA, Perth, Australia. Protein samples were trypsin digested, and the resulting peptides were extracted according to standard techniques.[20] Peptides were analyzed by electrospray ionization mass spectrometry using the Ultimate 3000 nano HPLC system (Dionex) coupled to a 4000 Q TRAP mass spectrometer (Applied Biosystems). Tryptic peptides were loaded onto a C18 PepMap100, 3 μm (LC Packings), and separated with a linear gradient of water/acetonitrile/0.1% formic acid (v/v).

Spectra were analyzed to identify proteins of interest using Mascot sequence matching software (Matrix Science) with taxonomy set to Viridiplantae (see the Supporting Information). All searches used Ludwig NR. The software was set to allow one missed cleavage and mass tolerances of ±1.2 Da for peptides and ±0.6 Da for fragment ions. The peptide charges were set at 1+, 2+, and 3+, and the significance threshold was set at $P < 0.05$. Generally a match was accepted when two or more peptides from the same protein were present in a protein entry in the Viridiplantae database.

RESULTS

Two-Dimensional Gel Electrophoresis of Protein Extracted after Reducing and Denaturing. The solubilization of wheat proteins in the reducing/denaturing conditions indicated that proteins in 70−100 kDa (p*I* 6−8), 32−37 kDa (p*I* 6.5−8.0), 20−24 kDa (p*I* 6.5−7.0), and 10−15 kDa (p*I* 6.5−7.0) ranges were relatively unaffected by the baking process (Figure 1A,B). This allowed the identity of proteins in the baked wheat bread to be assigned on the basis of their electrophoretic mobility and compared to the flour (Figure 1A,D). The comparison of normal wheat bread and lupin−wheat bread indicated that specific high and low molecular weight wheat proteins that characterize important features of bread could be extracted from the lupin−wheat bread under denaturing plus reducing conditions (Figure 1B,E).

In the case of lupin proteins, a wide range of proteins (10−100 kDa, p*I* 5−8.5) were found not to be extracted from

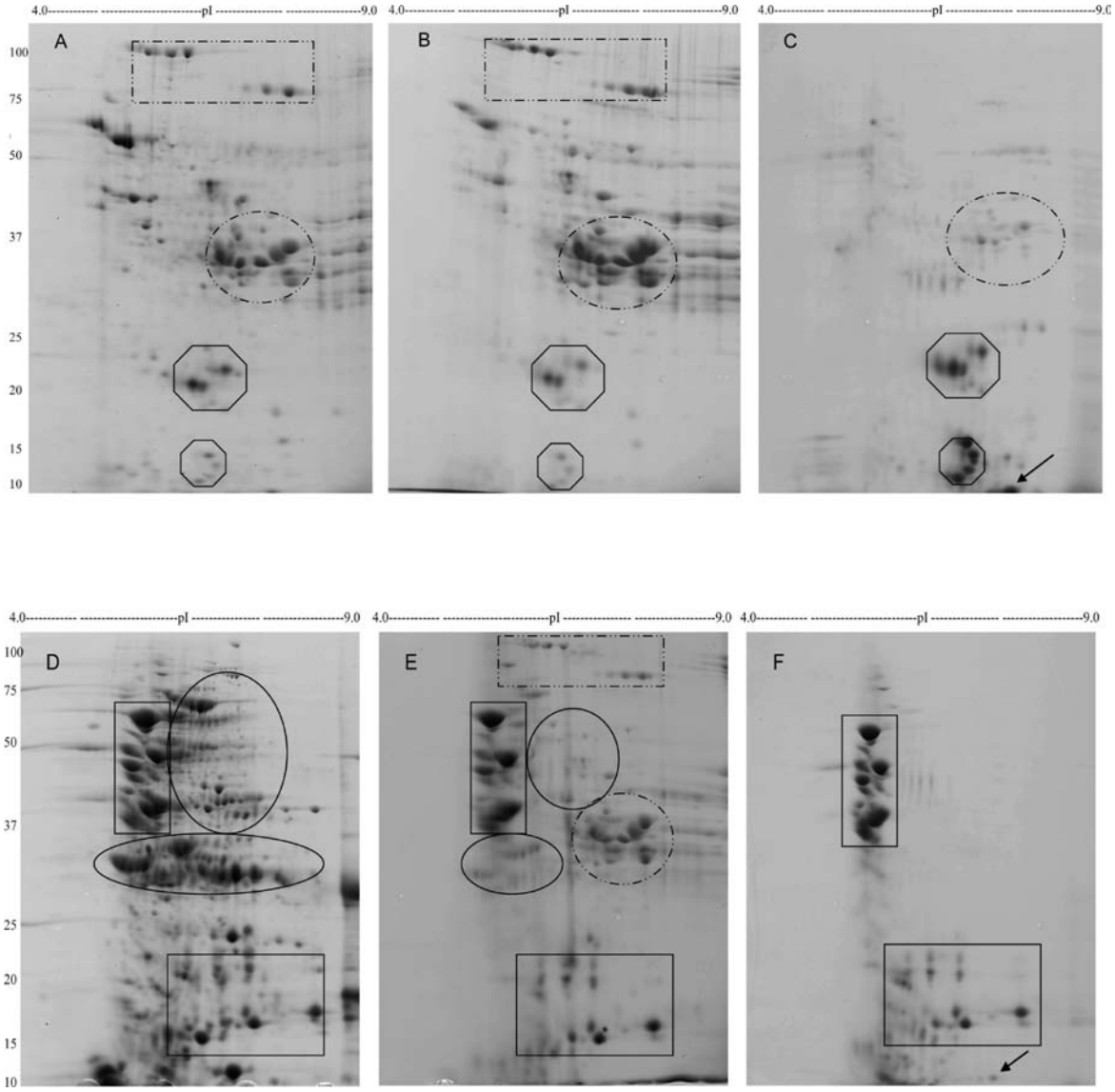


Figure 1. Protein profiles of lupin and wheat flours, lupin−wheat bread, and normal wheat bread by 2-D gel electrophoresis: (A) wheat flour with reduction and denaturing; (B) wheat bread with reduction and denaturing; (C) wheat bread without reduction and denaturing; (D) lupin flour with reduction and denaturing; (E) lupin−wheat bread with reduction and denaturing; (F) lupin−wheat bread without reduction and denaturing. Proteins enclosed by rectangles with continuous outlines indicate the α-conglutins. Proteins enclosed by circles with continuous outlines indicate the *β*-conglutins. Proteins enclosed by rectangles with dotted outlines indicate the HMW glutens of wheat. Proteins enclosed by circles with dotted outlines indicate the wheat gliadins. Proteins enclosed by octagons are LMW wheat proteins. The lines and numbers on the top of each gel indicate the pH values, and the numbers on the left of gels indicate molecular weights of the proteins in kDa. The arrow marks the only wheat protein extracted from lupin−wheat bread with nonreducing and nondenaturing buffer.

the lupin−wheat bread. The largest group of proteins in this class was in the molecular mass range 27−33 kDa (p*I* 5−8) (Figure 1D,E). In addition, some lupin proteins in the 37−85 kDa range (p*I* 6−7.5) were not extracted from lupin−-wheat bread. Most of these proteins were *β*-conglutins as identified by mass spectrometry (Table 1; Figure 2) and comparisons to previous studies in *L. angustifolious*.[17,21]

A group of proteins from lupin (molecular weight ranges 35−70 kDa, p*I* 5−6) was clearly extracted from lupin−wheat bread (Figure 1D,E). This group of lupin proteins plus the entities in the molecular weight range 15−22 kDa (p*I* 6−8) were identified as α-conglutin (Table 1; Figure 2). In addition, a few proteins corresponding to the *β*-conglutin with molecular mass ranges of 32−37 kDa (p*I* 4.5−5.5) and 45−60 kDa (p*I* 5.5−7) could also be extracted from lupin−wheat bread (Figure 1D,E). At least three *γ*-conglutins (Table 1; Figure 2) were extracted from lupin−wheat bread.

The qualitative difference between the 2-D gels (Figure 1D,E) indicating that the α-conglutins group of proteins were relatively more prominent in the protein complement extracted from bread relative to flour could be quantitated using standard software Progenesis Samespot. In Table 2 the output confirmed that the α-conglutin group at 35−70 kDa was relatively more prominent in the protein from lupin−wheat bread compared to lupin flour.

Two-Dimensional Gel Electrophoresis of Protein Extracted without Reducing and Denaturing (0.5 M NaCl Extraction). The extent of binding within the bread matrix was examined

Table 1. Identification of the Lupin and Wheat Proteins from Two-Dimensional Gels by Mass Spectrometry

origin of protein	extractability from lupin−wheat bread	spot numbers[a]	protein identification by MS/MS[b]	matching NCBInr accession (GI)
lupin	extracted at both reducing/ denaturing and nonreducing/ nondenaturing conditions	1	not clearly identified; holds the peptide TLTSLDFPILR, which is part of α-conglutin	
		23[a+], 31[a+], 36[a+], 37[a+]	α-conglutin	2313076
		11[a], 12[c], 15[c], 22[a], 30[c], 34[b], 35[b]	α-conglutin (legumin-like seed storage protein)	224184735
		24[c], 40[c]	α-conglutin (legumin-like protein)	85361412
		2[b], 39[a+]	β-conglutin	149208401
		13[b], 38[b]	γ-conglutin	662366
	extracted at reducing/ denaturing conditions	29[b]	α-conglutin	2313076
		3[b], 9[b], 16[c]	β- conglutin	149208401
		6[a+], 7[b], 8[b], 32[b]	β-conglutin	169950562
		27[a+]	β-conglutin	149208403
		25[b]	γ-conglutin	662366
	not extracted	4[b], 5[b], 50[a]	β-conglutin	149208403
		10[b]	β-conglutin	46451223
		33[b]	β-conglutin	169950562
		26[a+]	glyceraldehyde-3 phospahte dehydrogenase	62816190
		14[b]	δ-conglutin	116181
		28[c]	γ-conglutin	662366
wheat	extracted at reducing/ denaturing conditions	18[b]	HMW glutenin	269849175
		42[a]	HMW glutenin	162415983
		19[c]	γ-gliadin	209971935
		43[c]	α-gliadin	282721196
		45[a]	glyceraldehyde-3-phosphate dehydrogenase	148508784
		48[a+]	β-amylase	32400764
		21, 44, 46, 47	not identified	N/A
	not extracted	20[b]	superoxide dismutase	1654387

[a] Proteins were obtained from 2-D gels as shown in Figure 2. Spots from all protein groups significant to lupin−wheat bread were selected. Sequence coverage: a+, >30%; a, 20−29%; b, 10−19%; c, 4−9%. [b] β-Conglutin and α-conglutin are sometimes described as vicilin-like proteins and legumin-like proteins, respectively, but we have used β-conglutin and α-conglutin consistently to avoid confusion.

using a milder extraction procedure (0.5 M NaCl). As expected under these conditions, the HMW glutenin subunit proteins were not extracted (Figure 1C) from wheat bread because it is well-known that these proteins are bound within the bread gluten matrix by disulfide bonding (via the cysteine amino acids). In contrast, the wheat gliadins (32−37 kDa molecular weight range with p*I* 6.5−8.0) and low molecular weight protein groups (20−24 kDa molecular weight range with p*I* 6.5−7.0 and 10−15 kDa molecular weight range with p*I* 6.5−7.0) were extracted under this condition (Figure 1C). In the lupin−wheat bread the above-mentioned groups of wheat protein all became unextractable (Figure 1C,F). The only indication of a wheat protein being extractable came from the presence of the faint spot at 10 kDa, p*I* 7.5 (Figure 1C,F).

In the case of lupin proteins, all of the β-conglutins became unextractable in lupin−wheat bread (Figure 1D,F) using 0.5 M NaCl. In contrast, most α-conglutins (molecular weight range 35−70 kDa at p*I* 5−6; molecular weight range 15−22 kDa at p*I* 6−8) and two γ-conglutins appeared as extractable as observed for the reducing and denaturing conditions (Figure 1F; Table 1).

Two-Dimensional Gel Protein Identification by Mass Spectrometry (MS/MS). The mass spectrometric peptide sequence of the proteins of interest from the 2-D gel provided a successful identification of the proteins (Table 1). In total, 38 lupin proteins and 11 wheat proteins representing the major protein groups of lupin−wheat bread were studied. Most of the proteins (45 of 49) were matched positively with respective proteins in the database. Some protein spots such as 2 and 39 (Figure 2) were reported as α-conglutin previously, but in our identification they were more closely matched with β-conglutins. Moreover, one very dominant lupin protein, shown as spot 1 in Figure 2, was not clearly identified even though it was claimed to be an α-conglutin.[21] The peptide sequence of this protein from both the lupin flour and lupin−wheat bread gels provided 11 common peptides (Table 3), which could not be assigned to a known conglutin protein. In contrast, another protein (spot 22) (Figure 1) could be clearly identified as α-conglutin on the basis of the common peptides (Table 3) of protein sequences from lupin flour and lupin−wheat bread gels. Most of the β- and γ-conglutins showed relatively higher

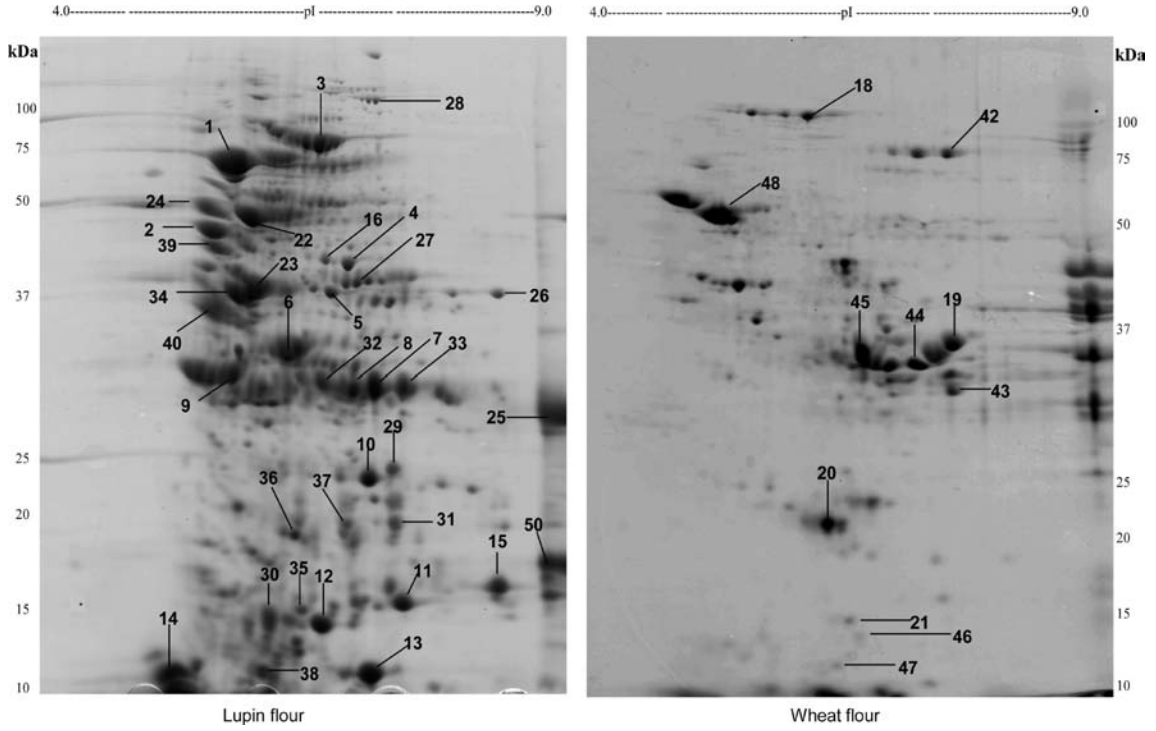
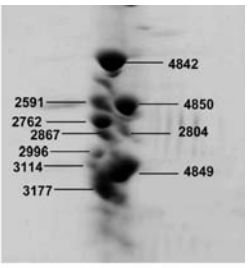


Figure 2. Two-dimensional gel map of lupin and wheat flour showing the protein spots having significance in lupin−wheat bread, which were identified by mass spectrometry.

Table 2. Differential Protein Intensity of the α-Conglutin Group (35−70 kDa) between Lupin Flour and Lupin−Wheat Bread That Quantitated the Qualitative Difference between the Two-Dimensional Gels

Spot number	Anova (p)	Average Normalised volumes (x10^6) Lupin-wheat bread	Lupin flour
2804	0.003	13.49	2.08
2996	0.017	14.16	5.25
3114	0.024	25.19	10.25
4849	0.026	102.70	38.27
3177	0.031	86.36	29.51
4842	0.040	78.13	33.28
2867	0.045	17.66	9.39
2591	0.065	41.46	20.12
2762	0.089	40.98	22.62
4850	0.214	73.42	43.70



sequence coverage of peptides to known corresponding proteins compared to α-conglutin due to the lack of information regarding this group of proteins in the database.

Direct Mass Spectrometric (MALDI-TOF) Survey of Protein Changes during Bread Baking. In the present research work a survey of protein modification was carried out using mass spectrometry (MALDI-TOF) for a higher resolution study of the low molecular weight proteins using nonreducing and nondenaturing (0.5 M NaCl) extraction. Many of the lupin and wheat proteins extracted from flour could be identified in the extracts from lupin−wheat bread (Figure 3A). A group of proteins in the molecular weight range of 13−16 kDa were very similar in the lupin flour and lupin−wheat bread extractions (Figure 3A) and may correspond to the small subunit of α-conglutin based on the comparison of 2-D gels and MALDI-TOF profiles of the samples, but further investigation of this possibility is required. Another group of proteins in the molecular weight range of 20−22 kDa was extracted from the lupin−wheat bread and were the same as those found in lupin flour, perhaps corresponding to the basic subunit of α-conglutin.[14,21]

At least five lupin proteins (at the molecular mass range of 7−9 kDa) corresponding to the large subunit of δ-conglutin[14] were not extracted from the lupin−wheat bread (Figure 3B). Similarly, two lupin proteins (24300 and 25860 Da) corresponding to the intermediate molecular weight subunit of β-conglutin[14] were not extracted from the lupin−wheat bread. In total, 14 of 24 lupin proteins became extractable from lupin−-wheat bread (Table 4). In the case of wheat protein, 25 of 38 lupin proteins (Table 4; Figure 3A) were found to be extractable from the lupin−wheat bread.

In addition, some new protein entities were identified (Figure 3C; Table 4). Proteins with molecular weights of 39030, 39230 (Figure 3C), and 19760 Da (Table 4) were present only in the lupin−wheat bread and could not be identified in either lupin flour, wheat flour, or wheat bread.

Table 3. Common Peptides As Identified by MS/MS Peptide Sequencing among the Same Proteins from Lupin Flour and Lupin−Wheat Bread

common peptides[a] of protein spot 1 (see Figure 2) from lupin flour and the corresponding spot from lupin−wheat bread	common peptides of protein spot 22 (see Figure 2) from lupin flour and the corresponding spot from lupin−wheat bread
AGPVR	AGMPK
ASLKVGEEEEEEEAGDGR	FYLAGNPEEEYPETQQQR
CAGQGR	GDEGQEEEETTTTTEER
CGAKVEFK	GGHEEEEVEEER
EQLATFR	GGHEEEEVEEERGR
GISILRR	GGKDH
IRNQEEFEQEIGR	GKPSESGPFNLR
KPSSPK	GSVVLSERGDGAAVPR
KYETTEQGR	HTRGDEGQEEEETTTTTEER
NKMSVIPYASAIGSIMYAMLCTR	IVEFQSNPNTLILPK
XEEXR	KGKPSESGPFNLR
	KITMPSSTQGFTY
	LLGFGINANENQR
	NFLAGSEDNVIR
	NNILSGFDPQFLSQALNIDEDTVHK
	NTLEATFNTR
	NTLEATFNTRYEEIQR
	QIIRVEEGLGVISPK
	QRVDTYWDLLSPK
	RFYLAGNPEEEYPETQQQR
	RGQEQSYQDEGVIVR
	TNRLENLQNYR
	VEEGLGVISPK
	YQAMKAGPDGEVVSLR

[a] These peptides could not be assigned to a known conglutin protein.

DISCUSSION

Recent papers have demonstrated that specific lupin protein bioactivities provide some health attributes. For example, γ-conglutin is capable of interacting with the mammalian protein hormone insulin and has effects in lowering blood glucose.[22] The other reported bioactivities of lupin protein include plasma cholesterol and triglyceride lowering effects,[23] antihypertensive properties,[24] and angiotensin converting enzyme (ACE) inhibitory activity.[25] Diversity in extractability of different conglutins (lupin proteins) from lupin−wheat bread as reported in the present study could account for some of the health attributes of these novel breads.

Two-dimensional gel electrophoresis was used as the primary basis for the protein analysis in this study. This allowed the broad range of intact proteins to be assayed and their extractability, as affected by baking, to be examined.[17,21,26] The MALDI-TOF-based analyses provided a high-resolution analysis of the lower molecular weight proteins.[18,27] The results showed that many of the proteins from both lupin and wheat remained qualitatively unchanged (judged by their electrophoretic behavior) in baked lupin−wheat bread, whereas others, such as the β-conglutins, were incorporated into the bread matrix and could not be extracted.

Decrease of Protein Extractability Due to Baking. The demonstrated decrease of both the lupin and wheat protein extractability caused by baking relates to the high temperatures during baking and the presence of a range of ingredients that would enhance the interaction and formation of protein linkages into a matrix.[28] Up to 80% loss of protein extractability due to baking in wheat bread has been reported.[13] Disulfide bonds were considered to be the major basis of the protein−protein interaction in baking and associated loss of extractability [13] The lack of extraction of β-conglutins from bread even under reducing and denaturing conditions indicates that covalent bonds other than disulfide bonds are involved in linking these proteins into the very high molecular weight protein matrix. For example, the reduction of protein extractability could result from tyrosine cross-linking among the proteins.[29] It has also been noted that lupin−wheat heteropolymer formation could be catalyzed by transglutaminase.[8] Protein binding to starch, as reported for soy protein interaction,[30] could also alter the extractability of the β-conglutins.

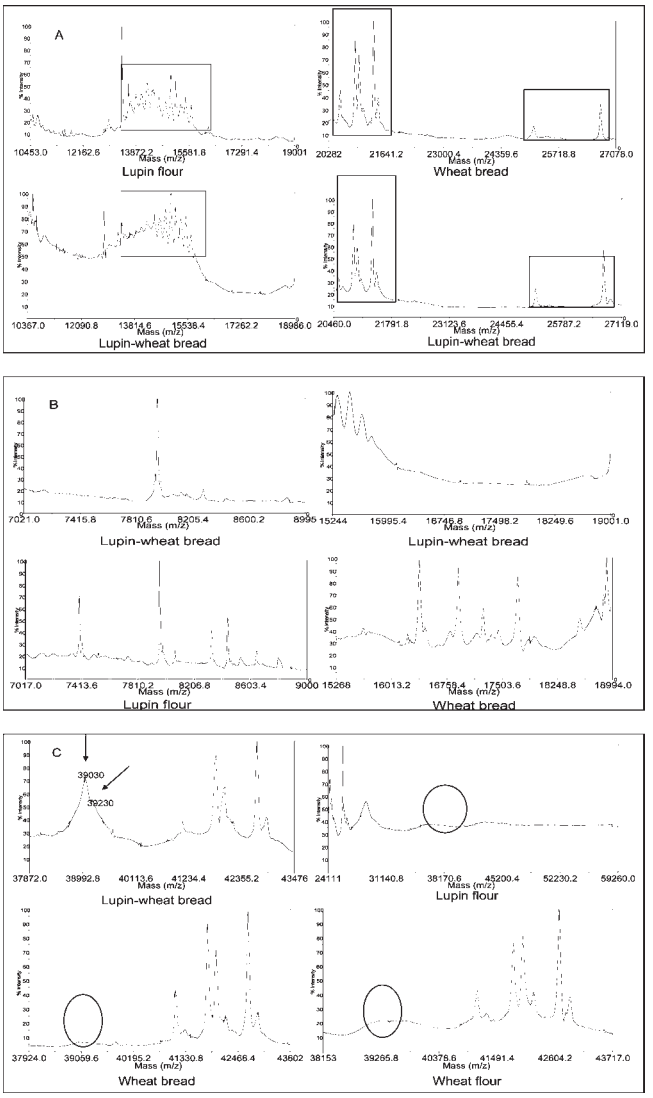


Figure 3. MALDI-TOF protein profiles of nonreduced and nondenatured (0.5 M NaCl) extracts showing different extractabilities of proteins from lupin−wheat bread: (A) boxes show some readily extractable lupin and wheat proteins from the lupin−wheat bread; (B) part of lupin and wheat proteins that could not be extracted from lupin−wheat bread; (C) proteins indicated by arrows are unique to lupin−wheat bread and could not be identified in lupin or wheat flour or wheat bread (as the corresponding molecular weight range shown by the circles).

Lupin and Wheat Protein Interaction. The current study revealed lupin and wheat protein interaction during the baking of lupin−wheat bread. The added proteins from lupin flours have been suggested to interact with the wheat gluten network by cross-linking.[4,6] In studies of the gluten network in soy−wheat breads, the soy protein retained in this network was held by strong association among proteins.[7]

Table 4. Comparative List of Proteins Identified by Direct Mass Spectrometry (MALDI-TOF) from Lupin−Wheat Bread, Normal Wheat Bread, and Lupin Flour following Nonreducing and Nondenaturing (0.5 M NaCl) Extraction[a]

lupin−wheat bread	wheat bread	lupin flour	lupin−wheat bread	wheat bread	lupin flour
–	51430	–	19550	19550	–
–	50180	–	19400	19408	19400
–	49000	–	17240	17240	–
42880	42880	–	–	17716	–
42690	42690	–	–	17247	–
42203	42203	–	–	16913	–
41990	41990	–	–	16387	–
41810	41810	–	15600	–	15600
41300	41300	–	15400	–	15400
41110	41110	–	15300	–	15300
39030	–	–	15100	–	15100
39230	–	–	14900	–	14900
33940	33940	–	14800	–	14800
31270	31270		14600	–	14600
–	–	28000	13417	–	13417
26900	26900	–	–	11596	–
26710	26710	–	–	–	8645
–	–	25860	–	–	8535
25130	25130	–	–	–	8442
–	–	24300	–	–	8329
21450	21450	–	–	–	8070
21349	21349	21300	8290	8290	–
–	21100	–	7960	–	7960
21000	21000	21000	–	–	7396
20900	20900	20900	–	7367	–
–	–	20845	–	7174	–
20560	20560	–	–	7032	–
20020	20020	–	–	6992	–
19880	19880	–	6600	–	6600
19760	–	–	5363	5363	–
19710	19710	–			

[a] Proteins are presented by their molecular weight in Da; − indicates the absence of protein in the corresponding sample.

The wheat proteins extracted from wheat bread indicate that they were unaffected by baking process and were expected to be extractable from lupin−wheat bread. However, a number of wheat proteins observed in wheat bread became unextractable from lupin−wheat bread. This observation indicates the occurrence of interactions of lupin protein with certain wheat proteins to produce a new polymer of high molecular weight that has a low extractability. In addition, some unchanged (judged by their electrophoretic behavior) proteins from lupin flour, wheat bread, and lupin−wheat bread indicated that not all proteins are involved in such interactions. Some soy proteins have been reported not to interact with wheat protein in bread baking,[7] which supports our observation.

Analysis of the low molecular weight proteins using MALDI-TOF showed 10 lupin proteins extracted from lupin flour could not be extracted from lupin−wheat bread and hence were deduced to be embedded in the bread matrix. In contrast, three new peaks appeared in lupin−wheat bread and are speculated to be new additions of either lupin−lupin or lupin−wheat protein interaction and are currently under further investigation.

Two Subgroups of Conglutins. In this study the two major subgroups of conglutins showed very different solubilities after the baking process and were identified as α-conglutin and β-conglutin by mass spectrometry. Under both reducing/denaturing and nonreducing/nondenaturing conditions most of the β-conglutins were not extracted from the lupin−wheat bread. These findings indicate that this subgroup of proteins is very sensitive to the baking process and are incorporated into a very high molecular weight matrix. The loss of extractability of β-conglutins from lupin−wheat bread suggested that they may not be available for generating an allergic response. However, the fact that most of the β-conglutins have been reported as allergenic proteins[17] indicates that the degree of binding into the bread matrix may not necessarily relate to allergenicity. It is, however, possible that the level of extraction of protein into a range of different buffers may provide a guide to their biological availability with respect to food attributes. For example, the easily extracted α-conglutins and one γ-conglutin are predicted to be more accessible for biological effects associated with the consumption of lupin−wheat products.

On the basis of thermal denaturation experiments, it has been suggested that α-conglutins (legumin-like) proteins were significantly more stable to denaturation than β-conglutins (vicillin-like) protein.[21] The structural features of these protein classes, which may therefore account for differences in extractability, could relate to differences in thermal stability. The high temperature during breadmaking could denature the vicillin-like protein β-conglutins more readily and provide greater opportunities for incorporation into the complex bread matrix. In contrast, the more thermally stable legumin-like α-conglutins would lead to greater retention of a folded structure and reduced exposure of internal amino acids to cross-linking and a relative independence from the lupin−wheat bread matrix. The relative independence of the legumin-like α-conglutins from the bread matrix would be consistent with being able to extract this protein class under mild conditions. Alternatively, some (currently unknown) feature of the amino acid sequences of α- and β-conglutins could result in large differences in proteolytic degradation during the breadmaking process.

Mass Spectrometric Identification of Lupin−Wheat Bread Proteins. Peptide sequencing of proteins by MS/MS showed positive identification for most of the β-, γ-, and δ-conglutins and a number of α-conglutins. Sequences of α-conglutins are still lacking in the database, and a single low molecular weight (131 amino acids; NCBInr 2313076) entity is available. As a result, additional information based on EST sequences was used for the identification of the α-conglutins. Four protein spots located in the predominantly α-conglutin region (Figure 1D; molecular weight range 35−70 kDa) were matched to legumin-like (NCBInr 85361412) and seed storage (NCBInr 224184735) proteins of lupin.

As expected, mass spectrometric peptide sequence of the same proteins from lupin flour and lupin−wheat bread provided a number of common peptides (Table 3) and confirmed the identification of the proteins. One of the most striking α-conglutins (spot 1) (Figure 2) showed 11 common peptides with its corresponding protein spot from lupin−wheat bread. However, there was no match of these peptides in the databases examined, which suggests the necessity for further investigation.

Most of the wheat proteins (including HMW glutenins and gliadins) extractable from lupin−wheat bread were identified,

although one group of wheat proteins (at 10−15 kDa molecular weight range with 6.5−7.0 p*I*) could not be identified due to the lack of sequence information in the database.

Among the lupin proteins, most of the α-conglutins were not affected by the baking process and could be readily extracted from the lupin−wheat bread even at nonreducing conditions. In contrast, most of the *β*-conglutins appeared to undergo interactions in baking that resulted in loss of extractability, which is speculated to be due to cross-linking and/or aggregation in the formation of large protein polymer. The higher thermal stability of α-conglutins relative to *β*-conglutins was suggested as one possible contribution to the distinct properties of these proteins because the unfolding of *β*-conglutins at lower temperature would allow more of the molecules to participate in network reactions.

■ ASSOCIATED CONTENT

Supporting Information. Details of the protein identification using mass spectrometric peptide sequence by matching with database. This material is available free of charge via the Internet at http://pubs.acs.org.

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■ ABBREVIATIONS USED

CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; EST, expressed sequence tag; IEF, isoelectric focusing; IPG, immobilized pH gradient; MALDI-TOF, matrix-assisted laser desorption/ionization mass spectrometry time-of-flight; NCBI, National Center for Biotechnology Information; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

■ REFERENCES

(1) Lee, Y. P.; Mori, T. A.; Puddey, I. B.; Sipsas, S.; Ackland, T. R.; Beilin, L. J.; Hodgson, J. M. Effects of lupin kernel flour-enriched bread on blood pressure: a controlled intervention study. *Am. J. Clin. Nutr.* **2009**, *89*, 766–772.

(2) Lee, Y. P.; Mori, T. A.; Sipsas, S.; Barden, A.; Puddey, I. B.; Burke, V.; Hall, R. S.; Hodgson, J. M. Lupin-enriched bread increases satiety and reduces energy intake acutely. *Am. J. Clin. Nutr.* **2006**, *84*, 975–980.

(3) Hall, R. S.; Thomas, S. J.; Johnson, S. K. Australian sweet lupin flour addition reduces the glycaemic index of a white bread breakfast without affecting palatability in healthy human volunteers. *Asia Pac. J. Clin. Nutr.* **2005**, *14*, 91–97.

(4) Doxastakis, G.; Zafiriadis, I.; Irakli, M.; Marlani, H.; Tananaki, C. Lupin, soya and triticale addition to wheat flour doughs and their effect on rheological properties. *Food Chem.* **2002**, *77*, 219–227.

(5) Zhang, Q.; Dong, Y.; An, X.; Wang, A.; Zhang, Y.; Li, X.; Gao, L.; Xia, X.; He, Z.; Yan, Y. Characterization of HMW glutenin subunits in common wheat and related species by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF-MS). *J. Cereal Sci.* **2008**, *47*, 252–261.

(6) Roccia, P.; Ribotta, P. D.; Pérez, G. T.; León, A. E. Influence of soy protein on rheological properties and water retention capacity of wheat gluten. *LWT − Food Sci. Technol.* **2009**, *42*, 358–362.

(7) Ribotta, P. D.; León, A. E.; Pérez, G. T.; Añón, M. C. Electrophoresis studies for determining wheat−soy protein interactions in dough and bread. *Eur. Food Res. Technol.* **2005**, *221*, 48–53.

(8) Bonet, A.; Blaszczak, W.; Rosell, C. M. Formation of homopolymers and heteropolymers between wheat flour and several protein sources by transglutaminase-catalyzed cross-linking. *Cereal Chem.* **2006**, *83*, 655–662.

(9) Ryan, K. J.; Homco-Ryan, C. L.; Jenson, J.; Robbins, K. L.; Prestat, C.; Brewer, M. S. Lipid extraction process on texturized soy flour and wheat gluten protein−protein interactions in a dough matrix. *Cereal Chem.* **2002**, *79*, 434–438.

(10) Gerrard, J. A.; Fayle, S. E.; Brown, P. A.; Sutton, K. H.; Simmons, L.; Rasiah, I. Effects of microbial transglutaminase on the wheat proteins of bread and croissant dough. *J. Food Sci.* **2001**, *66*, 782–786.

(11) Güemes-Vera, N.; Arciniega-Ruiz Esperza, O.; Dávila-Ortiz, G. Structural analysis of the *Lupinus mutabilis* seed, its flour, concentrate, and isolate as well as their behavior when mixed with wheat flour. *Lebensm.-Wiss. -Technol.* **2004**, *37*, 283–290.

(12) Dervas, G.; Doxastakis, G.; Hadjisavva-Zinoviadi, S.; Triantafillakos, N. Lupin flour addition to wheat flour doughs and effect on rheological properties. *Food Chem.* **1999**, *66*, 67–73.

(13) Singh, H. A study of changes in wheat protein during bread baking using SE-HPLC. *Food Chem.* **2005**, *90*, 247–250.

(14) Duranti, M.; Consonni, A.; Magni, C.; Sessa, F.; Scarafoni, A. The major proteins of lupin seed: characterisation and molecular properties for use as functional and nutraceutical ingredients. *Trends Food Sci. Technol.* **2008**, *19*, 624–633.

(15) Lampart-Szczapa, E. Preparation of protein from lupin seeds. *Food/Nahrung* **1996**, *40*, 71–74.

(16) Santos, C. N.; Ferreira, R. B.; Teixeira, A. R. Seed proteins of *Lupinus mutabilis*. *J. Agric. Food Chem.* **1997**, *45*, 3821–3825.

(17) Goggin, D. E.; Mir, G.; Smith, W. B.; Stuckey, M.; Smith, P. M. C. Proteomic analysis of lupin seed proteins to identify conglutin *β* as an allergen, Lup an 1. *J. Agric. Food Chem.* **2008**, *56*, 6370–6377.

(18) Chen, J.; Lan, P.; Tarr, A.; Yan, Y. M.; Francki, M.; Appels, R.; Ma, W. Matrix-assisted laser desorption/ionization time-of-flight based wheat gliadin protein peaks are useful molecular markers for wheat genetic study. *Rapid Commun. Mass Spectrom.* **2007**, *21*, 2913–2917.

(19) Nonlinear Dynamics Progenesis samespots normalisation; http://www.nonlinear.com/support/progenesis/samespots/faq/normalisation.aspx (March 15, 2011).

(20) Bringans, S.; Eriksen, S.; Kendrick, T.; Gopalakrishnakone, P.; Livk, A.; Lock, R.; Lipscombe, R. Proteomic analysis of the venom of *Heterometrus longimanus* (Asian black scorpion). *Proteomics* **2008**, *8*, 1081–1096.

(21) Sirtori, E.; Resta, D.; Brambilla, F.; Zacherl, C.; Arnoldi, A. The effects of various processing conditions on a protein isolate from *Lupinus angustifolius*. *Food Chem.* **2010**, *120*, 496–504.

(22) Magni, C.; Sessa, F.; Accardo, E.; Vanoni, M.; Morazzoni, P.; Scarafoni, A.; Duranti, M. Conglutin *γ*, a lupin seed protein, binds insulin in vitro and reduces plasma glucose levels of hyperglycemic rats. *J. Nutr. Biochem.* **2004**, *15*, 646–650.

(23) Sirtori, C. R.; Lovati, M. R.; Manzoni, C.; Castiglioni, S.; Duranti, M.; Magni, C.; Morandi, S.; D'Agostina, A.; Arnoldi, A. Proteins of white lupin seed, a naturally isoflavone-poor legume, reduce cholesterolemia in rats and increase LDL receptor activity in HepG2 cells. *J. Nutr.* **2004**, *134*, 18–23.

(24) Pilvi, T. K.; Jauhiainen, T.; Cheng, Z. J.; Mervaala, E. M.; Vapaatalo, H.; Korpela, R. Lupin protein attenuates the development of hypertension and normalises the vascular function of NaCl-loaded GotoeKakizaki rats. *J. Physiol. Pharmacol.* **2006**, *57*, 167–176.

(25) Yoshie-Stark, Y.; Bez, J.; Wada, Y.; Wasche, A. Functional properties, lipoxygenase activity, and health aspects of *Lupinus albus* protein isolates. *J. Agric. Food Chem.* **2004**, *52*, 7681–7689.

(26) Magni, C.; Scarafoni, A.; Herndl, A.; Sessa, F.; Prinsi, B.; Espen, L.; Duranti, M. Combined 2D electrophoretic approaches for the study of white lupin mature seed storage proteome. *Phytochemistry* **2007**, *68*, 997–1007.

(27) Muccilli, V.; Cunsolo, V.; Saletti, R.; Foti, S.; Masci, S.; Lafiandra, D. Characterization of B- and C-type low molecular weight glutenin subunits by electrospray ionization mass spectrometry and matrix-assisted laser desorption/ ionization mass spectrometry. *Proteomics* **2005**, *5*, 719–728.

(28) Xu, F.; Brown, K. M.; Dybdal, L.; Forman, T. M.; Fuglslang, C. C.; Wagner, P. Controlled stepwise reduction of disulfide bonds and heat-induced modification of wheat dough proteins. *Cereal Chem.* **1999**, *76*, 931–937.

(29) Tilley, K. A.; Benjamin, R. E.; Bagorogoza, K. E.; Okot-Kotber, B. M.; Prakash, O.; Kwen, H. Tyrosine cross-links: molecular basis of gluten structure and function. *J. Agric. Food Chem.* **2001**, *49*, 2627–2632.

(30) Ryan, K. J.; Brewer, M. S. In situ examination of starch granule−soy protein and wheat protein interactions. *Food Chem.* **2007**, *104*, 619–629.