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Analysis of *Lupinus albus* Storage Proteins by Two-Dimensional Electrophoresis and Mass Spectrometry

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A laboratory-prepared total protein extract (TPE) and a lupin protein isolate (LPI-E) produced in a pilot plant were submitted to a detailed two-dimensional (2DE) proteomic investigation. Recent findings have indicated that in an established rodent model of hyperlipidemia, moderate daily intakes of LPI-Es lead to a reduction of total and low-density lipoprotein cholesterol levels, and the knowledge of the actual composition of the protein sample used in that study is at the basis of further structure/action investigations. The experimental results indicate that the semi-industrial procedure used for the production of LPI-E damages only marginally the proteins. It does, however, cleave some disulfide bridges and induce mild proteolysis, as confirmed by the higher number of resolved protein spots in the low M_r and acidic p*I* region of the 2DE map. Out of 72 spots submitted to mass spectrometry and compared with available protein databases, 42 correspond to fragments of β-conglutin, the 7*S* globulin of lupin, spanning between positions 37 and 495 of the protein sequence. Using the bioinformatic tool BlastP, these peptides were compared to the α'-subunit of β-conglycinin, the 7*S* globulin of soybean, this being the most active hypocholesterolemic component of soybean protein, as shown by in vitro and in vivo experiments. At least 18 peptides derived from β-conglutin, having a percentage identity higher than 50% and a similarity percentage higher than 70% vs the α'-subunit of β-conglycinin, are likely candidates to be the biologically active components of lupin protein.

KEYWORDS: ***Lupinus albus*** **proteins; proteomics; posttranslational modifications; proteolysis**

INTRODUCTION

There is growing interest in the lipid lowering properties (*1*−*3*) and other potential human health benefits of proteins from grain legumes such as soyabean (*4*−*6*). This has stimulated research on other legume species in order to discover additional sources of functional food ingredients. The white lupin (*Lupinus albus*) has been the object of a number of studies in Western Europe, where there is some consumer resistance to soybean products for reasons including unfamiliarity, the presence of phytoestrogens, a typical beany "off-flavor", and the genetic modification issue (*7*).

White lupin, by contrast, has positive characteristics including similar protein content, a lower amount of antinutritional factors (*8*), and the virtual absence of phytoestrogens (*9*). Moreover, it is a traditional Mediterranean crop used for over 3000 years in human and animal nutrition (*10*).

Lupin seeds contain albumins and globulins in an approximate one to nine ratio. The globulins comprise the 7*S* and 11*S* globulins β-conglutin and α-conglutin and two minor components, γ- and δ-conglutin (*11*). γ-Conglutin is a sulfur-rich oligomeric glycoprotein, which accounts for about 5% of the total protein content (*12*). These proteins have been characterized at the molecular level, although deduced amino acid sequences are currently available only for β- (*13*) and γ-conglutin (*14*, *15*).

We have recently shown, using a rodent model of hyperlipidemia, that daily administration of lupin protein extract reduces total and low-density lipoprotein (LDL) cholesterol levels (*16*). Similar to soy proteins, a putative mechanism tested on isolated cultures is by up-regulation of LDL receptors in liver cells (*17*).

Ongoing studies with soybean proteins have provided an important indication for the identification of peptides potentially responsible for the plasma cholesterol reduction/LDL receptor up-regulation. In particular, recent evidence has indicated that most of the hypocholesterolemic effect is attributable to β-conglycinin (7*S* globulin). Moreover, its isolated α'-subcomponent, when given at pharmacological doses to rats (down to 10 mg/kg/day), reduces cholesterolemia to a similar extent as far higher doses of a synthetic hypolipidemic agent (*18*).

Proteomic analysis has indicated important differences between commercial soy protein preparations, some of which

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appear to be almost devoid of any 7*S* or 7*S* α′-subcomponents (*19*), suggesting that industrial preparation of protein isolates and concentrates may produce extensive degradation of functional components. Because white lupin kernels may become an important source of food ingredients for specific nutraceutical purposes, we have used two-dimensional electrophoresis (2DE) to compare a laboratory-prepared total protein extract (TPE) with one produced on a pilot plant scale (*20*). In addition, as a preliminary survey on structure/action relationships, we compared the sequence of lupin vicilin with the bioactive peptide α′ of soybean vicilin and verified whether the industrial process used for manufacturing the lupin protein isolate (LPI-E) was able to completely eliminate undesirable isoflavones.

MATERIALS AND METHODS

Samples. A laboratory-prepared TPE and a semi-industrial lupin protein preparation (LPI-E) from the Fraunhofer Institut für Verfahrenstechnik und Verpackung (Freising, Germany) (*20*) were analyzed. Both were obtained from defatted flaked white lupin kernels (variety Kiev), produced by Fraunhofer. In the industrial process (*20*), the deoiled flakes were first pre-extracted in cold water under acidic conditions, and the clarified acid extract was concentrated by cross-flow membrane filtration and spray-dried (this protein material, which contained mostly γ-conglutin, was not analyzed in the present study). The main storage protein fraction was obtained from the pre-extracted kernels by aqueous extraction under neutral conditions, and the extract was spray-dried (LPI-E). This preparation (LPI-E) is enriched in high molecular weight proteins, such as α-conglutin and β-conglutin, whereas γ-conglutin is a minor component (*20*).

Laboratory Preparation of the TPE. Ten grams of finely grounded, defatted flakes of white lupin were suspended in 200 mL of Tris-HCl buffer (pH 7.5, 4 °C) and stirred overnight. The solid residue was removed by filtration through a 60 mesh filter, and the solution was centrifuged at 7 °C for 45 min at 10000 rpm. Proteins were precipitated from the supernatant by treatment with $(NH_4)_2SO_4$ (4 °C, overnight), recovered by centrifugation (7 °C for 30 min at 10000 rpm), and freeze-dried. After extensive dialysis vs 10 mM Tris/HCl, the protein content of the TPE was assessed according to Bradford (*21*), using bovine serum albumine as standard.

Analysis of Isoflavones in TPE and LPI-E. Isoflavones (glycosyl derivatives of genistein and 2′-hydroxygenistein) were extracted and quantified by liquid chromatography−electrospray ionization−mass spectrometry (LC-ESI-MS) as described previously (*16*) and were below the detection limit (<0.1 nmol/g) in both TPE and LPI-E.

Electrophoretic Procedures. One-dimensional electrophoresis (sodium dodecyl sulfate polyacrylamide gel electrophoresis, SDS−PAGE) was run on 7.5−17.5% T polyacrylamide (PAA) gradients in the discontinuous buffer system of Laemmli (*22*).

Two-dimensional electrophoresis maps were obtained by IPG-DALT (*23*, *24*), with laboratory-made IPG gels. Proteins were separated by charge on a nonlinear pH 4−10 immobilized pH gradient (IPG) (*25*) in the presence of 8 M urea (cathodic application) and then according to size by SDS−PAGE on 7.5−17.5% PAA gradients. Gels were stained with 0.3% w/v Coomassie.

Protein samples for structural analysis (total extract) contained 250 μg and were either (i) nonreduced throughout the procedure, (ii) reduced between the first and the second dimension run, or (iii) reduced before the focusing step.

To optimize resolution and to increase the load in minor components for MS analysis, the sample (LPI reduced) was run at increasing concentrations on narrow pH ranges in the first dimension and on harder and softer PAA gradients in the second dimension. A similar "four corner" approach (high M_r, acidic; high M_r, basic; low M_r, acidic; low M_r, basic proteins) had been used by Fountoulakis and co-workers (*26*) for the analysis of *Haemophilus influenzae* proteome. In detail, 200 and 400 μg/lane were run on the 4−6 NL and 5−10 NL for the first dimension and on 7.5−12.5% T and 10−20% T PAA for the second dimension and 800 μg/lane was run on 4−10 NL IPG followed by 7.5−12.5% T and 10−20% T PAA.

Preparation of Samples for Mass Spectrometry. In-gel digestion with trypsin was performed according to published methods (*27*−*29*) modified for use with a robotic digestion system (Genomic Solutions, Huntington, United Kingdom). Cysteine residues were reduced with dithiothreitol and derivatized by treatment with iodoacetamide. The gel pieces were then dehydrated with acetonitrile and dried at 60 °C, prior to addition of modified trypsin (Promega, Madison, WI; 10 μL at 6.5 ng/μL in 25 mM ammonium hydrogen carbonate). After incubation at 37 °C for 8 h, the products were sequentially extracted with 25 mM ammonium hydrogen carbonate, 5% formic acid, and acetonitrile. Freeze-dried extracts were redissolved in 0.1% formic acid prior to ESI-MS/MS analysis.

Mass Spectrometry. Lyophilized extracts were redissolved in 0.1% formic acid prior to high-performance liquid chromatography−tandem mass spectrometry (HPLC-MS/MS) using a Micromass Q-TOF Instrument interfaced to a CapLC chromatograph (Waters, Manchester, United Kingdom). Samples were injected onto a 300 μm × 15 mm Pepmap C18 column (LC Packings, Amsterdam, NL) and eluted with an acetonitrile/0.1% formic acid gradient. The capillary voltage was set to 3500 V, and data-dependent MS/MS acquisitions were performed on precursor ions with charge states of 2, 3, or 4 over a survey mass range of 400−1300. The collision gas was argon, and the collision voltage was varied between 18 and 45 V depending on precursor charge and mass. Proteins were identified by correlation of uninterpreted tandem mass spectra to entries in SwissProt/TrEMBL, using ProteinLynx Global Server (Versions 1.1, Micromass). The database was created by merging the FASTA format files of SwissProt, TrEMBL, and their associated splice variants. No taxonomic, mass, or p*I* constraints were applied. One missed cleavage per peptide was allowed, and the fragment ion tolerance window was set to 100 ppm. Carbamidomethylation of cysteine was assumed, but no other modifications were considered. All matching spectra were reviewed manually, and in cases where the score reported by ProteinLynx Global Server was less than 100, additional searches were performed against the NCBI nr database (*30*) using MASCOT, which utilizes a robust probabilistic scoring algorithm (*31*). Where identifications were based on a single matching peptide, the sequences were confirmed by manual sequencing using the MassLynx program PepSeq. Measured parent and fragment masses were typically within 0.03 Da of their calculated values.

Software. BLASTP (*32*, *33*) operating on the NPS@ server (http://npsa-pbil.ibcp.fr/cgi-bin/simsearch_blast.pl) was used for database similarity search, SignalP (http://www.cbs.dtu.dk/services/SignalP/) for prediction of signal peptides (*34*, *35*).

RESULTS

Before 2DE analysis, both preparations were tested for the presence of lupin isoflavones (glycosyl derivatives of genistein and 2′-hydroxygenistein). They were below the detection limit (<0.1 nmol/g), indicating that the low concentrations present in raw seeds (*16*) are effectively eliminated by both isolation procedures.

Structural Analysis of Lupin Proteins. Figure 1 shows the two-dimensional pattern of the TPE of lupin kernels when the sample is either unreduced (**A**), reduced between the first and the second dimension (**B**), or reduced before the focusing step (**C**). IPGs are run in the presence of 8 M urea; hence, noncovalently bound oligomers are dissociated in all cases. Two groups of spots migrating with an apparent M_r of around 60 and 70 kDa under nonreducing conditions (solid arrow in **A**) after reduction shifted to rows of spots of lower M_r (empty arrows in **B** and **C**).

In most cases, rows rather than single spots are observed, i.e., series of components with very similar M_r values but varying p*I* values. Often, these spot rows are further arranged into clusters of rows of closely spaced M_r values. While all sectors of the map (from acidic to basic p*I* and from high to low M_r) contain some prominent features, the spot density is higher in the acidic region.

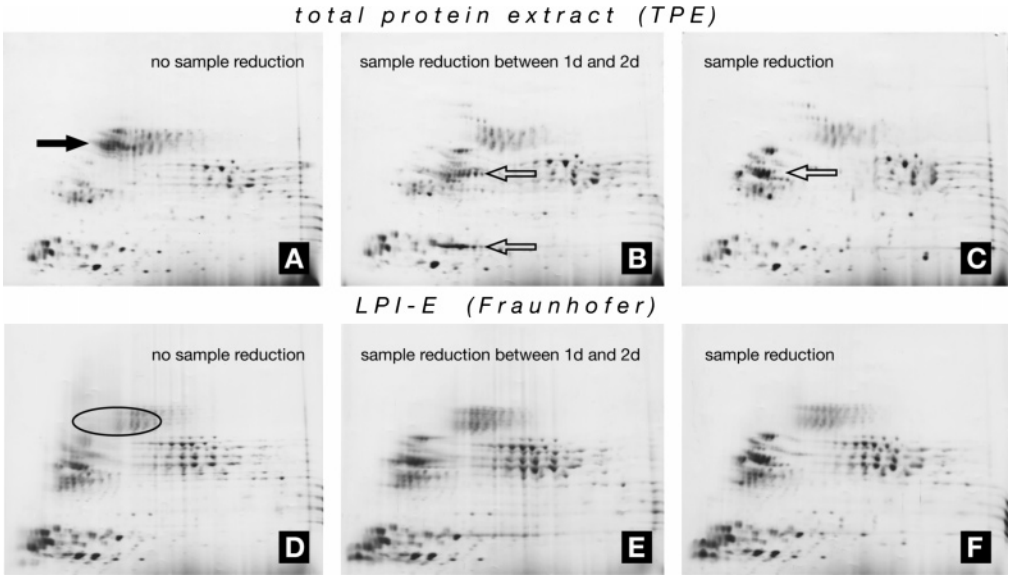

Figure 1. Two-dimensional electrophoresis maps of the total lupin protein extract TPE (**A**–**C**) and the industrial protein isolate LPI-E (**D**–**F**). The first dimension is on a 4–10 NL IPG, and the second dimension is on 7.5–17.5% T PAA. Sample redox states: **A** and **D** = not reduced; **B** and **E** = unreduced during the first and reduced during the second dimension run; and **C** and **F** = fully reduced. The solid arrow in **A** points to prominent spots that after reduction are split into two lower M_r components, marked by open arrows in panels **B** and **C**. For p*I* and M_r scales, please refer to **Figure 2**, containing a cropped version of panel **F**.

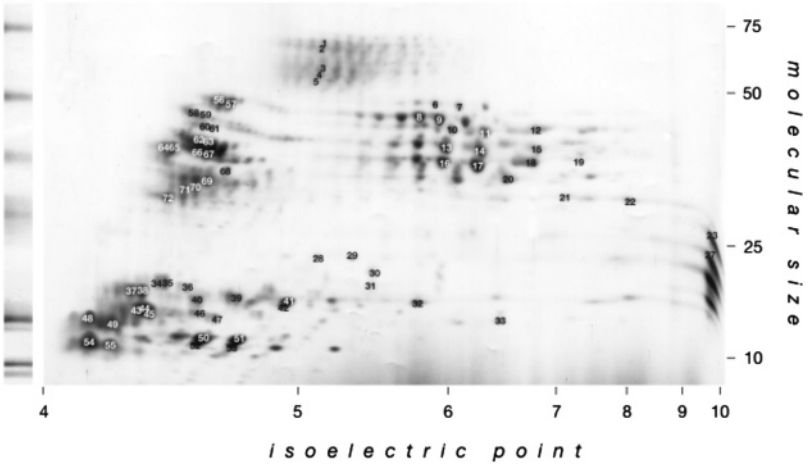

Figure 2. Reference map of fully reduced semi-industrial protein isolate LPI-E (cropped from panel **F** in **Figure 1**). p*I* and M_r scales are marked along *x*- and *y*-axes. The spots excised for identification by MS analysis are marked with numbers; the same numbers are associated with the MS result entries in **Table 1**.

Comparison between the Semi-industrial Preparation and the TPE. Panels **D**, **E**, and **F** of **Figure 1** show the two-dimensional patterns of LPI-E when the sample is either not reduced (**D**), reduced between the first and the second dimension (**E**), or reduced before the focusing step (**F**). In contrast to the TPE, reduction has little effect on the patterns obtained, indicating that the disulfide bridges have already been cleaved during preparation of the LPI-E. Comparing panels **C** and **F**, it appears that the heterogeneity of the sample, as assessed by the number of resolved protein spots, is greater in LPI-E than in TPE. This is particularly true in the low M_r and acidic p*I* region and to a lesser extent in the average M_r and basic p*I* regions.

Identification of Major Components of LPI-E. Because there are currently only 64 partial and complete sequences derived from *L. albus* deposited in SwissProt and TrEMBL (Release 45.3 and 28.3, December 2004), we used MS/MS rather than matrix-assisted laser desorption/ionization time-of-flight for protein identification, which frequently enables identification of orthologous proteins in the absence of a database sequence from the target organism.

The spots picked for MS analysis of LPI-E components are numbered in **Figure 2**, and the corresponding identifications are listed in **Table 1**. Of 72 spots analyzed, 47 were positively identified by correlation to database sequences. Of these 47, 42 correspond to fragments of β-conglutin, two were identified as γ-conglutin, and two were mixtures of β- and γ-conglutin. These identifications were facilitated by the availability of β- and γ-conglutin deduced amino acid sequences in public databases (*13*−*15*). Three proteins had peptides in common with pea legumin, and seven conglutinin spots were accompanied by additional peptides derived from nonstorage proteins, for example, carboxypeptidase H precursor (CBPH_LOPAM).

DISCUSSION

Two-dimensional electrophoresis and MS analysis clearly indicate that both preparations contain predominantly polypep-

Table 1. Identification of the Spots of the 2D Maps of LPI-E (Nonidentified Peptides Were not Included in the Table)

#	identity	sequenced peptides		coverage[a]
1	gi|46451223	SNEPIYSNK	EQEQQQGSPSYSR	97-463
	β-conglutin precursor (*L. albus*)	NPYHFSSQR	HSDADYVLVVLNGR	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
		LLGFGINADENQR		
2	gi|46451223	FQTLYK	LLGFGINADENQR	91-463
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	EQEQQQGSPSYSR	
		NPYHFSSQR	HSDADYVLVVLNGR	
		NTLEATFNTR	IVEFQSKPNTLILPK	
		ATITIVNPDRR	LAIPINNPGYFYDFYPSSTK	
		DQQSYFSGFSR		
3	gi|46451223	YEEIQR	LLGFGINADENQR	113-463
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	HSDADYVLVVLNGR	
		NPYHFSSQR	IPAGSTSYILNPDDNQK	
		ATITIVNPDRR	LAIPINNPGYFYDFYPSSTK	
		DQQSYFSGFSR		
4	gi|46451223	NPYHFSSQ	NTLEATFNTR	113-268
	β-conglutin precursor (*L. albus*)			
5	gi|46451223	SNEPIYSNK	HSDADYVLVVLNGR	91-344
	β-conglutin precursor (*L. albus*)	NPYHFSSQR	IPAGSTSYILNPDDNQK	
		NTLEATFNTR	IILGNEDEQEYEEQR	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
		EQEQQQGSPSYSR		
6	gi|46451223	NTLEATFNTR	IVEFQSKPNTLILPK	113-404
	β-conglutin precursor (*L. albus*)	NPYHFSSQR	IILGNEDEQEYEEQR	
		ATITIVNPDRR	LAIPINNPGYFYDFYPSSTK	
		DQQSYFSGFSR	AIYVVVVDEGEGNYELVGIR	
		HSDADYVLVVLNGR		
7	gi|46451223	YEEIQR	QAYNLEYGDALR	37-404
	β-conglutin precursor (*L. albus*)	SHERPEER	EQEQQQGSPSYSR	
		SNEPIYSNK	HSDADYVLVVLNGR	
		EQEEWQPR	IVEFQSKPNTLILPK	
		NTLEATFNTR	IPAGSTSYILNPDDNQK	
		NPYHFSSQR	LAIPINNPGYFYDFYPSSTK	
		ATITIVNPDRR	AIYVVVVDEGEGNYELVGIR	
8	gi|46451223	YEEIQR	QAYNLEYGDALR	113-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	HSDADYVLVVLNGR	
		NTLEATFNTR	IVEFQSKPNTLILPK	
		NPYHFSSQR	IPAGSTSYILNPDDNQK	
		ATITIVNPDRR	LAIPINNPGYFYDFYPSSTK	
		DQQSYFSGFSR		
9	gi|46451223	SNEPIYSNK	EQEQQQGSPSYSR	91-344
	β-conglutin precursor (*L. albus*)	NTLEATFNTR	HSDADYVLVVLNGR	
		NPYHFSSQR	IVEFQSKPNTLILPK	
		ATITIVNPDRR	IPAGSTSYILNPDDNQK	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
10	gi| 46451223	YEEIQR	HSDADYVLVVLNGR	
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	IVEFQSKPNTLILPK	
		NTLEATFNTR	IPAGSTSYILNPDDNQK	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
		QAYNLEYGDALR		
11	gi|46451223	YEEIQR	HSDADYVLVVLNGR	154-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	IVEFQSKPNTLILPK	
		NTLEATFNTR	IPAGSTSYILNPDDNQK	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
		QAYNLEYGDALR		
12	gi|46451223	YEEIQR	QAYNLEYGDALR	154-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	HSDADYVLVVLNGR	
		NTLEATFNTR	IVEFQSKPNTLILPK	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
13	gi|46451223	YEEIQR	HSDADYVLVVLNGR	91-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	IVEFQSKPNTLILPK	
		NPYHFSSQR	IPAGSTSYILNPDDNQK	
		NTLEATFNTR	IILGNEDEQEYEEQR	
		DQQSYFSGFSR	EEREQEQQQGSPSYSR	
		QAYNLEYGDALR	LAIPINNPGYFYDFYPSSTK	
		EQEQQQGSPSYSR		
14	gi|46451223	YEEIQR	EQEQQQGSPSYSR	91-355
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	HSDADYVLVVLNGR	
		NPYHFSSQR	IVEFQSKPNTLILPK	

Table 1 (Continued)

#	identity	sequenced peptides		coverage[a]
14	gi|46451223	NTLEATFNTR	IPAGSTSYILNPDDNQK	91-355
	β-conglutin precursor (*L. albus*)	DQQSYFSGFSR	IILGNEDEQEYEEQR	
		YGNFYEITPDR	IILGNEDEQEYEEQRR	
		QAYNLEYGDALR	LAIPINNPGYFYDFYPSSTK	
15	gi|46451223	YEEIQR	EQEQQQGSPSYSR	91-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	HSDADYVLVVLNGR	
		NTLEATFNTR	IVEFQSKPNTLILPK	
		DQQSYFSGFSR	IPAGSTSYILNPDDNQK	
		QAYNLEYGDALR	LAIPINNPGYFYDFYPSSTK	
16	gi|46451223	YEEIQR	IVEFQSKPNTLILPK	91-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	IPAGSTSYILNPDDNQK	
		NTLEATFNTR	IILGNEDEQEYEEQR	
		DQQSYFSGFSR	EEREQEQQQGSPSYSR	
		QAYNLEYGDALR	IILGNEDEQEYEEQRR	
		EQEQQQGSPSYSR	LAIPINNPGYFYDFYPSSTK	
		HSDADYVLVVLNGR		
17	gi|46451223	YEEIQR	HSDADYVLVVLNGR	91-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	IVEFQSKPNTLILPK	
		NPYHFSSQR	IPAGSTSYILNPDDNQK	
		NTLEATFNTR	IILGNEDEQEYEEQR	
		DQQSYFSGFSR	EEREQEQQQGSPSYSR	
		QAYNLEYGDALR	LAIPINNPGYFYDFYPSSTK	
		EQEQQQGSPSYSR		
18	gi|46451223	YEEIQR	HSDADYVLVVLNGR	91-344
	β-conglutin precursor (*L. albus*)	SNEPIYSNK	IVEFQSKPNTLILPK	
		NTLEATFNTR	IPAGSTSYILNPDDNQK	
		DQQSYFSGFSR	IILGNEDEQEYEEQR	
		QAYNLEYGDALR	LAIPINNPGYFYDFYPSSTK	
		EQEQQQGSPSYSR		
19	gi|46451223	FQTLYK	QAYNLEYGDALR	122-344
	β-conglutin precursor (*L. albus*)	YEEIQR	HSDADYVLVVLNGR	
		SNEPIYSNK	IVEFQSKPNTLILPK	
		NTLEATFNTR	IILGNEDEQEYEEQR	
		ATITIVNPDRR	LAIPINNPGYFYDFYPSSTK	
		DQQSYFSGFSR		
20	gi|46451223	SNEPIYSNK	DQQSYFSGFSR	113-344
	β-conglutin precursor (*L. albus*)	NPYHFSSQR	HSDADYVLVVLNGR	
		NTLEATFNTR	IVEFQSKPNTLILPK	
		ATITIVNPDRR	LAIPINNPGYFYDFYPSSTK	
21	gi|46451223	YEEIQR	ATITIVNPDRR	37-274
	β-conglutin precursor (*L. albus*)	SHERPEER	DQQSYFSGFSR	
		EQEEWQPR	HSDADYVLVVLNGR	
		NTLEATFNTR	IVEFQSKPNTLILPK	
		NPYHFSSQR	LAIPINNPGYFYDFYPSSTK	
22	gi|46451223	YEEIQR	DQQSYFSGFSR	45-274
	β-conglutin precursor (*L. albus*)	EQEEWQPR	HSDADYVLVVLNGR	
		NPYHFSSQR	IVEFQSKPNTLILPK	
		NTLEATFNTR	LAIPINNPGYFYDFYPSSTK	
		ATITIVNPDRR		
23	gi|46451223	YEEIQR	EQEQQQGSPSYSRR	91-274
	β-conglutin precursor (*L. albus*)	NTLEATFNTR	IVEFQSKPNTLILPK	
		DQQSYFSGFSR	IPAGSTSYILNPDDNQK	
		QAYNLEYGDALR	LAIPINNPGYFYDFYPSSTK	
		HSDADYVLVVLNGR		
24	gi|46451223	NPYHFSSQR	IVEFQSKPNTLILPK	113-268
	β-conglutin precursor (*L. albus*)	NTLEATFNTR	IPAGSTSYILNPDDNQK	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
		HSDADYVLVVLNGR		
25	gi|46451223	NPYHFSSQR	EQEQQQGSPSYSRR	91-268
	β-conglutin precursor (*L. albus*)	NTLEATFNTR	IVEFQSKPNTLILPK	
		ATITIVNPDRR	IPAGSTSYILNPDDNQK	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
		HSDADYVLVVLNGR		
26	gi|46451223	YEEIQR	HSDADYVLVVLNGR	91-274
	β-conglutin precursor (*L. albus*)	LENLQNYR	EQEQQQGSPSYSRR	
		NPYHFSSQR	IVEFQSKPNTLILPK	
		NTLEATFNTR	IPAGSTSYILNPDDNQK	
		ATITIVNPDRR	NTLEATFNTRYEEIQR	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	

Table 1 (Continued)

#	identity	sequenced peptides		coverage[a]
27	gi|46451223	FQTLYK	HSDADYVLVVLNGR	91-268
	β-conglutin precursor (*L. albus*)	NTLEATFNTR	IVEFQSKPNTLILPK	
		ATITIVNPDRR	IPAGSTSYILNPDDNQK	
		DQQSYFSGFSR	LAIPINNPGYFYDFYPSSTK	
		EQEQQQGSPSYSR		
31	gi|46451223	LAIPINNPGYFYDFYPSSTK		228-242
	β-conglutin precursor (*L. albus*)			
33	gi|11191819	VGFNTNSLK	HSIFEVFTQVFANNVPK	
	γ-conglutin (*L. albus*)	VGFNTNSLKSHAK	AGIALGTHQLEENLVVFDLAR	
34	gi|11191819	AGIALGTHQLEENLVVFDLAR		
	γ-conglutin (*L. albus*)			
34	gi|46451223	NTLEATFNTR	LLGFGINADENQR	
	β-conglutin (*L. albus*)			
35	gi|46451223	LLGFGINADENQR		451-463
	β-conglutin precursor (*L. albus*)			
36	gi|11191819	AGIALGTHQLEENLVVFDLAR		
	γ-conglutin (*L. albus*)			
36	gi|46451223	LLGFGINADENQR		451-463
	β-conglutin precursor (*L. albus*)			
41	gi|46451223	SNEPIYSNK	INEGALLLPHYNSK	324-408
	β-conglutin precursor (*L. albus*)	DKPSDSGPFNLR	AIYVVVVDEGEGNYELVGIRDQQR	
42	gi|46451223	SNEPIYSNK	AIYVVVVDEGEGNYELVGIR	324-408
	β-conglutin precursor (*L. albus*)	DKPSDSGPFNLR	AIYVVVVDEGEGNYELVGIRDQQR	
		INEGALLLPHYNSK		
43	gi|46451223	NFLAGSKDNVIR	AIYVVVVDEGEGNYELVGIR	385-495
	β-conglutin precursor (*L. albus*)	LLGFGINADENQR	AIYVVVVDEGEGNYELVGIRDQQR	
		AVNELTFPGSAEDIER		
44	gi|46451223	LLGFGINADENQR	AVNELTFPGSAEDIER	451-495
	β-conglutin precursor (*L. albus*)			
45	gi|46451223	SNEPIYSNK	LLGFGINADENQR	451-495
	β-conglutin precursor (*L. albus*)	NFLAGSKDNVIR	AVNELTFPGSAEDIER	
46	gi|46451223	LLGFGINADENQR		451-463
	β-conglutin precursor (*L. albus*)			
47	gi|46451223	NFLAGSKDNVIR	AVNELTFPGSAEDIER	414-495
	β-conglutin precursor (*L. albus*)	LLGFGINADENQR		
50	gi|46451223	SNEPIYSNK	YGNFYEITPDR	336-355
	β-conglutin precursor (*L. albus*)			
51	gi|46451223	SNEPIYSNK	YGNFYEITPDR	336-355
	β-conglutin precursor (*L. albus*)			
52	gi|46451223	SNEPIYSNK	LLGFGINADENQR	336-463
	β-conglutin precursor (*L. albus*)	YGNFYEITPDR		
53	gi|46451223	SNEPIYSNK	YGNFYEITPDR	336-355
	β-conglutin precursor (*L. albus*)			
57	gi|126161	LNALEPDNR		
	legumin A2 (*Pisum sativum*)			
60	gi|126161	LNALEPDNR		
	legumin A2 (*P. sativum*)			
67	gil126161	LNALEPDNR		
	legumin A2 (*P. sativum*)			
68	gi|46451223	SNEPIYSNK	AIYVVVVDEGEGNYELVGIRDQQR	336-408
	β-conglutin precursor (*L. albus*)	AVNELTFPGSAEDIER		
69	gil126161	LNALEPDNR		
	legumin A2 (*P. sativum*)			

[a] The putative signal peptide was assumed to have been proteolytically removed.

tides derived from different regions of β-conglutin by proteolytic cleavage. Because data-dependent MS data acquisition may detect only a subset of the peptides present in the digest of each excised spot, the sequence coverage obtained represents the minimum extent of each polypeptide. Comparison of the low M_r and acidic p*I* regions of the laboratory and industrial preparations shows that at least nine low M_r β-conglutin-derived peptides are present in the latter but absent from the TPE, suggesting that the industrial process induces some additional proteolysis. However, this degradation is marginal as compared to that observed in commercial soybean protein extracts (*19*).

In common with related plant proteins, β-conglutin would be expected to possess a signal peptide, although there is currently no experimental evidence for either its presence or the cleavage site. The prediction program SignalP 3.0 (*34*, *35*) suggests that the most likely point of cleavage is between positions 30 and 31, although other sites slightly upstream are also possible. The MS data unfortunately do not allow these possibilities to be resolved; the most upstream peptide experimentally detected (SHERPEER) starts at position 37, but the presence of a tryptic site (K) in position 32 means that all of the predicted N-terminal peptides are too small for detection by ESI-MS.

As the sequence of conglutin α-chain is not known, its identification by direct correlation of peptide mass spectra to database entries is not possible. However, the altered migration

Table 2. β-Conglutin Derived Peptides Having the Highest Similarity Percentage Higher than 70 % vs the α'-Subunit of β-Conglycinin[a]

β-conglutin peptides in 2DE		alignment to soybean 7S α'-subunit		
#	coverage	I	S	G
4, 24	113–268	52	76	10
21	37–274	51	76	10
22	45–274	51	76	10
23, 26	91–274	51	76	10
25, 27	91–268	51	75	10
31	228–242	60	75	
41, 42	324–408	59	80	1
43	385–495	57	67	
44, 45	451–495	73	80	
47	414–495	65	76	
50, 51, 53	336–463	64	76	
52	336–355	54	69	1
68	336–408	60	82	1

[a] I, identities; P, similarities; and G, gaps.

on reduction of the spots labeled with the open arrows in panel **C** and the similarity of some of them to pea A2 legumin indicate that they may reasonably be assigned to α-conglutin.

Some spots, which were more abundant in the TPE, were identified as γ-conglutin; this was very recently shown to display in vitro interaction with insulin, resulting in a reduced glycemic response to glucose loading (*36*). The increased concentration of this protein is presumably attributable to the absence of purification steps likely to decrease its concentration such as the acid extraction and membrane filtration used in the industrial process (*20*).

In vitro and in vivo data suggest that the most active hypocholesterolemic component of soybean protein is the α'-subunit of β-conglycinin (*18*). Sequence comparison using BLAST (*32*, *33*) shows 50% identity and 70% similarity between lupin β-conglutin and soy β-conglycinin α'-subunit. This level of similarity would be consistent with proteolytic peptides derived from β-conglutin being responsible for the observed hypocholesterolemic activity of lupin protein preparations. We therefore used BLAST to compare the specific peptide sequences identified in this study with the α'-subunit of β-conglycinin. Of 42 peptides tested, 18 had a percentage identity greater than 50% and a percent similarity above 70% (**Table 2**) and are thus good candidates for the hypocholesterolemic components of lupin proteins.

In conclusion, this investigation demonstrates that suitable industrial preparation of lupin protein extracts results in only marginal structural damage, which is important given the possible use of lupin proteins as ingredients of functional foods. In contrast to soy-derived materials, lupin protein preparations are isoflavone free, thus permitting a reduction of exposure to phytoestrogens.

ABBREVIATIONS USED

2DE, two-dimensional electrophoresis; HPLC-MS/MS, high-performance liquid chromatography−tandem mass spectrometry; IPG, immobilized pH gradient; LC-ESI-MS, liquid chromatography−electrospray ionization−mass spectrometry; LDL, low-density lipoprotein; LPI-E, lupin protein isolate; PAA, polyacrylamide; SDS−PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; TPE, total protein extract.

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LITERATURE CITED

(1) FDA. Food labeling health claims: Soy protein and coronary heart disease. Food and Drug Administration. Final rule. *Fed. Regist.* **1999**, *64*, 57699−57733.

(2) Sirtori, C. R.; Lovati, M. R.; Manzoni, C.; Gianazza, E.; Bondioli, A.; B., S.; Auwerx, J. Reduction of serum cholesterol by soy proteins: Clinical experience and potential molecular mechanisms. *Nutr. Metab. Cardiovasc. Dis.* **1998**, *8*, 334−340.

(3) Anderson, J. W.; Johnstone, B. M.; M. E.; C.-N. Meta-analysis of the effects of soy protein intake on serum lipids. *N. Engl. J. Med.* **1995**, *333*, 276−282.

(4) Fontaine, K. R.; Yang, D.; Gadbury, G. L.; Heshka, S.; Schwartz, L. G.; Murugesan, R.; Kraker, J. L.; Heo, M.; Heymsfield, S. B.; Allison, D. B. Results of soy-based meal replacement formula on weight, anthropometry, serum lipids and blood pressure during a 40-week clinical weight loss trial. *Nutr. J.* **2003**, *18*, 14.

(5) Allison, D. B.; Gadbury, G.; Schwartz, L. G.; Murugesan, R.; Kraker, J. L.; Heshka, S.; Fontaine, K. R.; Heymsfield, S. B. A novel soy-based meal replacement formula for weight loss among obese individuals: A randomized controlled clinical trial. *Eur. J. Clin. Nutr.* **2003**, *57*, 514−522.

(6) Deibert, P.; Konig, D.; Schmidt-Trucksaess, A.; Zaenker, K. S.; Frey, I.; Landmann, U.; Berg, A. Weight loss without losing muscle mass in pre-obese and obese subjects induced by a high-soy-protein diet. *Int. J. Obes. Relat. Metab. Disord.* **2004**, *28*, 1349−1352.

(7) Baker, G. A.; Burnham, T. A. Consumer response to genetically modified foods: Market segment analysis and implications for producers and policy makers. *J. Agric. Resour. Econ.* **2001**, *26*, 387−403.

(8) Muzquiz, M.; Pedrosa, M. M.; Cuadrado, C.; Ayet, G.; Burbano, C.; Brenes, A. Variation of alkaloids, alkaloid esters, phytic acid, and phytase activity in germinated seed of *Lupinus albus* and *L. luteus*. In *Recent Advances of Research in Antinutritional Factors in Legume Seeds and Rape Seeds, EAAP Publication n. 93*; Jasman, A. J. M., Ed.; Wageningen Press: Wageningen, The Netherlands, 1998; pp 387−390.

(9) Katagiri, Y.; Ibrahim, R. K.; Tahara, S. HPLC analysis of white lupin isoflavonoids. *Biosci., Biotechnol., Biochem.* **2000**, *64*, 1118−1125.

(10) Gladstones, J. S. Distribution, origin, taxonomy, history and importance. In *Lupins as Crop Plants: Biology, Production and Utilization*; Gladstone, I. S., Atkins, C., Hamblin, J., Eds.; CAB International: Wallingford, 1998; pp 1−37.

(11) Blagrove, R. J.; Gillespie, J. M. Isolation, purification and characterization of the seed globulins of *Lupinus albus*. *Aust. J. Plant Physiol.* **1975**, *2*, 13−27.

(12) Gueguen, J.; Cerletti, P. Proteins of some legume seeds: Soybean bean, pea, fababean and lupin. In *New and Developing Sources of Food Proteins*; Hudson, B. J. F., Ed.; Chapman and Hall: London, 1994; pp 145−193.

(13) Freitas, R. M.; Teixeira, A. NTrEMBL accession number Q6EBC1.

(14) Scarafoni, A.; Di Cataldo, A.; Vassilevskaia, T. D.; Bekman, E. P.; Rodrigues-Pousada, C.; Ceciliani, F.; Duranti, M. Cloning, sequencing and expression in the seeds and radicles of two *Lupinus albus* conglutin-gamma genes. *Biochim. Biophys. Acta* **2001**, *1519*, 147−151.

(15) Di Cataldo, A.; Vassilevskaia, T. D.; Bekman, E. P.; Rodrigues-Pousada, C.; Ceciliani, F.; Duranti, M. TrEMBL accession number Q9FSH9.

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

(17) Lovati, M. R.; C., M.; Canavesi, A.; Sirtori, M.; Vaccarino, V.; Marchi, M.; Gaddi, G.; Sirtori, C. R. Soybean protein diet

increases low-density lipoprotein receptor activity in mononuclear cells from hypercholesterolemic patients. *J. Clin. Invest.* **1987**, *80*, 1498−1502.
(18) Manzoni, C.; Duranti, M.; Eberini, I.; Scharnag, H.; Marz, W.; Castiglioni, S.; Lovati, M. R. Subcellular localization of soybean 7S globulin in HepG2 cells and LDL receptor up-regulation by its a constituent subunit. *J. Nutr.* **2003**, *133*, 2149−2155.
(19) Gianazza, E.; Eberini, I.; Arnoldi, A.; Wait, R.; Sirtori, C. R. A proteomic investigation of isolated soy proteins with variable effects in experimental and clinical studies. *J. Nutr.* **2003**, *133*, 9−14.
(20) Wäsche, A.; Müller, K.; Knauf, U. New processing of lupin protein isolates and functional properties. *Nahrung* **2001**, *45*, 393−395.
(21) Bradford, M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248−254.
(22) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680−685.
(23) Gianazza, E. Casting immobilized pH gradients. In *The Protein Protocol Handbook*, 2nd ed.; Walker, J. M., Ed.; Humana Press: Totowa, 2002; pp 169−180.
(24) Gianazza, E.; Eberini, I.; Villa, P.; Fratelli, M.; Pinna, C.; Wait, R.; Gemeiner, M.; Miller, I. Monitoring the effects of drug treatment in rat models of disease by serum protein analysis. *J. Chromatogr. B* **2002**, *771*, 107−130.
(25) Gianazza, E.; Giacon, P.; Sahlin, B.; Righetti, P. G. Nonlinear pH courses with immobilized pH gradients. *Electrophoresis* **1985**, *6*, 53−56.
(26) Langen, H.; Takacs, B.; Evers, S.; Berndt, P.; Lahm, H. W.; Wipf, B.; Gray, C.; Fountoulakis, M. Two-dimensional map of the proteome of *Haemophilus influenzae*. *Electrophoresis* **2000**, *21*, 411−429.
(27) Jeno, P.; Mini, T.; Moes, S.; Hintermann, E.; Horst, M. Internal sequences from proteins digested in polyacrylamide gels. *Anal. Biochem.* **1995**, *224*, 75−82.
(28) Wilm, M.; Shevchenko, A.; Houthaeve, T.; Breit, S.; Schweigerer, L.; Fotsis, T.; Mann, M. Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **1996**, *379*, 466−469.
(29) Picard, P.; Bourgoin-Grenech, M.; Zivy, M. Potential of two-dimensional electrophoresis in routine identification of closely related durum wheat lines. *Electrophoresis* **1997**, *18*, 174−181.
(30) http://www3.ncbi.nlm.nih.gov/entrez.
(31) Perkins, D. N.; Pappin, D. J.; Creasy, D. M.; Cottrell, J. S. Probability-based protein identification by searching sequence databases using mass spectrometry data. *Electrophoresis* **1999**, *20*, 3551−3567.
(32) Altschul, S. F.; Gish, W.; Miller, W.; Myers, E. W.; Lipman, D. J. Basic local alignment search tool. *J. Mol. Biol.* **1990**, *215*, 403−410.
(33) Combet, C.; Blanchet, C.; Geourjon, C.; Deleage, G. NPS@: Network protein sequence analysis. *Trends Biochem. Sci.* **2000**, *25*, 147−150.
(34) Nielsen, H.; Engelbrecht, J.; S., B.; von Heijne, G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **1997**, *10*, 1−6.
(35) Bendtsen, J. D.; Nielsen, H.; von Heijne, G.; Brunak, S. Improved prediction of signal peptides: SignalP 3.0. *J. Mol. Biol.* **2004**, *340*, 783−795.
(36) Magni, C.; Sessa, F.; Accardo, E.; Vanoni, M.; Morazzoni, P.; Scarafoni, A.; Duranti, M. Conglutin gamma, a lupin seed protein, binds insulin in vitro and reduces plasma glucose levels of hyperglycemic rats. *J. Nutr. Biochem.* **2004**, *15*, 646−50.

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