Extraction, Isolation, and Characterization of Globulin Proteins from *Lupinus albus*

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Ⓢ *Supporting Information*

ABSTRACT: Lupin has recently been added to the list of allergens requiring mandatory advisory labeling on foodstuffs sold in the European Union, and since December 2008, all products containing even trace amounts of lupin must be labeled correctly. Lupin globulins consist of two major globulins called α-conglutin (11S and "legumin-like") and β-conglutin (7S and "vicilin-like") and another additional two globulins, γ-conglutin and δ-conglutin, which are present in lower amounts. We report on a methodology to facilitate the extraction of each of these proteins using centrifugation and isolation by anion-exchange chromatography followed by size-exclusion chromatography. The isolated subunits were characterized using reducing and non-reducing polyacrylamide gel electrophoresis, western blotting, and peptide mass fingerprinting, all of which revealed that the individual protein subunits are highly pure and can be used as immunogens for the production of antibodies specific for each of the conglutin fractions, as well as standards, and the extraction protocol can be used for the selective extraction of each of the subunits from foodstuffs, thus facilitating a highly accurate determination of the lupin concentration. Furthermore, the subunits can be used to elucidate information regarding the toxicity of each of the subunits, by looking at their interaction with the IgE antibodies found in the serum of individuals allergic to lupin, providing critical information for the definition of the requirements of analytical assays for the detection of lupin in foodstuffs.

KEYWORDS: Lupin, conglutin, food allergen, peptide mass fingerprinting, anion-exchange chromatography

■ INTRODUCTION

Lupin is an herbaceous plant of the leguminous family, belonging to the genus *Lupinus*, which includes 450 species. Lupin seeds have been used as human food and animal feed since ancient times. There are four different species of agricultural interest, white lupin (*Lupinus albus*), blue lupin (*Lupinus angustifolius*), yellow lupin (*Lupinus luteus*), and Andean lupin (*Lupinus mutabilis*).[1] Lupin is considered a source of low-cost protein and can be cultivated in colder climates, making it attractive in comparison to other protein-rich plants,[1] and is attracting attention as an excellent food material with a high nutritional value.[2−4] Lupin or lupin flour is used in bread, cookies, pastry, pasta, and sauces and also in beverages as a substitute for milk or soy, and the seeds are widely used as snacks.[5−10]

The seed storage proteins of different species have been studied in detail from the turn of the century, when Osborne in 1924 classified them into four groups on the basis of their extraction and solubility characteristics: albumins, globulins, glutenins, and prolamins.[11,12] However, it is well-known that a clear-cut distinction between these groups of proteins is not always possible, with many proteins showing intermediate solubility behaviors.[13] The globulin fraction of lupin protein consists of two major globulins called α-conglutin (11S and "legumin-like"), accounting for about 33% of the total protein content in *L. albus*,[14,15] and β-conglutin (7S and "vicilin-like"), which

accounts for another 45%.[14,16] Finally, there are two additional globulins of minor quantity called γ- and δ-conglutin, which account for 5 and 12%, respectively, in *L. albus*.[10,14,15,17] Some reports have further sub-classified δ-conglutin into δ_1- and δ_2-conglutin (Table 1).[18,19]

Lupin, in the form of flour, seed, or dust, has been reported to produce a variety of different allergic responses, such as urticaria and angioedema,[20,21] contact urticaria,[22] oral allergy syndrome,[1] rhino conjunctivitis,[21,23] and anaphylaxis.[1,24−27] Lupin allergy apparently arises by either primary sensitization [1,23,28] or clinical cross-reactivity in individuals who are allergic to peanut.[15,21,29] These cross-allergic clinical reactions of peanut to other members of the leguminous family, such as soy, peas, beans, and lentils, occurs in about 5% of peanut-allergic patients but were found to be 68% with lupin.[29] Because lupin was officially admitted as a food ingredient in France in 1997, a high number of severe food allergy reactions to lupin have been reported.

In the work reported here, a procedure for lupin protein extraction, isolation, and characterization of lupin proteins is optimized, to obtain pure targets that can be used as standards,

Table 1. Summary of the Main Features of _L. albus_ Conglutins[19]

conglutin (protein family)	Svedberg velocity	UniProtKB/ TreEMBL (accession number)	percentage of total globulins (%)	native protein				upon reduction	
				M_r (kDa)	pI	quaternary structure	subunit size (kDa)	heavy chain	light chain
δ-conglutin (sulfur-rich)	2S	Q333K7	10−12	13	4.3	monomer		9	4
α-conglutin (legumin-like)	11S	Q53I54	35−37	330−430	5.6−5.9	hexamer	53	31	19
							60	36	19
							66	42	19
							70	46	19
β-conglutin (vicilin-like)	7S	Q53HY0 and Q6EBC1	44−45	143−260	5.9−6.2	trimer	19−60	no disulfide bonds	
γ-conglutin	7S	Q9FEX1 and Q9FSH9	4−5	200	7.9	tetramer	47	29	17

immunogens for specific antibody production, and can also find application in human toxicity studies.

MATERIALS AND METHODS

Reagents. Buffers, acids or bases, and salts were purchased from Sigma (Barcelona, Spain), and the electrophoresis reagents, staining solutions, and standards were purchased from Bio-Rad (Barcelona, Spain). Columns and chromatographic matrices were purchased in General Electric Healthcare (Barcelona, Spain). Trypsin was purchased from Roche Molecular Biochemicals (Barcelona, Spain), and the mass spectra standard calibrator kit was purchased from per septive Biosystems (Barcelona, Spain).

Instrumentation. Matrix-assisted laser desorption/ionization−time of flight mass spectrometry (MALDI−TOF MS) analyses were performed on a Voyager-DE STR instrument (PerSeptive Biosystems, Framingham, MA).

Plant Material. Dry seeds of white lupin (_L. albus_) were used. The dry cotyledons were milled (0.2 mm sieve) with a grinder (Moulinex, Moulinette). The resulting flour was defatted with _n_-hexane (35 mL/g of dry weight) under vigorous vortexing for 10 min at room temperature (RT). The defatted flour was centrifuged at 6000g for 10 min at RT, and the pellet obtained was dried under vacuum for 72 h, after decantation of _n_-hexane.

Extraction of Globulins with Dilute Salt Solutions. A diagram providing an overview of the extraction protocol can be seen in Scheme 1. The albumin fraction and other minor soluble constituents were extracted with water (adjusted to pH 8) containing 10 mM $CaCl_2$ and 10 mM $MgCl_2$ under vigorous vortexing for 10 min at RT. The suspension was centrifuged at 30000g for 15 min at 4 °C, and the supernatant containing the albumin fraction was collected. To minimize the residual albumin content present in the pellet, a second albumin extraction was performed and the supernatant was removed. The globulin fraction was extracted from the pellet with 100 mM Tris-HCl (pH 7.5) containing 10% (w/v) NaCl, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM ethylene glycol bis(α-aminoethyl ether)-_N,N,N′,N′_-tetraacetic acid (EGTA), by vigorous vortexing for 10 min at RT. The globulin-containing solution was centrifuged at 30000g for 15 min at 4 °C, and the supernatant obtained was dialyzed against phosphate-buffered saline (PBS) (pH 7.6) and stored at 4 °C.[10]

Anion-Exchange Chromatography (AEC). The different globulin fractions were isolated by AEC on a HiTrap ANX fast flow (FF) ion-exchange pre-packed column linked to a peristaltic pump. The column was equilibrated with 50 mM Tris-HCl (pH 7.5), loaded with 10 mL of the globulin fraction, and eluted with a linear gradient elution at a mobile phase flow of 0.5 mL/min, and the absorbance of the fractions was measured at 280 nm of wavelength. The sample (10 mL) was added

followed by a washing step of 20 min, and the gradient was established by increasing the concentration of NaCl every 2 min in steps of 0.01 M from 0 to 1 M. Under these conditions, γ-conglutin was not retained on the column and was immediately eluted. Fractions were eluted from 0 to 0.7 M NaCl, and the salt concentrations for elution of specific β-conglutin and combined α- and δ-conglutin fractions were optimized to be 0.3 and 0.45 M, respectively (Figure 1).

Fractions of the isolated subunits were lyophilized with a Freezone 1 Labconco apparatus at 0.066 mbar of vacuum and −50 °C. After lyophilization, the fractions were redissolved in 50 mM Tris-HCl (pH 7.5) and stored at 4 °C.
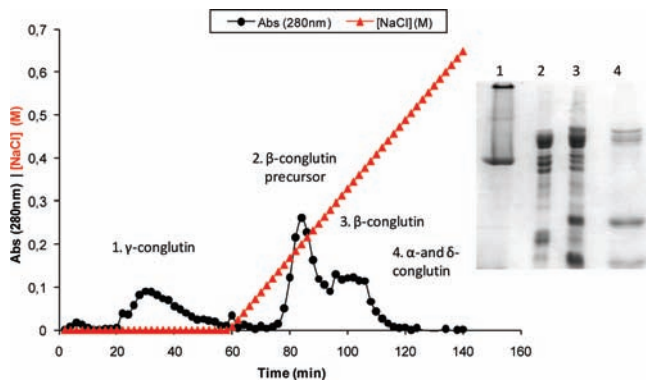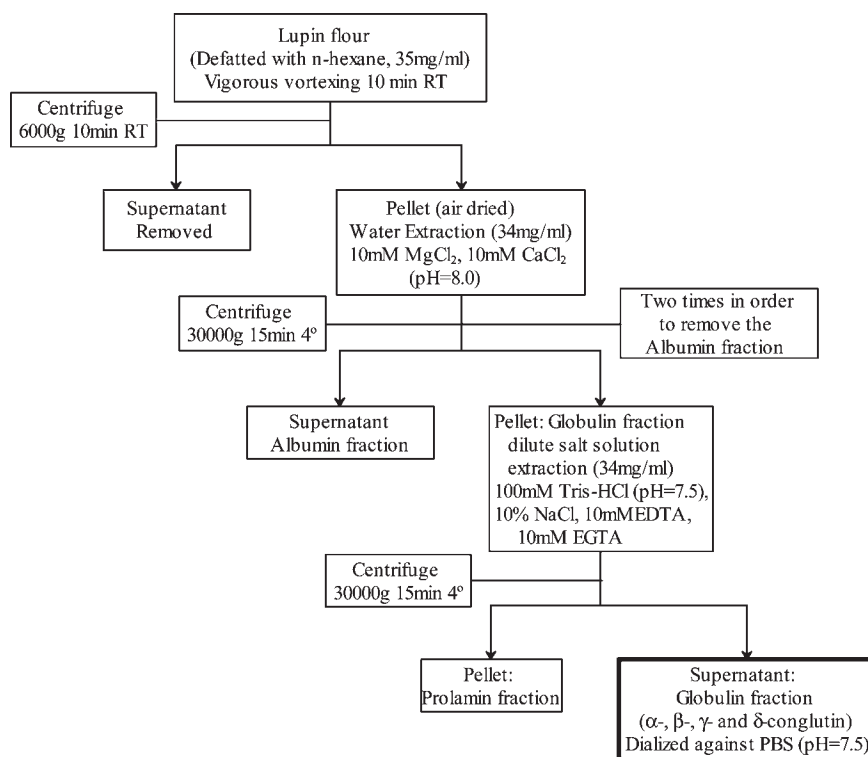
Size-Exclusion Chromatography (SEC) by Sephacryl-200 HR. α-Conglutin was purified from δ-conglutin by SEC using a self-prepared column (0.8 cm in diameter and 9 cm in height), packed with Sephacryl-200 HR and linked to a peristaltic pump. The column was equilibrated with 15 mM NaCl in 50 mM Tris-HCl (pH 7.5) and loaded with 0.2 mL of the combined α- and δ-conglutin fraction. The mobile phase flow was 0.1 mL/min. The fractions were collected every 2 min, and the absorbance of the fractions was measured at 280 nm.

Sodium Dodecyl Sulfate−Polyacrilamide Gel Electrophoresis (SDS−PAGE). Proteins from extracts were separated on SDS−PAGE (12% resolving gel and 4% stacking gel) performed in a vertical electrophoresis unit at an applied voltage of 70 V. When the tracking dye migrated to the bottom of the stacking gel, the voltage was then increased to 120 V until the tracking dye migrated to the bottom edge of the resolving gel. Gels were stained with Coomassie Brilliant Blue R-250 (0.05%, w/v) in methanol/acetic acid/water (25:10:65, v/v/v) and destained in the same solution without the dye.

Western Blotting. Proteins separated by 12% SDS−PAGE in reducing conditions were electroblotted onto a Immobilon-P membrane according to the method by Towbin et al.[30] on a Trans-blot electrophoretic transfer cell. The membranes were blocked in 5% bovine serum albumin (BSA) and washed 3 times in TBS-Tween at pH 7.5. Membranes were then soaked for 1 h at RT with 10% biotinylated rabbit anti-lupin antibody from the HAVen Lupin enzyme-linked immunosorbent assay (ELISA) kit, diluted 1:100 000 in 20 mM Tris-HCl buffer at pH 7.5 with 150 mM NaCl,[1] containing 1% (w/v) BSA, 0.5% (w/v) powdered milk, and 0.1% powdered sodium azide, and followed by the addition of streptavidin−horseradish peroxidase (HRP) (10 μg/mL) for 15 min at RT. Finally, 3,3′,5,5′-tetramethylbenzidine (TMB) liquid substrate was added until the precipitate was formed on the membrane for visualization.

Mass Spectrometric Analysis. To further characterize the isolated globulin proteins, peptide mass fingerprinting was performed for all of the bands obtained in the SDS−PAGE. Electrophoresis bands obtained were cut from the gel, and the proteins eluted. The isolated

Scheme 1. Scheme of the Extraction Protocol for Globulins with Dilute Salt Solutions

Lupin flour
(Defatted with n-hexane, 35mg/ml)
Vigorous vortexing 10 min RT

Centrifuge
6000g 10min RT

Supernatant
Removed

Pellet (air dried)
Water Extraction (34mg/ml)
10mM MgCl₂, 10mM CaCl₂
(pH=8.0)

Centrifuge
30000g 15min 4°

Two times in order
to remove the
Albumin fraction

Supernatant
Albumin fraction

Pellet: Globulin fraction
dilute salt solution
extraction (34mg/ml)
100mM Tris-HCl (pH=7.5),
10% NaCl, 10mMEDTA,
10mM EGTA

Centrifuge
30000g 15min 4°

Pellet:
Prolamin fraction

Supernatant:
Globulin fraction
(α-, β-, γ- and δ-conglutin)
Dialized against PBS (pH=7.5)

Figure 1. AEC: medium, ANX Sepharose 4 FF; bead size, $45-165\ \mu$m; type of gel, weak anion; charged group, $-N^+(C_2H_5)_2H$; sample, globulin extract; sample volume, 10 mL (1.7 mg/mL); buffer, 50 mM Tris-HCl at pH 7.5; fractions, eluted from 0 to 0.7 M NaCl at intervals of 16 mM; and flow, 0.5 mL/min. Note that the units of absorbance at 280 nm and the concentration of sodium chloride coincide on the y axis.

proteins were digested with trypsin, and the resulting peptide mixture, extracted from the polyacrylamide gel, was applied to MALDI−TOF and, finally, analyzed by MS, generating a peptide mass profile characteristic for the excised protein.

In-Gel Destaining, Reduction, Alkylation, Deglycosylation, and Digestion of Protein Samples. The bands of interest were manually excised from preparative Coomassie-stained 1-DE gels, depending upon their intensity and relative volume, destained by washing twice with 50% acetonitrile in 25 mM ammonium bicarbonate, and vacuum-dried. Bands were reduced with 10 mM dithiothreitol in 25 mM ammonium bicarbonate for 30 min at 56 °C and, subsequently, alkylated

with 55 mM iodoacetamide in 25 mM ammonium bicarbonate for 20 min in the dark. Gel pieces were alternately washed with 25 mM ammonium bicarbonate and acetonitrile and dried under vacuum. Gel bands were subsequently shrunk with acetonitrile and vacuum-dried. All gel pieces were incubated with 12.5 ng/μL sequencing-grade trypsin in 25 mM ammonium bicarbonate overnight at 37 °C. After digestion, the supernatants (crude extracts) were separated. Peptides were extracted from the gel pieces first into 50% acetonitrile and 1% trifluoroacetic acid and then into 100% acetonitrile. All extracts were pooled, and the volume was reduced by SpeedVac.[31]

MALDI−TOF MS. A total of 1 μL of each sample (extracted peptides) and then 0.4 μL of 3 mg/mL α-cyano-4-hydroxycinnamic acid matrix (Sigma) in 50% acetonitrile and 0.01% trifluoroacetic acid were spotted onto a MALDI target. Samples for MS were mixed 1:1 with α-cyano matrix (5 mg/mL in 50% acetonitrile and 0.3% trifluoroacetic acid) and spotted on the stained steel plate. Peptides were selected in the mass range of 750−3500 Da. All mass spectra were calibrated externally with the Sequazyme peptide mass standard kit and internally with trypsin autolysis peaks.[31]

■ RESULTS AND DISCUSSION

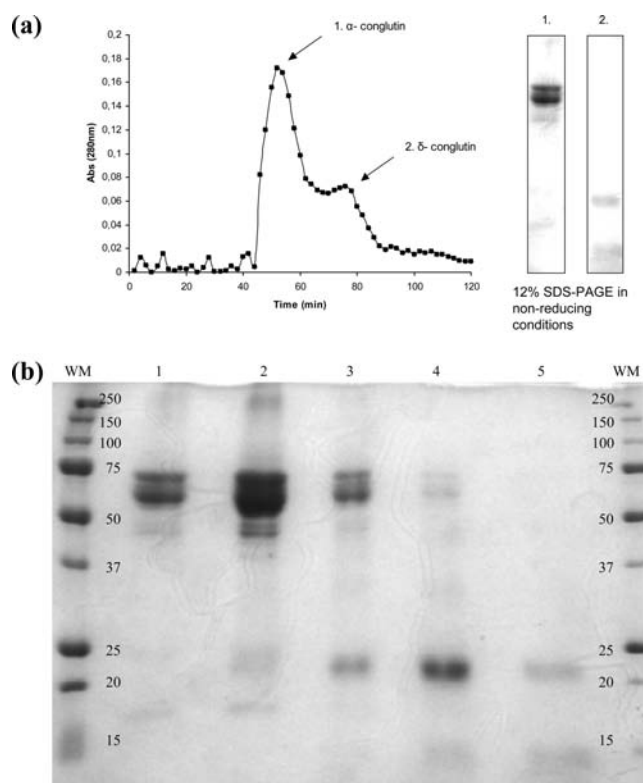**Extraction of Globulins and Isolation of Conglutin Subunits.** The protein extracts obtained showed the expected electrophoresis pattern (results not shown) for isolated, purified globulins, containing all of the α-, β-, γ-, and δ-conglutins. The purified globulin fraction was then applied to AEC for isolation of each of the globulin components. As previously reported by Dooper et al.[10] using AEC, the γ-conglutin is not retained on the column, the β-conglutin precursor and β-conglutin are eluted by a linear gradient of NaCl at 0.3 M, and at 0.45 M NaCl, the α- and δ-conglutins are co-eluted (Figure 1). As can be seen in lane 1 of
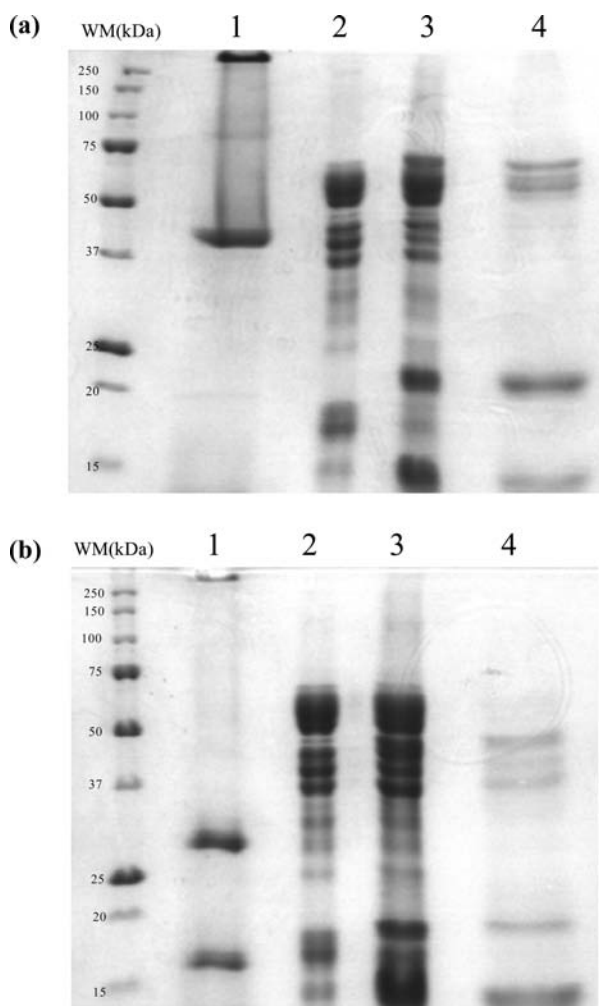
Figure 2. (a) Non-reducing and (b) reducing conditions of SDS—PAGE of the fractions obtained: WM, weight marker (kDa); lane 1, γ-conglutin; lane 2, β-conglutin precursor; lane 3, β-conglutin; and lane 4, α- and δ-conglutin.

Figure 3. (a) SEC: medium, Sephacryl S-200 HR; sample, globulin fraction previously eluted at 1 M NaCl by AEC; sample volume, 0.2 mL (1.34 mg/mL); column packed bed, 0.8 × 9 cm; buffer, 50 mM Tris HCl at pH 7.5 and 0.15 M NaCl; and flow, 0.1 mL/min. (b) SDS—PAGE (12%) in non-reducing conditions of the fractions obtained: WM, weight marker; lane 1, fraction 23—25, α-conglutin isolated; lane 2, fraction 26—29; lane 3, fraction 30—32; lane 4, fraction 33—36; and lane 5, fraction 37—44, δ-conglutin isolated.

Figure 2a, the electrophoresis pattern of the non-retained fraction of the AEC performed showed the expected γ-conglutin electrophoresis pattern, with a band obtained at 43 kDa of γ-conglutin, which, as can be seen in lane 1 of Figure 2b, upon reduction, is divided into its two subunits of 30 and 17 kDa. For the first eluted fractions at a concentration of 0.35 M NaCl, the bands obtained are indicative of β-conglutin (lane 3) and its precursor, obtained at a concentration of 0.25 M NaCl (lane 2) with bands ranging from 19 to 60 kDa, corresponding to the noncovalently linked subunits. The identity of the components was confirmed using the peptide mass fingerprinting. The expected α- and δ-conglutin electrophoresis pattern that was obtained in the eluted fraction at 0.45 M NaCl in 50 mM Tris-HCl (pH 7.5) shows bands at 70, 66, 60, and 53 kDa (lane 4 of Figure 2a), which, upon reduction, are split in the heavy chain of a size of 46, 42, 36, and 31 kDa, respectively, and light chain of 19 kDa (lane 4 of Figure 2b). A band of approximately 20 kDa and less than 15 kDa that can be ascribed to δ-conglutin is also observed under both reducing and non-reducing conditions.

The isolation of α-conglutin from δ-conglutin was not possible in a single purification step using AEC, and the most notable

difference between these globulins is their molecular weight, being 330—430 and 13 kDa, respectively (Table 1). SEC was chosen as the most appropriate technique to obtain pure proteins. As seen in Figure 3a, a clear isolation of the two proteins were obtained, and Figure 3b shows the purity of each of the isolated proteins, among others.

The protein bands were then analyzed using peptide mass fingerprinting, and the profile obtained for each band was then compared to the theoretical masses derived from the digestion *in silico* at the same enzyme excising sites of all protein sequences of both the Swissprot and Genbank databases. The proteins in the database were then ranked using peptide mass fingerprinting according to the number of peptide masses matching their sequence, within a given error tolerance in mass.[31]

The same procedure was applied to all of the bands obtained under both non-reducing and reducing conditions and positively confirms the isolation of the γ-conglutin, β-conglutin precursor, β-conglutin, and δ-conglutin subfractions, with very good sequence coverage and very high score (see the Supporting Information for detailed PMF analysis). It was possible to positively confirm the α-conglutin by PMF because it is only possible to find one fragment of this component in the databases coming from *L. angustifolius* [accession number of the National Center for Biotechnology Information (NCBI) is AAC49787, described as conglutin α (*L. angustifolius*) gi|2313076|gb|AAC49787.1|(2313076)] and one complete amino acid sequence

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for *L. albus* (accession number of UniProtKB/TrEMBL is Q53I54). However, correlating this fragment and amino acid sequence and taking into consideration the electrophoresis pattern and the mass spectra profile obtained, it can be proposed that it is isolated α-conglutin.

Western Blotting Analysis. As outlined in the Introduction, in individuals with lupin allergy, the globulin fractions have been suggested to be important allergens.[32] Furthermore, lupin has recently been added to the list of allergens requiring mandatory advisory labeling on foodstuffs sold in the European Union; thus, all products containing even trace amounts of lupin must be labeled correctly since December 2008 according to the European Commission Directive 2006/142/EC [European Commission (EC)] Annex IIIA of Directive 2000/13/EC,[33] in response to the increasing number of severe cases of lupin allergies reported during the past decade.[34]

Many studies have been carried out to identify which specific lupin fraction(s) are responsible for provoking the allergenic immune response. Initial studies outlining the isolation and characterization of protein bodies in *L. angustifolius* reported that γ-conglutin reacted with monoclonal antibodies, but the 2S sulfur-rich storage protein (δ-conglutin) did not produce any reaction.[35] Other studies, focused more on allergy to lupin flour, reported several IgE-binding proteins. The analysis of the serum of a patient who died after eating hot dog bread showed a high anti-peanut IgE level. Proteins with 38 and 15 kDa were responsible for the cross-reaction with the anti-peanut IgE of the patient, and a polyclonal anti-lupin serum detected three main protein bands at 65, 50, and 40 kDa in all extracts containing lupin. A cross-reaction against peanut proteins was negligible at high dilutions.[29]

More recent studies reported since 2005 maintain that γ-conglutin is the major allergen of lupin and that γ-conglutin polypeptides were also found to cross-react with IgG specific to the basic subunit of Ara h 3, a known peanut allergen of the 11S globulin family. This polypeptide of 43 kDa, which might coincide with the unreduced γ-conglutin, was also observed to produce cross-reactivity with anti-peanut IgE. The latest publications indicate that all of the α-, β-, γ-, and δ-conglutin subunits are candidate allergens and suggest a particularly strong allergenicity of α- and γ-conglutins. Furthermore, the results indicate the presence of cross-reactive allergens in lupin, peanut, and almond.[36,37]

A specific and sensitive analytical method for detecting and quantifying each one of the lupin conglutin protein is thus required for the protection of the consumers with food allergy from the danger of hidden allergens. The *L. angustifolius* conglutin β allergen has recently been designated Lup an 1 by the International Union of Immunological Societies (IUIS) Allergen Nomenclature Sub-committee.[38] However, as detailed above, there are reports indicating that all four subunits can provoke a toxic response. Currently, there are few commercial ELISA kits available on the market to detect lupin allergens;[1,10,39,40] however, these kits are not specific for each of the subunits, and none of them are capable of detecting the γ- and δ-conglutins. A real-time polymerase chain reaction (PCR) has also been used for the detection of lupin, increasing the sensitivity[41−43] but still incapable of specifically detecting each subunit, except for one report detailing the specific detection of α- and δ-conglutins as target immunogens.[43]

Using commercially available antibodies from the HAVen Lupin ELISA kit, western blotting analysis was carried out, and
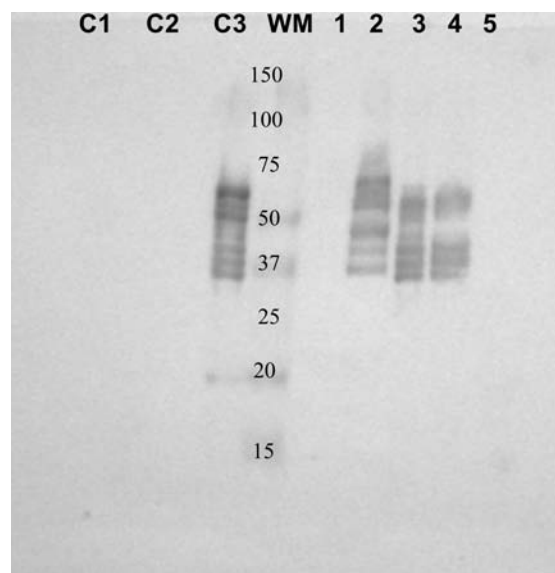
Figure 4. Western blot of the fractions obtained: lane 1, δ-conglutin; lane 2, α-conglutin; lane 3, β-conglutin; lane 4, β-conglutin precursor; lane 5, γ-conglutin; WM, kaleidoscope weight marker (8 μL, Bio-Rad); lane C1, PSA; lane C2, BSA; and lane C3, crude extract.

as can be seen in Figure 4, these antibodies interact with the β-conglutin precursor, as well as both of the α- and β-conglutin subunits, whereas no interaction with the δ- or γ-conglutin proteins is observed. These results can be expected because the antibodies used in the kit were produced using a mixture of uncharacterized β-, α-, and δ-conglutin proteins as the target immunogen. These results confirm the isolation of the α protein from the δ protein but also highlight that the kit does not detect δ- or γ-conglutin protein, despite the above-mentioned reports that the γ-conglutin protein also provokes toxicity, and furthermore, the assay detects the α-conglutin protein, even though there are reports that it does not provoke toxicity. It is clear that there is a definite requirement for an analytical assay that specifically detects each of the proteins.[38,44,45]

In conclusion, in this work, we report on the characterization of conglutin proteins isolated from lupin globulins. The isolated fractions were characterized using reducing and non-reducing electrophoresis, western blotting, and peptide mass fingerprinting, all of which confirmed the purity of each of the conglutin subunits. Furthermore, the peptide mass fingerprinting results provided new mass spectra for α-conglutin for a protein sequence not introduced in the Swissprot and Genbank databases.

These isolated pure proteins can be further used as immunogens for raising specific antibodies, as well as standards for the analytical detection of specific proteins. The protocol elucidated can also be used to selectively extract and purify specific globulin proteins from commercial foodstuffs for analysis. Furthermore, the isolated proteins can be used to elucidate information regarding toxicity of each protein by looking at their interaction with the IgE antibodies found in the serum of individuals allergic to lupin, such as reported by Goggin et al.,[38] who reported possible contamination of their isolated α-conglutin with β-conglutin, giving rise to a false-positive result, and the authors also indicate that the toxicity of the γ-conglutin needs to be further investigated.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information. Peptide mass fingerprinting of protein isolates (Annexes 1 and 2). This material is available free of charge via the Internet at http://pubs.acs.org.

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