JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Proteomic Analysis of Lupin Seed Proteins To Identify Conglutin β as an Allergen, Lup an 1

DANICA E. GOGGIN,[†] GISELA MIR,[‡] WILLIAM B. SMITH,[§] MARTIN STUCKEY,[||] AND PENELOPE M. C. SMITH*,[‡]

School of Plant Biology, University of Western Australia, 35 Stirling Highway, Crawley, Western Australia 6009, Australia, School of Biological Sciences, University of Sydney, New South Wales 2006, Australia, Clinical Immunology and Allergy, Royal Adelaide Hospital, North Terrace, South Australia 5000, Australia, and Clinical Immunology and Allergy, St. John of God Pathology, Hollywood Private Hospital, Monash Avenue, Nedlands, Western Australia 6009, Australia

Lupin products may be valuable as human foods because of their high protein content and potential anticholesterolemic properties. However, a small percentage of the population is allergic to lupin. In this study, we use *in vitro* IgE binding and mass spectrometry to identify conglutin β, a major storage protein, as an allergen in seeds of *Lupinus angustifolius* and *Lupinus albus*. Purification of conglutin β from *L. angustifolius* flour confirmed that serum IgE binds to this protein. Where IgE in sera recognized lupin proteins on Western blots, it recognized conglutin β, suggesting this protein is a major allergen for lupin. The *L. angustifolius* conglutin β allergen has been designated Lup an 1 by the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee.

KEYWORDS: Allergy; conglutin β; Lup an 1; IgE; lupin; storage protein

INTRODUCTION

Lupin seeds are a useful source of human food products because of their unique composition, being high in protein and dietary fiber but low in fat and starch. There are a number of other potential benefits to human health associated with the consumption of lupin seed components, such as decreases in blood glucose and cholesterol levels (e.g., refs *1–3*). The active component in the lupin protein was found to be conglutin γ, which comprises around 5% of total lupin seed protein (*1*). Conglutin β, one of the major seed storage proteins, is also a candidate for food improvement because of its similarity to soybean β-conglycinin, which has anticholesterolemic properties (*4*).

With the increasing inclusion of lupin products in human foods, particularly in Europe, where lupin was approved as a food ingredient in 1997, it has become clear that lupin seeds also contain allergenic proteins. Some individuals experience allergic reactions upon ingestion or inhalation of lupin products, with symptoms ranging from rashes and nausea to anaphylaxis (e.g., refs *5–8*). Many reports suggest there is cross-sensitization with peanut (*5, 6, 9*), although monosensitization to lupin also occurs (*7, 8, 10*). The prevalence of allergy to lupin is not clear, but it has been variously estimated that 30% (*9*) or 4% (*11*) of peanut-allergic individuals react to lupin. The prevalence of allergy to lupin in the absence of allergy to peanut is not known but appears low (*11, 12*).

The most common species of lupin consumed by humans is *Lupinus albus* L.; however, use of *Lupinus angustifolius* L. is increasing and *Lupinus luteus* L. seeds are also used in food production. These and other lupin species have two major (conglutin α and β) and two minor (conglutin γ and δ) seed storage proteins, which, with the exception of conglutin δ, are produced by cleavage of higher molecular-mass precursor proteins (*13*). In seeds, nuts, and grains consumed as food, it is usually storage proteins that cause allergic reactions upon ingestion (*14*). It is thus probable that one or more of the lupin seed storage proteins, with their stability under extremes of pH and temperature (*15*) and similarity in primary sequence to peanut allergens (*16*), are responsible for allergic reactions. Allergenic peanut storage proteins are represented by 11S globulins (Ara h 3, *14*), 7S globulins (Ara h 1, *17*), and 2S albumins (Ara h 2, *18*), and these classes of storage protein have also been identified as allergens in several species of tree nuts and legumes (reviewed in ref *14*). There have been indications that some of the lupin storage proteins bind IgE (*19–21*), but in most cases, the designation was based on IgE binding from only a few sera, making it difficult to determine the importance of particular allergens.

In this study, we have made a proteomic analysis of *L. angustifolius*, *L. albus*, and *L. luteus* seed flour and used mass spectrometry to identify the IgE-binding proteins. To confirm these results, lupin seed storage proteins from *L. angustifolius* (conglutins α, β, γ, and δ) were selectively purified and tested

* To whom correspondence should be addressed. Telephone: +61-2-9036-7169. Fax: +61-2-9351-4771. E-mail: p.smith@usyd.edu.au.
[†] University of Western Australia.
[‡] University of Sydney.
[§] Royal Adelaide Hospital.
[||] Hollywood Private Hospital.

10.1021/jf800840u CCC: $40.75 © XXXX American Chemical Society
Published on Web 07/12/2008

Table 1. Information about Volunteers Whose Sera Was Used in the Study[a]

patient number (age, sex)	symptoms	lupin wheal size (mm)	lupin-specific serum IgE (kU_A/L)	peanut-specific serum IgE (kU_A/L)	skin test reactions (wheal size, mm)[b]	clinical reactions to other allergens
1[c] (41, F)	DB, OAS, U	N/D	87.5	2.52	al (0), gb (0), ca (2) hn (3), bw (3), p (8), sb (3), pn (2),	none
2[c,d] (36, M)	N, V, DB, U, OAS	10	21.9	<0.35	gb (0), c (0), hn (0), o (0), p (0), pn (0), r (0), sb (0), w (0)	none
3[c] (31, F)	OAS, U	9	32.8	1.36	gb (0), c (0), hn (0), o (0), p (2), pn (5), r (0), sb (2), w (0)	none
4[c] (68, F)	DB	6	26.8	2.37	gb (0), c (4), hn (3), o (0), p (5), pn (5), r (2), sb (0), w (2), pc (3)	ryegrass, cats
5[d] (N/D, F)	DB, R	N/D	23.4	1.46	N/D	grass pollen
6[c] (29, F)	AN	9	55.9	<0.35	p (8), sb (3), pn (0)	none
7[c] (49,F)	U, AN	5	5.3	4.4	pn (2), sb (2), l (5) cp (6)	chickpea, lentil
8[c] (48, M)	AN	10	53	0.51	cp (8), l (10)	curry, specific ingredient N/D
9[c] (56, M)	N/D	6	N/D	N/D	gb (2), c (2), hn (0), o (0), p (3), pn (3), r (0), sb (3), w (0)	none
10[c] (53, F)	N/D	8	N/D	N/D	gb (0), c (0), hn (0), o (0), p (3), pn (0), r (0), sb (3), w (0)	none
11[c,d] (N/D, M)	R	9	N/D	N/D	N/D	none
12[d] (N/D, M)	DB, R	4	N/D	N/D	N/D	none
13 (31, F)	atopic control, no reaction to lupin		<0.35	<0.35		

[a] Abbreviations: N/D, not determined; AN, anaphylaxis; DB, difficulty breathing; N, nausea; OAS, oral allergy syndrome; R, rhinitis; U, urticaria; V, vomiting; al, almond; bw, buckwheat; ca, cashew; c, corn; gb, green bean; hn, hazelnut; o, oat; p, pea; pc, pecan; pn, peanut; sb, soybean; cp, chickpea; l, lentil; w, wheat. [b] A wheal diameter of ≥3 mm was considered a positive reaction. [c] Reacts to ingested lupin products. [d] Reacts to inhaled lupin products.

separately for IgE binding. We have concentrated on *L. angustifolius* because it is the major lupin grown in Australia and its allergenic proteins have not been characterized.

MATERIALS AND METHODS

Plant Material. Whole flour and protein or fiber isolates from *L. angustifolius* L. cvs Belara and Myallie were provided by the Department of Agriculture and Food, Western Australia, and by George Weston Foods (Sydney, Australia), respectively. Mature seeds of *L. angustifolius* cv Merrit, *L. albus* cv Kiev Mutant, and *L. luteus* cv TEO-105 were obtained from seed stocks at the School of Plant Biology, University of Western Australia.

Patient Sera. Sera used in the study was from 12 volunteers, who were mostly recruited from workplaces involved in lupin research or in processing lupin flour or had presented to medical clinics with allergic reactions (subjects 6−8). In some cases (**Table 1**), symptoms were noted on inhalation of lupin flour in the workplace, without any symptoms when exposed to other types of flour, and this correlated with a positive skin-prick test to lupin extract. In other cases, individuals had experienced symptoms after ingestion of lupin-containing foods and had a corroborating positive skin-prick test or blood test for lupin-specific IgE. Allergy to other foods that might have been ingested at the same time was excluded by the history and/or allergy testing. In the case of subject 1 (**Table 1**), although skin-prick testing data is not available, the clear clinical history of symptoms exclusively on exposure to lupin together with the positive serum IgE test was taken as sufficient evidence of lupin allergy. Some subjects had positive skin-prick tests or raised specific IgE levels to other legumes, such as peanut or pea, but were clinically tolerant to these foods (could ingest them without reaction), and the level of IgE was usually significantly lower than to lupin. Blood was collected from each volunteer and also from one atopic individual with a negative skin-prick test to lupin (nonallergic control). Informed consent was obtained from all volunteers. For most volunteers, a UniCAP assay (Phadia, Uppsala, Sweden) with allergens F335 (*L. albus*) and F13 (*Arachis hypogaea*, peanut) was performed.

Sample Preparation for Electrophoresis. Whole seed powder and isolates were defatted 3 times in 20 vol. cold (−20 °C) acetone. For sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE), 2 g of defatted material was extracted with 10 mL of 0.1 M Tris/0.5 M glycine (pH 8.7) at 45 °C overnight as described in Holden et al. (*22*); for two-dimensional (2D) electrophoresis, 50 mg of defatted material was extracted in 1 mL of IEF sample buffer [8 M urea, 4% (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 60 mM dithiothreitol (DTT), 2% (v/v) immobilized pH gradient (IPG) buffer (GE Life Sciences, Munich, Germany)] at room temperature for at least 2 h. Extracts were centrifuged at 12000*g* for 30 min at 4 °C, and the supernatant was used for electrophoretic analysis. The protein concentration was measured using the method of Bradford (*23*).

Purification of Conglutins from *L. angustifolius* Flour. Conglutins α, β, and γ were purified from defatted *L. angustifolius* flour using the classical method of Blagrove and Gillespie (*13*). Briefly, the flour was extracted in cold water to remove soluble albumins and then in 10% (w/v) NaCl to extract the water-insoluble storage proteins. These were subjected to successive ammonium sulfate precipitations and dialysis to separate cold-insoluble conglutin α from cold-soluble conglutin α and conglutin β and to separate conglutin γ from the other proteins. Cold-soluble conglutin α and conglutin β were separated by further ammonium sulfate precipitation, with the 64−66% ammonium sulfate cut of conglutin α and the 70−90% cut of conglutin β being used for analysis. Purified conglutins α, β, and γ were resuspended in 0.15 M sodium phosphate buffer (14 g L^{-1} Na_2HPO_4 and 8 g L^{-1} NaH_2PO_4 at pH 7) and precipitated with 3 vol. cold (−20 °C) acetone to remove salts before IEF. Precipitated protein was washed extensively with cold acetone before resuspension in IEF sample buffer for 2D PAGE or with 0.15 M sodium phosphate buffer for SDS−PAGE.

Conglutin δ was isolated according to Sironi et al. (*24*) in a separate procedure. Briefly, lupin storage proteins were solubilized by successive extraction at high (pH 8.5) and low (pH 4.5) pH. Conglutin γ was removed by precipitation with $ZnCl_2$, and conglutins α and β were removed by precipitation with a high salt (0.4 M NaCl)/ethanol solution. The remaining protein, highly enriched in conglutin δ, was cold-precipitated and resuspended in either IEF sample buffer or SDS−PAGE sample buffer.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis. Proteins were separated by tris(hydroxymethyl)aminomethane (Tris)−glycine SDS−PAGE (*25*), with a 12.5% polyacrylamide separating gel. To obtain accurate molecular masses of small protein subunits, purified conglutin γ and conglutin δ were also separated on Tris−*N*-[-tris(hydroxymethyl)methyl]glycine (Tricine) peptide gels (*26*), using a 16.5% polyacrylamide separating gel. Gels were stained with colloidal Coomassie Brilliant Blue G-250 or silver (*27*) or blotted onto a nitrocellulose membrane.

Two-Dimensional Electrophoresis. IPG strips [pH 3, 10 NL, 13 cm (GE Life Sciences)] were rehydrated with 350−1000 μg sample protein and focused for a total of 17 kV h according to the protocol provided by GE Life Sciences. Focused IPG strips were equilibrated twice in SDS equilibration buffer [50 mM Tris-HCl (pH 8.8), 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS, and 0.002% (w/v) bromophenol blue] containing 65 mM DTT for the first equilibration and 135

mM iodoacetamide for the second and then run on 16 × 18 cm, 12.5% polyacrylamide gels. Gels were either stained using colloidal Coomassie Brilliant Blue G-250 or electroblotted onto a nitrocellulose membrane.

Western Blotting and Immunodetection of Allergenic Proteins. Proteins separated by one- or two-dimensional SDS−PAGE were electroblotted onto 0.45 μm Protran nitrocellulose membrane (Schleicher and Scheull, Keene, NH) according to Towbin et al. (*28*). Total protein on membranes was detected by staining with Ponceau S (*29*). Allergenic proteins were identified on Western blots by probing with human sera. Blots were blocked in 0.2% (w/v) bovine serum albumin (BSA) in TBS buffer (50 mM Tris and 150 mM NaCl at pH 8) and then incubated overnight at room temperature in serum diluted $^1/_{10}$ in antibody solution (0.2% BSA and 0.1% Tween-20 in TBS). After extensive washing, the blots were incubated for 2 h in secondary antibody [$^1/_{3000}$ dilution in antibody solution for mouse monoclonal antihuman IgE conjugated to alkaline phosphatase (Sigma, Sydney, Australia) or $^1/_{15000}$ for goat antihuman IgE conjugated to horseradish peroxidase (Sigma)]. Binding of the secondary antibody was detected using the Immun-Star Chemiluminescent Protein Detection System (BioRad, Hercules, CA). In all cases, duplicate blots were probed with negative-control serum or with secondary antibody alone, to identify any nonspecific protein binding that could be misinterpreted as a positive result.

Mass Spectrometric Analysis of Protein Spots. Photographs of the 2D gels were resized to match the immunoblots so that IgE-reactive spots could be identified on the gels. Where closely spaced spots gave partially overlapping signals, the center of the spot on the immunblot was taken as the position of the spot on the gel. Protein spots of interest were excised from Coomassie Brilliant Blue stained 2D gels. Some spots were analyzed by Proteomics International, using trypsin digestion followed by electrospray time-of-flight mass spectrometry on a Q-Star Pulsar *i* mass spectrometer (Applied Biosystems, Foster City, CA). Other spots were analyzed by the Research School of Biological Sciences at the Australian National University using a Finnigan ProteomeX Work Station (ThermoFisher Scientific, Waltham, MA). Data analysis used the Mascot algorithm (MS/MS ion search, Matrix Science) with the MSDB database and a lupin database containing all *L. albus* and *L. angustifolius* sequences from GenBank and a number of in-house expressed sequence tags (ESTs) from *L. angustifolius*. A match was accepted where the MS/MS ion search predicted matches for two or more peptides from the same protein. Sequences that were used to predict the identity of spots but were not in GenBank have been submitted.

For purified conglutin β, spots were excised from SDS−PAGE or 2D gels and digested with trypsin in the gel. After a first matrix-assisted laser desorption/ionization−mass spectrometry (MALDI−MS) analysis on a QSTAR XL (Applied Biosystems), samples were derivatized with 4-sulfophenyl isothiocyanate (SPITC) reagent (Sigma) in 20 mM NH_4HCO_3 for 30 min at 55 °C, cleaned with PerfectPure tips (Eppendorf, Sydney, Australia), and peptides of interest were further fragmented and analyzed using MALDI MS/MS to determine the sequence.

Data from MALDI−MS were analyzed with Analyst software (Applied Biosystems), and searches were made using the Mascot algorithm, allowing one missed cleavage and considering cysteine carbamidomethylation and methionine oxidation, with tolerance set at ±50 ppm. MS/MS spectra were manually analyzed, and the amino acid sequences of individual peptides were deduced. These peptide sequences were used in BLAST searches for short, nearly exact matches on the nonredundant protein sequence database at National Center for Biotechnology Information (NCBI) or on an in-house lupin database.

RESULTS

Lupin Allergic Individuals. All of the lupin-allergic patients who were skin-prick-tested (11 of 12) showed a positive reaction (regarded as a wheal diameter of ≥3 mm) to lupin seed extract, and those for which the information was available had high to very high lupin-specific IgE levels in their sera (**Table 1**). Patient 1 (not skin tested) had had a previous positive lupin-specific serum IgE test and had reacted on a number of occasions to foods where the common ingredient was lupin. Some subjects with lupin allergy had low levels of IgE to peanut and/or positive skin-prick tests to other legumes. Responses to a patient questionnaire on previous allergic reactions revealed that only patient 7 had experienced allergic symptoms after ingesting other legume products (chickpea and lentil), although patient 2 had experienced an itchy throat after inhaling soy dust.

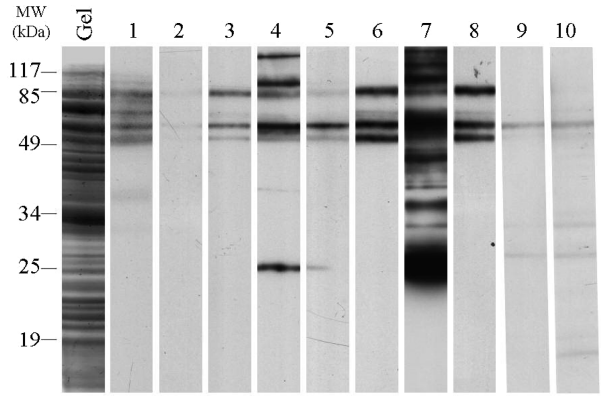

Figure 1. Immunoblot of *L. angustifolius* flour probed with sera from lupin-allergic individuals (lanes 1−8), serum from an individual who was not allergic to lupin (lane 9), or with secondary antibody alone (lane 10). The total protein pattern of lupin flour is shown to the left (gel).

Preliminary Characterization of Patient Sera. A total of 8 of the 12 sera collected from lupin-allergic individuals contained IgEs that bound to proteins on Western blots of lupin flour separated by SDS−PAGE (**Figure 1**). The majority of IgE-reactive bands were in the size range of 49−90 kDa. The control serum showed weak bands at approximately 25, 30, and 60 kDa. Because these bands often appear in our control probed only with secondary antibody, we consider these bands to be the result of nonspecific binding of secondary antibody (lane 10 in **Figure 1**).

Identification of Allergenic Proteins in Lupin. Seed proteins from *L. angustifolius*, *L. albus*, and *L. luteus* were separated by 2D electrophoresis. The different species showed distinct patterns of protein spots, but in all three species, the majority of IgE-reactive spots were at 40−65 kDa, with pI approximately 4.5−6 (**Figures 2** and **3**). In *L. angustifolius*, a second major group of IgE reactive spots was evident between 20 and 26 kDa, with approximate pI values from 5.5 to 7 (**Figure 2**). IgE-reactive spots analyzed by MS are indicated on the Coomassie-stained gels in **Figures 2** and **3**, and the identities of these spots are listed in **Table 2** (see the Supporting Information for peptides matched for each spot). Of the 86 IgE-reactive spots analyzed from the three lupin species (**Figures 2** and **3**), 31 were identified as conglutin β (30 from *L. angustifolius* and 1 from *L. albus*) and five as conglutin γ. Five nonreactive spots (numbered 87, 89, 92, 93, and 94), two of which (87 and 89) were highly abundant (**Figure 2**), were also sequenced from *L. angustifolius* and identified as conglutin β. For the 50 remaining IgE-reactive spots, 11 gave only one peptide match and the rest either could not be matched to any protein in the database (*16*) or there was no reliable sequence data obtained.

Analysis of Purified Conglutins. Conglutins α, β, γ, and δ purified from defatted *L. angustifolius* flour were analyzed by SDS−PAGE and 2D electrophoresis and immunoblotting, using combined sera as a probe. IgE bound to two proteins in the >50 kDa molecular mass range in the conglutin α preparation (**Figure 4**); however, MS/MS ion searches matched these proteins to conglutin β, and they likely represent contaminants in the sample. On the conglutin β blot, IgE reacted strongly

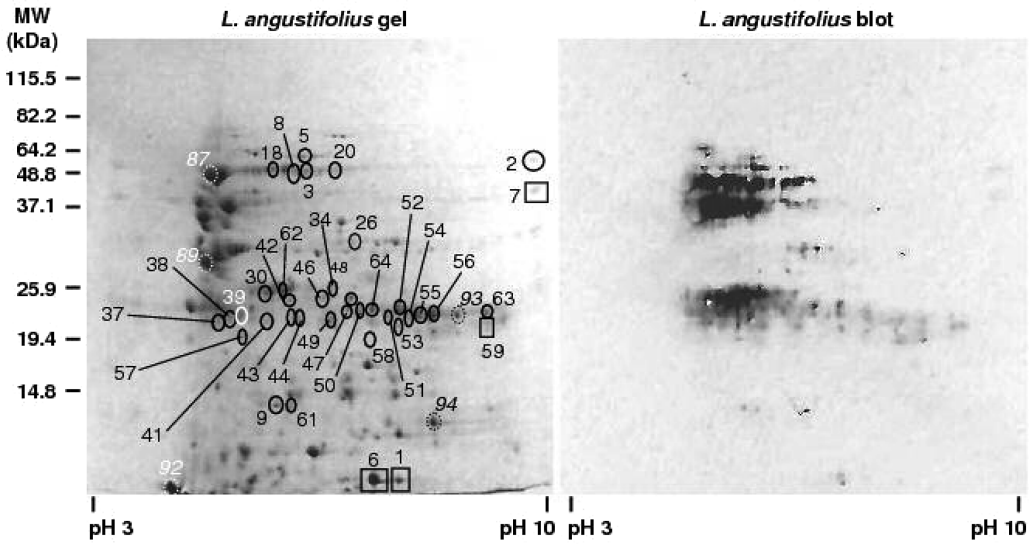

Figure 2. Two-dimensional electrophoretic analysis of *L. angustifolius* seed protein, showing total protein and immunoblots probed with combined sera. Protein spots for which identifications were made are enclosed by circles (solid for IgE-reactive conglutin β and dotted for nonreactive conglutin β) or rectangles (solid for conglutin γ).

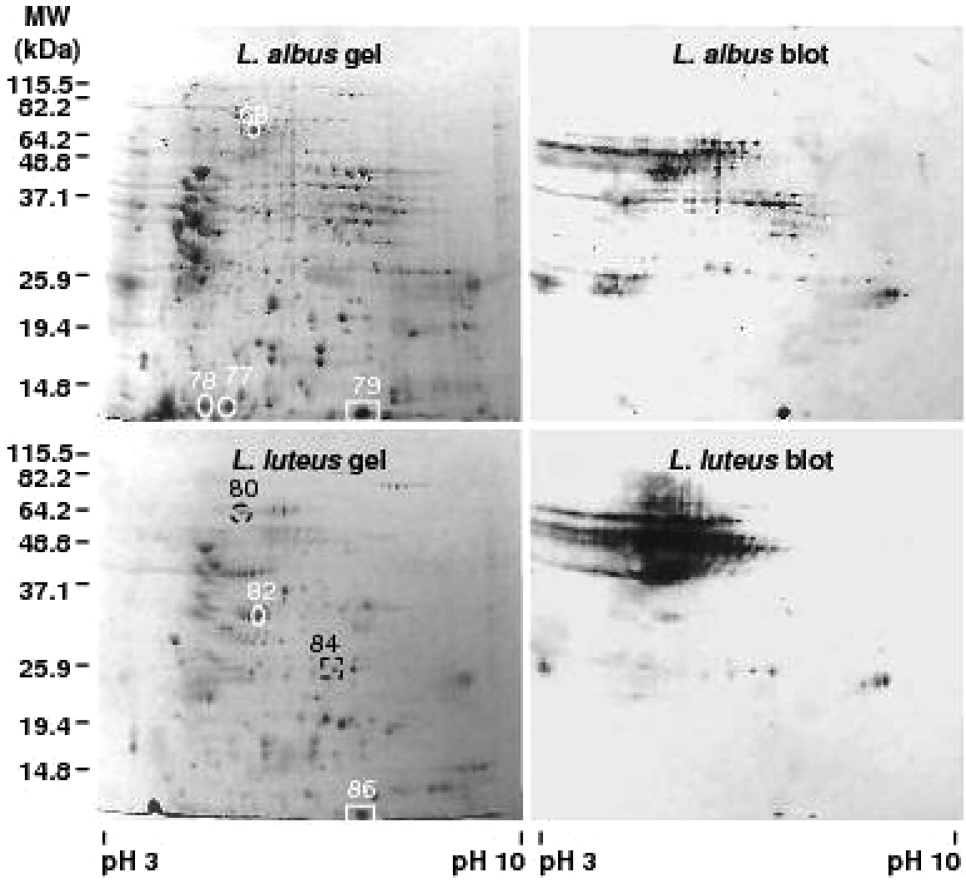

Figure 3. Two-dimensional electrophoretic analysis of *L. albus* and *L. luteus* seed protein, showing total protein and immunoblots probed with combined sera. Protein spots for which identifications were made are enclosed by circles (solid for IgE-reactive conglutin β, dotted for nonreactive conglutin β, and dashed for conglutin α) or rectangles [solid for conglutin γ and dashed for putative triacyl glycerol (TAG) factor protein].

with components that were >40 kDa, more weakly with the 25−31 kDa components, and not at all with those that were 12−16 kDa (**Figure 4**).

Both of the major subunits of conglutin γ (shown as 22 and 9 kDa proteins on Tris−glycine 2D electrophoresis and as 31 and 16 kDa proteins on Tris−Tricine SDS−PAGE) gave immunoreactive signals, as did some higher molecular-mass proteins (~50 kDa and pI 9−10), which were not abundant enough to be visualized by Coomassie staining (**Figure 4**). However, blots probed with secondary antibody alone gave strong signals for the 22 and 9 kDa conglutin γ proteins (**Figure 4**). IgE did not bind to either the 11 or 5 kDa subunits of purified conglutin δ (**Figure 4**).

Purified conglutin β separated by SDS−PAGE was probed with individual sera from lupin-allergic individuals and one nonallergic individual (**Figure 5**). Two bands of 60−70 kDa

Table 2. Identities of IgE-Reactive Lupin Proteins Excised from 2D Gels[a]

spot identity	spot number identified			closest match[b]
	L. angustifolius	*L. albus*	*L. luteus*	
conglutin β	2, 3, 8, 20, 43, 44, 47, 50−55, 59, 61, 63, 64			DT454368[c]
conglutin β	5, 18, 38, 39, 45, 46, 56, 57, 58			DT454323[d]
conglutin β	9, 26			EF455725[e]
conglutin β	30, 37, 42, 48			EF455724[f]
conglutin β		77		AJ966470[g]
conglutin γ	1			X65601[h]
conglutin γ	6, 7			L39786[i]
conglutin γ		79	86	AJ297490[j]

[a] Peptide matches for each spot are listed in the supporting data. [b] GenBank database ID of sequence from which closest match is derived. [c] UWA067 cDNA library from early and middle stages of seed development of *L. angustifolius*. [d] UWA021 cDNA library from early and middle stages of seed development of *L. angustifolius*. [e] *L. angustifolius* conglutin β seed storage protein (LSL2.2) mRNA, complete cds. [f] *L. angustifolius* conglutin β seed storage protein (UWA220) mRNA, partial cds. [g] *L. albus* partial mRNA for vicilin-like protein, clone LaJCMC675 (Genpept ID CAI84850). [h] *L. angustifolius* mRNA for conglutin γ. [i] LUPDNASEQ *L. angustifolius* conglutin γ gene, complete cds. [j] *L. albus* gene for conglutin γ, clone 32.

were recognized by all sera, while a band of 40 kDa was recognized by six of the eight allergic sera. The 25−31 kDa subunits that were faintly recognized by the combined sera in **Figure 4** were absent on these blots. Blots probed with secondary antibody alone gave one positive signal at ∼20 kDa (Figure S1 in the Supporting Information). The two bands that were recognized by IgE most strongly on the SDS−PAGE gel (**Figure 5**), and two IgE reactive spots of a similar size on the 2D gel of purified conglutin β (**Figure 4**) were analyzed by MALDI−MS. The mass spectra of all of the bands and spots were almost identical, suggesting the proteins have near identical sequences. The amino acid sequence of three peptides (YEEIQR, AGSKDNVI, and LLGFINADE) from two spots was determined. The closest match for the peptides was conglutin β (ABR21771, AAS97865, and in house EST sequences).

DISCUSSION

This study has identified conglutin β as an allergen in *L. angustifolius* and *L. albus* seeds based on mass spectrometric analysis of IgE-reactive protein spots on 2D gels and the IgE-specific reactivity of purified *L. angustifolius* conglutin β. Our analysis suggests conglutin β is a major allergen for *L. angustifolius*, because IgE from all lupin allergic individuals in which IgE could be detected ($^{8}/_{12}$) recognized the purified protein. The *L. angustifolius* allergen has been designated Lup an 1.0101 by the International Union of Immunological Societies (IUIS) allergen nomenclature subcommittee.

Conglutin β, the major storage protein in lupin (*13*), is a 7S globulin similar to vicilin and has significant sequence identity to Ara h 1 (*16*). Gayler et al. (*30*) previously identified three high-molecular-mass conglutin β precursor proteins in developing cotyledons using antibodies to the protein and *in vivo* radiolabeling. The results of the current study, in which 40 protein spots were identified as conglutin β, also suggest the presence of more than one precursor: 35 conglutin β spots bound IgE, while 5, including 1 abundant high-molecular-mass subunit, did not. In addition, 2D gels of purified conglutin β probed with IgE showed that the higher molecular-mass subunits were more likely to bind IgE than the lower molecular-mass subunits.

Our analysis of IgE-binding proteins from *L. albus* and *L. luteus* was not as comprehensive as for *L. angustifolius* but confirms the identification of *L. albus* conglutin β as an allergen (*21*) and suggests the same protein in *L. luteus* binds IgE. Only single peptide matches were predicted for *L. luteus* (see the Supporting Information), a result that is probably due to the lack of *L. luteus* conglutin β sequences in the databases that we searched. The matches that we obtained rely on identity between the *L. albus* or *L. angustifolius* sequences in the database and the *L. luteus* protein.

Conglutin γ appeared initially to be an allergenic protein in lupin, with five IgE-reactive spots from three lupin species being identified as conglutin γ and the purified protein from *L. angustifolius* giving a strong signal on Western blots. However, signals of equal or greater intensity were obtained with secondary antibody only (**Figure 4**) or with negative control serum, an interaction that could not be blocked by preprobing with secondary antibody or human IgG or eliminated by increasing the stringency of washing (results not shown). Initial screening of patient sera against total *L. angustifolius* seed protein showed that bands corresponding in size to the mature subunits of conglutin γ were detected by the negative control serum (faintly visible in lane 9, **Figure 1**, at ∼30 kDa) and also by the secondary antibody alone (**Figure 1** and Figure S1 in the Supporting Information). As conglutin γ makes up approximately 5% of total lupin seed protein (*31*), this corresponds to around 240 ng of conglutin γ protein per gel lane being bound by the nonspecific antibodies. Therefore, it is unlikely that overloading of purified conglutin γ protein on the 2D gels caused the nonspecific binding shown in **Figure 4**. However, IgE did bind specifically to spots at 50 kDa and pI 9−10 on the conglutin γ 2D gel (**Figure 4**), identified from a corresponding spot on the total flour gel (spot 7 in **Figure 2**) as a conglutin γ precursor. Therefore, it is possible that IgE binding to mature conglutin γ is masked by the nonspecific binding of secondary antibody to the protein. Skin-prick tests with purified *L. angustifolius* conglutin γ may resolve its status as an allergen.

There were a number of IgE-binding spots that could not be identified, because the MS/MS ion searches that we used rely on a comparison to amino acid sequences derived from a cDNA sequence (if the appropriate cDNA is absent from the database, the protein cannot be identified). Conglutin β may not be the only allergen to which the individuals tested react, because the IgE-reactive protein banding/spot patterns of total lupin flour and of purified conglutin β did not match exactly (note however that this may also result from selective purification of certain conglutin β subunits in the purification method that we used, *13*). In addition, if slightly different forms of conglutin β exist for which cDNA sequences are not available, they will not be identified by MS/MS ion searches. *L. angustifolius* conglutin δ is unlikely to be allergenic based on the results for IgE binding to purified protein and the fact that cDNA sequences were in our database.

A crucial question that remains to be answered is the difference between the proteins and/or epitopes recognized by patients who have IgE to lupin alone and those who have IgE to both lupin and peanut. In the latter group, it is assumed (but not yet proven) that peanut sensitization is primary and that lupin co-sensitization occurs through cross-reactivity. The subjects studied in this paper are likely to have been primarily sensitized to lupin (we assume this because they had known exposure or strong clinical reactivity) and some had low-level detectable IgE (by commercial assay) to peanut. Although there is similarity in the primary structures of Ara h 1 and conglutin β (*16*), it is unlikely that this explains cross-reactivity between

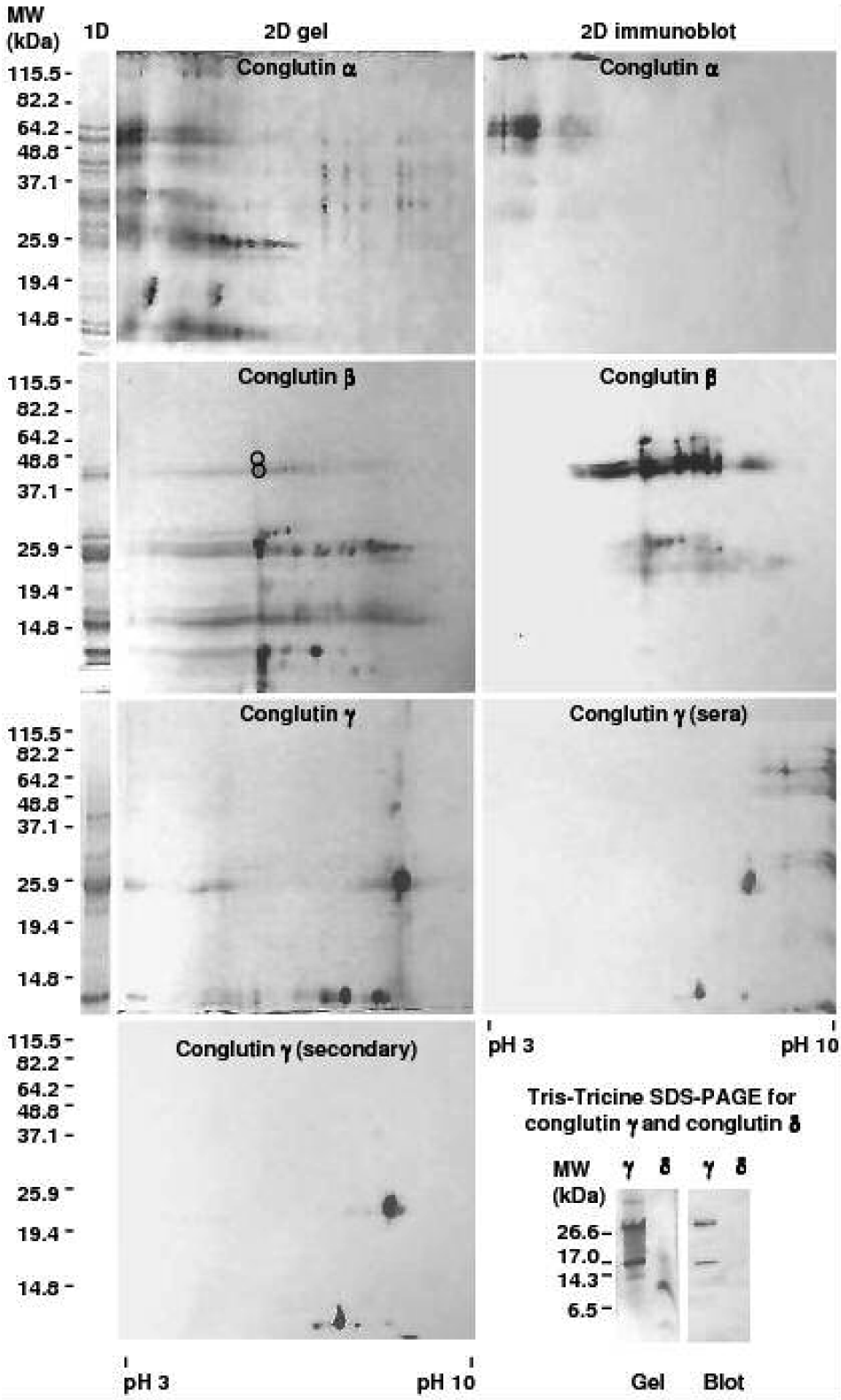

Figure 4. Analysis of purified conglutins, showing Tris−glycine SDS−PAGE banding patterns for total protein (lane 1D) alongside corresponding 2D gels and immunoblots for conglutin α, β, and γ (blots of conglutin γ were probed with sera or with secondary antibody alone). Conglutin β protein spots that were sequenced are circled. Additional Tris−Tricine SDS−PAGE and immunoblotting for conglutin γ and δ is shown at the bottom right.

peanut and lupin (*9*), because none of the lupin-allergic individuals in this study (all of whom reacted to conglutin β) reacted clinically to peanuts. It is possible that individuals that react to both peanut and lupin react to different lupin allergens, e.g., conglutin α (*21*), or that they react to different IgE epitopes on conglutin β, compared to the individuals that react to lupin alone. A final question of great importance is to determine whether particular proteins or epitopes are associated with clinical reactions (as opposed to the presence of IgE without allergic reactivity) or with the severity of the allergic reaction.

ABBREVIATIONS USED

2D, two-dimensional; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; BSA, bovine serum albu-

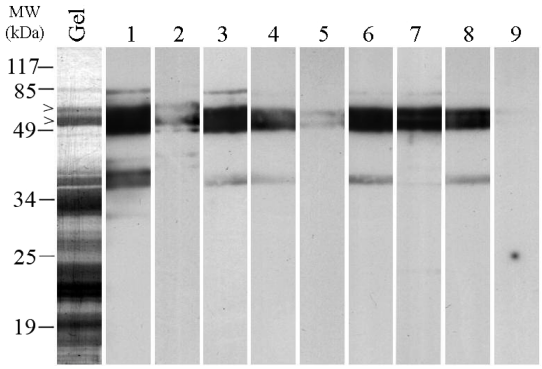

Figure 5. Immunoblot of purified conglutin β probed with sera from lupin-allergic individuals (lanes 1−8) and serum from an individual who was not allergic to lupin (lane 9). The total protein pattern of purified conglutin β is shown to the left (gel). Bands that were analyzed by MS (corresponding to the spots that were sequenced in **Figure 4**) are indicated by arrowheads.

min; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DTT, dithiothreitol; IEF, isoelectric focusing; IgE, immunoglobulin E; IPG, immobilized pH gradient; MALDI, matrix-assisted laser desorption/ionization; MS, mass spectrometry; SPITC, 4-sulfophenyl isothiocyanate; Tricine, *N*-[-tris(hydroxymethyl)methyl]glycine; Tris, tris(hydroxymethyl)-aminomethane.

ACKNOWLEDGMENT

The authors are grateful to R. Lipscombe and A. Livk, from Proteomics International, M. Djordjevic and M. Oakes, from the Research School of Biological Sciences, Australian National University, for mass spectrometric analysis of proteins, and B. Crossett from APAF at the University of Sydney for help with bioinformatic analysis of results. Thanks to S. Duckworth from George Weston Foods and S. Sipsas from the Department of Agriculture and Food, Western Australia, for providing lupin seed protein isolates. We thank L. Wienholt from the Royal Prince Alfred Hospital for doing the ImmunoCAP analysis of lupin-specific IgE in the serum.

Supporting Information Available: Detailed table of the peptide sequences that matched the MS spectra obtained from protein spots excised from the 2D gels, including accession numbers for the database sequences, and a figure showing secondary antibody binding to a Western blot of purified conglutins. This material is available free of charge via the Internet at http://pubs.acs.org.

LITERATURE CITED

(1) 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.

(2) 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.

(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) Wait, R.; Granazza, E.; Brambilla, D.; Eberini, I.; Morandi, S.; Arnoldi, A.; Sirtori, C. R. Analysis of *Lupinus albus* storage proteins by two-dimensional electrophoresis and mass spectrometry. *J. Agric. Food Chem.* **2005**, *53*, 4599–4606.

(5) Hefle, S. L.; Lemanske, R. F.; Bush, R. K. Adverse reaction to lupine-fortified pasta. *J. Allergy Clin. Immunol.* **1994**, *94*, 167–72.

(6) Faeste, C. K.; Lovik, M.; Wiker, H. G.; Egaas, E. A case of peanut cross-allergy to lupine flour in a hot dog bread. *Int. Arch. Allergy Immunol.* **2004**, *135*, 36–39.

(7) Parisot, L.; Aparicio, C.; Moneret-Vautrin, D. A.; Guerin, L. Allergy to lupine flour. *Allergy* **2001**, *56*, 918–919.

(8) Smith, W. B.; Gillis, D.; Kette, F. E. Lupin: A new hidden food allergen. *Med. J. Aust.* **2004**, *181*, 219–220.

(9) Moneret-Vautrin, D. A.; Guérin, L.; Kanny, G.; Flabbee, J.; Frémont, S.; Morisset, M. Cross-allergenicity of peanut and lupine: The risk of lupine allergy in patients allergic to peanuts. *J. Allergy Clin. Immunol.* **1999**, *104*, 883–888.

(10) Quaresma, R. R.; Viseu, R.; Martins, L. M.; Tomaz, E.; Inácio, F. Allergic primary sensitization to lupine seed. *Allergy* **2007**, *62*, 1473–1474.

(11) Shaw, J.; Roberts, G.; Grimshaw, K.; White, S.; Hourihane, J. Lupin allergy in peanut-allergic children and teenagers. *Allergy* **2008**, *63*, 370–373.

(12) Lindvik, H.; Holden, L.; Løvik, M.; Cvancarova, M.; Halvorsen, R. Lupin sensitization and clinical allergy in food allergic children in Norway. *Acta Paediatr.* **2007**, *97*, 91–95.

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

(14) Breiteneder, H.; Radauer, C. A classification of plant food allergens. *J. Allergy Clin. Immunol.* **2004**, *113*, 821–830.

(15) Álvarez-Álvarez, J.; Guillamon, E.; Crespo, J. F.; Cuadrado, C.; Burbano, C.; Rodríguez, J.; Fernández, C.; Muzquiz, M. Effects of extrusion, boiling, autoclaving, and microwave heating on lupine allergenicity. *J. Agric. Food Chem.* **2005**, *53*, 1294–1298.

(16) Guarneri, F.; Guarneri, C.; Benvenga, B. Identification of potentially cross-reactive peanut−lupine proteins by computer-assisted search for amino acid sequence homology. *Int. Arch. Allergy Immunol.* **2005**, *138*, 273–277.

(17) Burks, A. W.; Cockrell, G.; Stanley, J. S.; Helm, R. M.; Bannon, G. A. Recombinant peanut allergen Ara h 1 expression and IgE binding in patients with peanut hypersensitivity. *J. Clin. Invest.* **1995**, *96*, 1715–1721.

(18) Lehmann, K.; Hoffmann, S.; Neudecker, P.; Suhr, M.; Becker, W.-M.; Rösch, P. High-yield expression in *Escherichia coli*, purification, and characterization of properly folded major peanut allergen Ara h 2. *Protein Expression Purif.* **2003**, *31*, 250–259.

(19) Magni, C.; Herndl, A.; Sironi, E.; Scarafoni, A.; Ballabio, C.; Restani, P.; Bernardini, R.; Novembre, E.; Vierucci, A.; Duranti, M. One- and two-dimensional electrophoretic identification of IgE-binding polypeptides of *Lupinus albus* and other legume seeds. *J. Agric. Food Chem.* **2005**, *53*, 4567–4571.

(20) Dooper, M. M. B. W.; Holden, L.; Faeste, C. K.; Thompson, K. M.; Egaas, E. Monoclonal antibodies against the candidate lupin allergens α-conglutin and β-conglutin. *Int. Arch. Allergy Immunol.* **2007**, *143*, 49–58.

(21) Peeters, K. A.; Nordlee, J. A.; Penninks, A. H.; Chen, L.; Goodman, R. E.; Bruijnzeel-Koomen, C. A.; Hefle, S. L.; Taylor, S. L.; Knulst, A. C. Lupine allergy: Not simply cross-reactivity with peanut or soy. *J. Allergy Clin. Immunol.* **2007**, *120*, 647–653.

(22) Holden, L.; Fæste, C. K.; Egaas, E. Quantitative sandwich ELISA for the determination of lupine (*Lupinus* spp.) in foods. *J. Agric. Food Chem.* **2005**, *53*, 5866–5871.

(23) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilising the principle of protein dye-binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(24) Sironi, E.; Sessa, F.; Duranti, M. A simple procedure of lupin seed protein fractionation for selective food applications. *Eur. Food Res. Technol.* **2005**, *221*, 145–150.

(25) Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **1970**, *227*, 680–685.

(26) Schägger, H.; von Jagow, G. Tricine−sodium dodecyl sulphate−polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. *Anal. Biochem.* **1987**, *166*, 368–379.

(27) Rabilloud, T.; Charmont, S. Detection of proteins on two-dimensional electrophoresis gels. In *Proteome Research: Two-Dimensional Gel Electrophoresis and Identification Methods*, 1st ed.; Rabilloud, T., Ed.; Springer Verlag: Berlin, Germany, 2000; pp 107−126.

(28) Towbin, H.; Stachelin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: Procedure and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350–4354.

(29) Harper, S.; Speicher, D. W. Detection of proteins on blot membranes. In *Current Protocols in Protein Science*, Core ed.; Coligan, J. E., Dunn, B. M., Ploegh, H. L., Speicher, D. W., Wingfield, P. T., Eds.; John Wiley and Sons, Inc.: Hoboken, NJ, 1995; Vol. 2, pp 10.8.1−10.8.7.

(30) Gayler, K. R.; Boadle, B. G.; Snook, M.; Johnson, E. D. Precursors of storage proteins in *Lupinus angustifolius*. *Biochem. J.* **1984**, *221*, 333–341.

(31) Duranti, M.; Gius, C.; Scarafoni, A. Lectin-like activity of lupin seed conglutin γ, a glycoprotein previously referred to as a storage protein. *J. Exp. Bot.* **1995**, *46*, 725–728.

Received for review March 18, 2008. Revised manuscript received May 23, 2008. Accepted June 10, 2008. This work was funded by the Grain Foods Cooperative Research Center.

JF800840U

Supporting information

Table I. Matches to peptide sequences determined through Mascot MS/MS ion searches. The nucleotide sequence ID is listed. Unique peptide matches for other cDNAs have been listed if they differ from the peptides for the top scoring cDNA. Conglutin β is sometimes described as vicilin-like protein but we have used the term conglutin β for all cDNAs with strong sequence identity to avoid confusion. The descriptions of each accession used in Genbank are described in a footnote.

Spot no.	*Lupinus* sp.	Identification	Database ID	Peptides
1	*angustifolius*	**conglutin γ**	X65601[a]	AGIALGAHHLEENLVVFDLER
2	*angustifolius*	**conglutin β**	DT454368[b]	NFLAGSEDNVIR FGNFYEITPNR
			AJ966470[c]	LENLQNYR
3	*angustifolius*	**conglutin β**	DT454368[b]	NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR AIFVVLVDEGEGNYELVGIR
			AJ966470[c]	GQEQSHQDEGVIVR NPYHFNSQR
			AY500372[d]	DQQSYFSGFSR
			EF455724[e]	LENLQNYR

				EQEQQPQHGR
4	*angustifolius*	no match		
5	*angustifolius*	**conglutin β**	DT454323[f]	FGNFYEITPDR LLGFGINADENQR
			EF455724[e]	HSDADYILVVLNGR
6	*angustifolius*	**conglutin γ**	L39786[g]	VGFNSNSLK SRVGFNSNSLK VGFNSNSLKSYGK KISGGAPSVDLILDK ISGGAPSVDLILDKNDAVWR AGIALGAHHLEENLVVFDLER AGIALGAHHLEENLVVFDLERSR
7	*angustifolius*	**conglutin γ**	L39786[g]	VGFNSNSLK SRVGFNSNSLK ISGGAPSVDLILDKNDAVWR AGIALGAHHLEENLVVFDLER
8	*angustifolius*	**conglutin β**	DT454368[b]	QLDTEVK EQVQELR KYAQSSSR QQDEQEVR SNKPIYSNK QQDEQEVRR NFLAGSEDNVIR

				FGNFYEITPNR QLDTEVKGLTFPGSTEDVER LLGFGINADENQRNFLAGSEDNVIR
			AJ966470[c]	YEEIQR NTLEATFNTR DQQSYFSGFSR TNRLENLQNYR GQEQSHQDEGVIVR IVEFQSKPNTLILPK RGQEQSHQDEGVIVR NTLEATFNTRYEEIQR
			DT454323[f]	VSKEQVQELR
			EF455724[e]	EQEQGSSSSSR VSKEQIQELR
9	*angustifolius*	**conglutin β**	EF455725[h]	QQEEQER LENLQNYR EEEEHEPR NTLEATFNTR ATITIVNPDKR EQEQGSSSGSQR DQQSYFSGFSK QQEEQEREHR TNRLENLQNYR

				GQEQSHQDEGVIVR SQSEESQEEEHER EEREQEQGSSSGSQR IIEFQSKPNTLILPK RQEEEEEEEEWQPR LPAGTTSYILNPDDNQNLR
			EF455724[e]	YEEIQR QEEERER
10	*angustifolius*	no data		
11	*angustifolius*	no data		
12	*angustifolius*	no data		
13	*angustifolius*	no data		
14	*angustifolius*	no data		
15	*angustifolius*	no data		
16	*angustifolius*	no data		
17	*angustifolius*	no data		
18	*angustifolius*	**conglutin β**	DT454323[f]	FGNFYEITPDR LLGFGINADENQR AIFVVVVDEGEGNYELVGIR
			EF455724[e]	HSDADYILVVLNGR
19	*angustifolius*	no data		
20	*angustifolius*	**conglutin β**	DT454368[b]	FGNFYEITPNR
21	*angustifolius*	no data		

22	*angustifolius*	no data		
23	*angustifolius*	no match		
24	*angustifolius*	no match		
25	*angustifolius*	no data		
26	*angustifolius*	**conglutin β**	EF455725[h]	HSDADFILVVLNGR
27	*angustifolius*	no match		
28	*angustifolius*	no match		
29	*angustifolius*	no data		
30	*angustifolius*	**conglutin β**	EF455724[e]	LLGFGINANENQR NQQQSYFANAQPQQKQQR
			AJ966470[c]	NPYHFNSQR DQQSYFSGFSR
31	*angustifolius*	no match		
32	*angustifolius*	no data		
33	*angustifolius*	no data		
34	*angustifolius*	**conglutin β**	EF455725[h], EF455724[e]	LPAGTTSYILNPDDNQNLR
35	*angustifolius*	no data		
36	*angustifolius*	no match		
37	*angustifolius*	**conglutin β**	EF455724[e]	FGNFYEITPER NFLAGSEDNVISQLDR AIFIVVVDEGEGNYELVGIR

			DT454323[f]	LLGFGINADENQR
38	*angustifolius*	**conglutin β**	DT454323[f]	LLGFGINADENQR AIFVVVVDEGEGNYELVGIR
			DT454368[b]	FGNFYEITPNR
39	*angustifolius*	**conglutin β**	DT454323[f]	NFLAGSEDNVIR FGNFYEITPDR LLGFGINADENQR AIFVVVVDEGEGNYELVGIR
			DT454368[b]	GLTFPGSTEDVER
			EF455724[e]	GKPSESGPFNLR
40	*angustifolius*	no match		
41	*angustifolius*	**conglutin β**	DT454368[b]	FGNFYEITPNR
42	*angustifolius*	**conglutin β**	EF455724[e]	LLGFGINANENQR LPAGTTSYILNPDDNQNLR AIFIVVVDEGEGNYELVGIR
			DT454368[b]	FGNFYEITPNR
43	*angustifolius*	**conglutin β**	DT454368[b]	NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR NQQQSYFANAQPQQQQQR
			DT454323[f]	AIFVVVVDEGEGNYELVGIR

44	*angustifolius*	**conglutin β**	DT454368[b]	NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR AIFVVLVDEGEGNYELVGIR NPQAQDLDISLTFIEINEGALLLPHYNSK
45	*angustifolius*	**conglutin β**	DT454323[f]	FGNFYEITPDR AIFVVVVDEGEGNYELVGIR NPQAQDLDISLTFIEINEGALLLPHYNSK
			EF455724[e]	LPAGTTSYILNPDDNQNLR
46	*angustifolius*	**conglutin β**	DT454323[f]	AIFVVVVDEGEGNYELVGIR
			EF455724[e]	HSDADYILVVLNGR
			DT454368[b]	FGNFYEITPNR
47	*angustifolius*	**conglutin β**	DT454368[b]	FGNFYEITPNR LLGFGINADENQR AIFVVLVDEGEGNYELVGIR NPQAQDLDISLTFIEINEGALLLPHYNSK
48	*angustifolius*	**conglutin β**	EF455724[e]	LENLQNYR NTLEATFNTR HSDADYILVVLNGR LPAGTTSYILNPDDNQNLR
			DT454323[f]	AIFVVVVDEGEGNYELVGIR

49	*angustifolius*	**conglutin β**	DT454323[f]	AIFVVVVDEGEGNYELVGIR
50	*angustifolius*	**conglutin β**	DT454368[b]	EQVQELR NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR AIFVVLVDEGEGNYELVGIR
51	*angustifolius*	**conglutin β**	DT454368[b]	EQVQELR NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR NQQQSYFANAQPQQQQQR
			DT454323[f]	AIFVVVVDEGEGNYELVGIR
52	*angustifolius*	**conglutin β**	DT454368[b]	FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR NQQQSYFANAQPQQQQQR
			DT454323[f]	AIFVVVVDEGEGNYELVGIR
53	*angustifolius*	**conglutin β**	DT454368[b]	EQVQELR NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER

				LLGFGINADENQR NQQQSYFANAQPQQQQQR LSEGDIFVIPAGHPISINASSNLR
			DT454323[f]	AIFVVVVDEGEGNYELVGIR
54	*angustifolius*	**conglutin β**	DT454368[b]	EQVQELR QQDEQEVR NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR
			DT454323[f]	AIFVVVVDEGEGNYELVGIR
55	*angustifolius*	**conglutin β**	DT454368[b]	FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR NPQAQDLDISLTFIEINEGALLLPHYNSK
			DT454323[f]	AIFVVVVDEGEGNYELVGIR
56	*angustifolius*	**conglutin β**	DT454323[f]	NFLAGSEDNVIR LLGFGINADENQR AIFVVVVDEGEGNYELVGIR
			DT454368[b]	FGNFYEITPNR
57	*angustifolius*	**conglutin β**	DT454323[f]	NFLAGSEDNVIR FGNFYEITPDR LLGFGINADENQR

				AIFVVVVDEGEGNYELVGIR LSEGDIFVIPAGHPISINASSNLR
58	*angustifolius*	**conglutin β**	DT454323[f]	LLGFGINADENQR AIFVVVVDEGEGNYELVGIR NQQQSYFANAQPQQQQQR LSEGDIFVIPAGHPISINASSNLR
			DT454368[b]	FGNFYEITPNR
59	*angustifolius*	**conglutin β**	DT454368[b]	NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR
			EF455724[e]	AIFIVVVDEGEGNYELVGIR
60	*angustifolius*	no match		
61	*angustifolius*	**conglutin β**	DT454368[b]	FGNFYEITPNR
			DT454323[f]	AIFVVVVDEGEGNYELVGIR
62	*angustifolius*	**conglutin β**	AJ966470[c]	YEEIQR QLDREVK RQSGYER VLERFDQR NPYHFNSQR NTLEATFNTR DQQSYFSGFSR NFLAGSEDNVIR

				TNRLENLQNYR GQEQSHQDEGVIVR SVPAEATEVGLLVFST IVEFQSKPNTLILPK RGQEQSHQDEGVIVR NTLEATFNTRYEEIQR
			EF455724[e]	EQEQGSSSSSR LLGFGINANENQR HSDADYILVVLNGR LPAGTTSYILNPDDNQNLR NQQQSYFANAQPQQKQQR
			EF455725[h]	KHAQSSSGEGKPSESGPFNLR
63	*angustifolius*	**conglutin β**	DT454368[b]	YAQSSSR QLDTEVK EQVQELR QQDEQEVR SNKPIYSNK NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR NQQQSYFANAQPQQQQQR
			DT454323[r]	VSKEQVQELR

			AJ966470[c]	TNRLENLQNYR GQEQSHQDEGVIVR IVEFQSKPNTLILPK
			EF455725[h]	FQTYYR ATITIVNPDKR
64	*angustifolius*	**conglutin β**	DT454368[b]	YAQSSSR EQVQELR YAQSSSRK QQDEQEVR SNKPIYSNK QQDEQEVRR NFLAGSEDNVIR FGNFYEITPNR GLTFPGSTEDVER LLGFGINADENQR DQQRQQDEQEVR GLTFPGSTEDVERLIK NFLAGSEDNVIRQLDTEVK NQQQSYFANAQPQQQQQR QLDTEVKGLTFPGSTEDVER NQQQSYFANAQPQQQQQRER LLGFGINADENQRNFLAGSEDNVIR
			DT454323[f]	QLDREVK

65	*albus*	no data		
66	*albus*	no data		
67	*albus*	no data		
68	*albus*	**conglutin β**	AY500372[d], AJ966470[c]	LAIPINNPGYFYDFYPSSTK
69	*albus*	no data		
70	*albus*	no data		
71	*albus*	no match		
72	*albus*	no match		
73	*albus*	no data		
74	*albus*	no data		
75	*albus*	no data		
76	*albus*	no data		
77	*albus*	**conglutin β**	AJ966470[c]	ELTFPGSAEDIER LLGFGINAYENQR
78	*albus*	**conglutin β**	AY500372[d], DT454323[f], DT454368[b]	LLGFGINADENQR
79	*albus*	**conglutin γ**	AJ297490[i]	VGFNTNSLK SRVGFNTNSLK VGFNTNSLKSHGK ISGGVPSVDLIMDK KISGGVPSVDLIMDK

				HSIFEVFTQVFANNVPK AGIALGTHQLEENLVVFDLAR
80	*luteus*	**conglutin β**	EF455724[e]	AIFIVVVDEGEGNYELVGIR
81	*luteus*	no match		
82	*luteus*	**conglutin α**	AJ938034[j]	RPFYTNAPQEIYIQQGR
83	*luteus*	no match		
84	*luteus*	**putative TAG factor protein**	AF538688[k]	AVCNLFALEGATVIFTYVK
85	*luteus*	no match		
86	*luteus*	**conglutin γ**	AJ297490[i]	VGFNTNSLK HSIFEVFTQVFANNVPK AGIALGTHQLEENLVVFDLAR

[a]X65601 *Lupinus angustifolius* mRNA for conglutin γ. [b]DT454368 UWA067 cDNA library from early and middle stages of seed development of narrow leafed lupin. [c]AJ966470 *L. albus* partial mRNA for vicilin-like protein, clone LaJCMC675 (Genpept ID: CAI84850). [d]AY500372 *L. albus* β-conglutin precursor mRNA, complete cds (Genpept ID: AAS97865). [e]EF455724 *L. angustifolius* conglutin β seed storage protein (UWA220) mRNA, partial cds. [f]DT454323 UWA021 cDNA library from early and middle stages of seed development of narrow leafed lupin. [g]L39786 LUPDNASEQ *L. angustifolius* conglutin γ gene, complete cds. [h]EF455725 *L. angustifolius* conglutin β seed storage protein (LSL2.2) mRNA, complete cds. [i]AJ297490 *L. albus* gene for conglutin γ, clone 32. [j]AJ938034 *L. albus* mRNA for legumin-like protein, clone

LaJCMC295. [k]AF538688 Putative TAG factor protein (putative TAG-associated factor), *L. angustifolius*.

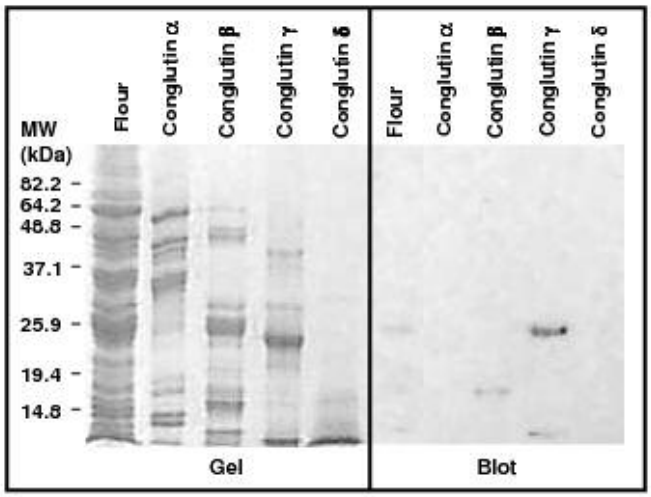

Figure S1. Secondary antibody binding to purified conglutins. Purified conglutin α, β, γ and δ from *L. angustifolius* were run on SDS-PAGE alongside total lupin flour. The left panel shows a Coomassie blue-stained gel, the right panel shows an identical immunoblot probed with secondary antibody only.