



Cross-reactivity between peanut and lupin proteins

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ABSTRACT

Peanut-allergic individuals may also react to lupin, which, for this reason, has been included in the EU list of food allergens. Since there is not yet any general consensus on the major allergen/s in lupin, the objective of this investigation was to compare the reactivity of the main lupin proteins towards the IgE of the sera of allergic patients. Both *Lupinus albus* and *Lupinus angustifolius* were investigated. ELISA's, Western blotting and mass spectrometry, including also *de novo* sequencing of the unknown lupin proteins, were used for identifying the IgE-binding proteins. Significant differences in the protein reactivities were observed. In particular, there was a direct relationship between the level of peanut-specific IgE's and the cross-reactivity to lupin proteins; also the reactivity of each serum appeared to be unique. Although numerous lupin proteins bind IgE's, our data suggest a predominant contribution of α -conglutinin in the reactivity of both *L. albus* and *L. angustifolius*.

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1. Introduction

Lupin has been included in the EU list of allergens to be declared in food labels (Commission Directive 2006/142/EC), and it is potentially life-threatening (NDA Scientific Panel on Dietetic Products, 2005) taking into account that at least 151 cases of lupin allergy have been described worldwide (Jappe & Vieths, 2010).

Allergic reactions to foods containing traces of lupin protein have been documented in patients already allergic to other legumes, such as peanut, soybean, lentils, beans and peas (Faeste, Løvik, Wiker, & Egaas, 2004; Kanny, Guérin, & Moneret-Vautrin, 2000; Leduc, Moneret-Vautrin, & Guérin, 2002; Moneret-Vautrin et al., 1999). A controlled study in peanut allergic patients suggested a cross-reactivity rate of about 30% (Moneret-Vautrin et al., 1999), but higher rates (68%) have also been reported (Leduc et al., 2002).

Although peanut allergic patients are likely to be the major risk group (Gayraud et al., 2009), sensitisation via ingestion has been suggested also in individuals without known peanut allergy (Novembre et al., 1999; Parisot, Aparicio, Moneret-Vautrin, &

Guerin, 2001; Quaresma, Viseu, Martins, Tomaz, & Inácio, 2007; Smith, Gillis, & Kette, 2004; Wassenberg & Hofer, 2007).

The clinical symptoms range from mild local reactions to systemic anaphylaxis. The ingested doses of lupin flour triggering clinical reactions range from 265 to 1000 mg (Kanny et al., 2000; Moneret-Vautrin et al., 1999). A detailed study (Peeters et al., 2007) reported that the minimal eliciting dose may be 1 mg or less for subjective symptoms (oral allergy symptoms) and 300 mg for objective symptoms. These doses are much higher than those identified for peanut allergy (Taylor et al., 2010; Wensing et al., 2002).

Lupin allergy is rapidly increasing in France, where the addition of lupin flour to wheat flour was firstly permitted in 1997 (Moneret-Vautrin et al., 1999): this seed was the fourth most frequent cause of severe food-associated anaphylaxis reported to the French Allergy Vigilance Network in 2002 (Moneret-Vautrin, Kanny, & Parisot, 2002). Around 1% of the UK population, including 250,000 pre-school children, suffers from peanut allergy and appears to be at risk since up to half of peanut-allergic population may be pre-sensitised for lupin allergens (Radcliffe, Scadding, & Brown, 2005). The first report on lupin anaphylaxis in UK was in 1999. Three cases of anaphylaxis have been also described in Australia (Smith et al., 2004). There is also some evidence of the allergenic potential of lupin after inhalation (Moreno-Ancillo, Gil-Adrados, Domínguez-Noche, & Cosmes, 2005; Novembre et al., 1999; Prieto et al., 2010), as a cause of occupational asthma and food allergy in exposed workers (Crespo et al., 2001; Parisot et al., 2001).

Abbreviations: IgE, immunoglobulin E; LPE, lupin protein extract; PPE, peanut protein extract; IAM, Iodoacetamide; CHAPS, 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate; IPG, immobilised pH gradient; TBS, Tris buffered saline; HRP, horseradish peroxidase; ECL, enhanced chemiluminescence.

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Despite several studies in the literature, there is no consensus about the presence of one or more major allergens in lupin. The storage globulins α -conglutin (legumin-like, 11 S), β -conglutin (vicilin-like, 7 S), γ -conglutin (7 S), and δ -conglutin (2 S-albumin) seem all to be responsible for the allergenic reactivity.

The generic term lupin refers to at least two major different species, *Lupinus albus* and *Lupinus angustifolius*. Since the latter has been only recently included in human foods, the data on its allergic potential are scarce (Goggin, Mir, Smith, Stuckey, & Smith, 2008). The scope of the present investigation was to compare the potential IgE binding of the main globulins of both lupin species and to identify reactive peptides. The sera from peanut-allergic patients were screened and different approaches were used to identify the IgE-reacting proteins/peptides, such as ELISA assays, Western blotting and mass spectrometry (HPLC-Chip-Ion Trap and HPLC-Chip-qTOF), including also *de novo* sequencing of unknown proteins.

2. Materials and methods

2.1. Samples

Dry lupin seeds from *L. albus* cv. Ares (A) and Multitalia (B); *L. angustifolius* cv. Arabella (C) and Boregine (D) were investigated: A and D were provided by the Fraunhofer-Institut für Verfahrenstechnik und Verpackung (Freising, Germany); B and C by Dr. Annicchiarico, CRA-ISCF (Lodi, Italy). Peanuts of the runner market type (Argentina) were provided by Imko Nut Products (Doetinchem, The Netherlands).

2.2. Patients

The sera were collected from 34 patients with positive case history of peanut allergy. The sera were provided by 5 hospitals in The Netherlands: University Medical Centre Utrecht (UMCU, Utrecht), Amsterdam Medical Centre (AMC, Amsterdam), Rijnstate Hospital (Arnhem), Gelderse Vallei Hospital (Ede), and Stichting Huisartsenlaboratorium Oost (SHO, Velp). The control sera were collected from 5 non-atopic, non-allergic healthy volunteers. [Supplementary Table 1](#) summarises the specific IgE levels of each patient determined by the ImmunoCAP (Phadia, Uppsala, Sweden), the allergy symptoms, and the results of their SPTs. The patients were carefully informed of the modalities and scope of the study and, after receiving all the information, they signed the written informed consent.

2.3. Laboratory preparation of total protein extracts from lupin and peanut seeds

Each legume protein was extracted in optimised conditions. Lupin protein extract (LPE) – Defatted lupin flour was extracted with 100 mM Tris–HCl, 0.5 M NaCl, pH 8.0, for 2 h at room temperature (RT), with gentle stirring. The slurry was centrifuged at 6000g, 4 °C, for 30 min, and the extracted proteins were stored at –80 °C until use.

Peanut protein extract (PPE) – The peanut proteins were extracted from defatted peanut meal by stirring in 15 mM sodium phosphate buffer, pH 6.2, with a 1:10 (w/v) ratio for 1 h at RT.

2.4. Purification of lupin globulin proteins

α -, β - and γ -Conglutins were purified according to a published method (Dooper, Holden, Faeste, Thompson, & Egaas, 2007), using a preparative HPLC 1200 (Agilent Technologies, Santa Clara, CA). The purity of the column fractions was confirmed by MS/MS anal-

ysis. Samples were denatured with 6 M urea, reduced with 1 M 1,4-dithiothreitol (DTT) (50 mol of DTT vs 1 mol of Cys) and alkylated with 1 M iodoacetamide (IAM) (200 mol of IAM vs 1 mol of Cys). The proteins were then digested with sequencing-grade trypsin (0.5 μ g/ μ l, Promega) with a ratio of 1:50 w/w enzyme/protein for 16 h at 37 °C, and the peptides were analysed using HPLC-Chip-Ion Trap (Agilent Technologies) (Sirtori, Resta, Brambilla, Zacherl, & Arnoldi, 2010).

2.5. Indirect ELISA

The 96-well MaxiSorb microplates (Fisher Scientific, The Netherlands) were coated with 500 ng/well (5 μ g/ml) of LPE, PPE and purified protein fractions in coating buffer (0.1% w/v BSA in PBS: 1.4 mM KH_2PO_4 , 8 mM Na_2HPO_4 , 2.7 mM KCl, 130 mM NaCl, pH 7.4), and incubated o/n at 4 °C. The negative control and a blank sample for non-specific secondary antibody binding were included. All subsequent incubations were performed at RT on a microplate shaker. The coating solution was removed and 200 μ l/well of blocking buffer (2% w/v BSA in PBS, pH 7.4) was added and incubated for 1 h. The microplates were washed after each incubation step in a microplate washer with 400 μ l/well of washing buffer (0.05% v/v Tween-20, 0.05% w/v BSA in PBS pH 7.4). The 1:3 serum dilutions in PBS containing 0.1% BSA were added at 100 μ l/well and incubated for 1.5 h. The wells were then incubated with 100 μ l of 1:500 monoclonal biotin-conjugated mouse anti-human IgE (BD Biosciences Pharmingen, USA) and 100 μ l of 1:10,000 streptavidin-poly-HRP (Sanquin, The Netherlands), in dilution buffer (0.1% w/v BSA in PBS, pH 7.4) both during 1 h. The enzymatic colour development started by the addition of 100 μ l/well 3,3',5,5'-tetramethylbenzidine substrate solution (KPL, Gaithersburg, MD) and the reaction was stopped by adding 100 μ l/well of 1 M H_3PO_4 . Colour development was measured at 450 nm using a microplate reader (Anthos 2020, Anthos Labtec Instruments, Austria). The measurements were performed in triplicate.

2.6. Inhibition ELISA

All sera were assessed by inhibition ELISA with PPE as inhibitor. Inhibition experiments were performed by coating 96-well MaxiSorb microplates with 5 μ g/ml LPE and blocking as described above. Diluted sera (1:2, 1:3, 1:5, and 1:10) were pre-incubated in triplicate with PPE at the final concentrations of 500–0.5 ng/ml o/n at 4 °C. Subsequently, the inhibitor mixtures (including also sera without inhibitor as control) were transferred to coated wells (100 μ l/well) and incubated for 1 h. The detection was performed in the same way as for indirect ELISA assay. Percentage inhibition was calculated according to:

$$\% \text{inhibition} = [1 - (\text{OD}_{450} \text{ serum with inhibitor} / \text{OD}_{450} \text{ serum without inhibitor})] \times 100$$

2.7. Bidimensional electrophoresis (2-DE)

LPE or desalted column fractions were diluted in IEF sample buffer (7 M urea, 2 M thiourea, 3% (w/v) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate hydrate (CHAPS), 1% ampholyte pH 3–10 and pH 4–8); the proteins were reduced with 65 mM DTT and alkylated with 200 mM IAM (Sirtori et al., 2010). Isoelectric focusing was performed on pH 3–10 non-linear IPG strips (Bio-Rad Laboratories Inc., USA), and the second dimension was done on 13% SDS-PAGE; Bio-Safe Coomassie (Bio-Rad) was used for the staining. Gels were scanned in a VersaDoc 3000 Imaging System (Bio-Rad). The

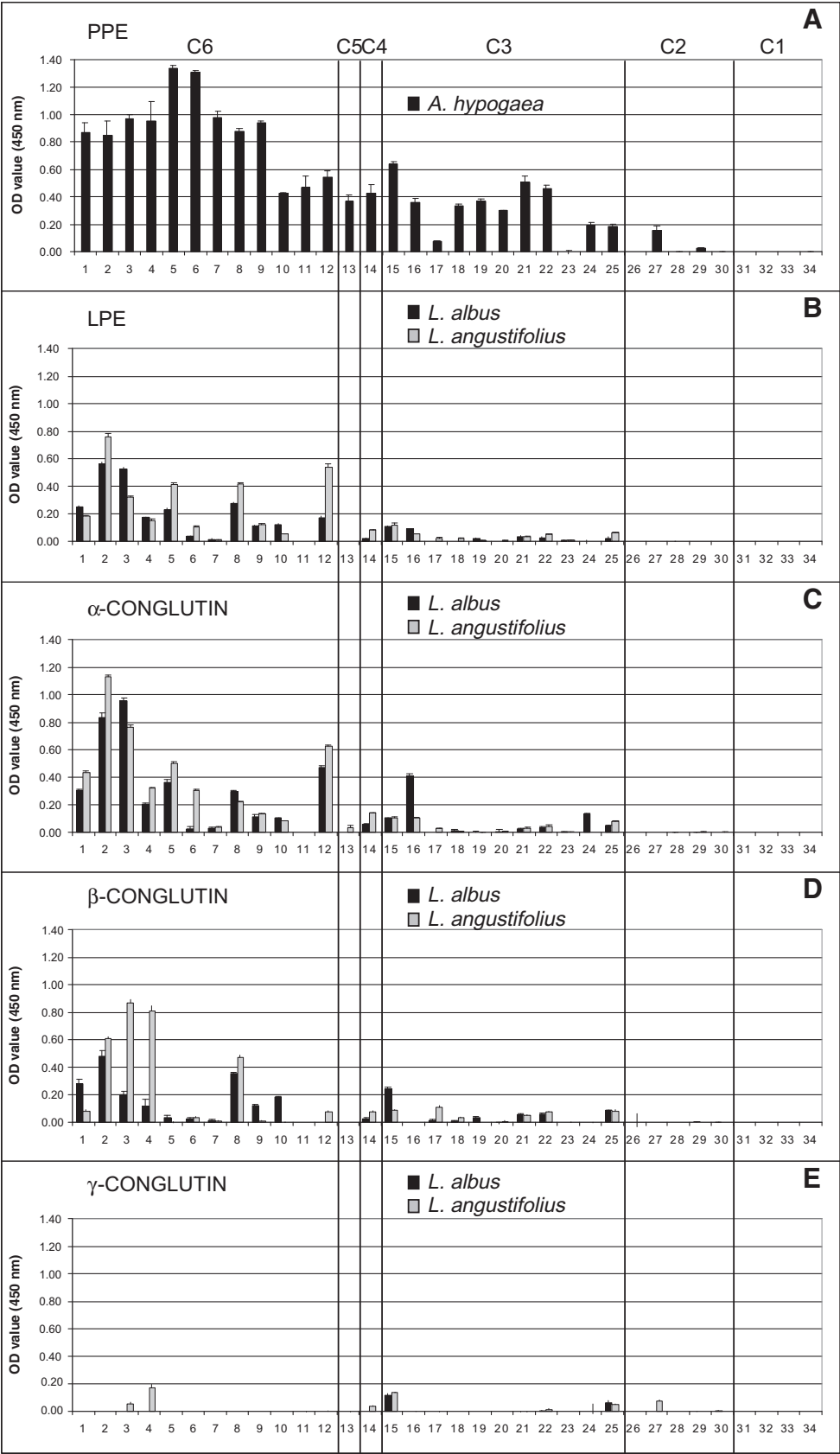


Fig. 1. Indirect ELISA assay using sera from 34 allergic patients. The samples tested were: (A) PPE; (B) LPE; (C) α -conglutinin; (D) β -conglutinin; (E) γ -conglutinin. The sera were divided in 6 groups according to the level of peanut-specific IgE (Pharmacia ImmunoCAP® RAST® classification).

software used to compare the different maps was PDQuest software (Bio-Rad). Each sample was run in duplicate: the for-

mer gel was Coomassie stained and the latter was used for the blotting.

2.8. Glycoprotein staining

The *in-gel* detection of glycosylated proteins was performed using the Glycoprofile™ III-Fluorescent Glycoprotein Detection Kit (Sigma, Italy). Following 2-DE, the proteins were fixed with fixing buffer (10% acetic acid, 30% methanol) and stained according to the experimental protocol of the kit. The stained gels were acquired with a VersaDoc 3000 Image System (Bio-Rad). The images were recorded with PDQuest software (Bio-Rad). ProteoProfile PTM marker (Sigma), containing glycosylated and non-glycosylated proteins, was used as a positive and negative control. Glycoprotein staining was performed in duplicate.

2.9. Western blotting and *in-gel* digestion of reactive spots

Proteins were electrophoretically transferred from 2D-gels to nitrocellulose membrane (0.2 µm, Bio-Rad) by applying a constant current of 250 mAmp for 2 h at RT on a Trans-blot electrophoretic Transfer Cell (Bio-Rad). The protein transfer efficiency was checked by amido black staining. The blots were blocked in TBS-Tween (20 mM Tris-HCl, pH 7.5, 500 mM NaCl, 0.05% v/v Tween-20) containing 10% (w/v) fat-free milk powder for 1 h at RT and then incubated o/n at RT with pooled sera (1:10 diluted) or a normal control serum pool. The membranes were then incubated with a monoclonal biotin-conjugated mouse anti-human IgE and developed with streptavidin-poly-HRP, using the enhanced chemiluminescence (ECL) assay (Bio-Rad). The membranes were washed 4 times in TBS-Tween after each incubation step. The reactive spots were identified by PDQuest software, excised from their relative 2D-gels and processed for mass spectrometry analysis as described elsewhere (Sirtori et al., 2010). All 2-DE analyses were performed in duplicate.

2.10. HPLC-Chip-Ion Trap and HPLC-Chip-qTOF analyses

All experiments were performed on an Agilent 1200 LC connected to an Agilent Ion Trap (SL series) mass spectrometer or to an Agilent G6520A Quadrupole Time-of-Flight (qTOF) mass spectrometer (Agilent Technologies). In both cases, the ionisation system was a HPLC-Chip Cube working in nanoflow electrospray positive ion mode. The Ion Trap and Quadrupole Time-of-Flight (qTOF) experimental conditions have been described in detail in a previous paper (Sirtori et al., 2010).

The protein database searches were performed with a Spectrum Mill MS Proteomics Workbench (Rev A.03.03), biweekly updated with trypsin specificity. In order to validate the peptide and protein matches, the Spectrum Mill default value for the peptide scores, forward-reversed scores, rank1-rank2 scores, percentage Scored Peak Intensity, and protein scores were considered (Brambilla, Resta, Isak, Zanotti, & Arnoldi, 2009).

The qTOF MS/MS spectra obtained from *L. angustifolius* digested spots were subjected to a manual *de novo* sequencing, because the amino acid sequence of α-conglutin is still unknown (Sirtori et al., 2010).

3. Results

3.1. Indirect ELISA for IgE response to lupin and peanut protein

An indirect ELISA assay was performed in order to investigate the potential IgE binding to immobilised lupin proteins. As a preliminary step, the LPE from different cultivars (*L. angustifolius* cv. Arabella and Boregine; *L. albus* cv. Ares and Multitalia) were screened with sera from 4 selected patients having a peanut-specific IgE level of >100 kU/l. The cultivars belonging to the same spe-

cies showed similar responses (data not shown), indicating that their proteins had comparable IgE binding affinity. When the two species were compared, all *L. angustifolius* cultivar strains were consistently found more responsive than the *L. albus* strains. Therefore, further experiments were performed with one selected cultivar for each species (Boregine and Ares, respectively).

Next, 34 patients were selected based on their positive peanut-specific IgE levels and their history of peanut allergy. Fig. 1 shows the IgE responses of each serum towards: PPE (panel A), LPE (panel B), purified α-conglutin (panel C), purified β-conglutin (panel D), and purified γ-conglutin (panel E). All the samples were tested in triplicate. The optical density at 450 nm (OD_{450}) ≥ 0.35 was used as a cut-off value to discriminate reactive from non-reactive sera (Dooper, Plassen, Holden, Lindvik, & Faeste, 2009).

The sera were divided in 6 groups according to the level of peanut-specific IgE: class 1 (0.35–0.7 kU/l); class 2 (0.71–3.5 kU/l); class 3 (3.51–17.5 kU/l); class 4 (17.51–50 kU/l); class 5 (50.1–100 kU/l); and class 6 (>100 kU/l) (Pharmacia ImmunoCAP® RAST® classification). The results of this screening showed different IgE binding reactivities towards either PPE, LPE, or the purified protein fractions based on their peanut-specific IgE levels.

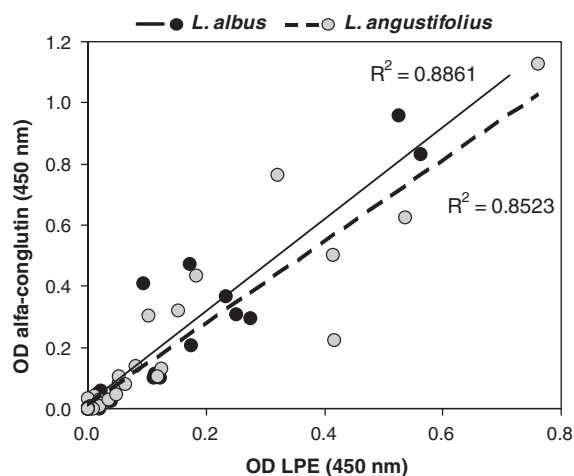


Fig. 2. Dependence of the reactivity of sera to α-conglutin vs LPE in both lupin species.

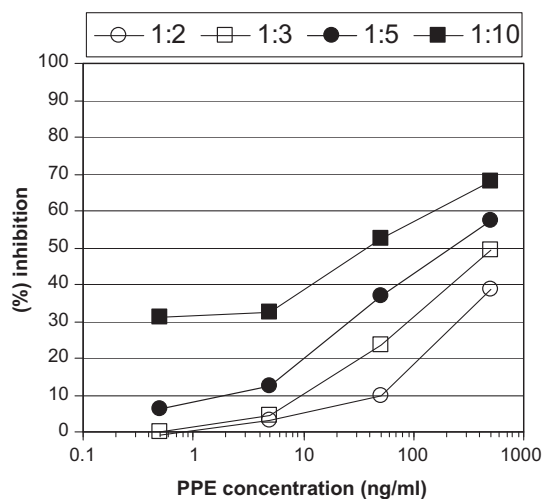


Fig. 3. Inhibition of IgE binding to LPE by PPE determined by inhibition ELISA. The OD values were obtained using a microplate reader at 450 nm and the analysis was performed in duplicate for each sample. The control wells contained no inhibitor.

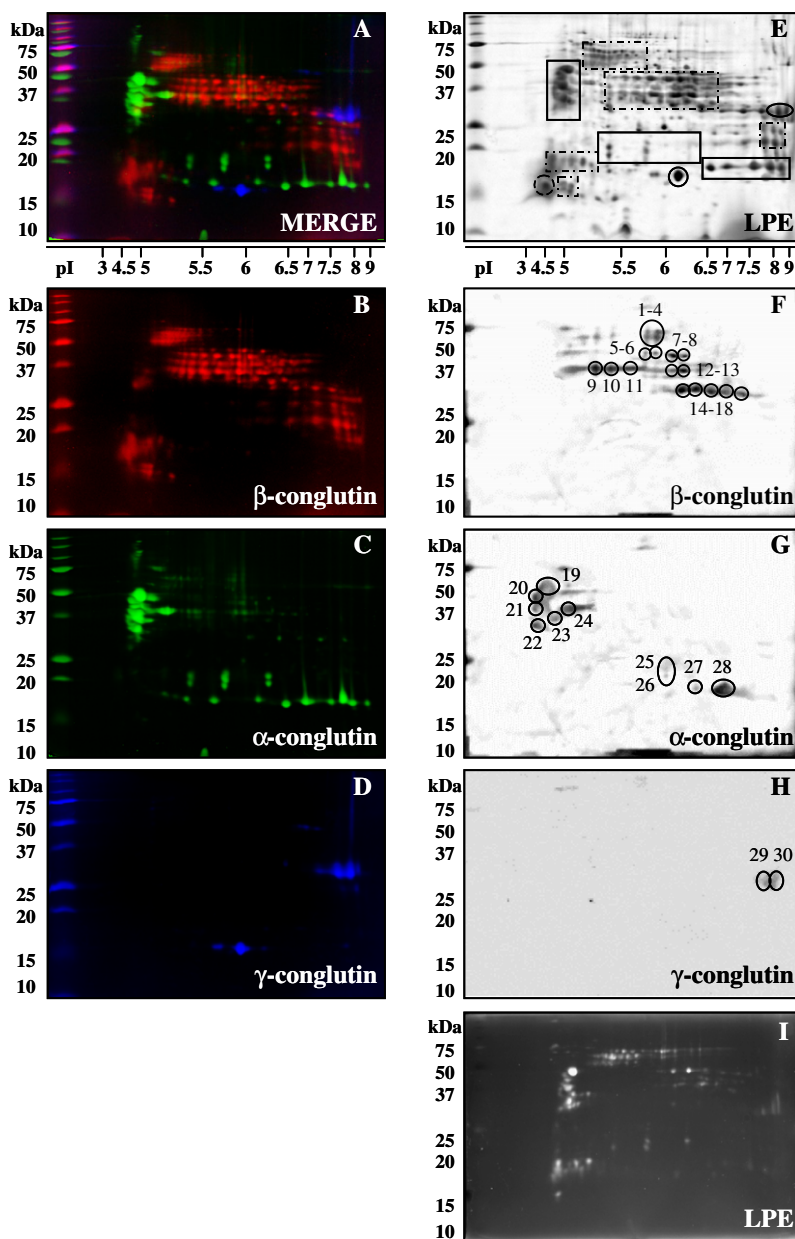


Fig. 4. *Lupinus albus*. (A) Merge obtained overlapping the coloured gels of the purified proteins. (B) β -Conglutinin 2D-gel. (C) α -Conglutinin 2D-gel. (D) γ -Conglutinin 2D-gel. (E) LPE 2D-gel. (F) β -Conglutinin 2D-WB. (G) α -Conglutinin 2D-WB. (H) γ -Conglutinin 2D-WB. (I) 2D-gel of LPE stained with a fluorescent dye (Sigma) selective for glycoprotein. The proteins were run in the first dimension on 7 cm, pH 3–10 non-linear IPG strips (Bio-Rad); the second dimension was performed on 13% denaturing SDS-PAGE gel. The standard marker (Precision Plus Protein; Bio-Rad) is indicated in kDa on the left. Solid line rectangles (—) indicate the acidic and basic subunits of α -conglutinin. Dash-dotted rectangles (---) indicate β -conglutinin. The dotted oval (---) highlights the heavy chain of δ -conglutinin. The solid line ovals enclose the large and small subunits of γ -conglutinin.

The reactivity towards LPE had the same trend as in the case of PPE (Fig. 1, panel B): the patients with the highest IgE level for peanut protein (>100 kU/l, class 6) reacted stronger with the lupin proteins, whereas all the sera with a low specific IgE level did not show any or only a low response to lupin protein. Only two patients (p7 and p11), belonging to class 6, showed drastically lower binding to LPE, despite the high reactivity to PPE.

Comparing the reactivity of the purified proteins, the sera showed a positive response to both α - and β -conglutinin, and a negative one to γ -conglutinin (Fig. 1, panels C–E). There were profound differences in the IgE reactivities of β -conglutinin from *L. albus* and β -conglutinin from *L. angustifolius*, whereas the IgE reactivities of α -conglutinins of both species were similar (except for sera 6 and 16). The α -conglutinins of both lupin species were recognised by sera

1, 2, 3, 5, and 12, whereas the sera 4 and 6 recognised only α -conglutinin of *L. angustifolius*, and the serum 16 recognised only α -conglutinin of *L. albus*. Sera 2 and 8 reacted towards the β -conglutinins of both species, whereas sera 3 and 4 recognised only β -conglutinin from *L. angustifolius*. No appreciable reaction towards γ -conglutinin was observed, although very weak responses ($0.12 \leq OD \leq 0.18$) were detected in sera 4 and 15.

Whereas weak correlations were found between LPE and β -conglutinin or between α - and β -conglutinin reactivity (data not shown), a nearly linear correlation was found between the LPE and α -conglutinin reactivity ($R^2 = 0.8861$ *L. albus*, and 0.8523 *L. angustifolius*) (Fig. 2). This may suggest an important contribution of α -conglutinin in the IgE-binding by the lupin total protein extract.

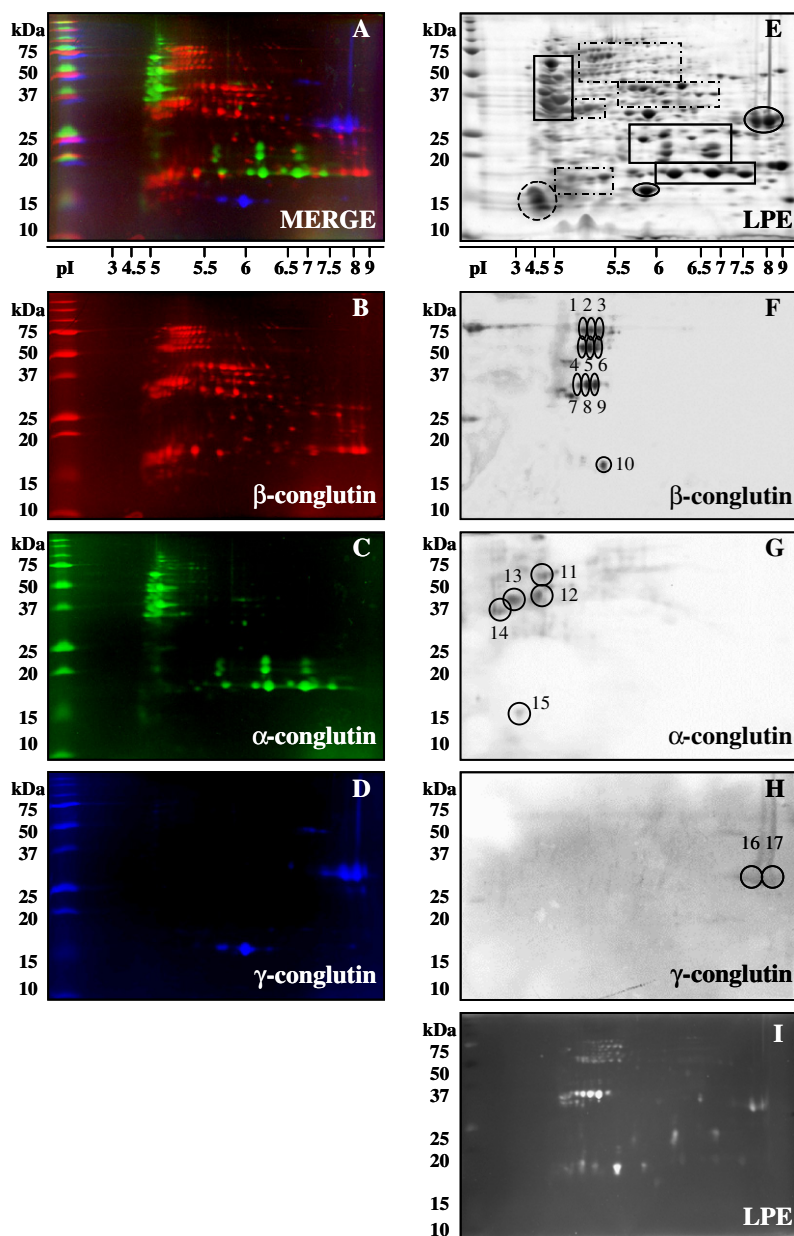


Fig. 5. *Lupinus angustifolius*. (A) Merge obtained overlapping the coloured gels of the purified proteins. (B) β -Conglutinin 2D-gel. (C) α -Conglutinin 2D-gel. (D) γ -Conglutinin 2D-gel. (E) LPE 2D-gel. (F) β -Conglutinin 2D-WB. (G) α -Conglutinin 2D-WB. (H) γ -Conglutinin 2D-WB. (I) 2D-gel of LPE stained with a fluorescent dye (Sigma) selective for glycoprotein. The proteins were run in the first dimension on 7 cm, pH 3–10 non-linear IPG strips (Bio-Rad); the second dimension was performed on 13% denaturing SDS-PAGE gel. The standard marker (Precision Plus Protein; Bio-Rad) is indicated in kDa on the left. Solid line rectangles (—) indicate the acidic and basic subunits of α -conglutinin. Dash-dotted rectangles (- · - · -) indicate β -conglutinin. The dotted oval (· · ·) highlights the heavy chain of δ -conglutinin. The solid line ovals enclose the large and small subunits of γ -conglutinin.

3.2. Evaluation of the cross-reactivity between lupin and peanut proteins

An inhibition ELISA was performed in order to compare the IgE binding capacities of lupin protein vs peanut protein by using pooled serum that was pre-incubated with serial dilutions of either PPE (experiment A) or LPE (experiment B) as inhibitors. The inhibition curves of experiment A obtained with different serum dilutions are reported in Fig. 3. These curves show a great inhibition (67.8%) at high sera dilution (1:10) and high PPE concentration (500 ng/ml), probably because in these conditions the antibodies were rapidly bound by the PPE. On the other hand, the percentage inhibition decreased considerably at higher antibody concentration (38.7%, using a 1:2 dilution). At the smaller PPE concentration

(0.5 ng/ml) the inhibitions ranged from 31.2% (1:10 sera dilution) to 0.8% (1:2 sera dilution). Instead, when pre-incubating the pooled serum with LPE (experiment B, data not shown) no inhibition was obtained, probably because this protein, having a very low IgE binding capacity, was not able to compete with the PPE binding. The much higher binding of PPE suggested that peanut was the prime sensitizer for these patients.

3.3. Identification of reactive lupin proteins by Western blotting coupled with mass spectrometry

In order to identify the lupin proteins responsible for IgE binding, Western blots were performed with the purified lupin fractions. Considering the expected large number of isoforms in the

Table 1The IgE-binding *L. albus* (A) and *L. angustifolius* (B) conglutins identified by means of both HPLC-Chip-Ion Trap and HPLC-Chip-qTOF.

Spot ^a	Identified protein ^b	NCBI nr accession ^c	Matched peptides ^d	Coverage (%) ^e	MW (kDa) theoretical ^f
A.					
19–26, 28	Legumin-like protein (<i>L. albus</i>)	85361412	9	29.69	58.41
25–28	Legumin-like protein (<i>L. albus</i>)	62816184	3	14.7	27.87
25–26, 28	Legumin-like (<i>L. albus</i>)	62816188	3	20	30.64
23	Legumin A (<i>Vicia sativa</i>)	483449	1	1.81	56.56
23	Legumin A2 precursor (<i>Pisum sativum</i>)	126161	1	1.73	59.27
1–18	β -Conglutin precursor (<i>L. albus</i>)	46451223	15	37.71	62.13
1–18	Vicilin-like protein (<i>L. albus</i>)	89994190	15	37.29	62.03
1–4, 12–18	β -Conglutin (<i>L. angustifolius</i>)	169950562	1	1.64	71.89
1–4, 12–18	β -Conglutin (<i>L. angustifolius</i>)	149208403	1	2.2	54.3
1–18	β -Conglutin (<i>L. angustifolius</i>)	149208401	2	4.6	61.47
29–30	γ -Conglutin (<i>L. albus</i>)	11191819	3	9.51	49.22
29	γ -Conglutin (<i>L. angustifolius</i>)	662366	1	6.01	48.92
B.					
11–14	Legumin (<i>L. angustifolius</i>)	<i>de novo</i> peptides	4	/	/
12–14	α -Conglutin (<i>L. angustifolius</i>), cupin domain	2313076	2	22.14	15.53
11	Legumin A2 precursor (<i>Pisum sativum</i>)	126161	1	1.73	59.27
11	Legumin A (<i>Vicia sativa</i>)	483449	1	1.81	56.56
12–14	Legumin-like protein (<i>L. albus</i>)	85361412	1	3.12	58.41
1–3, 4–10	β -Conglutin (<i>L. angustifolius</i>)	149208403	10	26.37	54.3
1–3, 4–10	β -Conglutin (<i>L. angustifolius</i>)	149208401	17	45.87	61.47
1–3, 4–10	β -Conglutin (<i>L. angustifolius</i>)	169950562	15	33.22	71.89
1–3, 4–9	β -Conglutin precursor (<i>L. albus</i>)	46451223	5	9.76	62.13
1–3, 4–10	Vicilin-like protein (<i>L. albus</i>)	89994190	6	12.24	62.03
16–17	γ -Conglutin (<i>L. angustifolius</i>)	662366	11	38.31	48.92
15	Conglutin delta-2 large chain (<i>L. angustifolius</i>)	116181	2	35	9.4

^a IgE reactive spots matched with protein spots from Coomassie-stained 2D-gels.^b Identified protein and the organism from which it proceeds.^c Database accession number according to NCBI nr.^d Number of peptides matched with Spectrum Mill MS Proteomics Workbench database (Agilent Technologies) and lists in Supplementary Tables II and III.^e Amino acid sequence coverage for the identified protein.^f Theoretical mass (kDa) of identified protein retrieved from the protein database.

protein extract, high resolution 2D spot maps of LPE, α -, β -, and γ -conglutins were prepared. The 2D-maps of *L. albus* and *L. angustifolius* protein are shown in Figs. 4 and 5, respectively. Panel E shows the LPE's, and panels B–D show the multi-channel viewers of the purified proteins, obtained with a specific tool of PDQuest Software. Panel A shows the merge obtained superimposing the coloured gels of the purified proteins: this virtual reconstruction corresponds well with panel E. This technical expedient was useful to better correlate the reactive spots with the corresponding proteins on the 2D-gel of LPE.

The Western blottings were performed with a pooled serum from lupin responsive patients, selected on the basis of ELISA results. For maximal resolution, blots were prepared for purified protein fractions only. These analyses highlighted several reactive spots, indicating the presence of different IgE-binding isoforms and supporting the concept that these isoforms are derived from a unique common ancestor polypeptide, which undergoes proteolytic cleavage, giving a complex mixture of polypeptides with different molecular weights and degrees of post-translational phosphorylation and glycosylation.

β -Conglutin, which is the most heterogeneous protein present in LPE, was positive in both species: in *L. albus* spots from 30 to 60 kDa and with a basic pI value were positive (Fig. 4, panel F); in *L. angustifolius* the majority of the reactive spots had acidic pH value and focused at 75, 50, 35, and 17 kDa (Fig. 5, panel F). In the case of the α -conglutins acidic subunit, a number of spots from 37 to 50 kDa were positive in *L. albus* (Fig. 4, panel G), whereas only 4 spots in the same MW range were positive in *L. angustifolius* (Fig. 5, panel G). The α -conglutins basic subunits were positive only for *L. albus*, in particular one prominent spot at pI 7.5. The δ -conglutins, which co-eluted in the α -conglutins fraction, seemed to be reactive only in *L. angustifolius*; in particular one isoform at 15 kDa (Fig. 5, panel G). In both species, only the large subunit of

γ -conglutins gave a positive response, whereas the small subunit showed no reactivity (Figs. 4 and 5, panel H).

For the identification of the IgE-binding protein subunits, the reactive spots were *in-gel* digested with trypsin and analysed via mass spectrometry, using both HPLC-Chip-Ion Trap and HPLC-Chip-qTOF approaches (Sirtori et al., 2010). Details of the amino acid sequences obtained are reported as supplementary data (Supplementary Tables II and III).

Identified IgE-binding peptide fragments matched with: legumin-like protein [NCBI nr 85361412, 62816184, 62816188 *L. albus*]; α -conglutins [NCBI nr 2313076, *L. angustifolius*]; β -conglutins precursor [NCBI nr 46451223, *L. albus*]; vicilin-like protein [NCBI nr 89994190, *L. albus*]; β -conglutins [NCBI nr 169950562, 149208403, 149208401, *L. angustifolius*]; γ -conglutins [NCBI nr 11191819, *L. albus*; 662366, 223005, *L. angustifolius*]; and δ -conglutins-2 large chain [NCBI nr 116181, *L. angustifolius*] (Table 1). Since *L. albus* and *L. angustifolius* proteins are highly homologous, in some cases the database search recognised a peptide as belonging to both species. The closest non-lupin identity matches to lupin α -conglutins were: legumin A [NCBI nr 483449, *Vicia sativa*] and legumin A2 precursor [NCBI nr 126161, *Pisum sativum*], both with 60% identity aligned against lupin legumin-like protein.

The peptides identified in the reactive spots were aligned against known allergenic peptides (Lewis, Grimshaw, Warner, & Hourihane, 2005; van Bostel, Koppelman, van den Broek, & Gruppen, 2008) belonging to the major peanut allergens. Supplementary Table IV summarises the results of the alignments obtained with the BLASTp Program: allergen Ara h 1 was aligned against β -conglutins precursor, vicilin-like protein and β -conglutins showing an identity between 50% and 76% and a homology between 63% and 93%. Allergens Ara h 3/4 were aligned against legumin-like protein, α -conglutins and *de novo* peptides showing an identity between 67% and 88% and a homology between 55% and 100%. Small

ler homologies were found between the lupin proteins and the allergen Ara h 2, the most relevant allergen in peanut (Porterfield et al., 2009) or allergen Ara h 6 (data not shown): Ara h 2 (NCBI nr: 6662248, 77756) showed an identity of only 39.1% and 40.9% when aligned respectively to *L. angustifolius* and *L. albus* δ -conglutinin (NCBI nr: 116181, 80221495); in the same way, allergen Ara h 6 (NCBI nr: 6662249) showed 44.4% identity against *L. albus* δ -conglutinin, and 41.7% identity against *L. angustifolius* δ -conglutinin.

3.4. Correlation between IgE reactivity and glycosylation profile of lupin proteins

Since lupin seed globulins all contain bound carbohydrates, it was useful to establish whether the glycosylated residues were involved in the lupin protein reactivity. It is in fact known that the carbohydrate present on many plant food proteins can induce anti-glycan IgE responses (Malandain, 2005). In order to detect the glycosylated spots, the 2D-gels of LPE were stained with a specific fluorescent dye. The panels I of Figs. 4 (*L. albus*) and 5 (*L. angustifolius*) highlight many different glycoproteins: several isoforms of β -conglutinin and the large subunit of γ -conglutinin were glycosylated in both species, whereas only the *L. albus* α -conglutinin was stained. Comparing these results with the Western blots, most of the reactive spots were glycosylated, as summarised in Supplementary Table V, strengthening the need to determine the degree of lupin protein glycosylation and to identify the specific glycoportion/moiety involved in the allergenic response.

4. Discussion

There is no general consensus about the identity of lupin allergens based on their IgE binding activity. Some authors suggest that IgE-binding lupin proteins possess MWs of 43–45 kDa and of 61–65 kDa, deduced from immunoblots (Holden, Sletten, Lindvik, Faeste, & Dooper, 2008; Moneret-Vautrin et al., 1999; Peeters et al., 2007), whereas others opt for MWs in the range from 35 to 55 kDa, with minor bands at 17, 21 and 24 kDa (Hefle, Lemanske, & Bush, 1994; Parisot et al., 2001). β -Conglutinin was identified as a major allergen (Goggin et al., 2008), but IgE reactivity towards γ -conglutinin and the α -conglutinin basic subunits were also reported (Magni et al., 2005). Collectively, these reports indicate that several lupin proteins are potential allergens. The majority of these studies focused on *L. albus*, whereas scarce data are available for *L. angustifolius* (Dooper et al., 2007; Holden et al., 2008). A recent paper has tried to fill this gap (Goggin et al., 2008), however the data were only partial, since the sequence of *L. angustifolius* α -conglutinin is still unknown and the IgE-reactive spots were not identified.

The important aspects of this work are that all the experiments were performed on purified lupin proteins; the traditional ELISA approach was complemented with Western blotting coupled with mass spectrometry in order to have a more detailed description of the entire pattern of IgE-binding proteins; and the *de novo* peptide sequencing allowed the identification of new peptides.

This study has confirmed the presence of several important reactive proteins in lupin. The IgE-binding profiles of α -, β - and γ -conglutinins were specific and individual for each serum, and strongly depending on the level of peanut-specific IgE in the sera, as evidenced by inhibition ELISA testing. The IgE reactivities to α -conglutinin, and to a lesser extent to β -conglutinin, were the most prevalent, possibly because these two proteins have high homology with their peanut equivalents, as has been suggested before (Holden et al., 2008; Moneret-Vautrin et al., 1999). The presence of a direct relationship between the LPE and the α -conglutinin allergenic reactivity suggests (Fig. 2) a predominant role of this protein in the allergenic response to lupin, which is in agreement with previous

reports (Holden et al., 2008; Moneret-Vautrin et al., 1999). Moreover, the protein alignments have shown that the IgE-binding lupin peptides from α -conglutinin and β -conglutinin are highly homologous to allergic peanut peptides reported in literature (Lewis et al., 2005; van Bostel et al., 2008), highlighting several conserved epitopes recognised by IgE antibodies.

In a recent work of our group a lupin protein isolate was submitted to industrial food processing with the aim to study the change of the protein bio-availability before and after strong thermal treatments (Sirtori et al., 2010). Many stable peptides after prolonged treatments were detected by shotgun proteomics; among them, the peptides DQSYFSGFSK [β -conglutinin, NCBI nr 149208401], NTLEATFNTR [β -conglutinin precursor, NCBI nr 46451223; β -conglutinin, NCBI nr 149208401], and RPFYTNAPQEIIYQQGR [legumin-like protein, NCBI nr 85361412; α -conglutinin, NCBI nr 2313076] were the same detected in the reactive spots, showing 72%, 70%, and 100% homology with peanut allergens, respectively. Indeed, resistance of the proteins to digestion and processing is supposed to be an important characteristic of food allergens (Bannon, 2004).

Together with α -conglutinin and β -conglutinin, a positive response was also observed in case of γ -conglutinin large subunit that was reactive in both species. However, the IgE binding to γ -conglutinin was not observed in the indirect ELISA, indicating the potential presence of folded epitopes, which were only exposed upon denaturation as in the case of Western blotting (Holden et al., 2008). Interestingly, the amino acid sequence of this protein does not align with any known peanut protein, suggesting that the IgE-binding epitopes of γ -conglutinin may be different. Possibly, the presence of both cross-reactive and unique allergens may help explaining the lupin allergy of some individuals without a known peanut allergy (Novembre et al., 1999; Quaresma et al., 2007).

The presence of glycosylated allergenic proteins argues for the possible contribution of carbohydrate determinants, since glyco-epitopes could potentially interfere with the IgE-binding and, thus, play a role in the cross-reactivity (Barre et al., 2008). This is particularly evident in the case of β - and α -conglutinin: β -conglutinin contains mannose units linked to the different subunits, whereas α -conglutinin is glycosylated in the acidic chain, which was reactive in both species.

Another important aspect to be taken into account is the geographical origin of the patients, which could determine the response profile, since the occurrence of food allergy is often associated with environmental risk factors, i.e. air pollution and aeroallergens (Roberts et al., 2005; Schroeder et al., 2009). In fact, many families of respiratory allergens are homologues of food proteins, i.e. Bet v 1 and profilins, due to the preservation of both surface residues and chain conformation of the proteins (Hoffmann-Sommergruber & Mills, 2009; Jenkins, Griffiths-Jones, Shewry, Breiteneder, & Mills, 2005). All patients investigated in this study were from The Netherlands. Tests on patients from Southern Europe, probably differently sensitised to pollen, might provide different results.

In conclusion, before promoting the use of lupin in innovative food products, it would be essential to obtain a better understanding of the allergenicity of its proteins and more studies are needed to establish the prevalence of allergic reactions to lupin in primarily peanut-sensitised allergic individuals as well as in other allergic populations. A still open question is whether primary lupin-sensitisation can occur: the allergen identification reported here would make such studies more feasible.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.foodchem.2010.11.073.

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1 **Supplementary Table I.** Results of skin prick tests (SPTs), allergy symptoms and IgE levels of the patients with diagnosed peanut allergy.
2 Symptoms were categorized as follows: 1) gastrointestinal (nausea, vomiting, diarrhea and abdominal pain); 2) lower respiratory (wheeze and
3 cough); 3) upper respiratory (nasal congestion, rhinorrhea, sneezing, tearing, and conjunctival injection); 4) cutaneous (pruritus, erythematous
4 morbilliform rash, and urticaria); 5) laryngeal edema (cough with itchy throat, hoarseness, and throat clearing); and ND (not determined). SPTs:
5 peanut 10 µg/mL, lupin 5% protein, control 0.9% NaCl.

6

Patient no.	Peanut specific IgE (kU/L)	Lupin specific IgE (kU/L)	SPTs (wheal response, mm)				Allergy symptoms
			Histamine	Control	Peanut	Lupin	
1	>100	29.4	20	0	17	5	1
2	>100	40.7	16	0	13	11	5
3	>100	37.3	14	0	9	5	1,2
4	>100	21.6	18	0	9	6	1,2
5	>100	25.6	13	0	12	7	5
6	>100	14.3	15	0	12	5	5
7	>100	16	12	0	8	3	5
8	>100	28.7	12	0	8	6	1
9	>100	20.4	17	0	6	5	5
10	>100	18	21	1	9	6	2,3
11	>100	13.7	19	0	6	2	1
12	>100	38.5	20	0	12	7	4
13	52	13.7	12	0	8	2	1
14	45.2	12.3	11	0	9	3	3,4
15	14	10.7	9	0	7	5	3,4
16	13.7	7.6	15	0	5	5	1
17	12.8	9.7	17	0	7	3	4
18	11.2	3.2	14	0	7	1	3

19	9.2	4	18	0	8	3	3
20	8	6.8	13	0	6	0	4
21	7.16	3.9	9	0	5	2	1
22	5.76	4.4	11	0	4	3	1
23	5.4	4.3	14	0	4	0	4
24	5.34	3.2	13	0	3	3	3
25	4	3.8	8	0	3	2	4
26	2.86	0.43	12	0	1	1	3-4
27	2.53	0.42	20	0	2	1	4
28	1.62	0.38	17	0	1	1	1
29	1.09	<0.35	18	0	2	0	4
30	0.82	<0.35	15	0	0	0	ND
31	0.71	<0.35	11	0	0	0	ND
32	0.62	0.52	13	0	0	0	ND
33	0.44	0.40	13	0	1	0	4
34	0.42	<0.35	15	0	1	0	4

1 **Supplementary Table II.** Identification of *Lupinus albus* peptides from reactive spots by HPLC-Chip-Ion Trap and database search performed
2 by Spectrum Mill MS Proteomic Workbench (Agilent Technologies). Details of amino acid sequence and NCBI nr access of identified proteins
3 are listed. β -conglutin or vicilin-like protein are different names to indicate the same protein. Several species have a positive match with the same
4 peptide sequences (*Lupinus angustifolius*, *Pisum sativum* and *Vicia sativa*).

no. spot	Peptide sequence	Protein identification	NCBI nr accession	Species
1-4	EQEQQQGSPSYSRR IVEFQSKPNTLILPK HSDADYVLVVLNGR ATITIVNPDR LAIPINNPGYFYDFYPSSTK DQSYFSGFSR NTLEATFNTR SNEPIYSNK LLGFGINADENQR	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	EQEQQQGSPSYSRR IVEFQSKPNTLILPK HSDADYVLVVLNGR ATITIVNPDR LAIPINNPGYFYDFYPSSTK DQSYFSGFSR NTLEATFNTR SNEPIYSNK	Vicilin-like protein	89994190	<i>Lupinus albus</i>
	NTLEATFNTR	β -Conglutin	169950562, 149208403, 149208401	<i>Lupinus angustifolius</i>
	EQEQQQGSPSYSRR IVEFQSKPNTLILPK HSDADYVLVVLNGR QAYNLEYGDALR IPAGSTSYILNPDDNQK LAIPINNPGYFYDFYPSSTK DQSYFSGFSR IILGNEDEQEYEEQR IILGNEDEQEYEEQR SNEPIYSNK	β -Conglutin precursor Vicilin-like protein	46451223 89994190	<i>Lupinus albus</i>
5-8	QAYNLEYGDALR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>

9-11	EQEQQQGSPSYSRR IVEFQSKPNTLILPK HSDADYVLVVLNGR ATITIVNPDR QAYNLEYGDALR IPAGSTSYILNPDDNQK LAIPINNPGYFYDFYPSSTK DQQSYFSGFSR IILGNEDEQEYEEQR AIYVVVVDEGEGNYELVGIR	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	EQEQQQGSPSYSRR IVEFQSKPNTLILPK HSDADYVLVVLNGR ATITIVNPDR QAYNLEYGDALR IPAGSTSYILNPDDNQK LAIPINNPGYFYDFYPSSTK DQQSYFSGFSR IILGNEDEQEYEEQR	Vicilin-like protein	89994190	<i>Lupinus albus</i>
	QAYNLEYGDALR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
12-13	EQEQQQGSPSYSRR IVEFQSKPNTLILPK HSDADYVLVVLNGR QAYNLEYGDALR IPAGSTSYILNPDDNQK LAIPINNPGYFYDFYPSSTK DQQSYFSGFSR NTLEATFNTR IILGNEDEQEYEEQR IILGNEDEQEYEEQR SNEPIYSNK	β -Conglutin precursor Vicilin-like protein	46451223 89994190	<i>Lupinus albus</i>
	QAYNLEYGDALR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
	NTLEATFNTR	β -Conglutin	169950562, 149208403, 149208401	<i>Lupinus angustifolius</i>
14-15	IVEFQSKPNTLILPK HSDADYVLVVLNGR ATITIVNPDR QAYNLEYGDALR LAIPINNPGYFYDFYPSSTK DQQSYFSGFSR NTLEATFNTR SNEPIYSNK	β -Conglutin precursor Vicilin-like protein	46451223 89994190	<i>Lupinus albus</i>

	QAYNLEYGDALR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
	NTLEATFNTR	β -Conglutin	169950562, 149208403, 149208401	<i>Lupinus angustifolius</i>
16-18	QAYNLEYGDALR DQSYFSGFSR NTLEATFNTR YGNFYEITPDR	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	QAYNLEYGDALR DQSYFSGFSR NTLEATFNTR ILLGNEDEQEYEEQR YGNFYEITPDR NFLAGSEDNVIR ELTFPGSAEDIER	Vicilin-like protein	89994190	<i>Lupinus albus</i>
	QAYNLEYGDALR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
	NTLEATFNTR	β -Conglutin	169950562, 149208403, 149208401	<i>Lupinus angustifolius</i>
19	QQPQENECQFQR LNALEPDNTVQSEAGTIETWNP RPFYTNAPQEIIYQQGR GIFGMIFPGCGETYEEPQSEK FYLSGNQEQEFLQYQEK	Legumin-like protein	85361412	<i>Lupinus albus</i>
20-21	QQPQENECQFQR LNALEPDNTVQSEAGTIETWNP GIFGMIFPGCGETYEEPQSEK FYLSGNQEQEFLQYQEK	Legumin-like protein	85361412	<i>Lupinus albus</i>
22	LNALEPDNTVQSEAGTIETWNP RPFYTNAPQEIIYQQGR GIFGMIFPGCGETYEEPQSEK FYLSGNQEQEFLQYQEK	Legumin-like protein	85361412	<i>Lupinus albus</i>
23	LNALEPDNTVQSEAGTIETWNP RPFYTNAPQEIIYQQGR FYLSGNQEQEFLQYQEK EGGIVEVK	Legumin-like protein	85361412	<i>Lupinus albus</i>
	INALEPDNR	Legumin A	483449	<i>Vicia Sativa</i>
	LNALEPDNR	Legumin A2 precursor	126161	<i>Pisum sativum</i>
24	QQPQENECQFQR LNALEPDNTVQSEAGTIETWNP RPFYTNAPQEIIYQQGR FYLSGNQEQEFLQYQEK	Legumin-like protein	85361412	<i>Lupinus albus</i>
25-26	HNIGESTSPDAYNPQAGR TLTSIDFPILGWLGLAAEHGSIYK	Legumin-like protein	85361412	<i>Lupinus albus</i>
	HNIGESTSPDAYNPQAGR	Legumin-like protein	62816184	<i>Lupinus albus</i>

	HNIGESTSPDAYNPQAGR TLTSIDFPILGWLGLAAEHGSIYK	Legumin-like	62816188	<i>Lupinus albus</i>
27	HNIGESTSPDAYNPQAGR SLSDNFR FLVPPPQSQLR	Legumin-like protein	62816184	<i>Lupinus albus</i>
28	HNIGESTSPDAYNPQAGR FLVPPPQSQLR	Legumin-like protein	62816184	<i>Lupinus albus</i>
	HNIGESTSPDAYNPQAGR FLVPPPQSQLR	Legumin-like protein	85361412	<i>Lupinus albus</i>
	HNIGESTSPDAYNPQAGR FLVPPPQSQLR	Legumin-like protein	62816188	<i>Lupinus albus</i>
29	TPLMQVPVLLDLNGK IPQFLFSCAPTFLTQK QGEYFIQVSAIR	Conglutin gamma	11191819	<i>Lupinus albus</i>
	ISSENFMVQAQDGVSLGFDGGVHAR	Conglutin gamma	662366	<i>Lupinus angustifolius</i>
30	TPLMQVPVLLDLNGK IPQFLFSCAPTFLTQK QGEYFIQVSAIR	Conglutin gamma	11191819	<i>Lupinus albus</i>

1 **Supplementary Table III.** Identification of *Lupinus angustifolius* peptides from reactive spots by HPLC-Chip-Ion Trap and database search
2 performed by Spectrum Mill MS Proteomic Workbench (Agilent Technologies). Details of amino acid sequence and NCBI nr access of identified
3 proteins are listed. β -conglutin or vicilin-like protein and α -conglutin or legumin-like protein are different names to indicate the same proteins.
4 Several species have a positive match with the same peptide sequences (*Lupinus albus*, *Pisum sativum* and *Vicia sativa*). The proteins that did not
5 show any match with the NCBI database were identified via HPLC-Chip-qTOF. As usual, since leucine (L) and isoleucine (I) have the same residue
6 mass (113.08407 Da), a correct attribution along the peptide sequence is very difficult: in this table both amino acids are reported for the *de novo*
7 peptides.

8

no. spot	Peptide sequence	Protein identification	NCBI nr accession	Species
1	LENLQNYR ATITIVNPKR LPAGTTSYILNPDDNQNL LAIPINNPPPGK DQSYFSGFSK NTLEATFNTR KEQIQELR	β -Conglutin	149208403	<i>Lupinus angustifolius</i>
	LENLQNYR IVEFQSNPNTLILPK HSDADYILVVLNGR ATITIVNPKR QAYNLEYGDALR LPAGTTSYILNPDDNQNL LAIPINNPSNFYDFYPSSTK DQSYFSGFSK NTLEATFNTR GKPSESGPFNL FGNFYEITPER NFLAGSEDNVISQLDR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>

	LENLQNYR ATITIVNPKR LPAGTTSYILNPDDNQNL LAIPINNPGK DQSYFSGFSK NTLEATFNTR KQIQELR NFLAGSEDNVIK	β -Conglutin	169950562	<i>Lupinus angustifolius</i>
	LENLQNYR QAYNLEYGDALR NTLEATFNTR LLGFGINADENQR SNEPIYSNK	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	LENLQNYR QAYNLEYGDALR NTLEATFNTR NFLAGSEDNVIR SNEPIYSNK	Vicilin-like protein	89994190	<i>Lupinus albus</i>
2	LENLQNYR ATITIVNPKR LAIPINNPPPGK DQSYFSGFSK KQIQELR	β -Conglutin	149208403	<i>Lupinus angustifolius</i>
	LENLQNYR HSDADYILVVLNGR ATITIVNPKR QAYNLEYGDALR DQSYFSGFSK GKPSESGPFNLR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
	LENLQNYR ATITIVNPKR LAIPINNPGK DQSYFSGFSK KQIQELR	β -Conglutin	169950562	<i>Lupinus angustifolius</i>
	LENLQNYR QAYNLEYGDALR LLGFGINADENQR	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	LENLQNYR QAYNLEYGDALR NFLAGSEDNVIR	Vicilin-like protein	89994190	<i>Lupinus albus</i>

3	LENLQNYR ATITIVNPDKR LAIPINNPPGK DQSYFSGFSK LPAGTTSYILNPDDNQNL NTLEATFNTR KQIQELR	β -Conglutin	149208403	<i>Lupinus angustifolius</i>
	IVEFQSNPNTLILPK LENLQNYR HSDADYILVVLNGR ATITIVNPDKR QAYNLEYGDALR LPAGTTSYILNPDDNQNL DQSYFSGFSK NTLEATFNTR NFLAGSEDNVISQLDR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
	LENLQNYR ATITIVNPDKR LAIPINNPGK LPAGTTSYILNPDDNQNL DQSYFSGFSK NTLEATFNTR KQIQELR	β -Conglutin	169950562	<i>Lupinus angustifolius</i>
	LENLQNYR QAYNLEYGDALR NTLEATFNTR SNEPIYSNK	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	LENLQNYR QAYNLEYGDALR NTLEATFNTR SNEPIYSNK	Vicilin-like protein	89994190	<i>Lupinus albus</i>
4-6	LENLQNYR ATITIVNPDKR LPAGTTSYILNPDDNQNL DQSYFSGFSK KQIQELR	β -Conglutin	149208403	<i>Lupinus angustifolius</i>

	LENLQNYR ATITIVNPKR QAYNLEYGDALR LPAGTTSYILNPDDNQNL DQSYFSGFSK GKPSESGPFNL	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
	LENLQNYR ATITIVNPKR LPAGTTSYILNPDDNQNL DQSYFSGFSK KQIQEL	β -Conglutin	169950562	<i>Lupinus angustifolius</i>
	LENLQNYR QAYNLEYGDALR LLGFGINADENQR	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	LENLQNYR QAYNLEYGDALR NFLAGSEDNVIR	Vicilin-like protein	89994190	<i>Lupinus albus</i>
7-9	LENLQNYR ATITIVNPKR LPAGTTSYILNPDDNQNL DQSYFSGFSK NTLEATFNTR GQEQSHQDEGVIVR KQIQEL HAQSSSGEGKPSSESGPFNL	β -Conglutin	149208403	<i>Lupinus angustifolius</i>
	LENLQNYR IVEFQSNPNTLILPK ATITIVNPKR QAYNLEYGDALR LPAGTTSYILNPDDNQNL LAIPINNPSNFYDFYPSSTK DQSYFSGFSK NTLEATFNTR ILLGNEDEQEDEEQR GQEQSYQDEGVIVR FGNFYEITPER LLGFGINANENQR NQQQSYFANAQPQQKQQR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>

	LENLQNYR ATITIVNPKR LPAGTTSYILNPDDNQNL DQQSYFSGFSK NTLEATFNTR GQEQSHQDEGVIVR KQIQELR HAQSSSGEGKPSGPFNLR QQDEQEEYEQGEVEVR LLGFGINANENQR NFLAGSEDNVIK ELTFPGSIEDVER NQQSYFANAQPQQQQR	β -Conglutin	169950562	<i>Lupinus angustifolius</i>
	LENLQNYR QAYNLEYGDALR NTLEATFNTR LLGFGINADENQR	β -Conglutin precursor	46451223	<i>Lupinus albus</i>
	LENLQNYR QAYNLEYGDALR NTLEATFNTR GQEQSHQDEGVIVR	Vicilin-like protein	89994190	<i>Lupinus albus</i>
10	GQEQSHQDEGVIVR HAQSSSGEGKPSGPFNLR	β -Conglutin	149208403	<i>Lupinus angustifolius</i>
	AIFIVVDEGEGNYELVGIR	β -Conglutin	149208401	<i>Lupinus angustifolius</i>
	GQEQSHQDEGVIVR HAQSSSGEGKPSGPFNLR AIFIVVDEGEGNYELVGIR	β -Conglutin	169950562	<i>Lupinus angustifolius</i>
	GQEQSHQDEGVIVR	Vicilin-like protein	89994190	<i>Lupinus albus</i>
11	VEEGI/LGVI/LSPK	Legumin	De novo peptide	<i>Lupinus angustifolius</i>
	LNALEPDNR	Legumin A2 precursor	126161	<i>Pisum sativum</i>
	INALEPDNR	Legumin A	483449	<i>Vicia sativa</i>
12-14	VEEGI/LGVI/LSPK FYI/LAGNPEEEYPETQQQR NNI/LI/LSGFDPQFI/LSQAI/LNI/LDEDTVHK WQEQQEEEEEEKEEPR	Legumin	De novo peptide	<i>Lupinus angustifolius</i>
	TLTSLDFPILR HNIGQSTSPDAYNPQAGR	Conglutin alpha	2313076	<i>Lupinus angustifolius</i>
	PFYTNAPQEIIYIQQR	Legumin-like protein	85361412	<i>Lupinus albus</i>
15	QQEQQLEGELEK ALQQIYESQSECEGR	Conglutin delta-2 large chain	116181	LUPAN (<i>L. angustifolius</i>)
16	VPQFLFSCAPSFLAQK	Conglutin gamma	662366	<i>Lupinus angustifolius</i>

	QFSVCLSR QGEYFIQVNAIR AVGPFGLCYDSR ISGGAPSVDLILDK ISGGAPSVDLILDKNDVWR ISSENFMVQAQDGVSLGFVDGGVHAR AGIALGAHHLEENLVFDLER VGFNSNSLK TCSNLFDLNNP(-)			
17	VPQFLFSCAPSFLAQK QGEYFIQVNAIR HLVIPTK	Conglutin gamma	662366	<i>Lupinus angustifolius</i>

1 **Supplementary Table IV.** Alignments between lupin peptides identified in reactive spots and peanut allergic peptides. A) *L. albus* β -conglutin
2 precursor [NCBIInr 46451223], and vicilin-like protein [NCBIInr 89994190] aligned against allergen Ara h 1, clone P41B [NCBIInr 1168391]. B) *L.*
3 *angustifolius* β -conglutin [NCBIInr 169950562, 149208403, 149208401 aligned against allergen Ara h 1, clone P41B. C) *L. albus* legumin-like
4 protein [NCBIInr 85361412, 62816184, 62816188] aligned against allergens Ara h 3/Ara h 4 [NCBIInr 21314465]. D) *L. angustifolius* α -conglutin
5 [NCBIInr 2313076], and *de novo* peptides aligned against allergens Ara h 3/Ara h 4. Start amino acid position and both identity and homology %
6 (calculated with BLASTp Program) of each peptide are reported. The identity percentage of *de novo* peptides was not calculated since it is difficult
7 to attribute leucine or isoleucine along the peptide sequences, having them the same residue mass (113.08407 Da).

8

A.					
Peanut peptide	Lupin peptide	Spot no.	Alignment	Identity (%)	Homology (%)
IVQIEAKPNTLVLPK	IVEFQSKPNTLILPK	1-15	arah1, 214 IVQIEAKPNTLVLPK IV+ ++KPNTL+LPK lupin, 154 IVEFQSKPNTLILPK	66	93
HADADNILVIQQGQ	HSDADYVLVVLNGR	1-15	arah1, 229 HADADNILVIQQGQ H+DAD +LV+ G lupin, 169 HSDADYVLVVLNGR	50	71
IPSGFISYILNRHDNQN	IPAGSTSYILNPDDNQK	5-13	arah1, 266 IPSGFISYILNRHDNQN IP+G SYILN DNQ lupin, 206 IPAGSTSYILNPDDNQK	65	71
DQSSYLQGFSR (Lewis et al., 2005; van Boxtel et al., 2008)	DQQSYFSGFSR	1-18	arah1, 308 DQSSYLQGFSR DQ SY GFSR lupin, 233 DQQSYFSGFSR	72	72
NTLEAAFNAE (Lewis et al., 2005; van Boxtel et al., 2008)	NTLEATFNTR	1-4, 12-18	arah1, 319 NTLEAAFNAE NTLEA FN lupin, 259 NTLEATFNTR	70	70
LLGFGINAENNHHR (van Boxtel et al., 2008)	LLGFGINADENQR	1-4	arah1, 528 LLGFGINAENNHHR LLGFGINA+ N R lupin, 451 LLGFGINADENQR	76	84
IFLAGDKDNVID (van Boxtel et al., 2008)	NFLAGSEDNVIR	16-18	arah1, 542 IFLAGDKDNVID	67	75

			FLAG +DNVI lupin, 464 NFLAGSEDNVIR		
DLAFPGSGEQVEK (Lewis et al., 2005; van Bortel et al., 2008)	ELTFPGSAEDIER	16-18	arah1, 560 DLAFPGSGEQVEK +L FPGS E +E lupin, 483 ELTFPGSAEDIER	54	70
B.					
IVQIEAKPNTLVLPK	IVEFQSNPNTLILPK	1, 3, 7-9	arah1, 214 IVQIEAKPNTLVLPK IV+ ++ PNTL+LPK lupin, 139 IVEFQSNPNTLILPK	60	86
FQNLQNR	LENLQNYR	1-9	arah1, 207 FQNLQNR +NLQN+R lupin, 146 LENLQNYR	63	88
HADADNILVIQGGQ	HSDADYILVVLNGR	1-3	arah1, 229 HADADNILVIQGGQ H+DAD ILV+ G lupin, 154 HSDADYILVVLNGR	57	71
IPSGFISYILNRHDNQNL	LPAGTTSYILNPDDNQNL	1, 3-9	arah1, 266 IPSGFISYILNRHDNQNL +P+G SYILN DNQNL lupin, 191 LPAGTTSYILNPDDNQNL	68	79
DQSSYLQGFSR (Lewis et al., 2005; van Bortel et al., 2008)	DQQSYFSGFSK	1-9	arah1, 308 DQSSYLQGFSR DQ SY GFS lupin, 233 DQQSYFSGFSK	63	63
NTLEAAFNAE (Lewis et al., 2005; van Bortel et al., 2008)	NTLEATFNTR	1, 3, 7-9	arah1, 319 NTLEAAFNAE NTLEA FN lupin, 259 NTLEATFNTR	70	70
LLGFGINAENNR (van Bortel et al., 2008)	LLGFGINANENQR	7-9	arah1, 528 LLGFGINAENNR LLGFGINA N R lupin, 436 LLGFGINANENQR	76	76
IFLAGDKDNVIDQIEK (van Bortel et al., 2008)	NFLAGSEDNVISQLDR	1, 3	arah1, 542 IFLAGDKDNVIDQIEK FLAG +DNVI Q++ lupin, 449 NFLAGSEDNVISQLDR	56	75
DLAFPGSGEQVEK (Lewis et al., 2005; van Bortel et al., 2008)	ELTFPGSIEDVER	7-9	arah1, 560 DLAFPGSGEQVEK +L FPGS E VE lupin, 468 ELTFPGSIEDVER	62	69
C.					
QQPEENACQFQR (Lewis et al., 2005)	QQPQENECQFQR	19-21, 24	arah3/4, 25 QQPEENACQFQR QQP+EN CQFQR lupin, 29 QQPQENECQFQR	83	91
LNAQRPDNRIESEGYYIETWNP	LNALEPDNTVQSEAGTIETWNP	19-24	arah3/4, 37 LNAQRPDNRIESEGYYIETWNP LNA PDN ++SE G IETWNP lupin, 41 LNALEPDNTVQSEAGTIETWNP	68	77
KNIGRNRSPDIYNPQAG	HNIGESTSPDAYNPQAG	25-28	arah3/4, 364 KNIGRNRSPDIYNPQAG	71	76

			NIG + SPD YNPQAG lupin, 74 HNIGESTSPDAYNPQAG		
RPFYSNAPQEIFIQQGR (Lewis et al., 2005; van Bostel et al., 2008)	RPFYTNAPQEIIYQQGR	19, 22-24	arah3/4, 82 RPFYSNAPQEIFIQQGR RPFY+NAPQEI+IQQGR lupin, 86 RPFYTNAPQEIIYQQGR	88	100
GYFGLIFPGCPSTYEPPAQ (Lewis et al., 2005)	GIFGMIFPGCGETYEEPQE	19-22	arah3/4, 99 GYFGLIFPGCPSTYEPPAQ G FG+IFPGC TYEEP lupin, 103 GIFGMIFPGCGETYEEPQE	68	74
FNLAGNHEQEFLRYQQ (Lewis et al., 2005)	FYLSGNQEQEFLQYQE	19-24	arah3/4, 196 FNLAGNHEQEFLRYQQ F L+GN EQEFL+YQ+ lupin, 186 FYLSGNQEQEFLQYQE	68	87
D.					
VRGGLRILSPDRK	VEEGI/LGVI/LSPK	11-14	arah3/4, 298 VRGG L RI L SPD G + + + SP lupin, VEEGI/LGVI/LSPK	/	55
FNLAGNHEQEFLRYQQQ	FYI/LAGNPEEEYPETQQQR	12-14	arah3/4, 196 FN L AGNHEQEFLRYQQQSR F L AGN E+E+ QQQ lupin, FYI/LAGNPEEEYPETQQQR	/	70
NIFSGFTPEFLAQAFQVDDRQI	NNI/LI/LSGFDPQFI/LSQAI/LNI/L DEDTVHK	12-14	arah3/4, 258N I F SGFTPEF L AQAF N I SGF P+F L +QA lupin, NNI/LI/LSGFDPQFI/LSQAI/L arah3/4, Q V DDRQILQ + D+ + lupin, NI/LDEDTVHK	/	72
NIGRNRSPDIYNPQAG (Lewis et al., 2005)	HNIGQSTSPDAYNPQAGR	12-14	arah3/4, 360 KNIGRNRSPDIYNPQAGS NIG++ SPD YNPQAG lupin, 68 HNIGQSTSPDAYNPQAGR	67	78

Supplementary Table V. Correlation between reactive spots and glycosylated spots. The first column lists the reactive spots identified in the western blots; the second column summarizes the protein identity of each spot; the third column lists the numbers of the reactive spots that were also positive to glyco-staining. In *L. angustifolius* α -conglutin acidic subunit and δ -conglutin were not glycosylated.

Spot no.	Protein identity	Glycosylated spots
<i>L. albus</i>		
From 1 to 18	β -conglutin	1-4, 7, 8, 12-18
From 19 to 24	α -conglutin acidic subunit	19-23
From 25 to 28	α -conglutin basic subunit	25, 26
29, 30	γ -conglutin	29, 30
<i>L. angustifolius</i>		
From 1 to 10	β -conglutin	1-3, 7-10
From 11 to 14	α -conglutin acidic subunit	/
15	δ -conglutin	/
16, 17	γ -conglutin	16, 17