RESEARCH ARTICLE

Characterization of lupin major allergens (*Lupinus albus* L.)

Eva Guillamón[1], Julia Rodríguez[2], Carmen Burbano[1], Mercedes Muzquiz[1], Mercedes M. Pedrosa[1], Beatriz Cabanillas[2], Jesús F. Crespo[2], Ana I. Sancho[3], E. N. Clare Mills[3] and Carmen Cuadrado[1]

[1] Departamento de Tecnología de Alimentos, SGIT-INIA, Madrid, Spain
[2] Servicio de Alergia, Hospital Universitario 12 de Octubre, Madrid, Spain
[3] Food Materials Division, Institute Food Research, Norwich Research Park, Norwich, UK

White lupin is considered to be a rich source of protein with a notable content of lysine and is being increasingly used in bakery, confectionery, snacks and pastry products due to its multifunctional properties, in addition to its potential hypocholesterolemic and hypoglycemic properties. However, lupin seed flour has been reported as a causative agent of allergic reactions, especially in patients with allergy to peanut since the risk of immunological cross-reactivity between lupin and peanut is higher than with other legumes. Previously, we had identified two proteins as major lupin allergens (34.5 and 20 kDa) as determined by IgE immunoblotting using sera of 23 patients with lupin-specific IgE. The aim of this study was to purify and characterize the two major lupin allergens. The results using *in vitro* IgE-binding studies and MS analysis have shown that the 34.5 kDa allergen (Lup-1) is a conglutin β (vicilin-like protein) while the 20 kDa allergen (Lup-2) corresponds to the conglutin α fraction (legumin-like protein). The high level of amino acid sequence homology of Lup-1 and Lup-2 with the major allergens of some legumes explains the IgE cross-reactivity and clinical cross-reactivity of lupin and other legumes.

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1 Introduction

White lupin flour is increasingly being used in a variety of food products, mostly as an additive to wheat flour or as a substitute for soy flour, especially in bread and pasta products, because of its nutritional (rich in protein and fiber, poor in fat and gluten free), functional and nutraceutical properties [1–3].

White lupin seeds contain albumin and globulin fractions in a ratio of approximately one to nine. Globulins are the major protein component of lupin seed storage protein and comprise two major protein types, conglutin β (vicilin like-protein or acid 7*S* globulin, 43.4%) and conglutin α (legumin like-protein or 11S globulin, 33%), and two minor components, conglutin δ (2S sulfur-rich albumin, 12.5%) and conglutin γ (basic 7S globulin, 6%) [4]. According to Duranti *et al.* [5], conglutin β shows the greatest structural variation, being composed of over 20 polypeptide chains with no disulfide bridges, covering a broad range of molecular masses (M_r 15–65 kDa); this globulin component is similar to soybean β-conglycinin (7*S*), which has hypocholesterolemic activity [6]. Conglutin α is composed of a heterogeneous mixture of subunits linked by two disulfide bridges (acid subunits M_r 54–47 kDa and basic subunit of M_r 20 kDa). Conglutin γ consists of disulfide-linked M_r 30 and 17 kDa polypeptides, which can reduce blood glucose in hyperglycemic rats, probably by binding insulin [2], and conglutin δ, which is made up of two disulphide-linked polypeptide chains of M_r 9 and 4 kDa.

Reports of allergic reactions to lupin following ingestion have been growing with the increased use of lupin proteins in

Correspondence: Dr. Eva Guillamón, Centro para la Calidad de los Alimentos, SGIT-INIA, C/José Tudela, Campus Universitario "Duques de Soria," Soria, Spain
E-mail: eva@inia.es
Fax: +34-9752-33-205

dietary products, either as primary lupin allergy [7–9] or as a result of cross-reactivity to other legumes [10], especially peanut [11–15]. Furthermore, occupational IgE-mediated allergy has been also reported in exposed workers after inhalation of or contact with lupin flour [16]. In 2007, the insertion of lupin on the list of ingredients that must be labeled on prepackaged foodstuffs pursuant to EU law [17] irrespective of the level of inclusion is a precautionary measure introduced to protect consumers from the risks posed by the accidental consumption of lupin-containing foods.

Some IgE-binding proteins of lupin seeds of M_r 13-108 kDa have been described [7, 11, 18], with a M_r 43 kDa protein presumable to be responsible for the IgE cross-reactivity with peanut [11]. However, white lupin allergens have yet to be fully characterized, although the lupin globulins conglutin α and γ have been identified as candidate allergens [19]. Recently, conglutin β has been suggested as a major allergen of *L. angustifolius*, being designated Lup an 1 by the International Union of Immunological Societies allergen nomenclature subcommittee [20]. Previously, we have reported two major allergens from white lupin with molecular weights of 34.5 and 20 kDa, identified using sera from 23 patients with lupin-specific IgE [21]. Furthermore, the thermostability of these lupin allergens was demonstrated, even in combination with pressure in instantaneous controlled pressure drop (DIC) treatment [22].

The aim of this work was to purify, identify and characterize the 34.5 and 20 kDa major allergens of *Lupinus albus* and describe homologies with other major legume allergens.

2 Materials and methods

2.1 Plant material

Sweet white lupin seeds (*L. albus* var Multolupa), supplied by the Servicio de Investigaciones Agrarias (Badajoz, Spain), were manually dehulled and the cotyledons were used to obtain the protein extract.

2.2 Sera

A serum pool was prepared using sera from four patients diagnosed with actual allergy to lupin, as described elsewhere [21]. Individual sera showed specific IgE levels from 0.38 to 100 kilounits/L (median = 17.33 kilounits/L), as quantified by the CAP-fluorescent enzyme immunoassay system (Pharmacia Diagnostic, Uppsala, Sweden).

2.3 Lupin protein extracts

Cotyledons were ground to pass through a 1-mm sieve (Tecator, Cyclotec 1093), and the flour was defatted with *n*-hexane (34 mL/g of flour) for 4 h, shaken and air-dried after filtration. The resulting defatted flours were extracted twice in a solution of 0.05 M Tris-HCl (pH 8.0) buffer with salt (0.5 M NaCl) at a 1:10 w/v ratio for 1 h at 4°C by stirring according to the method described by Álvarez-Álvarez *et al.* [21]. The extracts were centrifuged at 27 000 × *g* for 20 min at 4°C, and the combined supernatants were dialyzed against distilled water for 48 h at 4°C using dialysis membranes with a cutoff of 3.5 kDa (Spectra/Por, Serva, Heidelberg, Germany) and freeze-dried.

The soluble protein content of all extracts was determined by the Bradford dye-binding assay (Bio-Rad, Hercules, CA, USA) using bovine serum albumin (Sigma, St. Louis, MO, USA) as a standard.

2.4 Purification of conglutins

Storage protein fractions were isolated according to a modification of the method of Blagrove and Gillespie [23]. After extracting lupin flour with water (2 h at 4°C), and discarding albumins of the supernatant, the remaining globulins were desalted on PD-10 column (Pharmacia), previously equilibrated with 0.05 M Tris-HCl (pH 7.5). The desalted extract was subjected to anion-exchange fast protein liquid chromatography (ÄKTA) on the POROS 20HQ (4.6 mm (diameter) × 100 mm) column, previously equilibrated with 0.05 M Tris-HCl (pH 7.5). The protein fractions were eluted with a NaCl gradient from 0 to 1 M in 30 min obtaining three main globulins, namely conglutin γ (0–0.33 M in 8 min), conglutin β (0.33–0.49 M in the next 6 min) and conglutin α (0.49–0.65 M, 6 min later) and then, a gradient from 0.65 M to 1 M during 10 min. The flow rate was 1.5 mL/min and elution was monitored at 220 and 280 nm.

The two major allergen proteins used in sequence analysis were obtained passing the total globulin fraction through a BioLogic LP chromatography system (Bio-Rad) on BioGel P-100 medium column (Bio-Rad). The column was equilibrated with 0.5 M Tris-HCl buffer, pH 8, 0.5 M NaCl. The flow rate was 0.16 mL/min.

2.5 Protein electrophoresis and immunoblotting

SDS-PAGE was carried out as described by Laemmli [24] on 12 and 15% polyacrylamide gels using MINI PROTEAN III system (Bio-Rad). For reducing conditions, 2% v/v of 2-betamercaptoethanol was added to the sample buffer (Bio-Rad) and then heated at 100°C for 10 min. Proteins were visualized with Coomassie brilliant blue R250 staining.

Western blotting was performed by electrophoretic transfer to poly(vinylidene difluoride) membranes (Bio-Rad) at 220 mA during 1.5 h, at room temperature, essentially according to the method of Towbin *et al.* [25]. The

membrane was blocked with phosphate-buffered saline (pH 7.4) with 0.1% v/v Tween-20 containing 5% w/v fat-free milk powder at room temperature and then incubated overnight at room temperature with serum pool (1:3 dilution, v/v). A polyclonal goat anti-human IgE conjugated with alkaline phosphatase (diluted 1:500, v/v) was used as secondary antibody (Caltag, Burlingame, CA, USA). Following three washings in phosphate buffered saline with 1% v/v Tween-20, the blots were visualized using alkaline phosphatase conjugate substrate kit (Bio-Rad).

Coomassie-stained gels and membranes were scanned, and the molecular weight of the bands was assessed using Quantity One software (Bio-Rad) and the low-range SDS-PAGE protein mixture (SIGMA) as standard.

2.6 Mass spectrometric analysis of the major lupin allergens

Coomassie blue-stained spots corresponding to major allergens were excised from the gel and analyzed at Proteomic Service of Severo Ochoa Center (CSIC-Universidad Autónoma de Madrid, Spain) by a peptide mass fingerprinting method using MALDI-TOF/MS analysis. 2,5-Dihidroxybenzoic acid was used as the matrix.

The peptide mass fingerprint spectra were matched to the National Center for Biotechnology Information no redundant database entries by using the Mascot search engine. The National Center for Biotechnology Information no redundant was searched for tryptic peptides. The mass value was monoisotopic and protein mass 100 kDa with peptide tolerance $\pm$ 50 ppm. One missed cleavage *per* peptide was allowed. Carbamidomethylation of cysteine was the only or just assumed modification and the considered variable modifications were oxidation of methionine, acetylation of N-term and Gln to pyro-Glu conversion.

The multiple alignments of allergen sequences were generated by Clustal W software version 2.0.10.

The unidentified proteins by peptide mass fingerprinting were also analyzed by LC/ESI-ion trap MS-dynamic exclusion mode to determine the amino acid sequence. The MS dates were identified using TurboSequest software.

Manual *de novo* sequencing was performed with the quality MS/MS spectra and the generated sequences were searched for homology in FASTA. The level of identity between characterized legume allergens and the putative allergens in lupin was analyzed.

3 Results

3.1 Analysis of lupin protein fractions

Three globulin fractions, conglutin γ, β and α were separated by anion-exchange chromatography using an increasing salt concentration. The conglutin γ fraction did not bind to the POROS 20HQ column at pH 7.5; the conglutin β fraction eluted with 0.3–0.5 M NaCl and the conglutin α fraction eluted with 0.5–0.65 M NaCl (data not shown).

Figure 1 shows the characteristic SDS-PAGE profile of the total lupin proteins, albumins, globulins and the three main globulin fractions, conglutin β, α and γ, after isolation by anion-exchange chromatography. A polypeptide with a molecular weight of 34.5 kDa was detected in total lupin protein, globulin and conglutin β (open arrows, lanes La, G and β). This protein, hereafter termed Lup-1, corresponded to one of the two major white lupin allergens previously reported in Álvarez-Álvarez *et al.* [21]. Other conglutin β peptides from 15 to 65 kDa were also apparent (lane β). The pattern of conglutin α polypeptides (lane α) comprised several bands of M_r ~31–45 kDa and another of M_r 20 kDa that corresponded to the acidic and basic subunits, respectively, of the other major white lupin allergen, hereafter termed Lup-2. This 20-kDa band was detected in a total extract of lupin protein, the globulin fraction and conglutin α (solid arrows, lanes La, G and α). Two band proteins of 18 and 30 kDa were visualized in the conglutin γ lane of the Fig. 1.

The purified conglutin β, α and γ fractions were analyzed by immunoblotting (Fig. 2). Several conglutin β peptides from 31 to 59 kDa reacted to IgE, including the 34.5 kDa major allergen (Lup-1), which was a strong IgE-reactor (empty arrow, lane β). However, only the basic subunit protein of conglutin α of 20 kDa (Lup-2) reacted to IgE (solid arrow, lane α) and two immunoreactive bands of 17 and 30 kDa were detected in the conglutin γ lane.

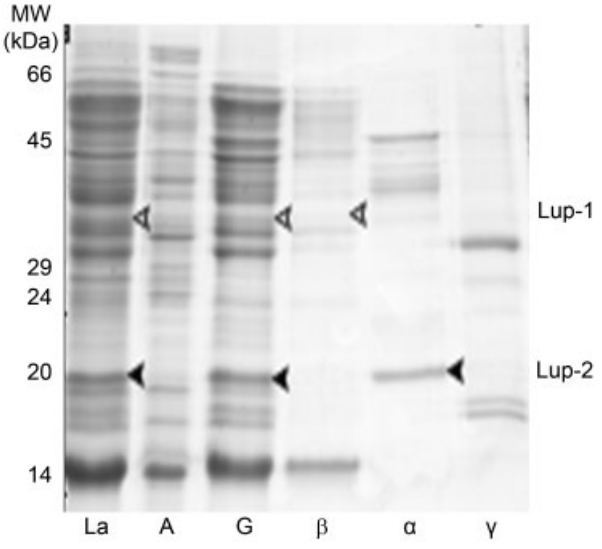

Figure 1. SDS-PAGE patterns of lupin total storage protein (La); albumin fraction (A); globulin fraction (G); conglutin β, conglutin α and conglutin γ from anion-exchange chromatography. Empty and solid arrows indicated the location of 34.5 kDa allergen (Lup-1) and 20 kDa (Lup-2), respectively.

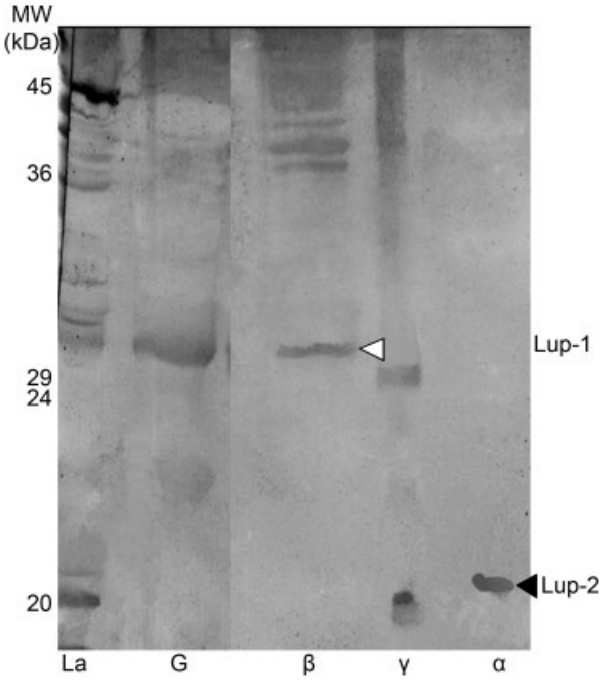

Figure 2. IgE immunoblotting analysis of lupin total storage protein (La); globulin fraction (G); conglutin β, conglutin γ and conglutin α from anion-exchange chromatography using serum pool from four patients allergic to lupin (20 μg protein *per* lane). Empty and solid arrows indicated the location of 34.5 kDa allergen (Lup-1) and 20 kDa (Lup-2), respectively.

3.2 Identification of Lup-1

The major allergen Lup-1 (34.5 kDa) band from conglutin β fraction (Figs. 1 and 2) was tryptic digested in order to carry out the analysis by MALDI-TOF. The study of MALDI-TOF/MS using Mascot allowed to match with vicilin-like protein (61.99 kDa) (gi|89994190) with the largest score (162), conglutin β precursor (62 kDa) (gi|46451223) with a 140 score and BLAD (20.39 kDa) (gi|77994351) with the smallest score (91), all of them belonging to the vicilin fraction from *L. albus* (Table 1).

Figure 3 shows the alignment of amino acid sequences of Lup-1 with vicilin-like protein (gi|89994190) (sequence coverage over 50%) and other legume allergens. To characterize the extent of the homologies and the potential cross-reactivity between Lup-1 and vicilin-like legume allergens, a sequence comparison was undertaken using FASTA [26] (Table 2). With this approach, the amino acid sequence of Lup-1 was found to be highly homologous with that of Lup an 1 from *L. angustifolius* (60% sequence identity and around 70% sequence similarity) and with Pis s 1 from *Pisum sativum* and Len c 1.01 from *Lens culinaris* (40% identity and 70% similarity) (Table 2). The homology was lower (Table 2) with Ara h 1 from *Arachis hypogaea* and Gly m Bd 28 K from *Glycine max*.

Table 1. Mascot search results of Lup-1 from lupin

Identity	Mass (Da)	Score[a)]	Protein identification
gi|89994190	61 994	162	Vicilin-like protein (*L. albus*)
gi|46451223	62 092	140	β-conglutin precursor (*L. albus*)
gi|77994351	20 392	91	BLAD (*L. albus*)

Protein scores greater than 79 are significant ($p < 0.05$).
a) Score: $10 \times \log(p)$, where p is the probability that the observed match is a random event.

3.3 Identification of Lup-2

It was impossible to identify Lup-2 by fingerprinting because the scores were in the greatest statistical uncertainty. Therefore, the IgE immunoreactive 20-kDa band (Lup-2) from conglutin α fraction was subjected to LC/ESI-ion trap MS-dynamic exclusion mode. The quality MS/MS spectra (1106.84, 1521.00 and 1427.00 m/z) of Lup-2 were used to determine its *de novo* sequence (Fig. 4). The three generated sequences were used to find homology with other proteins by database searching (FASTA). The sequences GQL(I/L)VVPQNFVV (Fig. 4B) and PAEVLANAFGLR of Lup-2 (Fig. 4A) showed high homology (100 and 90.9%, respectively) with the 11S basic subunit from lupin (gi|85361412) and seed storage protein (*Lupinus angustifolius*) (gi|224184735), respectively. The third sequence of Lup-2 (Fig. 4C) was found to have a high homology with *Bacillus thuringiensis* (gi|49328240), which could be due to a microbial contamination; however, this sequence was also homologous to legumin K (TNDRAAVSHV) from *P. sativum* (gi|126171) (http://www.ebi.uniprot.org/entry/LEGKPEA). When we compared the three obtained Lup-2 sequences against known allergen sequences corresponding to 11S seed storage globulin (legumin) fraction of other legumes (Table 3), it was observed that the sequence similarity between Ara h 3 from peanut and glycinin G1 from soybean was 100% [10]. The homology results showed that the sequence GQL(I/L)VVPQNFVV of Lup-2 (Fig. 4B) was highly conserved in Ara h 3 (gi|47933675) and subunit G1 of soybean conglycinin (gi|121276) (Fig. 5) [10].

4 Discussion

In our previous study about lupin allergy, we had identified two lupin proteins, Lup-1 (34.5 kDa) and Lup-2 (20 kDa), as major allergens using sera from 23 patients [21]. In the present work, the results of SDS-PAGE, immunoblot and mass fingerprinting analysis coincided in the identification of Lup-1 as a polypeptide of conglutin β fraction.

Lup-1 was matched (Table 1) with a vicilin-like protein from *L. albus* and conglutin β precursor sequenced by Monteiro *et al.* (gi|46451223). This precursor polypeptide

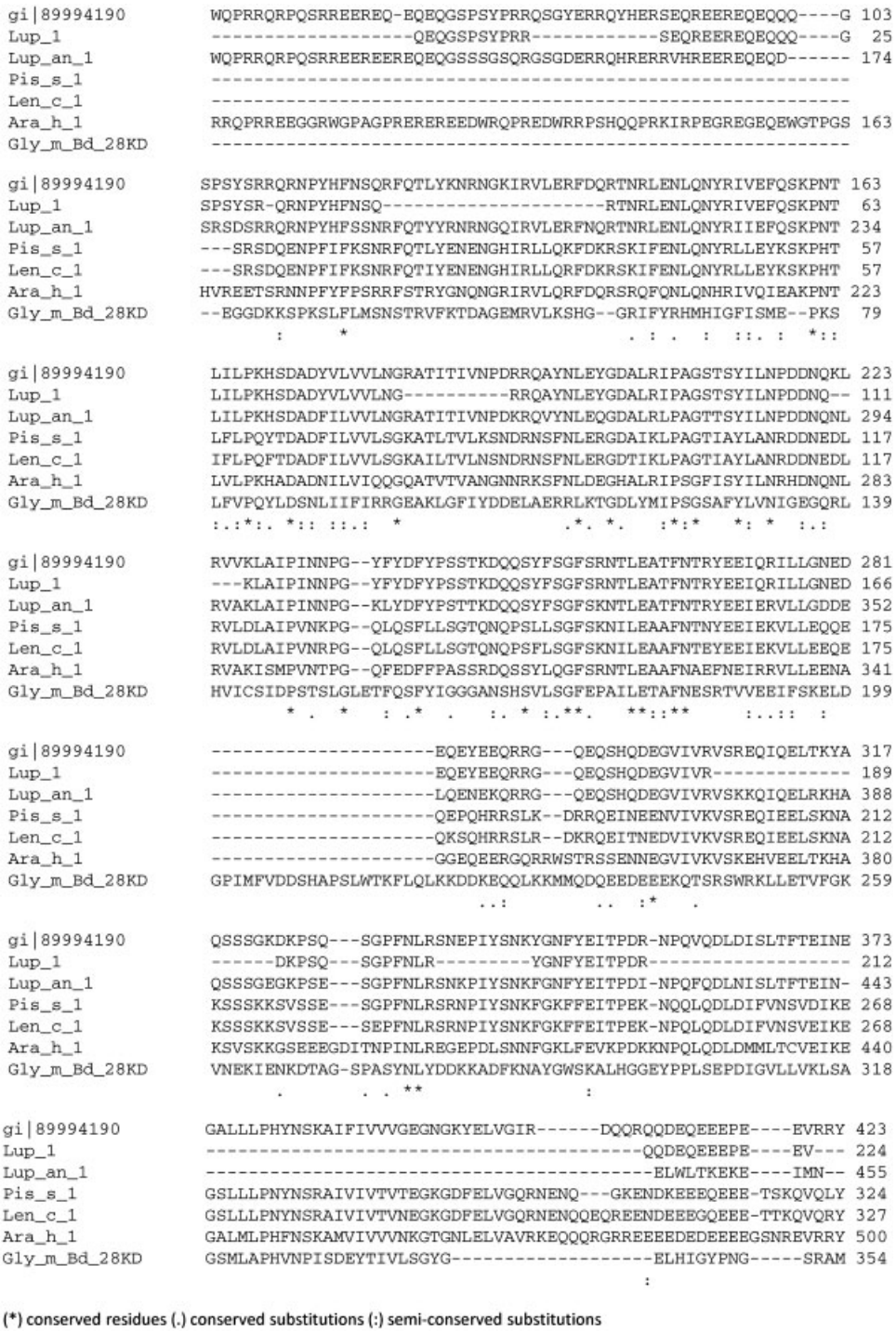

Figure 3. Alignment of amino acid sequences of Lup-1 from *L. albus* against (gi|89994190) from *L. albus*, Lup an 1 from *L. angustifolius* (gi|149208403), Pis s 1 from *P. sativum* (gi|42414626), Len c1 from *L. culinaris* (gi|29539109), Ara h 1 from *A. hypogaea* (gi|1168391) and Gly m Bd 28 K from *G. max* (gi|12697782). (*) conserved residues; (.) conserved substitutions; (:) semi-conserved substitutions.

gives rise to the conglutin β polypeptides with 14–59 kDa including a 34.5-kDa band as a consequence of post-translational proteolysis in the seed. The BLAD protein (20 kDa) is an intermediate product of conglutin β catabolism, with lectin-type activity possibly involved in plant defense [27]. Conglutin β has recently been identified as a major allergen (Lup an 1) in *L. angustifolius* seed by proteomic analysis and was recognized by serum IgE from most of 12 lupin-allergic patients' sera [20]. Several different vicilin-like proteins have been identified as major allergens of some legumes and of other plant-derived foods, including Ara h 1 (*A. hypogaea)* [28], Len c 1 (*L. culinaris*) [29], Gly m Bd 28 K y 60 K (*G. max*) [30, 31], Gly m 5 and 6 [32], Ana o 1 (*Anacardium occidental)* [33], Ses i 3 (*Sesamun indicum*) [34] and Jug r 2 (*Juglans regia)*

Table 2. Amino acid sequence comparision using FASTA database of Lup-1 and the major allergens of some legumes

Identity	Protein identification	% Identity	% Similarity
gi|149208403	Lup an 1 (*Lupinus angustifolius*)	60.1	69.1
gi|42414626	Pis s 1 (*Pisum sativum*)	42.2	70.4
gi|29539109	Len c 1.01 (*Lens culinaris*)	40.2	69.2
gi|1168391	Ara h 1 (*Arachis hypogaea*)	39.2	66.6
gi|12697782	Gly m Bd 28K (*Glycine max*)	26.1	58.8

[35]. The high sequence homology among vicilins from legumes (peanut, pea, lentil, and soybean) is responsible for the IgE cross-reactivity detected between these grain legumes [36–38].

The homologies identified between Lup-1 and legume IgE-binding vicilins from lentil, peanut, soybean and pea (Fig. 3) are consistent with those reported by Wait *et al.* [6], using two-dimensional electrophoresis and MS characterization of *L. albus* protein extracts. Lup-1 was identified as the lupin conglutin β precursor (gi|46451223) and was highly homologous to soy β-conglycinin, in particular with a α′ subunit sequence (gi|9967361), which is considered to be the main agent of the hypocholesterolemic activity of soybean [39].

MS confirmed that the 20-kDa Lup-2 allergen was a peptide of lupin conglutin α. This fact is consistent with the findings of Magni *et al.* [19] who observed that the 20-kDa band, which strongly reacted with serum IgE from lupin-sensitized patients, corresponded to the basic subunit of conglutin α (lupin legumin-like protein). They also demonstrated the cross-reactivity between this protein and other allergens, specifically with Ara h 3 of peanut [10]. The GQL(I/L)VVPQNFVV sequence of Lup-2 described here is highly conserved in lupin, soybean and peanut allergens (glycinin G1 and Ara h 3, respectively) (Fig. 5). Therefore, this peptide could be a candidate cross-reactive IgE epitope of Lup-2 allergen with soybean and peanut, even though it does not coincide with the known epitopes sequenced hitherto described for peanut or soybean; this fact could be due to the use of sera, which probably did not contain cross-reactive allergens of legumes in these studies [40, 41].

Recently, it has been pointed out that the structural homology of the legumin allergens of peanut (Ara h 3) and some tree nuts (Jug r 4 of walnut, Cor a 9 of hazelnut and Ana o 2 of cashew nut) might explain the IgE cross-reactivity sometimes observed between these proteins despite their different botanical origins [42]. The immune-cross-reactivity between lupin and other legumes has been demonstrated [10, 19], although such IgE reactivity may not always be related to clinical cross-reactivity. However, it has been observed in lupin, peanut and pea [7, 9, 11, 12, 15]. This serological cross-reactivity could be due to the high sequence

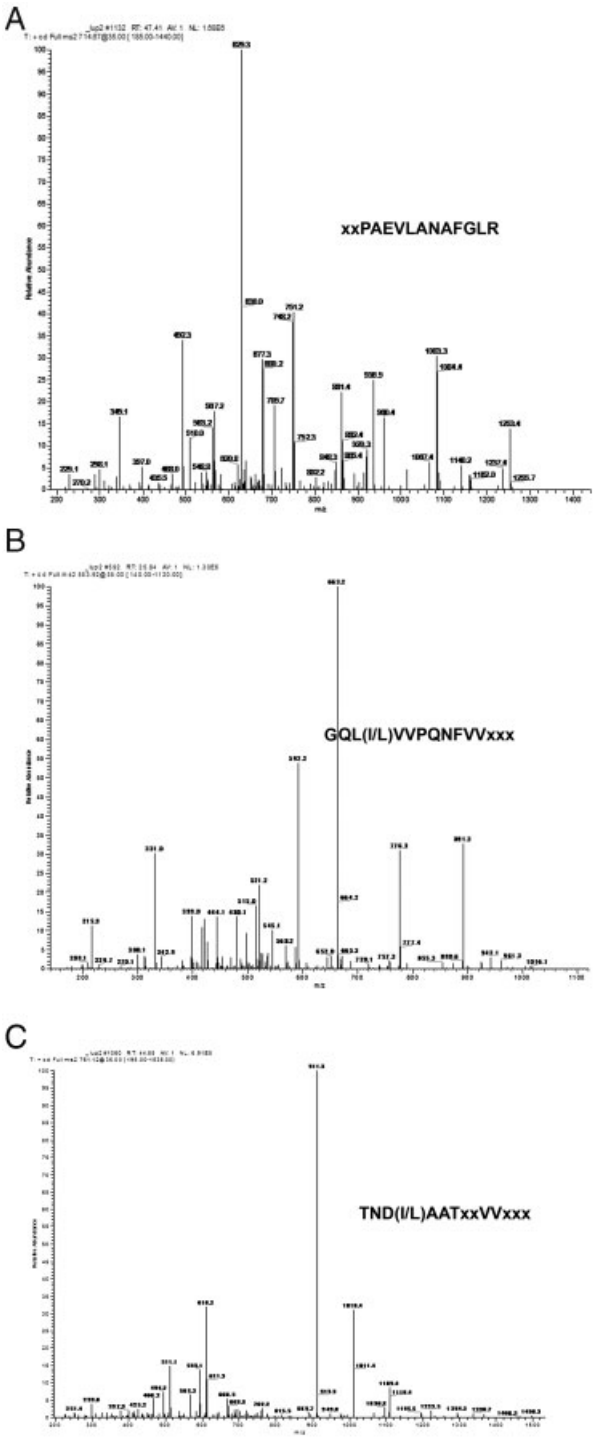

Figure 4. HPLC/ESI-MS/MS spectra of the 1106.84 (A), 1427.00 (B) and 1521.00 (C) *m/z* peptide of Lup-2 and *de novo* amino acid sequences.

homology found among some of their allergens according to Guarneri *et al.* [43] and Barre *et al.* [42].

The Lup-2 allergen, corresponding to basic subunit of conglutin α showed greater heat stability than Lup-1; a harsh

Table 3. Amino acid sequence comparision using FASTA database of Lup-2 and the major allergens of some legumes

Identity	Protein identification	% Identity	% Similarity
gil121276	Glycinin G1 (*Glycine max*)	75.0	100.0
gil47933675	Ara h 3 (*Arachis hypogaea*)	75.0	100.0

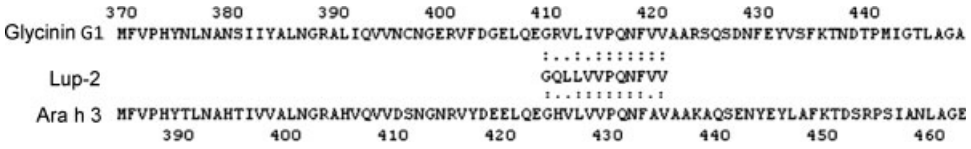

Figure 5. Comparative amino acid sequence alignments of the homologous segments of allergens glycinin G1 (*G. max*) (gil121276), Ara h 3 (*A. hypogaea*) (gil47933675) and Lup-2. (.) Identical residues and (:) similar residues in the three sequences.

autoclave treatment (138°C for 20 min) is required to eliminate Lup-2 [21]. Legumins are composed of one or several acid bands linked by disulphide bonds to one basic subunit. The presence of disulphide bonds in a protein is one of the structural characteristics involved in conferring heat stability, together with the cupin barrel itself [44]. The disulphide bonds in the fractions, which contain Lup-1 (7S globulin) and Lup-2 (11S globulin) could account for the differences on the effect of heating in their allergenic properties. In peanut and soybean, the basic subunit of their legumin is also more immunoreactive than the respective acid subunits [45–47].

In our previous works, the thermal stability of lupin allergens has been demonstrated [21, 22]. It has also been reported on other legume allergens with high sequence homology to Lup-1 (Ara h1, Len c 1 and Pis s 1) and Lup-2 (Ara h3, G1) [48]. In this study, homology between lupin major allergens and other legume allergens support cross-reactivity between them. In a similar fashion, vicilin allergens such as Ara h 1 and Lup-1 also share high homology and thermal stability [49].

Recent studies indicate that *ex vivo* basophile activation test can be a useful tool to assess the reactivity of a raw and roasted allergen in clinically reactive patients [50]. In future research, it would be interesting to evaluate the thermal stability of lupin allergens in lupin-reactive patients. Moreover, inhibition experiments should be performed with sera from patients reactive to lupine and other legumes in order to assess cross-reactivity.

In the present work, two lupin major allergens Lup-1 and Lup-2 have been identified as a polypeptide of conglutin β and basic subunit of conglutin α, respectively. The knowledge of the linear epitopes of lupin major allergens, Lup-1 and Lup-2, is of paramount importance to help identify the causal agents in clinical diagnosis of lupine allergy and to implement specific techniques to identify the presence of lupin flour allergens as ingredients in food in order to protect patients with lupin allergy. It is also important to determine the overall conformation of the IgE-binding epitopes on the molecular surface and study possible IgE-binding cross-reactivity among lupin and other allergens.

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The authors have declared no conflict of interest.

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