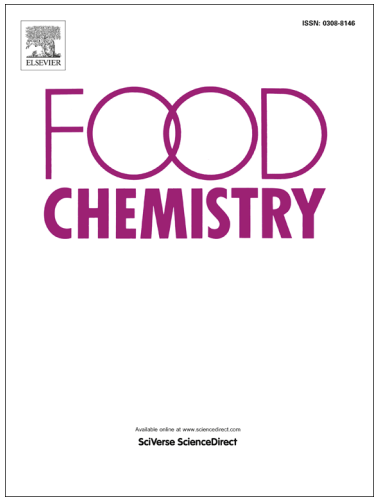

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Characterization of narrow-leaf lupin (*Lupinus angustifolius* L.) recombinant major allergen IgE-binding proteins and the natural β-conglutin counterparts in sweet lupin seed species

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ABSTRACT

β-conglutin has been identified as a major allergen for *Lupinus angustifolius* seeds. The aim of this study was to evaluate the binding of IgE to five recombinant β-conglutin isoforms (rβ) that we overexpressed and purified and to their natural counterparts in different lupin species and cultivars. Western blotting suggested β-conglutins were the main proteins responsible for the IgE reactivity of the lupin species and cultivars. Newly identified polypeptides from "sweet lupin" may constitute a potential new source of primary or cross-reactive sensitization to lupin, particularly to *L. albus* and *L. angustifolius* seed proteins. Several of them exhibited qualitative and quantitative differences in IgE-binding among these species and cultivars, mainly in sera from atopic patients that react to lupin rather than peanut.

IgE-binding was more consistent to recombinant β2 than to any of the other isoforms, making this protein a potential candidate for diagnosis and immunotherapy.

KEYWORDS: Conglutins; Cross-allergenicity; Diagnosis; Food allergy; IgE-binding Activity; Immunotherapy; Seed storage proteins; Sweet lupin; Recombinant allergen; Vicilin.

1. Introduction

Lupin is a popular pulse worldwide. From more than 450 species of the *Lupinus* family, only lupins known as "sweet lupins", such as white lupin (*L. albus*), yellow lupin (*L. luteus*) and blue lupin, also known as narrow-leaf lupin (NLL) (*L. angustifolius*) are being used in food production. NLL flour is used in bakery products and other food to improve the nutritional value, with additional health related benefits of a high percentage of protein, low fat and starch content, reasonable levels of dietary fibre and the absence of gluten (Villarino et al., 2016).

As is the case for all edible legume seeds, the major protein fraction of lupin seeds is storage proteins, comprised of α- (legumin like-protein or 11S globulin) and β- (vicilin like-protein or acid 7S globulin) conglutin as the two major globulin fractions, and γ- (basic 7S globulin), and δ- (2S sulphur-rich albumin) conglutin in lower amounts (Foley et al., 2011; 2015).

Sweet lupin seeds seem to be particularly promising as a source of innovative food ingredients due to a protein content similar to soybean and an adequate composition of essential amino acids (Duranti, 2006). Foods based on sweet lupin proteins, including bakery products, pasta formulations and gluten-free products, are gaining more attention from industry and consumers because of a number of health-promoting benefits that have been ascribed to lupin seed components, e.g. prevention of cardiovascular disease, reduction of blood glucose and cholesterol levels (Arnoldi et al., 2015).

On the other hand, with the rapid introduction of novel foods and new ingredients in traditional foods, the number of people allergic to lupin is also rising. Food allergy is a serious and growing problem in many parts of the world. Food allergy is estimated to affect about 1-3% in the general population and 3–8% among children (Prescott et al., 2013). There is some limited evidence that legume sensitization may be a dynamic process, where symptoms may start with reactions to single legume proteins, with individuals becoming progressively sensitized to other legumes, such as lentil, beans, lupin and pea (Matheu et al., 1999).

In recent years, reports on sensitization or immunoglobulin E (IgE)- mediated allergic reactions to lupin, either as a primary response or as a result of cross-reactivity with other legumes, particularly peanut (Mennini et al., 2016; Ballabio et al., 2013), have increased, and this parallels the increasing number of food applications for this legume. Lupin flour has also been recognized as a cause of occupational airborne allergy (van Kampen et al., 2015), where IgE-mediated allergy was reported after inhalation or contact with lupin flour (Prieto et al., 2010).

The frequency of sensitization and allergic reactions to lupin in the general population is unknown. However, as lupin becomes more prevalent as an alternative protein source for human use, it can be expected that demand for it will increase and more consumers will be exposed to lupin antigens. Serological cross-reactivity of IgE with other legume antigens, particularly those from peanut, has also been reported in both non-occupational and occupational settings (Campbell et al., 2010). Allergic reactions to lupin can be triggered via ingestion of lupin in some peanut-allergic individuals (Peeters et al., 2009), although triggering via ingestion, inhalation and occupational exposure in individuals without peanut allergy has also been reported (Peeters et al., 2007).

In consideration of the increasing number of clinical cases of lupin allergy reported in the literature, in 2008, lupin was added to the list of foods that must be labelled as an allergen in pre-packaged foods as advised by the European Food Safety Authority (EFSA) (http://www.efsa.europa.eu/).

IgE-binding proteins described for lupin seeds ranged from 13 to 108 kDa (Hefle et al., 1994; Moneret-Vautrin et al., 1999; Peeters et al., 2007; Goggin et al., 2008; Foley et al., 2011). Different polypeptides belonging to the main lupin protein families α-conglutins (~ 55kDa), and β-conglutins (~13 to 80kDa), are the most likely involved in the *in vitro* and *in vivo* allergenic responses (Ballabio et al., 2013). Moreover, conglutin β has been identified as a major allergen in *L. angustifolius* (Goggin et al., 2008), as well as in *L. albus* (Guillamon et al., 2010), being designated Lup an 1 and Lup a 1, respectively, by the International Union of Immunological Societies allergen nomenclature subcommittee (http://www.allergen.org/), and included in the allergen database

Allergome (http://www.allergome.org/). In addition, three other proteins have been included in this allergen database for both species, corresponding to α-, γ- and δ-conglutins (http://www.allergome.org/). However, the number and the polypeptide composition of these major allergen proteins is yet to be investigated in other lupin species or in the major cultivars used for food production.

In this study, we present molecular data aiming to identify and compare the protein profiles of β-conglutin, a major group of lupin allergen proteins among lupin species including *L. albus* and *L. angustifolius*. We also aimed to compare the IgE reactivity to β-conglutin polypeptides from different lupin species, as well as to recombinant β-conglutin proteins encoded by different β-conglutin genes in NLL.

2. Materials and methods

2.1. Construction of expression plasmids

β1-, β2-, β3-, β4- and β6-conglutins were over-expressed using the pET28b construct (Novogen, www.novogen.com) containing an N-terminal poly-histidine (6xHis) tag and thrombin cleavage site for removal of the tag when necessary. A pUC57 vector containing conglutin beta genes coding for conglutins with GenBank accession numbers HQ670409 (β1), HQ670410 (β2), HQ670411 (β3), HQ670412 (β4) and HQ670414 (β6), were synthesized to include optimal codon usage for bacterial expression (GenScript). The beta genes, β1, β2, β3, β4 and β6 were cloned into the NcoI/XhoI restriction site of the bacterial expression vector, pET28b and transformed into Rosetta™ 2(DE3) pLysS Singles™ Competent Cells (Novagen).

2.2. β-conglutin protein over-expression

All β-conglutin recombinant proteins were expressed in Rosetta™ 2(DE3) pLysS Singles™ Cells (Novogen). Protein expression was performed using an auto-induction method (Studier, 2005) with small modifications. Briefly, a colony of *E. coli* containing the expression construct was isolated

and grown for 20h in ZY-medium plus kanamycin at 50 µg/ml at 37 ºC and continuous shaking (200 rpm). The culture was diluted 1:150 in ZYM-5055 medium (tryptone 1%, yeast extract 0.5%, Na_2HPO_4 25mM, KH_2PO_4 25mM, NH_4Cl 50mM, Na_2SO_4 5mM, glycerol 0.5%, glucose 0.05%, α-lactose 0.2%, $MgSO_4$ 2mM) and grown for a further 5h before subsequently inducing over-expression of the proteins by adjusting the temperature to 19 °C for another 20 h. Cells were collected by centrifugation at 5000 x g at 4 ºC. The bacterial cell pellet was rinsed twice with phosphate buffered saline (PBS), pH 7.5, the supernatant was removed and the cell pellet was flash frozen using liquid nitrogen. The bacterial cell pellet was stored at -80 ºC until further use.

2.3. Purification of recombinant β-conglutin proteins

Protein purification from bacterial pellets was performed following the manufacturers' recommendations for His-tagged proteins (Qiagen).

Briefly, the first step was the lysis of the bacterial cells followed by nickel affinity chromatography using Ni-NTA spin columns (Qiagen) that interact with the histidine (6xHis) tags at the C-terminus of the recombinant β-conglutin proteins, since the interaction between Ni-NTA and the 6xHis tag of the recombinant proteins does not depend on tertiary structure. After elution of 6xHis-tagged proteins from the column with an increasing imidazole concentration gradient (10 to 300 mM), 2.5 ml fractions were collected.

Fractions containing protein were analyzed using SDS-PAGE and fractions showing a single band corresponding to the expected molecular weight were pooled, and dialyzed 5 times against Tris-HCl 100 mM, pH 7.5, 150 mM NaCl to eliminate the imidazole reagent. The protein was concentrated using a 30 kDa Amicon centrifuge filter (Millipore, www.emdmillipore.com). The aliquots were flash-frozen in liquid nitrogen and kept at -80°C until further use.

Protein purity was >95% as determined by densitometry analysis of the SDS-PAGE gel image. An aliquot of each protein was used to measure their concentration using Bradford assays (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as a standard. The β-conglutin purification yields ranged between 2-7 mg/ml.

2.4. Patients

Twenty seven patients with positive skin prick test (SPT) and three with negative SPT were utilised in this study. Thirteen patients had known clinical reactivity to lupin and exhibit specific serum IgE to lupin (class 2 to 5; ImmunoCAP, Phadia, Uppsala, Sweden). Of these, nine patients reacted to lupin but not peanut and were recruited from workplaces involved in lupin research or in processing lupin flour or had presented to medical clinics with allergic reactions (Goggin et al., 2008). Five patients were peanut allergic individuals who had a positive food challenge to lupin and their sera were collected at the Allergy Unit at the Royal Prince Alfred Hospital, Sydney, Australia. Another fourteen exhibited specific serum IgE to peanut (class 2 to 4) but their reactivity to lupin was not known. They were recruited at the Allergy Unit, "San Cecilio" University Hospital, Granada, Spain. ImmunoCAP class ≥1 was interpreted as positive (Ballabio et al., 2013).

The patients´ sera are referred to as S1 – S30, and their clinical characteristics and specific serum IgE values are given in Supplementary Table S1. These diagnoses were based on anamnesis, positive SPTs and high IgE levels. Informed consent was obtained from all patients before they were included in the study, which was approved by the Committee for Human Medical Research Ethics, Sydney University, Australia; and the Research Ethics Committee of the Granada province, Andalusia Health System (Spain).

2.5. Extraction of globulin protein enriched fractions from lupin seeds

Mature seeds from *L. angustifolius* (cv. Tanjil and Unicrop), *L. angustifolius* accession number P27255 (cv. wild-type), *L. albus* (cv. Andromeda and Kiev), *L. mutabilis* (cv. ID30) and *L. luteus* (cv. Pootalong) were used for protein extraction.

Proteins were extracted from 2 g of mature seeds from each lupin variety. The seeds were ground to fine powder, which was resuspended in 70 ml of n-pentane (Sigma) to remove lipids by vigorous stirring for 2 h at RT. Defatted flour from the previous step, was centrifuged at 10000 x g for 15 min at RT, and the pellet was dried for 80 h after decantation of the supernatant.

The albumin protein fraction was extracted by re-suspension of the defatted dried pellet in an aqueous solution containing 20 mM $MgCl_2$, 10 mM KCl and 20 mM $CaCl_2$, in a ratio of 40 mg/ml (w/v), stirring the solution for 1.5 h at RT and keeping the pH at 8.5-9. The pellet containing the globulin fraction was recovered by centrifugation at 30000 x g for 30 min at 4ºC. The previous two steps were repeated for a second time. The pellet was resuspended in a solution containing 100 mM Tris-HCl pH 7.5, 20 % NaCl, 20 mM EDTA, 20 mM EGTA, in a ratio of 40 mg/ml (w/v), plus PIC 1:200 (v/v) and 0.5 mM PMSF and the solution was stirred for 1h at RT. The solution containing the globulin fraction (supernatant) was recovered by centrifugation at 30000 x g for 30 min at 4ºC. The supernatant was dialyzed three times for 24 h at 4 ºC in PBS pH 7.5 plus PIC 1:200 (v/v) and 0.5 mM PMSF. Samples were mixed with Laemmli buffer, boiled at 95 ºC for 5 min and stored at -80 ºC.

2.6. SDS-PAGE protein separation, immunoblot and quantitative analysis of protein bands

Total protein (10 µg per sample) was separated by SDS-PAGE on 4–20% Mini-PROTEAN® TGX™ precast Gels (Bio-Rad) using the Mini-PROTEAN® Tetra Cell apparatus (Bio-Rad). After electrophoresis, proteins were stained with Coomassie Brilliant Blue according to standard procedure. Proteins were electroblotted onto a PVDF membrane using a Mini-Trans-Blot Electrophoretic Transfer Cell system (Bio-Rad). The membranes were blocked for 2 h in blocking solution containing 5 % (w/v) non-fat dry milk in Tris-buffered saline (TBS) buffer, pH 7.4. Immunodetection of β-conglutin proteins was carried out by incubation with rabbit polyclonal antiserum developed in this study (see below), diluted 1:1000 in TBS buffer containing 5 % (w/v) non-fat dry milk and 0.5% Tween-20. A horseradish peroxidase (HRP)-conjugated anti-rabbit IgG (Bio-Rad) served as the secondary antibody, and was diluted 1:3000 in TBS buffer and 0.5% Tween-20, and then incubated for 2 h, followed by three washing steps of 15 min each with TBS containing 0.5% Tween-20. The chemiluminescence signal was detected in X-ray films (Kodak) after incubation of the membrane with SuperSignal® West Pico Chemiluminescent substrate (Thermo Scientific).

IgE reactivity was assayed following the same steps, using sera from thirteen lupin and fourteen peanut allergic patients' sera, diluted 1:10 and 1:20, respectively, in blocking solution. A goat anti-human IgE-HRP conjugate was used as the secondary antibody. Chemiluminescent detection of antibody binding used the Immun-StarTM WesternC^{TM} Chemiluminescence Kit (Bio-Rad) according to the manufacturer´s instructions, and was visualized using the LI-COR C-DiGit Chemiluminescence Western Blot Scanner.

Quantitative (densitometry) analysis of protein bands from recombinant or natural β-conglutin proteins or those that showed IgE reactivity with patients' sera was performed using ImageJ v1.47 (http://imagej.nih.gov/ij/). A t-test was used to compare the means of recombinant protein quantification scores.

The MW of each polypeptide recognized by anti-β–conglutin antibody was calculated based on the migration distances of the corresponding bands on SDS-polyacrylamide gels. The image was analyzed to obtain the relative migration distance (R_f) values for each band, were R_f is defined as the migration distance of the protein through the gel divided by the migration distance of the dye front. A standard curve was constructed based on the values obtained for proteins of known MW and used to determine the MW of unknown bands. $MW = 10^y$; $y = bx+a$; $x = R_f$ in the standard curve.

2.7. Production of a polyclonal antiserum against β-conglutin proteins

Synthesis of a synthetic peptide used in the development and production of the polyclonal serum against β-conglutin proteins was done by Agrisera (Sweden), resulting in 25 mg of this peptide (purity >95%) with the following sequence: VDEGEGNYELVGIR. This sequence matches 100% to the NLL β-conglutin gene sequences deposited in the GenBank database (accession numbers HQ670409 to HQ670412, HQ670414). The synthetic peptide was conjugated to a KHL carrier. Sera were taken at different times prior to or following immunization from several rabbits and were tested in immunoblot experiments. The final polyclonal serum was purified by affinity chromatography and IgG quantification was by ELISA.

2.8. Statistical analysis

The immunoblot quantitative results were obtained by densitometry analysis using ImageJ software v. 1.38e (http://imagej.nih.gov/ij/). The data were statistically evaluated using Duncan's multiple range tests after one-way ANOVA using Stat Graphics statistical software (http://www.statgraphics.com/). Statistically significant differences were considered at the $p<0.05$ level.

3. Results

3.1. Characterization of IgE-binding to recombinant β-conglutin proteins from NLL

In order to investigate IgE binding to NLL recombinant β proteins, five recombinant beta-conglutin (rβ) isoforms were expressed using the pET28 vector in *E. coli* Rosetta™ 2(DE3) pLysS Singles™ Competent Cells and purified by affinity chromatography (purity > 95%) (Fig. 1). After SDS-PAGE, the recombinant proteins showed an apparent molecular mass of ~70 kDa (Fig. 1a). To further confirm the identity of these five proteins as rβ (Fig. 1b), immunoblotting was performed using an antibody developed against a synthetic peptide (Method section 2.7) with a sequence that is conserved in all these rβ proteins. Comparable densitometry results were obtained when chemiluminescence results were analyzed using ImageJ software (Method section) (Fig. 1b), thus equal loading of each protein in the SDS-PAGE and immunoblot replicates was confirmed. Comparable amounts of recombinant protein of each form were tested for their capacity to bind IgE from 13 sera from patients with known reactivity to lupin [9 not reactive to peanut and 4 reactive to peanut and lupin (see methods)], plus a control non-allergic patient serum (Supplementary Table S1) in immunoblot experiments (Fig. 2). No IgE-binding could be observed in control samples (BSA) (data not shown).

Marked differences were observed in the ability of each rβ form to bind IgE from the different patients' sera. Five patient's sera (S1, S3, S6, S8, S9) recognized every rβ form (densitometry value ≥ 5 units), but the intensity of the resulting signal varied for the different rβ

forms. Sera 8 and 12 displayed the weakest IgE binding (Fig. 2), with S4 and S5 reacting to rβ2 and 4, while S11 exhibited very low but equal reactivity to the five beta isoforms. Four patients' sera (S2, S3, S6, S8) exhibited strong IgE-binding to rβ1 (densitometry value ≥ 15 units); S2, S3, S5, S6, S7 and S8 to S9 to rβ2; patients S1, S3 and S9 to rβ3; patients S1 to S8, and S9 to rβ4; and S1, S3 and S9 had strong IgE-binding to rβ6 (Fig. 2).

Statistical analysis was performed in order to establish differences between these five rβ isoforms based on the differential reactivity of these sixteen patients' sera. We performed one-way ANOVA followed by Duncan's multiple range test to show statistically significant differences of reactivity between rβ4 and rβ1, rβ3 and rβ6; and rβ2 and rβ6.

We also investigated the binding of IgE from sera of fourteen patients diagnosed with peanut allergy to NLL recombinant β proteins for which lupin reactivity had not been determined. As shown in Figure 3 differences in the ability of each rβ form to bind IgE were observed. Four patient's sera (S17, S18, S25 and S28) recognized every rβ form (densitometry value ≥ 5 units). However, IgE in sera S16 to S19 exhibited the highest intensity binding (at least 3 values ≥ 30 units), and these were also the sera showing the highest intensity IgE-binding or reactivity. On the other hand, sera S20, S24, S26 and S29 displayed the weakest IgE binding (Fig. 3), and IgE from serum 9 reacted only to rβ4. IgE from eight out of fourteen patients' sera (S16 to S19, S21, S23, S27 and S28) exhibited strong IgE-binding to rβ1 (at least 3 values ≥ 30 units); IgE from sera S16 to S19, S22, S23 and S28 to rβ2; from S21 to S23, S25, S27 and S28 to rβ3; IgE from all patients' sera but S20 and S22 bound to rβ4; and IgE from S16 to S20, S25 and S28 had strong IgE-binding to rβ6 (Fig. 3).

Duncan's multiple range test performed after one-way ANOVA showed statistically significant differences of reactivity only between rβ3 vs. rβ4.

3.2. Analysis of protein extracts from various lupin species using SDS-PAGE and immunoblotting

In order to examine the protein species profiles and differences in the protein composition of extracts from lupin species (and cultivars) used in this study [*L. angustifolius* (cv. Tanjil, and

Unicrop), *L. angustifolius* accession number P27255 (cv. wild-type), *L. albus* (cv. Andromeda and Kiev), *L. mutabilis* (cv. ID30), and *L. luteus* (cv. Pootalong)], total protein was extracted from mature seeds, SDS-PAGE was performed and the proteins separated and visualized with Coomassie Brilliant Blue staining (Fig. 4).

Overall, in all lupin protein extracts, proteins with molecular weights from ~15 to 75 kDa were present in different ratios depending on the lupin variety (Fig. 4a). No significant amount of protein was detected above 100 kDa. Five groups of protein, based on the abundance of polypeptides separated by SDS-PAGE, were identified: 75k Da and above, 50 to 75 kDa, 30 to 50 kDa, 17 to 25 kDa, and below 17 kDa.

Considerably less protein between 50 and 75 kDa was observed in *L. albus* cvs. Andromeda and Kiev (lanes 1 and 3, Fig. 4), and *L. angustifolius* cv. Tanjil (lane 6) and Unicrop (lane 7), compared to protein extracts from *L. mutabilis* or *L. luteus*. Interestingly, *L. angustifolius* cultivars Tanjil (lane 6) and Unicrop (lane 7) displayed a large difference in polypeptide content in this size range when compared to wild-type (P27255, lane 5). These proteins are in the size range expected for unprocessed β-conglutin polypeptides (mature protein before proteolytic processing that occurs as seeds mature) [28] from *L. angustifolius*, particularly those for which we have focused the over-expression and purification in the present study (GenBank accession numbers HQ670409 to HQ670412, and HQ670414; theoretical MW range (68 – 75kDa). Unprocessed α-conglutin protein (a major allergen in the genus *Lupinus*) in *L. angustifolius* is also in the same MW range (~64, ~67 and ~74kDa) as the β-conglutin proteins but this is also likely to be proteolytically processed in mature seed (Magni et al., 2007).

Major differences in both the number of polypeptides (protein profile) and their abundance (intensity of protein bands) were observed in the 30 to 50 kDa mass range, with up to 14 polypeptides observed. When comparing different cultivars, particular differences were displayed when *L. albus* cv Andromeda was compared to *L. albus* cv Kiev, as well as between species, such

as *L. mutabilis* and *L. luteus*. No appreciable differences were observed for the three *L. angustifolius* cultivars (Fig. 4a).

A large variability in the number and intensity of protein bands in the MW range from 17 to 25 kDa was observed in *L. mutabilis* compared to *L. luteus*. On the other hand, comparable protein profiles were shown when comparisons were made between cultivars of the species *L. angustifolius* or *L. albus*.

In the 10 to 17 kDa size range, the protein profiles were comparable among all the lupin cultivars analyzed. In this size range polypeptides corresponding to α-, β- and δ- (~17.5 and ~10.5 kDa) conglutin proteins are also likely to be present. A higher intensity of bands was noticeable in *L. luteus*, compared to the other lupin species. Below 10 kDa there was a prominent protein band in *L. luteus* when it was compared to the rest of the species analyzed (Fig. 4a).

We identified polypeptide bands that corresponded to β-conglutin by probing blots with an antibody developed against a synthetic peptide, with a sequence that is conserved in all seven β-conglutins from *L. angustifolius*. The antibody is likely to have specific binding to β-conglutins as no reactive bands were detected using pre-immune serum as a control (data not shown). Since there is proteolytic processing of β-conglutin precursors in seeds to produce the mature β-conglutin polypeptides (Duranti et al., 1992), this helped to identify the range of MWs where mature β-conglutin proteins were present in the seed protein extracts from these lupins, as well as which β-conglutins from other lupins shared a similar peptide to that used for producing the antibodies (Fig. 4b). It should be noted that some β-conglutin polypeptides may not bind the antibody as the region containing the peptide to which it was raised may be cleaved as the seed storage proteins mature.

At least 25 different reactive polypeptide bands were identified among the different lupin species and cultivars (Supplementary Table S2), some which were of a similar size in the different lupin species. Specifically, we identified six protein bands that bound the antiserum between 50 and 75 kDa, seven bands between 37 and 49 kDa, seven bands between 25 to 36 kDa, two bands

between 20 and 24 kDa, and three bands between 15 and 19 kDa (Supplementary Table S2, Fig. 4b).

L. albus cv. Andromeda and Kiev (white Lupin) (Fig. 4b, lanes 1 and 3) protein extracts showed differences in reactivity in two bands above 50 kDa, both of which were missing in Kiev, while the intensity of a reactive band at 17 kDa (Fig. 4b, lines 1 and 3) was stronger in Andromeda. Species such as *L. mutabilis* cv. ID30, *L. luteus* cv. Pootalong (yellow lupin) and *L. angustifolius* cv P2527S (wild-type) exhibited the strongest reactivity for a group of β-conglutins ranging in size from 50 to 75 kDa (Fig. 4b, lanes 2, 4 and 5). *L. albus* cv. Andromeda and Kiev displayed similar reactivity for β-conglutins between 33 and 43 kDa (Fig. 4b, lanes 1 and 3), and similar sized polypeptides were present at reduced levels in *L. luteus* cv Pootalong (Fig. 4b, lane 4). Similarly, *L. mutabilis* cv. ID30 differs in the pattern of reactive bands between 26 and 33 kDa compared to the *L. angustifolius* varieties (lanes 2, 5, 6 and 7, respectively). Finally, and with the exception of *L. luteus*, all other lupin species exhibited a prominent reactive band at 17 kDa.

3.3. Characterization of IgE-binding to natural β-conglutin proteins among lupin species

To investigate the IgE binding to natural proteins from lupin species and cultivar extracts, we used ten sera from patients diagnosed with allergy to lupin, and five sera from patients diagnosed with allergy to peanut and a positive food challenge to lupin. Both sets of sera showed IgE binding in immunoblotting experiments using lupin protein extracts enriched in conglutin proteins, although those from the peanut allergic individuals generally showed weaker binding. Identification of possible allergenic β-conglutin proteins (IgE-reactive bands) with the patient's sera was assessed by comparison of protein profiles in Fig. 5 with reactive bands recognized by anti-β-conglutin antiserum (Fig. 4b, Supplementary Table S2).

IgE from S4 to S6, and S10 displayed clear reactivity to β-conglutin proteins in the range of 75 to 50 kDa (68.1, 62.9 and 51.9), particularly for cultivars in lanes 4 to 6; S2, S4, S6 and S10 in the range 49 to 25 kDa (49.3, 37 and 28.1); and S2, S4 to S6, and S10 below 25 kDa (21.7 and 20.6), particularly for cultivars in lanes 4 and 6 (Fig. 5). However for some sera (S3 to S6), the IgE

seems to be directed more towards the *L. angustifolius* and *L. luteus* β-conglutins than to those of other species. In contrast the IgE from S1 and S9 have similar reactivity to β-conglutins from all species and cultivars.

S2 shows a different pattern of IgE binding to most of the other sera tested (Fig 5). Although there was binding to some larger proteins, these do not appear to match the proteins recognized by the anti-β-conglutin antibody. In particular there is strong binding to proteins of 21.7 and 28.1 kDa in most species and cultivars. These do not bind IgE in *L. mutabilis*. The 21.7 kDa protein is also recognized by IgE from S10 and S12, and the 28.1 kDa in Unicrop with weaker IgE-binding.

IgE from S14 shows binding to only a small number of proteins, which are not present in all species (Fig 5).

To identify characteristic polypeptides binding to IgE from patients with diagnosed allergy to peanut, we screened immunoblots of lupin proteins from different species and/or cultivars with five sera. Figure 6 showed a general pattern of bands that is comparable for the five sera with only a small number of bands differing between blots (summarized in Supplementary Table S2). A clean blot was obtained with a non-allergic serum (Fig. 6). Some of the proteins recognized by the IgE seem to correlate with those bound by the anti-β-conglutin antibody. In addition the 21.7 and 28.1 kDa proteins recognized by S2 are also identified in both *L. albus* cultivars and *L. angustifolius* cultivars Tanjil and Unicrop. The most intense binding of IgE is seen for proteins in *L. albus* cultivars and *L. luteus*. IgE binding to proteins in *L. angustifolius* is much weaker.

4. Discussion

Several lupin seed proteins are potential allergens, and β-conglutin proteins from *L. angustifolius* and *L. albus* have been included as allergens in the allergome (http://www.allergome.org/). Characterization of β-conglutins is complicated by the fact that i) the different β-conglutin protein isoforms show more structural variation compared to other lupin seed storage proteins and when compared to other vicilin-like proteins, despite the limited amino acid sequence polymorphism

(74% the highest), this may increase the number and potential types of epitopes (Jimenez-Lopez et al., 2015; Lima-Cabello et al., 2016); ii) it has a complex polypeptide composition, with over 25 polypeptide chains with no disulphide bridges, covering a broad range of molecular masses (Mr 15–75 kDa) (Hefle et al., 1994; Moneret-Vautrin et al., 1999; Peeters et al., 2007; Foley et al., 2011) that result from proteolytic cleavage of multiple β-conglutin precursor polypeptides (Foley et al., 2011; Duranti et al., 1992); and iii) the putative combination of different subunits that define the quaternary (multimeric) structure of these conglutins in different *Lupinus* cultivars and species (Wait et al., 2005; Magni et al., 2007).

The use of high purity recombinant proteins in immunological test systems for diagnosis of allergy is of great utility, although purification processes are difficult and time-consuming, particularly for β-conglutins. This family of proteins is difficult to purify from natural extracts, as isolated proteins are often contaminated with other globulins, and separation methods cannot isolate all members of individual families or individual isoforms, e.g. β-conglutins (Nadal et al., 2011). Since β-conglutin protein isoforms show relatively limited polymorphism (74 – 99% identical) a limited number of lupin-specific IgE-binding epitopes may be involved in the differential recognition of these isoforms (Duranti et al., 1992).

There is no general consensus about the identity and protein composition of lupin allergens, and the variability increases depending on the varieties analyzed (current study). We performed immunoblots with an anti-β-conglutin antibody (IgG), and IgE from lupin- and/or peanut allergic patients. The variability in lupin seed proteins was reflected in the results obtained in these experiments, where there were large differences in the number of polypeptides of wide ranging MW recognized by human IgE (Wait et al., 2005; Magni et al., 2007) in the different lupin species and/or cultivars.

The largest differences between cultivars were found between P25275 (Wild-type) compared to cv. Tanjil and Unicrop in *L. angustifolius*, particularly in the protein group around 75 to 50 kDa (current study). These bands were likely to represent β-conglutins given their strength of

reactivity with the anti-β-conglutin antiserum used in this study (Fig. 4-6). Since most of the proteins recognized as β-conglutin are derived from processing of precursor proteins after translation, the differences in size of the processed proteins suggests there are differences in the way the precursors are processed in the different lupin species.

When analyzing the different lupin species and cultivars we identified up to 25 different polypeptides in the range of 75 to 15 kDa that correspond to β-conglutin reactive bands (Fig. 4, Fig. 5, Fig. 6 and Supplementary Table S2). Many of these proteins are recognised by IgE, confirming β-conglutin as a potential allergen in all the species analysed (*L. albus*, *L. mutabilis*, *L. luteus* and *L. angustifolius*) (Ballabio et al., 2013; Goggin et al., 2008; Bublin and Breiteneder, 2014; current study). Our results show IgE binding to β-conglutin proteins of comparable size to those identified as binding IgE in a number of other studies (Supplementary Table S3), e.g. reactive bands at 71 kDa (Parisot et al., 2001), 59kDa (Parisot et al., 2001; Brennecke et al., 2007; Lindvik et al., 2008; Quaresma et al., 2007; Ayşenur et al., 2012; Ballabio et al., 2013), 50kDa (Wüthrich B. 2008), 38kDa (Moneret-Vautrin et al., 1999; Lindvik et al., 2008; Quaresma et al., 2007), or 34 kDa (Hefle et al., 1994; Moneret-Vautrin et al., 1999; Parisot et al., 2001; Quaresma et al., 2007) found in *L. albus*.

In addition to the polypeptides commonly recognized in the current and previous studies (Supplementary Table S3), we can highlight many other β–conglutin polypeptides not previously described (compare Supplementary Table S2 and S3) as potential allergens in all species of the sweet lupin group. Proteomic analysis to pinpoint the particular region of the proteins conserved in the IgE-binding polypeptides may help explain this complexity by identifying polypeptides that differ in size but include common amino acid sequences in the different lupin species.

Lupin allergy may arise by cross-reactivity in people who are already allergic to another member of the legume family, in particular peanut (Hefle et al., 1994; Moneret-Vautrin et al., 1999) or may also provoke adverse effects by primary sensitization (Hefle et al., 1994; Moneret-Vautrin et al., 1999; Goggin et al., 2008; Parisot et al., 2001; Peeters et al., 2007). The results suggested that

for sensitization in this small group, β-conglutin or Lup an 1, was the major cross-reactive lupin allergen. Hefle et al (1994) reported that the IgE-binding proteins of a lupine extract appeared to have approximate molecular weights of 21 kDa and 35 to 55 kDa. Moneret-Vautrin et al (1999) showed that the most IgE-reactive protein in lupin flour had a molecular weight of 43 kDa for their patients with peanut allergy and also identified bands at 13, 38 and 65 kDa that were not cross-reactive with peanut. Another study with serum of a patient without peanut allergy but with allergic symptoms to airborne lupin flour showed binding to proteins with molecular weights of 34, 59 and 71 kDa as the most prominent IgE-binding proteins, with minor bands of 17 and 24 kDa (Parisot et al., 2001). Our data showed that most of the patients with peanut allergy and positive food challenge to lupin showed weak IgE binding to protein bands of lupin. One of the lupin and peanut allergic individuals and all the peanut allergic patients we tested who were sensitized to lupin but with unknown clinical reactivity showed IgE binding at MW ranges below 15 kDa and between 37-50 kDa for all species but *L. albus*; and in between 51-75 kDa for *L albus* and *L angustifolius* (except for P27255). Some of the polypeptides identified in different species/cultivars in this work may have epitopes that are responsible for cross-reactivity between patients sensitized to different lupin and/or other legumes such as peanut. Most of the studies so far on cross-reactivity between peanut and lupin allergens rely on small numbers of peanut allergic individuals, often with no information on whether they are clinically reactive to lupin. To address the question of cross-reactivity properly it is important that some larger studies are attempted that identify groups of individuals allergic to peanut or lupin and to seed proteins from both legumes. It may then be possible to identify specific polypeptides and/or specific epitopes recognized by the different groups. As a first approach to that question, computational biology based approaches (differential algorisms) made it possible to predict several specific and commonly-shared potential epitopes (Jimenez-Lopez et al., 2015).

So far, an effective specific immunotherapy against food allergens is still in the infancy. The main approach to food allergy has been avoidance of food, application of symptomatic medication

or desensitization strategies resulting in increased tolerance to the respective food (Hamad and Burks, 2017). Comparative analyses of recombinant food allergens and their natural counterparts may offer new insights into the structural basis of allergenicity. As possible sources of desensitization in food allergy therapy, food allergens have to be over-expressed to be used as recombinant proteins (Nowak-Węgrzyn and Sampson, 2011).

Allergen specific immunotherapy (ASIT) is the only known causative treatment of allergic diseases. The prerequisites to be able to use recombinant allergens for this are i) to identify possible allergens (particularly the main families or groups of allergens) of a food and ii) having them available in recombinant form, something made easier by genome sequencing projects, such as the one for *L. angustifolius* L. (Kamphuis et al., 2015; http://www.lupinexpres.org) , and iii) the recombinant peptides are active and have the same IgE-binding capacity as the natural analogue (Assenberg et al., 2013). In our study, we were able to express and purify five β-conglutins proteins from *L. angustifolius* (purity >95%) (Fig. 1), with theoretical MWs of 71.9, 70.7, 70.1, 68.2 and 64.4 kDa (Uniprot database, http://www.uniprot.org/), which are the longest currently known sequences of β-conglutins from *L. angustifolius* (Kamphuis et al., 2015; http://www.lupinexpres.org). The successful purification of these recombinant β-conglutin proteins allowed their IgE-binding capacities to be compared to the natural proteins from *L. angustifolius*, as well as β–conglutin proteins from different lupin varieties (Assenberg et al., 2013). In this study, we have found that among all the recombinant β-conglutins, rβ2 is the recombinant protein that most consistently binds IgE from patients' sera. This is a desirable feature if rβ2 was used as a molecular tool in diagnosis and "desensitization" therapy for patients suffering lupin food allergy. Moreover, IgE from most sera of patients with known lupin allergy that we tested (92 %) recognized rβ2, as did IgE from 71 % of lupin sensitized peanut allergic individuals. Almost all these individuals also had IgE that recognized proteins in the MW range of 60-75 kDa that in *L. angustifolius* (and other sweet lupins) would correspond to unprocessed conglutin β. These are desirable features for recombinant protein implementation in diagnosis and therapy (Jeong et al., 2011).

Pure recombinant food allergens could be used as reference material to standardize extracts for *in vitro* uses, i.e. ELISA, and *in vivo* skin tests (Smoldovskaya et al., 2016). Diagnostic results obtained with standardized preparations would be reproducible and more precise, and tests could be carried out with a panel of recombinant food allergens that represent major allergenic components of a food, which would be the case for rβ polypeptides in lupin enriched food, since conglutins constitute 55% of the seed total proteins (Valenta et al., 2015). In addition, assays using single recombinant allergens will make it possible to compile a patient's individual sensitization pattern, whereas with the use of protein extracts, only sensitization to the whole food is determined (Smoldovskaya et al., 2016). Thus, knowledge of the sensitization patterns to recombinant proteins will make it possible to generate prognosis statements, as well as preventive recommendations.

Recombinant allergens provide new opportunities to refine the diagnostic procedures for IgE mediated allergy. In addition to pointing out the offending allergen source, it is now also possible to identify the actual protein components to which patient-IgE is directed. Thus, *in vitro* tests based on recombinant allergens are useful tools to collect information on symptom triggers at the molecular level. Recombinant *in vitro* tests make it possible to study more complicated phenomena, such as geographic differences in clinical reactivity and cross-reactions towards seemingly distant allergens. Overall, in light of the positive health promoting properties of lupins (Arnoldi et al., 2015; Lima-Cabello et al., 2017), it will be essential to get a better understanding of the allergenicity of its individual proteins and more studies are needed to establish the prevalence of allergic reactions to lupin species and cultivars in different allergic populations.

5. Conclusion

Using sera from lupin and peanut atopic patients to study recombinant purified β-conglutin proteins and their natural counterparts among “sweet lupin” species and cultivars allowed the identification of new polypeptides exhibiting clear qualitative and quantitative differences in IgE-binding among these species and cultivars. These IgE-reactive polypeptides may have the potential to constitute a

new source of primary or cross-reactive sensitization to lupin. In addition, recombinant β-conglutins were demonstrated to be an excellent tool to examine the IgE-binding capacity in comparison to the natural proteins from the "sweet lupin" group. In this regard, we found that rβ2 is the protein with the most consistent IgE-binding, which is an important and essential feature for proteins to be used in diagnosis and immune therapies to food allergies.

The results of this work underline the importance of studying the different lupin species used in plant-derived food for a full-characterization of the large number of peptides involved in molecular allergy. Further studies should be conducted to better understand whether the IgE binding of these proteins is based on shared or specific epitopes and the clinical importance of this plethora of IgE-reactive proteins and also on the use of recombinant β-conglutins in the research and treatment of lupin allergic disease.

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Conflict of interest statement

The authors have declared that no competing interests exist.

Authors' contribution

Conceived and designed the experiments: JCJ-L. Performed the experiments: JCJ-L, RCF, EB, VCC and ELC. Analysed the data: JCJ-L, RCF, KBS, JDA and PMCS. Contributed reagents/materials/analysis tools: JCJ-L, JFF, KBS, JDA and PMCS. Wrote the paper: JCJ-L, RCF, KBS, JDA and PMCS.

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FIGURE LEGENDS

Fig. 1. Purified recombinant β-conglutin isoforms from *L. angustifolius* L.

(a) SDS-PAGE of five over-expressed and purified recombinants β-conglutins exhibiting purity higher than 95%, loading 10µg of each rβ protein and subjected to 4-20% gradient separation. Molecular weights are indicated at the left side in kilodaltons (kDa). (b) Immunoblot analysis corresponding to the above recombinant conglutins using the anti-β-conglutin antiserum generated in the current study.

Quantitative densitometry analysis of reactive bands showed the relative intensity of each polypeptide recognized by the anti-β-conglutin serum in the immunoblot. Uniprot accession number for each β-conglutin isoform corresponds to β1: HQ670409; β2: HQ670410; β3: HQ670411; β4: HQ670412; β6: HQ670414.

Fig. 2. Immunoblots of purified recombinant β-conglutins probed with IgE from sera of lupin allergic patients. IgE-binding capacity to rβ has been highlighted with plotted bars from the densitometry analysis of these reactive bands after immunoblotting experiments with sixteen sera. 1: *L. albus* cv. Andromeda; 2: *L. mutabilis* cv. ID30; 3: *L. albus* cv. Kiev; 4: *L. luteus* cv. Pootalong; 5: *L. angustifolius* cv. P27255 (wild-type); 6: *L. angustifolius* cv. Tanjil; 7: *L. angustifolius* cv. Unicrop.

Fig. 3. Immunoblots of purified recombinant β-conglutins probed with IgE from sera of peanut allergic patients.

IgE-binding capacity to rβ has been highlighted with plotted bars from the densitometry analysis of these reactive bands after immunoblotting experiments with fourteen sera.

1: *L. albus* cv. Andromeda; 2: *L. mutabilis* cv. ID30; 3: *L. albus* cv. Kiev; 4: *L. luteus* cv. Pootalong; 5: *L. angustifolius* cv. P27255 (wild-type); 6: *L. angustifolius* cv. Tanjil; 7: *L. angustifolius* cv. Unicrop.

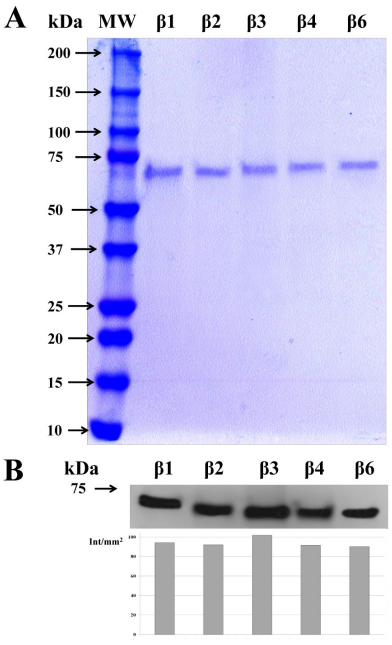
Fig. 4. SDS-PAGE and immunoblot experiments of protein extracts from lupin species. (a) Proteins profiles of protein extracts enriched in conglutin families of proteins were obtained by SDS-PAGE. Quantitative (densitometry) analysis of protein bands showed the relative abundance of each polypeptide. Molecular weights are indicated at the left side in kilodaltons (kDa). (b) Immunoblot analysis of protein extracts performed with anti-β-conglutin antiserum displaying the polypeptides recognized by the antiserum (arrows). Quantitative (densitometry) analysis of reactive bands showed the relative intensity of each polypeptide recognized by the serum. Dots represent the MW of the different (25) polypeptides reactive with anti-β-conglutin antiserum. Dotted lines represent the MWs.

1: *L. albus* cv. Andromeda; 2: *L. mutabilis* cv. ID30; 3: *L. albus* cv. Kiev; 4: *L. luteus* cv. Pootalong; 5: *L. angustifolius* cv. P27255 (wild-type); 6: *L. angustifolius* cv. Tanjil; 7: *L. angustifolius* cv. Unicrop.

Fig. 5. Representative blots showing IgE-binding from serum of lupin atopic patients. Thirteen sera (S1 to S12, S14) from patients diagnosed allergic to lupin were used to probe protein extracts enriched in the globulin family of proteins and representative blots from ten allergenic patients (S2 to S6, S9 to S12, S14) are shown. Protein reactive bands highlighted with arrows corresponded to polypeptides previously recognized using the specific anti-β-conglutin antiserum from Fig. 4b. Molecular weights are indicated at the left side in kilodaltons (kDa). Controls (S13, and S15).

1: *L. albus* cv. Andromeda; 2: *L. mutabilis* cv. ID30; 3: *L. albus* cv. Kiev; 4: *L. luteus* cv. Pootalong; 5: *L. angustifolius* cv. P27255 (wild-type); 6: *L. angustifolius* cv. Tanjil; 7: *L. angustifolius* cv. Unicrop.

Fig. 6. Representative blots showing IgE-binding from serum of peanut atopic patients. Fourteen sera (S16 to S29) from patients diagnosed allergic to peanut were used to probe protein extracts enriched in the globulin family of proteins and representative blots from five allergic patients (S16, S20, S21, S22 and S26) are shown. Protein reactive bands highlighted with arrows corresponded to polypeptides previously recognized using the specific anti-β-conglutin antiserum from Fig. 4b. Molecular weights are indicated at the left side in kilodaltons (kDa). Control (S30). 1: *L. albus* cv. Andromeda; 2: *L. mutabilis* cv. ID30; 3: *L. albus* cv. Kiev; 4: *L. luteus* cv. Pootalong; 5: *L. angustifolius* cv. P27255 (wild-type); 6: *L. angustifolius* cv. Tanjil; 7: *L. angustifolius* cv. Unicrop.



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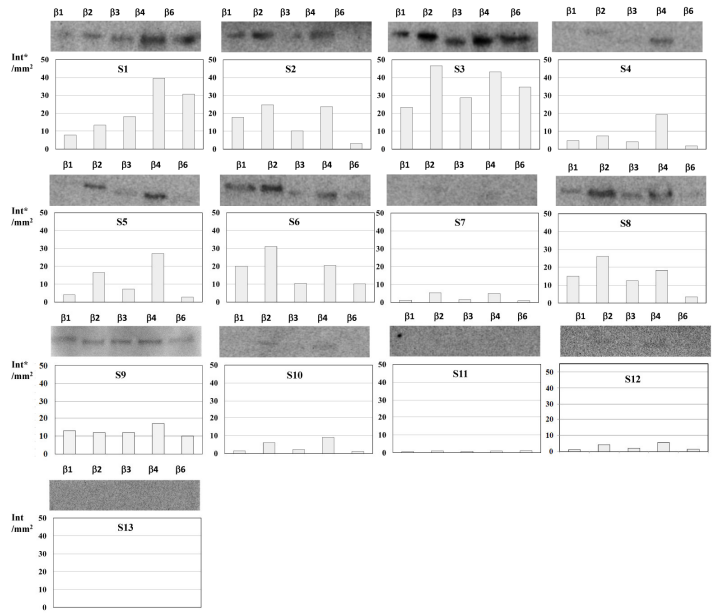

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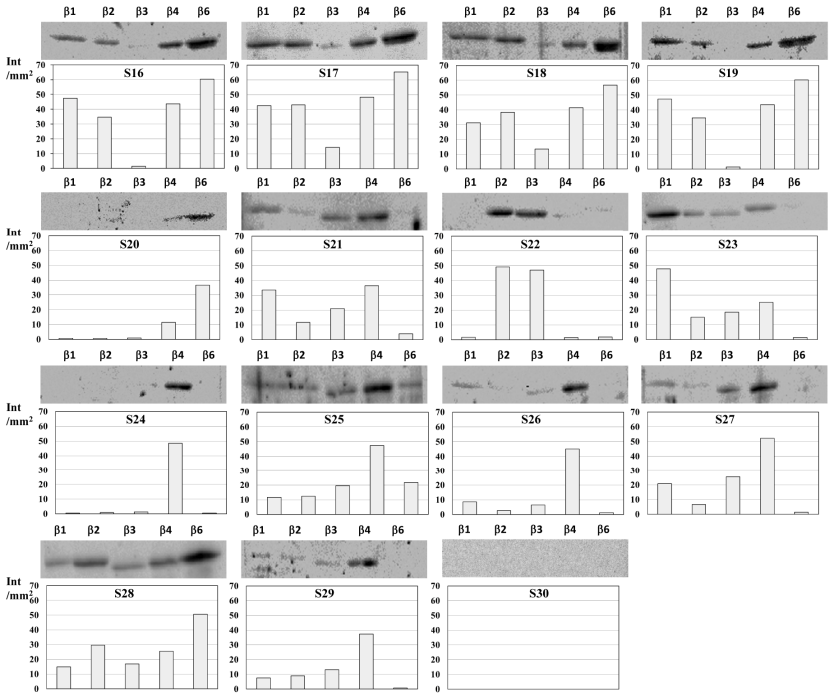

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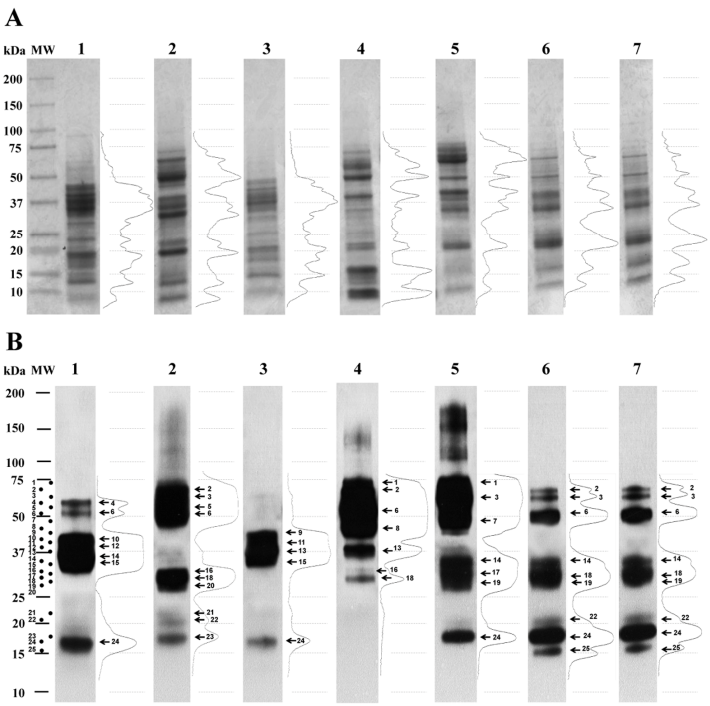

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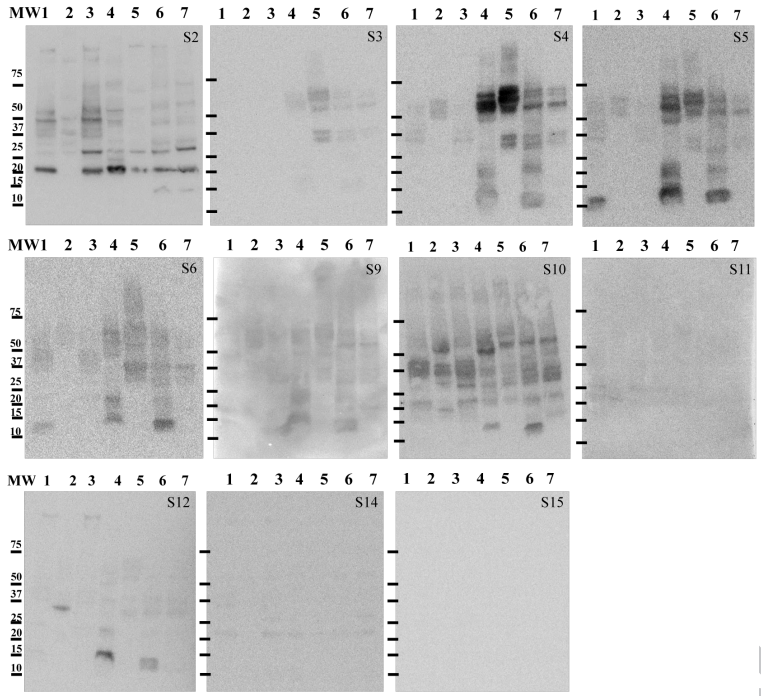

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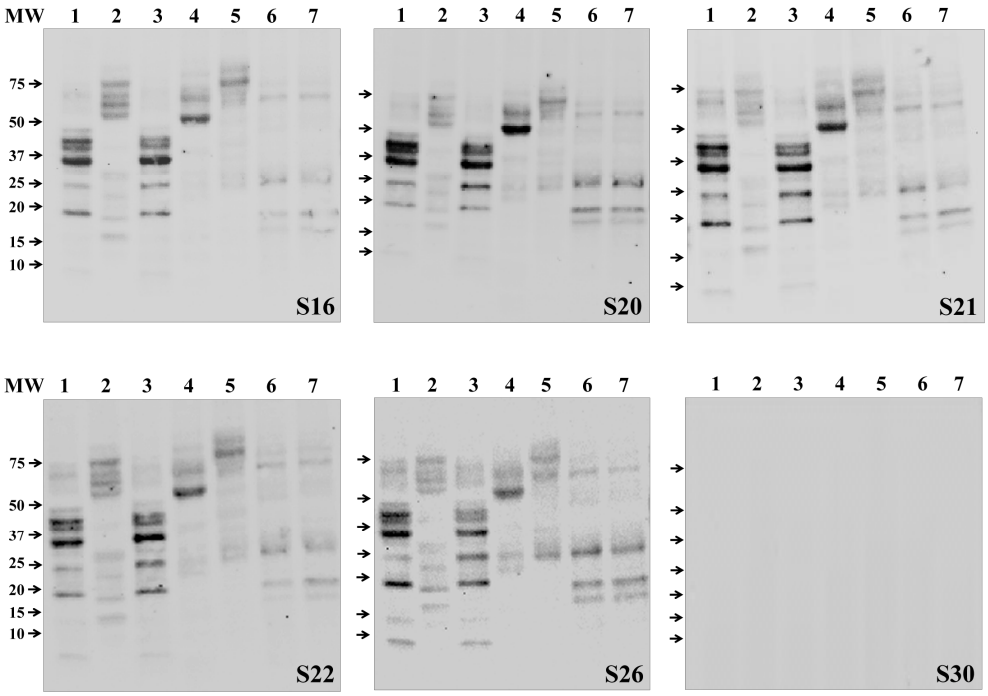

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Highlights

- Several newly identified polypeptides belonging to β-conglutin family may constitute a new source of lupin primary and/or cross-reactive sensitization to lupin.
- Lupin species and cultivars exhibited qualitative and quantitative differences in IgE-binding, particularly in atopic patients´ sera reacting to lupin.
- IgE-binding capacity to five recombinant purified β-conglutin (rβ) protein isoforms from *L. angustifolius* was examined and compared.
- rβ2 may be a suitable candidate to be used for diagnosis and immunotherapy for patients with allergy to lupin.