Lupin Allergy: Uncovering Structural Features and Epitopes of β-conglutin Proteins in *Lupinus Angustifolius* L. with a Focus on Cross-allergenic Reactivity to Peanut and Other Legumes

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Abstract. The use of sweet lupins as a new food is resulting in an increasing number of cases of allergy reactions, particularly in atopic patients with other pre-existing legume allergies. We performed an extensive *in silico* analysis of seed β-conglutins, a new family of major allergen proteins in lupin, and a comparison to other relevant food allergens such as Ara h 1. We analyzed surface residues involved in conformational epitopes, lineal B- and T-cell epitopes variability, and changes in 2-D structural elements and 3D motives, with the aim to investigate IgE-mediated cross-reactivity among lupin, peanut, and other different legumes.

Our results revealed that considerable structural differences exist, particularly affecting 2-D elements (loops and coils), and numerous micro-heterogeneities are present in fundamental residues directly involved in epitopes variability.

Variability of residues involved in IgE-binding epitopes might be a major contributor to the observed differences in cross-reactivity among legumes.

Keywords: β-conglutins, Computational Biology, Epitopes, Diagnosis, Food Allergy, Legume Seeds, *Lupinus angustifolius* L., Protein Structure Modeling, IgE-binding, Immunotherapy, Recombinant Allergen, Vicilin-Like Proteins.

1 Introduction

Lupin is a popular PULSE (the edible seeds of plants in the legume family) worldwide, which has traditionally been consumed as source of proteins since long ago.

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From more than 450 species of the *Lupinus* family, only lupin known as ''sweet lupins'' such as white lupin (*Lupinus albus*), yellow lupin (*Lupinus luteus*), and blue lupin (*Lupinus angustifolius*) are being used in food manufacturing. Flour of raw lupin is increasingly used as food ingredient because of its nutritional value (rich in protein and fibre, poor in fat and gluten-free) [1].

Furthermore, ingestion of lupin-containing foods has been associated with the prevention of obesity, diabetes, and eventually cardiovascular disease. Recently, hypocholesterolaemic properties have been demonstrated for lupin conglutin γ proteins, which may decrease the risk of cardiovascular disease [2].

Lupin belongs to the *Fabaceae* family. As all edible legume seeds, the major protein fraction of lupin seeds is associated with storage proteins, which could be classified in the cupin and prolamin superfamilies, based in structure, solubility and/or sedimentation properties.

The two major lupin storage proteins are α-conglutin (legumin-like or 11S globulin), and ß-conglutin (vicilin-like or 7S globulin). Vilicin proteins are characterized by two cupin (barrel-shaped) domains constituted by α-helices. Another family with a cupin-like structure, γ-conglutin (basic 7S-globulin), displays tetrameric structure integrated by two different disulphide-linked monomers. In contrast, δ-conglutin (2S sulphur-rich albumin) contains 2 disulphide-linked proteins with the typical cysteine-rich prolamin structure [3].

Sweet lupin seeds seem to be particularly promising as a source of innovative food ingredients due to averaged protein content similar to soybean and an adequate composition of essential amino acids. Foods based on sweet lupin proteins include flour for bakery, pasta formulations, gluten-free products and other food items, which are gaining more attention from industry and consumers because the large number of health-promoting benefits described above [2].

On the other hand, with the rapid introduction of novel foods and new ingredients in traditional foods, the number of reports of allergic reactions to lupin proteins is also rising, either as primary lupin allergy or as a result of cross-reactivity to other legume proteins, particularly peanut, soybean, lentil, bean, chickpea, and pea [4]. The most common clinical pattern of lupin allergy is the triggering of an allergic reaction via ingestion of lupin in peanut-allergic individuals, although most commonly triggered via ingestion, inhalation and occupational exposure in individuals without peanut allergy has also been reported. The prevalence varies considerably between studies, but a prevalence of about 1-3% in the general population and 3–8% among childrens is the consensus [5]. Considering the increasing number of clinical cases of lupin allergy reported in the literature, lupin was added in 2008 to the list of foods that must be labelled in pre-packaged foods as advised by the European Food Safety Authority (EFSA) (http://www.efsa.europa.eu/).

Overall, cross-reactivity is the result of IgE-binding to commonly shared epitopes among proteins, i.e. different legume seed proteins, with conserved steric domains (conformational epitopes), and/or amino acid sequences (lineal epitopes).

Given the increase in the number of cases of lupin allergy and the frequency of cross-reactivity with other legume seed proteins, the possible involvement of individual major lupin proteins, i.e. β-conglutins, and their counterparts from other legumes in cross-allergy is of major concern and of great interest to investigate.

In the present study, we add to our results an extensive *in silico* analysis including allergen structure modeling based epitopes (T- and B-cells) identification, aiming to uncover common-shared and specific epitopes, and providing a comprehensive insight of the broad cross-allergy among legume proteins, as well as specific allergic reactions to lupin β-conglutins. This is an important step towards understanding the molecular basis of the allergy phenomenon, particularly cross-reactivity, and towards the development of safe and efficacious diagnosis tools and immunotherapy to lupin-related food allergy.

2 Methods

2.1 Allergen Sequences

We retrieved allergen sequences necessary for the present study from GenBank/ EMBL Database: β-conglutin 1 or Lup an 1 (F5B8V9), β-conglutins 2 to 7 (F5B8W0 to F5BW5), Ara h 1 (P43237, P43238), Gly m 5 (O22120), Len c 1 (Q84UI0, Q84UI1), Gly m β-conglycinin (P25974), Vig r 2 (Q198W3, B1NPN8).

2.2 Phylogenetic Analysis of Food Allergen Sequences

Allergen protein sequences from legumes (lupin, peanut, soybean, Mung bean, lentil, chickpea, pea, mezquite) were retrieved and used to perform a phylogenetic analysis. Sequences alignments were performed by using ClustalW multiple sequence alignment tool (www.ebi.ac.uk/Tools/clustalw) according to Jimenez-Lopez et al. [6]. Trees were visualized using Treedyn (www.treedyn.org).

2.3 Template Assessment

All allergen sequences were searched for homology in the Protein Data Bank (PDB). Suitable homologous templates were selected by using Swiss-Prot database (swiss-model.expasy.org) and BLAST server (ncbi.nlm.nih.gov/) employing fold recognition.

2.4 Proteins Homology Modeling

Sequences were modelled through SWISS-MODEL via the ExPASy web server (swissmodel.expasy.org), by using the top PDB closest template structures previously assessed. Models refinement of 3D structural errors, and structural assessment were performed using stereo-chemical and energy minimization parameters [7].

2.5 Structural Comparison and Evolutionary Conservational Analysis

Allergen proteins structure comparison was performed by superimposition to calculate average distance between their Cα backbones. Protein models were submitted to ConSurf server (consurf.tau.ac.il) to generate evolutionary related conservation scores, in order to identify functional region in the proteins. Functional and structural

key residues were confirmed by ConSeq server (conseq.tau.ac.il). 2-D and 3D were visualized and analyzed using PyMol software (www.pymol.org).

2.6 Solvent Accessible Surface Area and Poisson–Boltzmann Electrostatic Potential

Solvent accessible surface area (SASA), defined as the percentage of surface area of a biomolecule that is accessible to a solvent for each residue was calculated by using the GETAREA v1.1. program (curie.utmb.edu/getarea.html). The electrostatic Poisson-Boltzmann (PB) potentials for the built structures were obtained [7,8].

2.7 Allergenicity Profile Assessment

Allergenicity of lupin and other legume allergen sequences was checked by a full FASTA alignment in the Structural Database of Allergenic Proteins (SDAP) (Fermi.utmb.edu/SDAP). Allergenicity profile was assessed by combination of different parameters: hydrophobicity, antigenicity and SASA [9]. Values of absolute surface area (ASA) of each residue were also calculated by DSSP program (swift.cmbi.ru.nl/gv/dssp), and transformed to relative values of ASA and visualized by ASAView (www.netasa.org/asaview).

2.8 Linear and Conformational B-cell Epitopes Analysis

For determination of linear (continuous) epitopes, the allergen proteins sequences were submitted to ABCpred (uses artificial neural networks, www.imtech.res.in/raghava), BepiPred 1.0b (based on hydrophobicity scale with a Hidden Markov Model, www.cbs.dtu.dk), BCPREDS (uses support vector machine, ailab.cs.iastate.edu/ bcpreds), Bcepred (based on a combination of physico-chemical properties, www.imtech.res.in/raghava), and COBEpro (uses support vector machine, scratch.proteomics.ics.uci.edu). Linear and discontinuous antibody epitopes based on a protein antigen's 3D structure were predicted using Ellipro (http://tools.immuneepitope.org/tools/ElliPro/iedb_input/, discontinuous epitopes are defined based on PI values and are clustered based on the distance R (Å) between residue's centers of mass, tools.immuneepitope.org), and Discotope (tools.immuneepitope.org) webservers.

The epitopes identified frequently by most of the tools were selected [9,10].

2.9 T-cell Epitopes Identification and Analysis

The identification of MHC Class-II binding regions for all the allergen sequences was performed by using neuronal networks and quantitative matrices derived from published literature. Promiscuous peptides binding to multiple HLA class II molecules were selected. The analysis was made by using the TEPITOPE software (www.bioinformation.net/ted), with a threshold of 5% for the most common human HLA-DR alleles [DR1, DR3, DR4, DR7, DR8, DR5 and DR2] among Caucasian population [10], and covering a large proportion of the peptides that bind with human HLA.

3 Results

3.1 Searching for Allergen Proteins Templates

We used the Swiss-model server to identify the best possible templates to build allergen structures, finding high scores and very low E-values (ranging 12E−34 to 7E−42) for the templates retrieved from Protein Data Bank (PDB) database and used for homology modeling: lupin β-conglutins (1uijA, 2eaaB), Ara h 1 (3s7i, 3s7e), Gly m 5 (1uijA), Len c 1 (1uijA), Gly m β-conglycinin (1uijA), Vig r 2 (2eaaB).

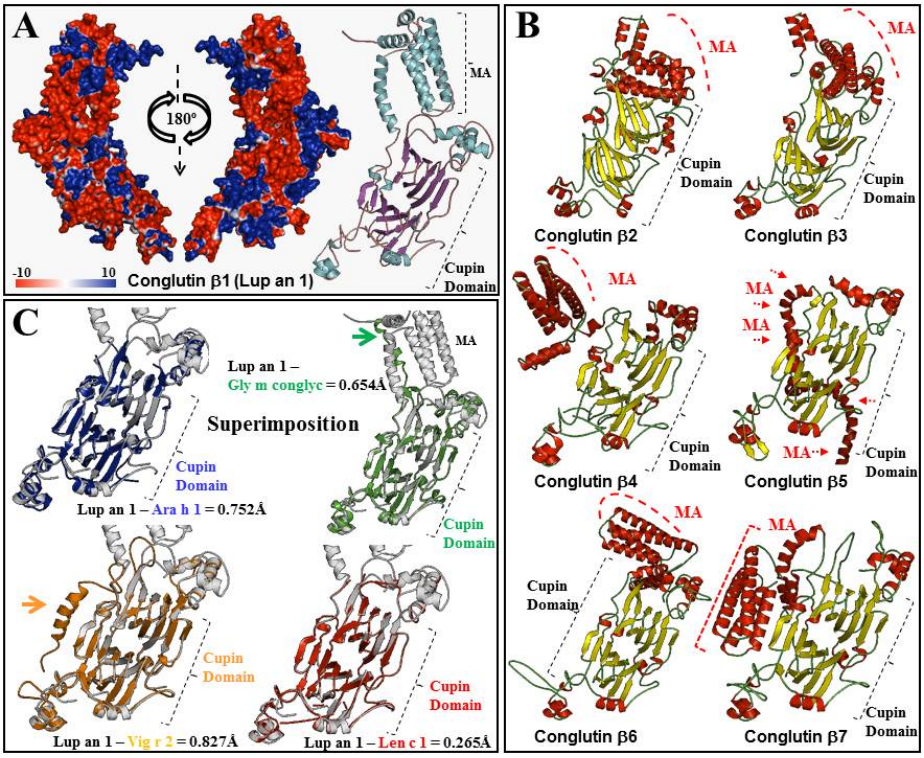

Fig. 1. Lupin β-conglutins structural analysis. A) Cartoon and surface representation views of conglutin β1 rotated 180°, showing the surface electrostatic potential clamped at red (-10) or blue (+10). 2-D elements (α-helices, β-sheets, coils) were depicted in cartoon model, showing main proteins domains (mobile arm and cupin domain). B) 3D structures of conglutins β2 to β7 were depicted as a cartoon diagram. α-helices, β-sheets and coils are depicted in red, yellow and, green respectively, integrating main proteins domains. C) Superimpositions showed the close structural relationship with allergens from other legumes such as peanut (Ara h 1), soybean (β-conglycinin), Mung bean (Vig r 2), and lentils (Len c 1). **Å** = Armstrong; **MA** = mobile arm.

Figure 1 showed that lupin β-conglutins are characterized by a surface negatively charged, a domain from the Cupin superfamily constituted by 2 barrels of 8-10 α-helices each, and a mobile arm, which position may be different depending of the β-conglutin form. One of these barrels followed the Rossmann fold structure, typically found in oxidoreductase enzymes.

2-D elements comparison by superimposition among allergens showed a comparable low values (< 1Å) of structural differences, when compared Cupin superfamily domain, since the mobile arm is absent in these allergens. Overall, β-conglutins were found structurally close to Len c 1 and most distantly related to the Gly m 5 allergen.

3.2 Structural Assessment of the β-conglutin 1 to 7, Gly m 5, Len c 1, Gly m Conglycinin, and Vig r 2 Structural Models

Different molecular tools (stereochemistry, energy minimization) were used to assess the quality of the models built for this study. A general quality assessment parameter as *QMEAN* displayed adequate values for all models. Most of the residues of the main chain of built models were located in the acceptable regions of the Ramachandran plot shown by *Procheck* analysis. In addition, Z-scores returned from *ProSa* indicated that structures showed negative interaction energy and within the lowest energy range. In addition, the Z-scores were within the range usually found for templates used for allergen structure modeling.

3.3 Phylogenetic Analysis

We analyzed the relationships between lupin β-conglutin proteins and allergens from other species. The data clearly reveal five established groups/clusters. We have identified 5 main groups, where β-conglutins were grouped with allergens of 7S-globulin nature (Fig. 2).

3.4 Identification of Highly Antigenic Regions in Plant Profilins

Physicochemical parameters such as hydrophobicity, accessibility, exposed surface, and antigenic propensity of polypeptide chains have been used to identify continuous epitopes (see methods section). In our analysis, antigenicity determinants were assigned by locating the positive peaks in hydrophilicity plots, and identifying the regions of maximum potential of antigenicity (data not shown).

We identified up to 8 regions in lupin β-conglutins, with high potential of antigenicity, 7 regions in Ara h 1, 7 regions in Gly m 5, 8 regions in β-conglycinin, 7 regions in Vig r 2, 4 in Len c 1, and 5 in Pis s 2 (data not shown). These regions with high antigenicity correlated well with the lineal T- and B-cell and conformational epitopes identified and analyzed in the present study.

The highest differences in terms of antigenicity regions polymorphism correspond to lupin β-conglutins, while the lowest variable allergen was Len c 1 (data not shown).

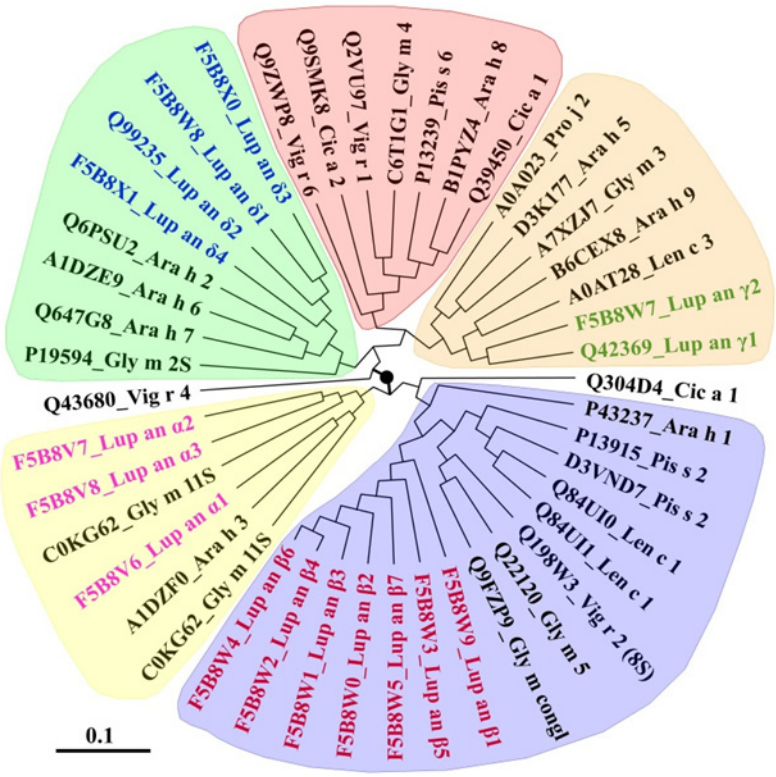

Fig. 2. Phylogenetic analysis of food allergens. Neighbor-joining (NJ) method was used to perform a phylogenetic analysis of 45 legume allergens from lupin (conglutins α1–3, β1–7, γ1–2, and δ1–4), peanut (Ara h 1, 2, 3, 5, 6, 7, 8, and 9), soybean (Gly m 5, and Gly m conglycinin), lentil (Len c 1, and 3), pea (Pis s 2, and 6), Mung bean (Vig r 1, 2, 4, and 6), Mezquite (Pro j 2), and chickpea (Cic a 1, and 2).

3.5 Analysis of B-cell Epitopes

12 antigenic lineal regions prone to B-cell binding were analyzed in conglutins β1, 7 for β 2 and β 7, 5 for β3, 4 for β 4, β 5, β 6. In addition, we identified 6 antigenic regions in Ara h 1, 7 in Gly m 5, 11 in β -conglycinin, 4 in Len c 1, 10 in Pis s 2, and 8 in Vig r 2 (Fig. 3). Comparative analysis of these regions showed that 5 lineal epitopes in conglutin β 1 are located in the mobile arm, 3 of them overlapping with a big conformational epitopic area (black color, Fig. 3) and 2 lineal epitopes independent. Furthermore, β 2 and β 5 present 3 conformational epitopic areas, 1 in β 3, β 6 and β 7, 2 in β 4, related to the differential mobile arm structure.

The biggest difference as structural feature between the β -conglutins and the other legume allergens is the presence of the mobile arm in N-terminal region of the lupin β -conglutins and the epitopes which integrate. Number of epitopes and polymorphism analysis of lineal and conformational B-cell epitopes in other legume allergens

showed a wide range of variability in both, the number and the sequence identity of these epitopes (data not shown).

3.6 Identification of T-cell Epitopes

We have identified a variable number of anchor motifs to HLA-DR in the sequences of lupin β-conglutins (8 main T-cell epitopes), and their counterparts in five species of legumes.

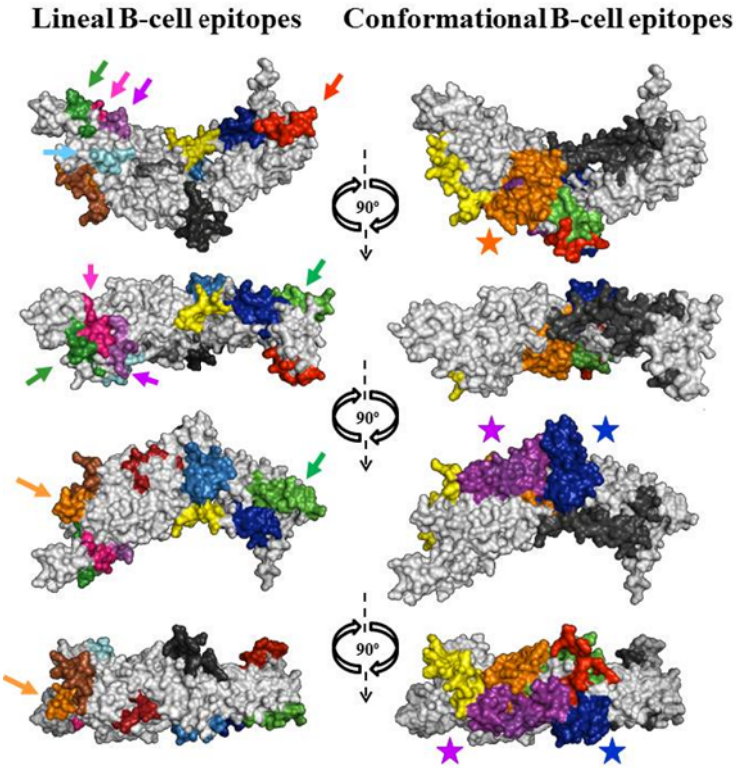

Fig. 3. B-cell epitopes analysis in lupin β-conglutins and their legume proteins counterparts. Cartoon representation of Lup an 1 allergen showing in various colors lineal and conformational B-cell epitopes in its surface. Arrows and stars represent specific lineal and conformational epitopes, respectively, which do not overlap with each other.

T1 was the "solo" epitope in the mobile arm of β-conglutins (Fig. 4), exhibiting a large surface orientation. This epitope is common for other legume allergens such as peanut (Ara h 1), soybean (β-conglycinin), Mung bean (Vig r 2), and pea (Pis s 2). The rest of epitopes identified in β-conglutins were located in the globular (Cupin-like) domain of these proteins. Some of these epitopes were differentially shared with other legume allergens, i.e. T2 is the most commonly shared epitope, and T8 only

commonly located in allergens of soybean. In addition, each of the allergen analyzed has specific epitopes not found in other species (Fig. 4). Most of these lineal epitopes displayed 50% or more of their residues not exposed to the surface (T2 to T8).

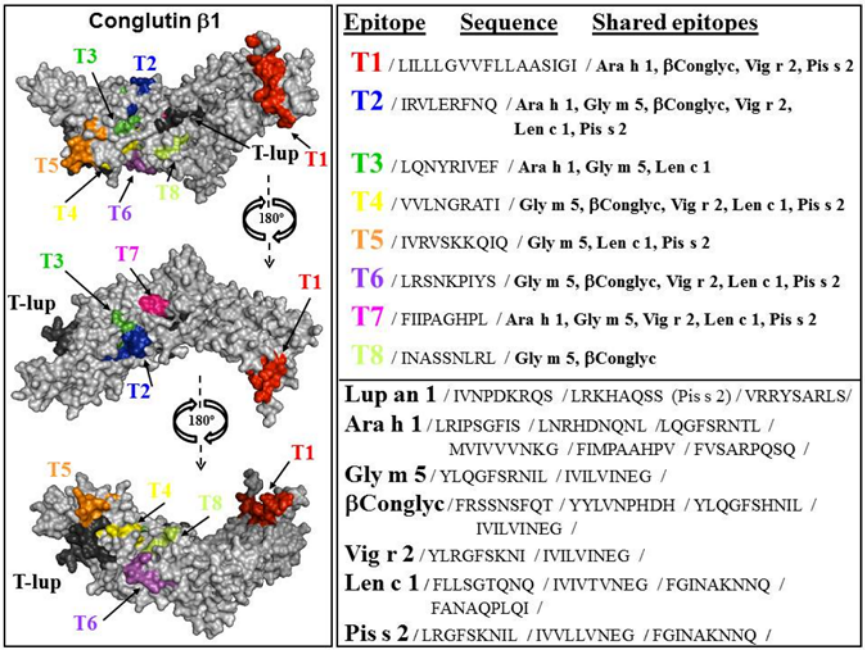

Fig. 4. T-cell epitopes comparison between lupin β-conglutins and their legume proteins counterparts. T-cell epitopes depicted on the three views rotated 180° conglutin β1 protein surface, respectively, following a color code for epitopes identification (T1 to T8, upper right square). Epitopes identified belonging to exclusive legume specie have been listed in the figure (bottom right square).

4 Discussion

The immune-cross-reactivity between lupin seed proteins and other legumes is starting to be analyzed, and knowledge at molecular level is scarce.

In *Lupinus angustifolius* L., Lup an 1 (conglutin β 1) has been recently identified as a major allergen by using proteomic analysis and was recognized by serum IgE from most of 12 lupin-allergic patients' sera [11], which matched with a vicilin-like (7S-type) protein.

Knowledge about the linear epitopes of lupin major allergens is of crucial importance to help identify the trigger agent in clinical diagnosis (trials) of lupin allergy and to develop and implement molecular tools in order to identify the presence of lupin allergens as ingredients in food, in order to protect patients with lupin allergy and other cross-allergenic reaction. Sequence homology between lupin major allergens and other legume allergens support cross-reactivity between them. However, in

the present study has been identified commonly shared T- and B-cell lineal and conformational epitopes in lupin and allergens from other legumes, which are located in the globular (Cupin Superfamily) characteristic domain. The largest number of epitopes has been identified in conglutin β 1, which may be the reason why Lup an 1 is currently the main allergen among the beta forms. Several of these epitopes are common to other legume proteins. However, others are not well-conserved, finding a noticeable degree of polymorphism. We have identified surface patterns (conformational epitopes), as well as multiple regions (B- and T-cell epitopes) in legume allergens, including lupin, exhibiting differences in length and variability. Furthermore, we have found shared common B- and T-cell epitopes among these legume allergens, as well as epitopes differentially distributed in specific allergens. The variability in their surface residues might contribute to generate areas of the protein enable of being differentially recognized as Th2- inducing antigens. Depending on the location of these polymorphic residues, recognition by IgE/IgG may be also affected [12].

Thus, we propose that the presence of several of these epitopes (T- and B-cell) is the main reason for cross-allergenicity reactions among legume proteins, which however react differentially with lupin β -conglutins forms and between them. The extension of the reactions may be directly linked to the residues variability of these epitopes. It has been reported serological cross-reactivity among legume allergens [4], and Lup an 1 (Ara h1, Len c 1 and Pis s 1). IgE reactivity may not always be related to clinical cross-reactivity (leading to allergy symptoms), which has been observed in lupin, peanut and pea. In this regard, we have found that six T-cell epitopes are shared between Lup an 1 and Len c 1. From these, four epitopes are commonly found in Ara h 1 and Pis s 1 as well. Furthermore, one of these four epitopes is the "T- solo" or T1 located in the mobile arm of β -conglutins. This epitope may play a key role in specific cross-reactivity between legume seeds proteins and lupin β -conglutins as one of the four main families (α, β, γ, δ) of seed storage proteins in lupin.

Molecular modeling of proteins throughout computational biology tools help identifying specific regions, which could be candidates for the development of peptide-based immunotherapeutic reagents for allergy, while conserved regions could be responsible of the cross-reaction between allergens [10]. Epitopes prediction based on knowledge derived from structural surface features such as increased solvent accessibility, backbone flexibility, and hydrophilicity [7,9,10]. Such predictions were found to correlate well with antigenicity in the present study. At structural level, antigenic determinants may be integrated by 2-D structure elements, which protrude from the surface of the protein, such as coils and loops [10]. Our results have shown that conformational epitopes are these more affected by 2-D structure elements, which are mostly integrated by short α-helices and coils (Fig. 1 and 3). Variability in sequence and length of these 2-D elements may additionally increase the differences and the extension of the cross-allergenic reactions between legume allergens [13].

On the other hand, linear B- and/or T- cell epitopes may play most important roles in cross-reactivity between food allergens [14], since food processing or digestion may increase the number or the accessibility of IgE binding epitopes. Thus, some food allergens have been described to lead to a loss of some or all the B-cell epitopes (but not the T-cell epitopes) by denaturalization/digestion [15]. In a similar fashion,

vicilin-like allergens such as Ara h 1 and Lup an 1 also share thermal stability. B- and T-cell responses have a defining and differential recognition of antigenic epitopes, and their localization in the allergen does not necessarily coincide. T-cell receptor recognizes only the linear amino acid sequence [16]. In contrast, B-cell epitopes recognized by IgE antibodies are either linear or conformational and are located on the surface of the molecule accessible to antibodies. The extension of the epitope may range from 5 to 8 or longer amino acids for IgE to be able of binding to the epitope [17-20]. However, we have identified lineal B-cell epitopes in lupin β-conglutins and the other legume allergens with a wide range of amino acid lengths, and overlapping with conformational epitopes.

5 Conflict of Interest

The authors confirm that this article content has no conflicts of interest.

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