Vicilin and the basic subunit of legumin are putative chickpea allergens

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IgE-mediated reactions to food allergens constitute a major health problem in industrialized countries. Chickpea is consumed in Mediterranean countries, and reportedly associated with IgE-mediated hypersensitivity reactions. However, the nature of allergic reactions to chickpea has not been characterized. A serum pool from paediatric patients allergic to chickpeas was used to detect IgE-binding proteins from chickpea seeds by immunoassay and immunoblot inhibition assay. Protein samples enriched in chickpea legumin and vicilin were obtained by anion exchange chromatography, and were identified by mass spectrometric analysis.

IgE-immunoassays of globulin fractions from chickpeas revealed that vicilin (50 kDa) and the basic subunit of legumin (20 kDa) were bound by IgE from patient sera. Pea and lentil protein extracts strongly inhibited the IgE binding to chickpea globulin.

We speculate that vicilin and the basic subunit of legumin are major chickpea allergens. Also, the globulin fraction of chickpea likely cross-reacts with the allergenic proteins of pea and lentil.

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1. Introduction

Food allergy affects 5% of children in westernized countries (Sicherer & Sampson, 2010). Most food allergies are thought to be caused by eight food groups: eggs, milk, tree nuts, peanuts, soybeans, wheat, fish and crustaceans (Sicherer & Sampson, 2010). The list and the order of incidence vary from country to country.

Several allergens have been identified in the legume family, many of which belong to the vicilin- and legumin-type proteins. Major food allergens include vicilins of walnuts (Jug r 2), peas, lentils (Len c 1.0101) and lupins (Lup a) (Teuber, Jarvis, Major food allergens include vicilins of walnuts (Jug r 2), peas, lentils (Len c 1.0101) and lupins (Lup a) (Teuber, Jarvis, 1999). (Sanchez-Monge et al., 2004), (Guillamón et al., 2010), (Beyer, Grishina, & Sampson, 2002), Wallowitz et al., 2006).

Despite the intensive research devoted to identifying and characterising allergens in legumes, little is known about the allergenicity of chickpeas. Chickpea is an abundant crop in Mediterranean and Asian countries, and according to a 2010 market survey, chickpea consumption in the United States has increased by 35% over a period of 21 months. Chickpea’s possible association with IgE-mediated hypersensitivity reactions, especially among children, merits serious attention (Crespo, Pascual, Burks, Helm, & Esteban, 1995). There are hardly any reports on chickpea allergy in countries or regions where chickpea is a staple food (India, the Middle East, Southern Europe, Central and South America and the USA). However, in Spain allergy to legumes, including chickpeas, is the fifth most common food allergy in children (Crespo et al., 1995). Chickpea albumin and globulin fractions have been characterized (Liu, Hung, & Bennett, 2008) and certain characteristics of the vicilin-type globulin of chickpea have been reported (Tavano & Neves, 2008). Specific IgE-binding protein fractions in the immunoblotting patterns of crude and boiled chickpea extracts were detected (Martínez San Ireneo et al., 2008), and the effect of boiling and autoclaving on chickpea allergenicity have been examined (Cuadrado et al., 2009). In addition, chickpea 2S and Pa2 albumin were found to evoke positive reactions in chickpea-sensitive individuals (Vioque et al., 1999), (Vioque et al., 1998).

Reactivity to more than one legume is quite common, although cross-reactivity in various geographic areas differs. Some allergens may share common epitopes or similar sequences and/or structural features (Ivanciuc, Garcia, Torres, Schein, & Braun, 2009).
These homologies can cause cross-reactions that usually occur in related molecular species of the same family, for example lentils and peas (Martínez San Ireneo, Ibáñez, Fernández-Caldas, & Carnés, 2008). However, cross-reactivity between members of different botanical families have also been reported, such as in the latex-fruit syndrome (Blanco, 2003).

The aim of this study was to identify specific protein components in chickpea that are potentially responsible for the allergenic response in humans.

2. Materials and methods

2.1. Serum from patients

Serum samples were obtained from 16 children with multiple legume and nut allergies (Table 1). A written informed consent was obtained from the next of kin, caregivers or guardians on behalf of all the participants in this study, as part of the overall agreement for routine medical treatment. Each case was diagnosed on the basis of positive skin prick test responses and positive specific serum IgE to chickpea, as well as a clinical history of allergic reaction following chickpea ingestion or a positive response to an open food challenge. Specific IgE levels to chickpeas were quantified with the CAP-FEIA System (Pharmacia Diagnostics). All individual sera showed specific IgE levels above 5 kUA/L. As a negative control we used pooled sera from non-allergic individuals.

2.2. Protein fractionation

Chickpea albumin and globulin were extracted as previously described (Franco, Ferreira, & Teixeira, 1997), with minor modifications. Briefly, chickpea was milled (0.2 mm sieve) and the resulting meal was defatted with n-hexane (34 mL/g of flour) for 4 h with agitation, decanted and air-dried. The defatted chickpea flour was extracted with water containing 10 mM CaCl₂, 10 mM MgCl₂, and 1 mM PMSF, with stirring for 4 h (34 mL/g of flour) (pH 8.0). The slurry was centrifuged at 30,000 g (4 °C, 1 h), and the supernatant containing the albumin fraction was discarded. The pellet, containing the total globulin fraction, was stirred for 4 h with 0.1 M Tris–HCl buffer (pH 7.5), containing 10% (w/v) NaCl, 0.05% (w/v) NaN₃, 10 mM EDTA, 10 mM EGTA and 1 mM PMSF (34 mL/g of flour). The solution was centrifuged at 30,000 g (4 °C, 1 h), and the globulins were precipitated by adding ammonium sulphate (561 g/L), followed by centrifugation at 30,000 g (4 °C, 20 min). The pellet was resuspended in 50 mM Tris–HCl buffer (pH 7.5) (5.7 mL/g of flour) and was desalted using HiPrep 26/10 desalting column (GE Healthcare) equilibrated with the resuspension buffer. Protein concentration was determined by the Bradford method (Bradford, 1976).

2.3. Purification of legumins and vicilins

Legumins and vicilins were purified from the total globulin fraction using the Fast Performance Liquid Chromatography system (ÄKTAexplorer™, GE Healthcare), as previously described (Freitas, Teixeira, & Ferreira, 2007). Briefly, 20 mL of Globulin fraction was loaded on a Mono Q 10/100 column (GE Healthcare) at flow rate of 2 mL/min, previously equilibrated with 15 column volume of 50 mM Tris–HCl buffer (pH 7.5) and bound proteins were steps eluted with 0.3, 0.4 and 0.6 M NaCl in a flow rate of 2 mL/min. Absorbance was monitored at 280 nm and various fractions were collected. Fractions corresponding to chromatogram peaks were pooled, electrophoresed on gel, and immunodetected with a pool of sera from patients with chickpea allergy.

2.4. Gel electrophoresis and immunodetection

Protein samples were separated by SDS–PAGE in 12% acrylamide gels as previously described (Laemmli, 1970). The gel was stained with 0.1% comassie blue and destained in water:methanol:acetic acid solution (50:40:10). For immunodetection, fractionated samples were electrotransferred to PVDF membranes (Millipore) as previously described (Diaz-Perales et al., 1999). After washing and blocking, membranes were incubated overnight at 4 °C with individual, pooled or control sera (dilution 1:70), and then with monoclonal anti-human IgE, alkaline-phosphatase-conjugated (Sigma; 1:500 dilution) at room temperature for 1.5 h, and developed by adding disodium 2-chloro-5-(4-methoxy-spiro{1,2-dioxetane-3,2-(5-chloro)tricyclo[3.3.1.1]decane}-4-yl)-1-phenyl phosphate (Sigma).

Immunoblot inhibition assays were carried out by the same method, except that the sera were pre-incubated for 3 h at 25 °C with 35 μg of pea or red lentil protein extracts. Incubation with bovine serum albumin (BSA) served as negative controls.

2.5. Identification of putative chickpea allergens by mass spectrometry

The 20, 50 and 70 kDa protein bands of the globulin fraction were cut from the corresponding electrophoretic gels and digested with trypsin. The resulting tryptic peptides were resolved by reverse-phase chromatography (J&W Scientific). Mass spectrometry (MS) was performed with an ion-trap mass spectrometer (Orbitrap, Thermo Scientific). The MS data were clustered and analysed using Discoverer software (Thermo-Finnigan), using two search algorithms: Sequest (Thermo) and Mascot (Matrix science), searching against the plants part of the NCBI nr database, and a decoy database (in order to determine the false discovery rate). Gel digestion and MS were conducted in the Smoler Proteomics centre, Technion—Israel Institute of Technology, Haifa, Israel. Peptides identified by Mascot were filtered with a significance threshold of 0.05. The Mascot score is derived from the individual ion-scores for each peptide, and all peptides in our MS report are above ion score of 30, which is considered certain (data not shown). Peptides that were identified by Sequest were filtered according to an Xcorr of 2 for doubly charged peptides, 2.5 for triply charged peptides and 3 for quadruply charged peptides. Since there are only a few hundred proteins for chickpea in the database, other proteins can only be identified if the database contains homolog proteins from other plants. Amino acid sequence of the candidate allergens was compared to known plant allergens using the BLAST algorithm, FASTA database and MUSCLE program.

Table 1

<table>
<thead>
<tr>
<th>Food Allergies</th>
<th>Chickpea-specific IgE (kU/L)</th>
<th>Subject No.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lentils, hazelnut, walnut</td>
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<td>1</td>
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<tr>
<td>Lentils, peas, peanuts</td>
<td>24</td>
<td>2</td>
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<tr>
<td>Lentils</td>
<td>26</td>
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<td>Lentils, peas</td>
<td>28</td>
<td>4</td>
</tr>
<tr>
<td>Lentils, peas, peanuts</td>
<td>37</td>
<td>5</td>
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<tr>
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</tr>
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<td>Lentils, peas, peanuts, hazelnut</td>
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<td>7</td>
</tr>
<tr>
<td>Lentils</td>
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<td>8</td>
</tr>
<tr>
<td>Lentils, peas, peanuts, hazelnut</td>
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<td>9</td>
</tr>
<tr>
<td>Lentils, peanuts, walnut</td>
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<td>10</td>
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<tr>
<td>Lentils, peas, peanuts</td>
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<tr>
<td>Lentils, peas</td>
<td>125</td>
<td>16</td>
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</table>
3. Results

3.1. Detection of allergens in total chickpea globulins

The electrophoretic profile of total chickpea globulin was characterized by bands ranging from ~15 to ~75-kDa (Fig 1A). Multiple reactive protein bands were identified by immunoassays of chickpea-globulin with the serum pool obtained from patients allergic to chickpea (Fig 1B). The same pattern appeared with the individual patient’s serum (Fig 1B). Specific IgE antibodies reacted more frequently with three protein bands with molecular weights of ~70, 50, and 20 kDa, and showed a less pronounced reaction with a 30 kDa band. Sera from non-allergic patients were used as a negative control and did not react with any protein bands (Fig 1B).

3.2. Immunoblot inhibition assay

Immunoblot inhibition assay using protein extracts of pea, and red lentil showed an overall decrease of IgE binding to the reactive globulin bands (Fig 2), indicating the presence of common IgE epitopes in chickpea globulin and proteins of these legumes. A less accentuated decrease, however, was noted at the 20 kDa band, which may indicate that this protein of chickpea shares less epitopes with peas and lentils proteins of the same molecular weight.

3.3. Enrichment of putative chickpea-globulin allergens

The chromatogram in Fig 3A shows the elution profile of the chickpea globulin fractions from the Mono Q FPLC column. Fractions obtained from the FPLC separation (A–H), corresponding to peaks I, II and III, were examined by immunoblotting against serum pool after separation on a SDS-12% acrylamide gel (Fig 3B). Fractions that showed positive reactions (peaks I and II) were separated, used to cut the putative allergenic bands of 70, 50 and 20 kDa (Fig 3C) and subjected to mass spectrometry analysis. Fractions corresponding to peak III did not react with the tested sera (data not shown).

3.4. Identification of putative chickpea-globulin allergens

MS analysis of the putative allergenic bands of 20, 50 and 70 kDa of chickpea-globulin is presented in Table 2. For each band analysed, results are presented according to the order of the Mascot score each protein received.

The 20 kDa band is comprised of a legumin-type protein. The legumin type globulins exit as a mixture of trimers and hexamers, comprising subunits of Mr ~50,000–60,000. These subunits generate a basic (Mr ~20,000) and acidic (Mr ~30,000–40,000) polypeptides. The protein with the highest Mascot score was chickpea legumin (Accession No. Q9SMJ4), and we speculate it is the basic subunit of legumin. In order to validate the extent of homologies and the potential cross-reactivity between the chickpea legumin and the different legumin type allergens, we compared their amino acid sequences using FASTA database. Using this approach, we found that chickpea legumin most closely resembled Gly m 6 of soybeans (P04776) and Lup α (alpha conglutinin) of lupins (Q53I54) (56.9% and 56.6% amino acid identity, respectively), but also with Jug r 4 of common walnut (Q2TPW5), Cor a 9 of hazelnut (Q8W1C2), Pis v 5 of pistachio (B7SLJ1) and Ana o 2 of cashew (Q8GZP6) (45.6%, 45.1%, 44.5%, and 45.2% amino acid identity, respectively). Fig 4 shows the alignment of amino acid sequences of chickpea-legumin and the allergenic legumin-type proteins of Gly m 6 (Fig 4A), Jug r 4 and Cor a 9 (Fig 4B). Three sequences of chickpea legumin detected by MS analysis share...
some resemblance with the Gly m 6, Jug r 4 and Cor a 9 epitopes (Beardslee, Zeece, Sarath, & Markwell, 2000; Robotham et al., 2009); YQQEGEEEENEGGNIFSGFK, DFLEDALNVNR and LQGRNEDDEEKGAIVK are presented in Fig 4.

All proteins identified in the 50 kDa band belonged to the vicilin-type family. The protein that received the highest Mascot score and had the highest number of matched peptides was the lentil allergen Len c 1.0101 (AJ551424.1). Provicilin precursor from chickpea (CAA36188.1) was also identified in this band. Applying FASTA tool for amino acid sequence comparison revealed that chickpea provicilin highly resembles Len c 1.0101, Pis s 1 (Q702P1), Pis s 2 (P13915) of peas, Lup an 1 (BOY7F7) and Lup a 1 (Q53HY0) of lupin (75.5%, 65.3%, 65%, 59.8% and 58.7% amino acid identity, respectively).

The 70 kDa band is comprised of vicilin-type protein (Len c 1.0101). However, luminal-binding protein was also identified in this band (Accession No. P49118), although it received a relatively low (but significant) Mascot score. Putative luminal binding protein from Corylus avellana (Accession No. CAC14168.1) was also identified in this band, which is speculated to be a cross-reactive plant allergen (Gruehn, Suphioglu, O’Hehir, & Volkmann, 2003). Both proteins belong to the Hsp70 family, have a MW of ~73 kDa, and BLAST results show they share an identical amino acid sequence (data not shown).

4. Discussion

In this study we showed that chickpea legumin- and vicilin-type proteins are recognized by specific IgEs of chickpea-allergic patients, and are therefore putative chickpea allergens.

The 20 kDa band of chickpea globulin is comprised of a legumin-type protein. Also known as 11S globulin, the legumin-type proteins have been confirmed as allergens in peanuts (Ara h 3) (Burks et al., 1992), soybeans (Gly m 6) (Holzhauser et al., 2009) and recently in L. albus (Lup a_conglutin) (Guillamón et al., 2010). The high sequence homologies between chickpea legumin and other legumin-allergens from soybeans and L. albus, as found in the FASTA database, could be responsible for the cross-reactivity observed between these legumes. Furthermore, cross-reactions may also occur between chickpea and allergens of other botanical families, such as hazelnut and walnut, although not always with overt clinical symptoms. The sera of patients included in our study contained IgE’s not only against legumes but also against hazelnut and walnut (Table 1). This further supports the data obtained in the FASTA comparison presented here, which shows a relatively high degree of sequence identity between chickpea legumin and legumin-allergens of walnut and hazelnut (Jug r 4 and Cor a 9, respectively). Among the sequences of chickpea legumin detected by MS analysis, three appeared to resemble some of the epitopes of Gly m

<table>
<thead>
<tr>
<th>MW in gel (kDa)</th>
<th>Protein identification</th>
<th>Theoretical MW (kDa)</th>
<th>No. of tryptic peptides matched</th>
<th>Coverage</th>
<th>Score (Mascot)</th>
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<td>20</td>
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<td>129.39</td>
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6, Jug r 4 and Cor a 9 (Fig 4). These sequences could therefore be candidate IgE binding epitopes of chickpea-legumin.

The 50 kDa band was matched with a vicilin-type protein, and the FASTA database shows it shares high sequence homology with the lentil allergen Len c 1.0101, and Pis s 1 and 2 of green peas, all of which are vicilin-like allergens. The homologies described here for the first time, in addition to the immunoblot inhibition assay (Fig 2), which shows clear inhibition of the chickpea 50 kDa band by lentils and peas, could provide a suitable explanation for the clinical observation that approximately 70% of Spanish paediatric patients allergic to lentils show associated reactions to chickpeas and/or peas (Ibanez, Martinez, Sanchez, & Fernandez-Caldas, 2003).

The 70 kDa band of chickpea-globulin was identified mainly as a vicilin type protein. However, we cannot ignore the fact that luminal binding protein was also identified in that band, in spite of the relatively low score this protein received in the MS analysis. Our results may be correlated with the identification of a novel lentil allergen Len c 1.0101, and Pis s 1 and 2 of green peas. We propose a molecular basis for the clinically observed cross-reactivity between chickpea, lentils and peas, as evidenced here by the identity between amino-acid sequences of the vicilin-like protein of chickpea that was described here and the lentil allergen Len c 1.0101 and the pea allergens Pis s 1 and 2. We also speculate that chickpea-legumin might cross-react not only with legume allergens, such as Gly m 6 and Lup a, but also with allergens of different botanical families, such as Cor a 9 and Jug r 4. Further studies are needed to identify the specific epitopes of these potentially allergenic proteins.

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