ORIGINAL ARTICLE

Immunoglobulin E Cross-Reactivity Between Lupine Conglutins and Peanut Allergens in Serum of Lupine-Allergic Individuals

M M BW Dooper,1 C Plassen,1 L Holden,1 H Lindvik,2 CK Fæste1

1 National Veterinary Institute, Oslo, Norway
2 Voksentoppen Children’s Center for Asthma and Allergy, Oslo, Norway
M M BW Dooper and CK Fæste contributed equally to the study

Abstract

Background: Lupine is used increasingly in food products. The development of lupine allergy in peanut-allergic patients is believed to occur as a result of cross-reactivity between lupine and peanut proteins.

Objective: To investigate the degree of immunoglobulin (Ig) E cross-reactivity between allergens in lupine and peanut.

Methods: We investigated IgE cross-reactivity between lupine α- , ß- , γ- , and δ-conglutins and the major peanut allergens Ara h 1, Ara h 2 and Ara h 3 using enzyme-linked immunosorbent assay with sera from patients with coexisting peanut and lupine allergy.

Results: Peanut proteins inhibited IgE binding towards α- conglutins, δ-conglutins, and, to a lesser degree, ß-conglutins, while no IgE cross-reactivity with γ-conglutin was observed. Ara h 2 most potently inhibited IgE binding to lupine and δ-conglutins, while Ara h 1 most potently cross-reacted with ß-conglutin. Ara h 3 was apparently not involved in these mechanisms.

Conclusions: The present study reveals IgE cross-reactivity between the 2S albumins Ara h 2 and δ-conglutin, and the 7S vicilin-like Ara h 1 and β-conglutin, which are possibly based on homologies between phylogenetically related proteins. Ara h 2 was the most potent inhibitor of IgE binding to lupine conglutins.

Key words: IgE. Conglutinin. Cross-reactivity. Lupine. Peanut.

Resumen

Antecedentes: El lupino es utilizado crecientemente en productos alimentarios. Se cree que el desarrollo de la alergia a lupino en pacientes alérgicos a cacahuete ocurre como resultado de la reactividad cruzada entre las proteínas del lupino y cacahuete.

Objetivo: Investigar el grado de reactividad cruzada de la inmunoglobulina (Ig) E entre los alérgenos del lupino y cacahuete.

Métodos: Investigamos la reactividad cruzada IgE entre la α- , ß- , γ- y δ-conglutinas de lupino y los alérgenos mayores del cacahuete Ara h 1, Ara h 2 y Ara h 3 empleando el ensayo de enzimoinmunoanálisis adsorbente con sueros de pacientes con coexistencia de alergia a cacahuete y lupino.

Resultados: Las proteínas del cacahuete inhibieron la unión IgE frente a α-conglomerina, δ-conglomerina, γ, en menor medida, ß-conglomerina, mientras que no se observó reactividad cruzada IgE entre el lupino y la γ-conglomerina. El Ara h 2 inhibió en mayor potencia la unión IgE a lupino y δ-conglomerina, mientras que el Ara h 1 reaccionó de forma cruzada con mayor potencia con la ß-conglomerina. El Ara h 3 no estaba implicado aparentemente en estos mecanismos.

Conclusiones: El presente estudio revela reactividad cruzada IgE entre las 2S albúminas Ara h 2 y δ-conglomerina, y la 7S vicilina Ara h 1 y ß-conglomerina, que posiblemente se basen en la homología entre proteínas filogenéticamente relacionadas. El Ara h 2 fue el inhibidor más potente de la unión IgE a las conglutinas del lupino.

Introduction

Sweet lupine flour from 4 agriculturally grown species (Lupinus albus, Lupinus angustifolius, Lupinus luteus, Lupinus mutabilis) is increasingly used in baked goods and health foods in Europe. In parallel, the number of food-induced episodes of lupine allergy has increased [1]. Although lupine allergy has been reported to occur by primary sensitization [2-4], it is more often the consequence of cross-reactivity in patients with an existing peanut allergy [5-7]. Moreover, several studies indicate that the risk of clinically manifest cross-reactivity in peanut-allergic patients is rather high after exposure to lupine [5,8,9]. Cross-reactivity is generally a result of homologous epitopes in proteins with conserved amino acid sequences or steric domains [10].

Both lupine and peanut (Arachis hypogaea) belong to the Leguminosae plant family. Like other legumes, lupine seeds contain storage proteins that belong to the cupin and prolamin superfamilies [11]. Both α-conglutin from the legumin-like 11S globulin family and β-conglutin from the vicilin-like 7S family include 2 cupin domains. Another cupin is γ-conglutin, a basic protein with homogeneous tetramers consisting of 2 different disulfide-linked monomers [12]. In contrast, δ-conglutin is a 2S albumin containing 2 disulfide-linked proteins with the typical cysteine-rich prolamin structure [13].

The molecular properties of peanut proteins have been studied extensively. Several kernel proteins have been identified as allergens and have been classified as Ara h 1 to Ara h 9 [14-16]. In studies assessing the relevance of individual peanut allergens, Ara h 1, Ara h 2, and Ara h 3 were identified as major allergens [16,17]. Ara h 1 and Ara h 3 and 4 belong to the 7S vicilin-like and 11S legumin-like families, respectively. Ara h 2 is a 2S albumin.

Given the increase in the number of cases of lupine allergy and the assumed frequency of cross-reactivity with peanut, the involvement of individual major lupine and peanut allergens in these processes is of interest. In the present study, we add to our previous results on lupine conglutins [18] by using fractionated lupine and peanut allergens in inhibition experiments with the sera of lupine-allergic patients.

Materials and Methods

Patients

Six patients with positive skin prick test (SPT) and specific serum IgE results to lupine flour (≥2 kU/L, ImmunoCAP, Phadia AB, Uppsala, Sweden) were recruited at Voksentoppen Children’s Center for Asthma and Allergy (Rikshospitalet University Hospital, Oslo, Norway). All patients reacted positively to an open oral or double-blind placebo-controlled food challenge with lupine-containing pancakes or muffins (Table). Their clinical characteristics and medical histories have been published elsewhere in more detail [18]. Blood was obtained 2 weeks after challenge. Informed consent was obtained from all patients before they were included in the study, which was approved by the Regional Committee for Medical Research Ethics, Southern Norway.

<table>
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<tr>
<th>Patient</th>
<th>Age, y</th>
<th>Sex</th>
<th>Total IgE (kU/L)</th>
<th>Specific IgE Lupin (kU/L)</th>
<th>Specific IgE Peanut (kU/L)</th>
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<td>21.2</td>
<td>&gt;100</td>
</tr>
</tbody>
</table>

Abbreviations: f, female; Ig, immunoglobulin; M, male.

Materials

Lupine seeds from Langustifolius were kindly provided by Frank & Miedendorf Food Products (Twello, The Netherlands). Unprocessed peanuts (Arachis hypogaea, Jumbo runners variety) were a gift from Kims (Toten, Norway). Human serum albumin (HSA) (Calbiochem, La Jolla, California, USA) and β-lactoglobulin (Sigma-Aldrich, Steinheim, Germany) were used as negative control proteins.

Protein Extraction From Lupine and Peanut

Lupine and peanut protein extracts were prepared by a previously described method for the isolation of lupine globulins [19]. In short, homogenized lupine seeds or peanuts (2 g) were defatted with hexane (10 mL) by vigorous vortexing (5 min at room temperature) and centrifuged, and the pellets were air-dried. Lupine and peanut proteins were dissolved in 50 and 20 mM Tris, pH 7.5, respectively, by vigorous vortexing (5 min at room temperature). The samples were centrifuged at 6000g for 5 minutes at room temperature. The aqueous layers were collected and centrifuged at 39 200g for 20 min at room temperature. The supernatants were filtered through a low protein binding filter unit (0.45 μm, Millipore, Molsheim, France). Protein extracts were stored in aliquots at −20°C.

Lupine Protein Fractions

Procedures for the isolation of globulins and the generation of protein fractions containing α- or β-conglutins have been described elsewhere [19]. γ-Conglutin was isolated by soaking Langustifolius seeds in deionized water for 16 hours and heating the solution at 60°C for 4 hours. Precipitated γ-conglutin was obtained by centrifugation and dissolved in 50 mM Tris buffer, pH 7.5. δ-Conglutin was isolated as described elsewhere [20]. In short, defatted flour from milled Langustifolius seeds was extracted in deionized water, pH 8.0-8.5, and centrifuged, and the proteins in the supernatant were precipitated by lowering the pH to 4.5. After centrifugation, the pellet (containing α-, β- and δ-conglutins) was resuspended in a 0.1-M calcium carbonate buffer, pH 4.0, containing 0.4 M sodium chloride and 40% ethanol. α- and β-conglutin were removed by centrifugation and δ-conglutin was collected by cold precipitation.
Peanut Protein Fractions

The peanut extract was fractionated into fractions containing Ara h 1, Ara h 2, and Ara h 3 by anion exchange chromatography on a Resource Q column (GE Healthcare, Uppsala, Sweden) using fast protein liquid chromatography (FPLC; GE Healthcare, Uppsala, Sweden) according to a published method [21]. In short, the column was equilibrated with 50 mM Tris-HCl (pH 7.5) loaded with approximately 5 mg of the peanut protein, and eluted with a continuous, linear gradient of NaCl (0 to 1 M). The mobile phase flow was 1.5 mL/min and the detection wavelength was 280 nm.

Total Protein Determination

Total protein concentrations were determined according to the Lowry method (DC protein assay; Bio-Rad, Hercules, California, USA) using bovine serum albumin as a standard.

Gel Electrophoresis and Immunoblotting

Protein extracts (5-10 µg) were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions using 4-12% Bis-Tris pre-cast gels (Invitrogen, Carlsbad, California, USA). Gels were either stained with SimplyBlue Safestain (Invitrogen) or the separated proteins were electrophoretically transferred from the gel onto nitrocellulose membranes. Membranes were blocked with Tris-buffered saline containing 0.05% Tween 20 and 3% human serum albumin (HSA) for 1 hour, following incubation with patient serum (diluted 1:20) overnight at 4°C. For detection of IgE, membranes were developed with a signal-enhancing 2-step procedure: incubation with rabbit anti-human IgE (1:6000; DakoCytomation, Glostrup, Denmark) was followed by a second incubation with goat anti-rabbit IgG horseradish peroxidase (HRP) conjugate (1:5000, DakoCytomation), each for 1 hour. IgE binding was finally revealed with 3,3',5,5'-tetramethylbenzidine (TMB) substrate solution (Zymed, San Francisco, California, USA). All washing and incubation steps were performed at room temperature with gentle shaking.

Indirect ELISA

High-binding 96-well microtiter plates (Corning Inc, Corning, New York, USA) were coated overnight at 4°C with 0.05 M carbonate-bicarbonate buffer, pH 9.6 (Sigma-Aldrich), containing 4 µg/mL protein extract (100 µL/well). Plates were washed with phosphate-buffered saline (PBS), pH 7.6, containing 0.05% Tween 20 (PBS-T) and blocked with 1% HSA in PBS-T (200 µL/well). Test and control sera, diluted with 1% HSA in PBS-T (1:25, 50 µL/well), were incubated in triplicate for 1 hour at room temperature under gentle shaking. For detection of IgE, wells were incubated with rabbit anti-human IgE (1:15 000, 100 µL/well, DakoCytomation) followed by incubation with goat anti-rabbit IgG HRP conjugate (1:10 000, 100 µL/well, Zymed). Binding was finally revealed with K-Blue TMB substrate solution (75 µL/well, Neogen, Lexington, Kentucky, USA), the reaction was stopped with 2 M H₂SO₄ (50 µL/well), and optical density (OD) was read at 450 nm.

Inhibition ELISA

All sera were assessed by inhibition ELISA with total peanut protein extract. However, only sera reaching an OD 450 nm ≥0.35 in the indirect ELISA were included in inhibition assays with the fractionated peanut proteins. Inhibition experiments were performed repeatedly by coating 96-well microtiter plates with 4 µg/mL lupine protein extract or lupine protein fractions and blocking as described above. Diluted sera were pre-incubated in triplicate with peanut protein extract or peanut protein fractions at final concentrations of 0.2, 1, 5, 25, 125, and 625 µg/mL for 2 hours at room temperature under gentle shaking. Subsequently, the inhibitor mixtures (including sera with no inhibitor as zero-inhibition control) were transferred to coated and blocked wells (100 µL/well) and incubated for 1 hour. The assay was completed as described above. Percentage inhibition was calculated using the following formula:

\[
\% \text{ inhibition} = 100 - \left( \frac{\text{OD}_{450} \text{of serum without inhibitor}}{\text{OD}_{450} \text{of serum with inhibitor}} \right) \times 100
\]

Results

IgE Binding Profiles to Lupin Conglutins

Sera from 6 lupine-allergic patients (Table 1) were used to study IgE binding to total lupine protein and the individual conglutins by indirect ELISA (Figure 1, left column). Sera showed considerable variation in affinity to the individual conglutins. Using OD 450 nm >0.35 as a cutoff value, IgE was observed to bind to α-conglutin in 5 sera, to δ-conglutin in 4 sera, and to β-conglutin in 1 serum, whereas no patients showed considerable binding to γ-conglutin. A comparison of affinity to the different conglutins in the respective sera revealed that binding was highest to δ-conglutin in patients 1, 2, and 3, while binding was highest to α-conglutin in patients 4, 5, and 6. In patient 5, IgE binding to total lupine extract was marginally stronger than to the individual allergens for which binding intensities were all under the set cutoff value. Therefore, patient 5 was omitted from the allergen cross-reactivity analyses.

Inhibition of IgE Binding to Lupine Conglutins by Peanut Proteins

Inhibition of IgE-binding to lupine extract and the 4 lupine protein fractions by total peanut extract resulted in individual inhibition patterns for the 6 patients (Figure 1, right column). With the highest peanut concentration, IgE binding to lupine proteins decreased by 50-70% for patients 1, 3, 5, and 6, by 80% for patient 2, and only 10% for patient 4. IgE binding to δ-conglutin was inhibited most effectively in the sera of patients 1 (80%), 2 (95%), and 3 (80%). In these patients, IgE binding to α-conglutin was also inhibited strongly (70-85%). In patient 5, however, IgE binding to β-conglutin was inhibited most (75%), and patient 6 demonstrated inhibition in the range of 40-60% for all fractions. Patient 4 showed very little peanut-lupine cross-reactivity (overall
Figure 1. IgE responses (means of triplicate measurements; standard deviations are indicated) against total lupine protein extract (Lupine) and α-, β-, γ- and δ-conglutins in sera from all 6 patients (P1-P6) determined by indirect ELISA (left column). Inhibition of IgE binding to lupine protein extract and α-, β-, γ- and δ-conglutins in patient sera (P1-P6) by peanut protein extract determined by inhibition ELISA (right column). The peanut concentrations (µg/mL) are shown on top of the bars. ELISA indicates enzyme-linked immunosorbent assay; Ig, immunoglobulin.
inhibition of about 10%), and was excluded from further inhibition experiments.

Profiles of IgE Binding to Peanut Allergens

Peanut proteins were fractionated by FPLC into 3 fractions containing the major peanut allergens Ara h 1, Ara h 2, and Ara h 3 (Figure 2). The dominant protein bands were excised from the gel, digested with trypsin and confirmed by proteomic analysis using matrix-assisted laser-desorption ionization time-of-flight mass chromatography (MALDI-ToF-MS) (Bruker Daltonik, Bremen, Germany) and the Mascot search engine (Matrix Science, Boston, Massachusetts, USA) for comparison with the NCBInr-protein database (data not shown). Ara h 1 migrated as a single band of 63 kDa containing the homotrimeric subunits of the 7S vicilin-like protein. The 2S albumin Ara h 2 generated a band duplet at 18 kDa and 20 kDa, whereas 3 subunits of the 11S legumin-like Ara h 3 were located at 40 kDa, 44 kDa, and 45 kDa, and its basic polypeptide at 20 kDa. However, additional protein bands of weak intensity were visible on the gel, and these were either degradation products of the major bands or indicated incomplete fractionation.

IgE binding to peanut allergens was investigated in the sera from all 6 patients using indirect ELISA (Figure 3a). All sera except that of patient 4 showed binding to the total peanut protein extract and to the individual allergens Ara h 1, Ara h 2, and Ara h 3. Patients 1 and 2 demonstrated almost equal affinity for all 3 allergens, while patients 3, 5, and 6 bound most to Ara h 2, somewhat less to Ara h 1, and least to Ara h 3.

The results obtained by immunoblotting (Figure 3b) using denatured proteins were similar, although they showed more differentiated binding patterns. IgE binding to total peanut protein was relatively weak or nearly imperceptible (patients 4 and 6). Patients 1, 2, and 3 bound stronger to Ara h 1 and Ara h 2 than to Ara h 3, whereas patient 6 reacted almost only with Ara h 2. Patient 5 showed affinity to all 3 peanut fractions, as did patient 4, albeit in a much reduced way, yet clearly discernible by immunoblotting.

Inhibition of IgE Binding to α-Conglutin by Peanut Allergens

In initial experiments using Ara h 1, Ara h 2, and Ara h 3 concentrations in the range of 100 to 0.1 µg/mL and the sera from patients 1, 2, 3, and 6, saturation was observed at 10 µg/mL (data not shown). Therefore, subsequent experiments were performed in the concentration range 5 to 0.0016 µg/mL (Figure 4). In patients 1, 2, and 3, IgE-binding to α-conglutin was inhibited at a maximum of approximately 70% by the 3 peanut allergens. However, the curve progressions looked different. The strongest inhibition at lower concentrations was observed for Ara h 2. The next strongest was observed for Ara h 1, whereas Ara h 3 showed inhibition activity only at the highest concentrations. Patient 6 did not show significant α-conglutin IgE-binding inhibition, reaching 30% only with the highest peanut allergen concentrations tested.
Figure 3. A, IgE responses (means of triplicate measurements; standard deviations are indicated) against total peanut protein extract and Ara h 1, Ara h 2 and Ara h 3 in sera from all 6 patients (P1-P6) determined by indirect ELISA. B, Immunoblot using sera from all 6 patients (P1-P6) on total peanut protein extract (P) and the major peanut allergens Ara h 1, Ara h 2, and Ara h 3. Protein sizes (kDa) by SeeBluPlus2 pre-stained molecular weight marker are indicated on the right side of the gel. ELISA indicates enzyme-linked immunosorbent assay; Ig, immunoglobulin.
**Figure 4.** Inhibition of IgE binding to α-conglutin by Ara h 1, Ara h 2 or Ara h 3 in patient sera P1, P2, P3 and P6 determined by inhibition ELISA. ELISA indicates enzyme-linked immunosorbent assay; Ig, immunoglobulin.

* Control wells did not contain any inhibitor proteins.

**Figure 5.** Inhibition of IgE binding to β-conglutin by Ara h 1, Ara h 2 or Ara h 3 in patient serum P3 determined by inhibition ELISA. ELISA indicates enzyme-linked immunosorbent assay; Ig, immunoglobulin.

* Control wells did not contain any inhibitor proteins.

**Figure 6.** Inhibition of IgE binding to δ-conglutin by Ara h 1, Ara h 2 or Ara h 3 in patient sera P1, P2, and P3 determined by inhibition ELISA. ELISA indicates enzyme-linked immunosorbent assay; Ig, immunoglobulin.

* Control wells did not contain any inhibitor proteins.
Discussion

Patients with peanut allergy are often sensitized to several legume species [22], and lupine allergy in particular has been clinically confirmed in several studies [5,7,8,23]. In the present study, IgE cross-reactivity between the main lupine and peanut allergens was studied by continuing a previous IgE profiling study of 6 patients with lupine and peanut allergy [18]. In this patient cohort, the legumin-like α-conglutin, the vicilin-like β-conglutin, and the 2S albumin-related δ-conglutin demonstrated higher allergenicity than the basic γ-conglutin. These findings are consistent with those of one of the first studies on lupine allergy [5], where a 43-kDa band and a 13-kDa band, probably representing α-conglutin and δ-conglutin, respectively, showed the highest IgE binding intensity. However, the present and other studies indicate that proteins in the 38-kDa bands [5,6,24] and the 65-kDa bands [4] in β-conglutin might also have considerable allergenic potential.

In the present study, IgE binding to lupin conglutins was inhibited by 50-70% by total peanut extract, which is consistent with the results of previous inhibition studies [25]. The lupine proteins α-conglutin and δ-conglutin showed the highest allergenicity and were inhibited most effectively by peanut in nearly all the sera. The exception was patient 4, whose sera did not show relevant IgE cross-reactivity between peanut and lupine. This patient was also highly allergic to hazelnut (>100 kUa/L) and showed an unusual binding profile to lupine, reacting only to α-conglutin bands ranging from 20 kDa to 43 kDa [18]. Binding to peanut was also limited, showing only weak affinity to the basic 20-kDa protein of Ara h 3, the Ara h 2 duplet at 18 kDa and 20 kDa, and the 63-kDa band of Ara h 1. Patient 4 probably reacted to lupine and peanut due to cross-sensitization by pre-existing hazelnut allergy. Peanuts and tree nuts have been shown to contain homologous IgE-binding epitopes, as is the case with the 11S legumin-like hazelnut allergens Cor a 9 and Ara h 3 [26].

Evaluation of serum affinity to fractionated peanut allergens under native and denaturing conditions in ELISA and immunoblotting, respectively, revealed that IgE binding was strongest to Ara h 2, followed by Ara h 1, and finally Ara h 3 in patients 1, 2, 3, 5, and 6. Several studies comparing the potency of the 3 major peanut allergens using skin prick tests, the basophil activation test, or IgE-binding emphasize the importance of Ara h 1 and Ara h 2 [16,17], whereas Ara h 3 is considered to be less allergenic because of its instability [21,27]. Therefore, the observed lack of cross-reactivity between Ara h 3 and lupine conglutins might be a consequence of sample preparation.

Immunoblotting enabled the individual IgE binding patterns to be studied for each patient. The allergenicity of the major peanut bands was detectable, as was binding to several bands of lower molecular weights, probably resulting from degradation.

The combined results from the 3 peanut allergen/lupin conglutin cross-inhibition experiments with selected sera show that Ara h 2 was the most potent inhibitor of IgE binding to α- and δ-conglutin, whereas Ara h 1 was the most potent inhibitor of binding to β-conglutin. For the 2S albumin pair Ara h 2/δ-conglutin and the 7S vicilin-like pair Ara h 1/β-conglutin, cross-reactivity probably resulted from homologous epitopes in the amino acid sequences. Additionally, Ara h 2 inhibited IgE-binding to α-conglutin at relatively low concentrations, which may result from shared epitopes or potential carry-overs in the peanut protein fractions. The expected cross-reactivity between the 11S legumins Ara h 3 and α-conglutin was only observed at high Ara h 3 levels. Ara h 1 did not significantly inhibit binding to α-conglutin, which supports the hypothesis of dissimilar IgE binding epitopes in Ara h 3 and Ara h 1 [27]. However, these results should be verified by including a larger number of patients and appropriate control groups. The clinical relevance of the findings should be confirmed by oral challenge studies with pure recombinant peanut allergens.

In short, the incidence of peanut-lupine cross-allergy is rising [5-8], and cross-sensitization between the major peanut and lupine allergens seems to be relatively frequent [5,8]. The results of the present study indicate that lupine β- and δ-conglutins cross-react with the peanut proteins Ara h 1 and Ara h 2, respectively. In addition, cross-reactivity between α-conglutin and Ara h 2 was observed, whereas γ-conglutin and Ara h 3 seemed to be involved in lupine-peanut cross-reactivity to a lesser degree.

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Christiane K. Faeste
National Veterinary Institute
Postboks 750 Sentrum, N-0106 Oslo
Norway
E-mail: christiane.faste@vetinst.no