Cloning and characterization of profilin (Pru du 4), a cross-reactive almond (*Prunus dulcis*) allergen

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Background: The identity of allergenic almond proteins is incomplete.
Objective: Our objective was to characterize patient IgE reactivity to a recombinant and corresponding native almond allergen.
Methods: An almond cDNA library was screened with sera from patients with allergy for IgE binding proteins. Two reactive clones were sequenced, and 1 was expressed. The expressed recombinant allergen and its native counterpart (purified from unprocessed almond flour) were assayed by 1-dimensional and 2-dimensional gel electrophoresis, dot blot, and ELISA, and screened for cross-reactivity with grass profilin.
Results: The 2 selected clones encoded profilin (designated Pru du 4) sequences that differed by 2 silent mutations. By dot-blot analyses, 6 of 18 patient sera (33%) reacted with the recombinant Pru du 4 protein, and 8 of 18 (44%) reacted with the native form. ELISA results were similar. Almond and ryegrass profilins were mutually inhibitable. Two-dimensional immunoblotting revealed the presence of more than 1 native almond profilin isoform. The strength of reactivity of some patients' serum IgE differed markedly between assays and between native and recombinant profilins.
Conclusion: Almond nut profilin is an IgE-binding food protein that is cross-reactive with grass pollen profilin and is susceptible to denaturation, resulting in variable reactivity between assay types and between patients.
Clinical implications: Serum IgE of nearly half of the tested patients with almond allergy reacts with almond nut profilin. Because most patients also had pollinosis, the well-known cross-reactivity between pollen and food profilins could account for this pattern of reactivity. (J Allergy Clin Immunol 2006;118:915-22.)

Key words: *Allergen, almond allergy, profilin, food allergy, recombinant allergen, pan-allergen*

Tree nuts are one of the ''big eight'' allergenic foods[1] and include cashew nut, almond, pistachio, walnut, hazelnut, and Brazil nut (see review[2]). Allergic reactions to tree nuts range from mild oral symptoms, often seen in the pollen-food syndrome, to life-threatening anaphylaxis (see review[3]). The prevalence of allergy to tree nuts in the United States is estimated at 0.2% in young children and 0.5% in adults.[4] A variety of allergens from cashew nut, walnut, hazelnut, and Brazil nut have been isolated, cloned, and characterized.[2] Thus far, several native almond allergens have been identified and characterized to varying degrees, including members of the legumin,[5-7] vicilin,[8] and 2S albumin[8] families, but none has been cloned and tested for reactivity with almond-allergic sera.

Two well-studied low-molecular-weight tree nut allergens are 2S albumin and lipid transfer protein (see reviews[2,9,10]), which have molecular weights in the range of 12 to 14 kd and 9 to 10 kd, respectively. Profilin is another small (12-16 kd), ubiquitous eukaryotic protein that is recognized as allergenic in pollens, vegetables, and fruits. Profilin can also be allergenic in seeds, nuts, and latex, often as a consequence of cross-reactivity with IgE directed to pollen profilin.[9-11] Profilins are best known for binding to monomeric actin (G-actin) and regulating the polymerization of actin into filaments (see review[12]). Profilin sequence homology is high (70% to 85%) among plants, and, in some plants, multiple isoforms have been identified.[13] The emerging evidence suggests that there is some degree of tissue specificity to the expression of profilin isoforms, which is reflected in their pattern of sequence homology.[12-15] Thus, the fruit and seed profilin sequences cluster together, whereas the pollen isoforms are composed of several disparate clusters.[15] Of the various plant food allergenic protein families, profilins rank third behind the prolamin and Bet v 1 families with respect to the number of allergens thus far identified.[16]

Many plant profilins exhibit significant cross-reactivity, apparently because of conserved amino acid sequences and shared IgE-reactive epitopes, prompting the designation of profilins as pan-allergens.[17-21] Evidence for both linear and conformational epitopes has been reported.[22-24] The

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Abbreviations used
2-DE: Two-dimensional electrophoresis
DBPCFC: Double-blind, placebo-controlled food challenge
MBP: Maltose binding protein
nRGPP: Ryegrass pollen profilin

cosensitization of patients to hazelnut pollen and nut profilins was first verified with the aid of cross-reactive rabbit anticelery profilin antibodies.[17] Thus it is not surprising that profilin from 1 plant source can cross-sensitize an individual to the tissues of multiple plant species, and pollen profilins can sensitize individuals to food profilins.[15,25]

Here we purify native almond profilin, express a recombinant form of the profilin, demonstrate reactivity with serum IgE from patients with almond allergy, and show cross-reactivity with ryegrass pollen profilin.

METHODS

Human sera

Blood samples were drawn, after informed consent, from patients with self-reported allergic reactions to almonds. The reactions were not confirmed by oral challenges. Eleven of the 18 patients reported potentially life-threatening reactions to almonds with symptoms of bronchospasm, hypotension, or throat swelling/laryngoedema. Seven patients reported more mild reactions, particularly oral angioedema or pruritus to raw but not cooked almonds, and 3 of the 7 reported reactions to multiple fresh fruits. The latter 7 patients were included, even though food challenges were not performed, because of the known association between IgE anti-profilin and the pollen-food syndrome.[26] The 3 patients with self-reported allergy to multiple fresh fruits also reported pollinosis, as did 15 of the 18 overall.

The sera were stored at −70°C. The study was approved by the institutional review board of the University of California, Davis. The presence of almond-reactive IgE was confirmed by Phadia CAP-FEIA assay (Phadia, Inc, Uppsala, Sweden), modified RAST, or immunoblot. Patient characteristics are shown in this article's Table E1 in the Online Repository at www.jacionline.org.

Almond extract and native profilin

Defatted Nonpareil almond flour, and an aqueous protein extract thereof, were prepared as previously described.[27] Almond profilin was purified by affinity chromatography on poly-(L-proline)–Sepharose 4B (GE Healthcare, Uppsala, Sweden).[28-30] Briefly, 100 mg poly(L-proline) of molecular weight 1000 to 10,000 (Sigma-Aldrich, St Louis, Mo) was coupled to 15 mL CNBr-activated Sepharose 4B.[28-30] The poly-(L-proline)–Sepharose 4B was packed into a glass column (1.6 × 7.5 cm), and almond extract (from 20 g defatted almond flour) was passed through the column at 4°C. The column was first eluted by using extraction buffer containing 3 mol/L urea to remove profilactin (profilin-actin) complexes, followed by elution of profilin with extraction buffer containing 7 mol/L urea. The 7 mol/L urea fraction was dialyzed against distilled water at 4°C, and concentrated by using Centricon-3 (Millipore, Billerica, Mass) (molecular weight cutoff, 3 kd) concentrators. A total of ~0.5 mg purified profilin was recovered. Protein purity was verified by SDS-PAGE (data not shown). Ryegrass pollen profilin was similarly prepared from 4.0 g ryegrass pollen.

Recombinant profilin production

Almond cDNA library construction was performed by using mRNA derived from immature almond kernels as previously described in detail for cashew library generation.[31,32] The library was amplified and screened with sera from patients with almond allergy. IgE-reactive clones were picked, plaque-purified, and stored at 4°C.

Inserts from the selected phage clones were amplified with M13 forward and reverse primers by PCR and sequenced as previously described.[31] Similarity searches and alignments of deduced amino acid sequences were performed on Genetics Computer Group software using the BLAST 2.0 program (www.ncbi.nlm.nih.gov/BLAST/). Leader peptide prediction was performed by using the SignalP V1.1 World Wide Web Prediction Server (www.cbs.dtu.dk/services/SignalP/).

As described in detail (for cashew nut),[31] the cDNA coding sequences were ligated into a maltose-binding protein (MBP) fusion expression vector pMAL-c2 (New England BioLabs Inc, Beverly, Mass), into which a thrombin cleavage site had been engineered. The cDNA insert was produced by PCR amplification of the portion of the sequence extending from the presumed first codon (following the leader peptide) through to the last codon before the stop codon using the following primers: 5′gtcctctagaatgtcgtggcagcagtacg3′ (forward) and 5′gcttctgcagttacagaccctgctcgataag3′ (reverse).

Competent *Escherichia coli* BL21 (DE3) cells (Novagen Inc, Madison, Wis) were transformed and induced with isopropyl-D-thiogalactopyranoside. The cells were harvested and lysed with mild sonication. The lysate supernatant was passed over an amylose affinity column and the fusion protein eluted with 10 mmol/L maltose. Uncleaved recombinant protein was concentrated and either stored (briefly) at 4°C until use or frozen at −70°C. The fusion protein was cleaved with thrombin (Sigma-Aldrich) at 1 mg fusion protein per unit of thrombin. Cleaved and purified recombinant (r)Pru du 4 was used in the dot-blot and ELISA assays.

One-dimensional electrophoresis (SDS-PAGE) and immunoblotting

Aqueous almond extract samples were boiled in reducing sample buffer and subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (10-15 μg/4 mm well width) as previously described.[31] Proteins were transferred onto 0.2 μm Immobilon-P^{SQ} nitrocellulose transfer membrane (Millipore Corp, Billerica, Mass) using a Mini Trans-Blot Transfer Cell (BioRad Laboratories, Inc, Hercules, Calif). Immunoblotting to test for patient serum IgE reactivity was performed as previously described.[31] Horseradish peroxidase mouse-antihuman IgE (Invitrogen/Zymed Laboratories Inc, Carlsbad, Calif), diluted 1:15,000 in 5% nonfat dry milk in Tris-buffered saline with Tween 20, was used as the secondary enzyme-labeled antibody. Reactive bands were visualized by using SuperSignal West Femto Maximum Sensitivity Substrate (Pierce Biotechnology Inc, Rockford, Ill) and subsequent exposure to Kodak X-OMAT X-ray film (Kodak, New York, NY) for 10 seconds to 2 minute.

Two-dimensional gel electrophoresis, immunoblotting, and N-terminal sequencing

Two-dimensional gel electrophoresis was performed by using ZOOM IPGRunner System (Invitrogen Life Technologies, Carlsbad, Calif). The almond extract samples (300 μg in 10 μL) were diluted in 155 μL sample rehydration buffer (8 mol/L urea, 2% CHAPS, 0.5% (vol/vol) ZOOM Carrier Ampholytes (pH 3-10) nonlinear, 0.002% bromophenol blue, 20 mmol/L dithiothreitol) and were then applied to immobilized pH gradient gel strip (pH 3-10 NL, Invitrogen Life Technologies) incubated o/n at RT. Isoelectric focusing was performed by using the ZOOM IPGRunner Mini-cell (Invitrogen Life Technologies) as described by the manufacturer.

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