

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;117:1016-1028.)

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

Tree nuts are one of the "big eight" allergenic foods¹ and include cashew nut, almond, pistachio, walnut, hazelnut, and Brazil nut (see review²). Allergic reactions to tree nuts range from mild oral symptoms, often seen in the pollen-food syndrome, to life-threatening anaphylaxis (see review³). 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 albumin⁸ 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 review¹²). 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

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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

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 co-sensitization of patients to hazelnut pollen and nut profilins was first verified with the aid of cross-reactive rabbit antiserum to 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)-Sephacrose 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)-Sephacrose 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'gtcctctagaatgtcgtggcagcagctacg3' (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 $\mu\text{g}/4$ mm well width) as previously described.³¹ Proteins were transferred onto 0.2 μm Immobilon-P[®] 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 minutes.

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

Two-dimensional gel electrophoresis was performed by using ZOOM IPGRRunner 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.

The second dimension electrophoresis was performed by using Novex 4–20%Tris-Glycine gels (Invitrogen Life Technologies) in an Xcell SureLock Mini-Cell (Invitrogen Life Technologies) as described by the manufacturer. The 2-dimensional electrophoresis (2-DE) gel was then transferred to 0.2 μ m Immobilon-P^{SO} membrane, and the profilin isoforms were detected by using rabbit anti-maize profilin antiserum (anti-ZMPro5, generously provided by Dr Chris J. Staiger, Purdue University, West Lafayette, Ind) using the Enhanced Chemiluminescence Plus kit (GE Healthcare, Piscataway, NJ) and subsequent exposure to Biomax X-ray film for 10 seconds to 2 minute (Kodak, Rochester, NY). In addition, the gel was either stained with colloidal Coomassie stain (Sigma-Aldrich) or transferred to ProBlot PVDF membrane (Applied Biosystems, Foster City, Calif) for N-terminal sequence analysis using a ABI 477A Protein Sequencing System (Applied Biosystems).

Dot-blot analysis

Defined quantities of almond extract and purified native and recombinant profilin were applied to nitrocellulose membrane using a 96-well Bio-Dot microfiltration apparatus (BioRad Laboratories, Hercules, Calif). BSA served as a negative control. The membranes were then cut into strips, each of which contained the desired sets of protein dots, and probed as described. For inhibition dot blots, the inhibitor (nPru du 4 or native ryegrass pollen profilin nRGPP) concentration used was 10 μ g/mL.

ELISA

Almond extract (40 μ g/mL), rPru du 4 (20 μ g/mL), and native almond profilin (20 μ g/mL) diluted in coating buffer (0.1 mol/L carbonate-bicarbonate buffer, pH 9.6) were coated (50 μ L/well) onto the wells of 96-microwell round bottom polyvinyl microtiter ELISA plates (Seracluster “U” Vinyl, #2797; Costar, Cambridge, Mass) at 4°C o/n. Plates were washed 3 times between steps with PBS/0.1% Tween 20. Blocking was performed by using 5% nonfat dry milk in PBS/0.1% Tween-20 at 37°C for 1 hour. Sera from patients with almond allergy (listed in this article’s Table E1 in the Online Repository at www.jacionline.org; diluted 1:10, 50 μ L/well) were added and incubated o/n at 4°C. After washing, bound IgE was reacted with horseradish peroxidase-conjugated mouse anti-human IgE (Zymed Laboratories Inc) at a dilution of 1:1000 and incubated for 1 hour at 37°C. IgE reactivity was detected by colorimetric reaction using *o*-phenylenediamine (Zymed Laboratories Inc) and H₂O₂ as substrate. OD was measured in a KC4 v2.5 ELISA reader (Bio-Tek Instruments Inc, Winooski, Vt) at 495 nm.

RESULTS

cDNA library screening and gene characterization

A screening of the cDNA library with a pool of human almond-allergic sera (patients #7, 8, and 34) yielded 2 clones (a 924-bp fragment, clone 1 [AY081850], and a 742-bp fragment, clone 2 [AY081852]), which, on translation, had identical 131 amino acid coding sequences but differed by 2 silent substitutions (A $\leftrightarrow$ T and A $\leftrightarrow$ G at positions 310 and 322, respectively; see this article’s Fig E1 in the Online Repository at www.jacionline.org).

When analyzed by a GenBank BLAST search, the sequence was homologous with plant profilins. Consistent with the allergen nomenclature,³³ the allergen was designated Pru du 4. The almond profilin polypeptide had a calculated molecular mass of 14,061 d, a pI of 4.67, and an A_{280nm} at 10 mg/mL of 11.8.

Protein sequence homology comparisons

This article’s Table E2 in the Online Repository at www.jacionline.org lists the proteins having the highest degrees of identity and similarity to almond profilin and represents products from a diversity of tree and plant species. Those in the same family, such as peach and sweet cherry, have very high degrees of similarity and identity (98% to 99%). Even those more distantly related species, such as muskmelon and orange, displayed identity and similarity of $\geq$ 97% and 98%, respectively, to almond profilin. Many other plant profilins displayed $\geq$ 80% similarity and $\geq$ 90% identity. Almost all of the plant profilins listed in this article’s Table E2 in the Online Repository at www.jacionline.org have been previously identified as allergens.

Production of recombinant profilin

For immunologic characterization, we cloned and expressed clone 1 almond profilin in *E. coli*. The resulting ~55 kd almond profilin fusion protein was affinity purified and digested with thrombin to yield a polypeptide of ~14 kd (ie, the expected molecular mass of profilin) as well as the 41 kd MBP polypeptide.

Purification of native profilin

Although profilin is ubiquitous, it is present in minute amounts in plant tissues. Nevertheless, we attempted purification, based on the natural affinity of profilin for polyproline, to compare with the recombinant almond profilin. Approximately 25 μ g profilin was obtained per gram defatted almond flour (excluding profilactin complexes). In contrast, a yield of ~2.5 to 3 mg protein of cleaved and purified recombinant profilin was obtained per liter of culture medium. The yield of ryegrass pollen profilin was ~40 μ g per gram of pollen.

Immunologic analysis of almond profilin

Because of the well-known property of cross-reactivity among plant profilins, we attempted to identify the almond profilin using rabbit Ab to maize profilin (ZMPro5). Both cleaved and uncleaved recombinant molecules and total almond extract were reactive (Fig 1, A). To identify the corresponding peptide in immunoblot, the sera of 3 patients with almond allergy (patients #11, 41, and 34) were reacted with electrophoresed and blotted almond extract and compared with similar blots inhibited by nPru du 4 (Fig 1, B). The inhibition of antisera with nPru du 4 resulted in the loss of a protein band at 14 kd as expected.

SDS-based immunoblotting is known to denature proteins substantially and diminish or abolish the reactivity of some constituent epitopes, and IgE-binding epitopes of some profilins are known to be largely conformational.^{34,35} We confirmed that SDS treatment and/or

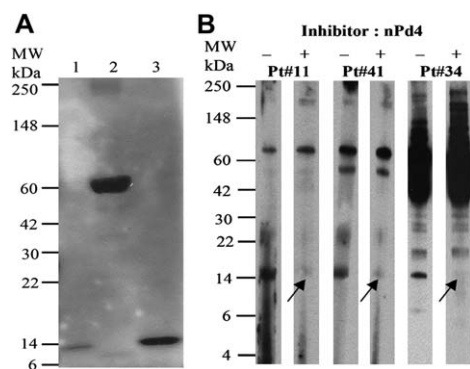


FIG 1. Immunoreactivity of rabbit anti-maize profilin polyclonal antibody (ZMPPro5) (**A**) with total almond extract (lane 1), rPru du 4-MBP fusion protein (lane 2), and cleaved and purified rPru du 4 (lane 3), and inhibition immunoblot of almond extract (**B**) probed with patient sera (patients #11, 41, 34) and with (+) and without (-) added nPru du 4 inhibitor. Arrows indicate positions of inhibited bands. MW, Molecular weight; Pt, patient.

boiling of almond profilin substantially reduced or eliminated binding to some patients' IgE (data not shown). Consequently, we screened the sera of patients with almond allergy for reactivity to recombinant and native profilin by dot blot under nondenaturing conditions as shown in Fig 2. Of the 18 tested sera from patients with histories of mild to life-threatening reactions to almond, 8 (44%) demonstrated IgE reactivity to the nPru du 4. The qualitative IgE reactivity to nPru du 4 was strong with 6 (33%) and moderate with 2 sera (11%; Fig 2). On the other hand, sera from 6 of 18 (33%) patients demonstrated IgE reactivity to the rPru du 4. Of these 6 reactive sera, 2 (11%) showed strong IgE reactivity (from patients #34 and 41), and 4 sera (from patients #2, 8, 18, and 37) exhibited weak IgE reactivity.

Antigen used in ELISA is even less likely to be denatured than in dot blotting. ELISA with rPru du 4 (as a fusion protein) using 18 almond-allergic sera indicated that 8 of 18 (44%) were positive (≥ 0.6 absorbance) (Fig 3). Four of the 8 sera were strongly positive (patients #2, 8, 18, and 41), and 4 were moderately positive (patients #5, 7, 11, and 34). Similarly, ELISA with native profilin showed 9 of 18 (50%) almond-allergic sera were positive, 5 strongly positive (patients #8, 11, 18, 34, and 41), and 4 moderately positive (patients #2, 5, 7, and 37). Overall, IgE reactivity of almond allergic sera to nPru du 4 gave stronger signals compared with those of rPru du 4. It is possible that these differences in IgE binding reflect variations in antigen coating efficiency or epitope accessibility under the assay conditions rather than the innate differences in epitope expression.

There was general concordance between positive IgE binding to the dot blot and ELISA in most patients (patients #2, 8, 18, 34, and 41), although some unexpected patterns were noted. For example, IgE from patient #7 demonstrated reactivity to both nPru du 4 and rPru du 4 in ELISA but no detectable reactivity to either protein in the dot-blot assay. Patient #11 displayed high reactivity with nPru du 4 in both assays but failed to react with rPru du 4

in dot blot even though the dot blot signal with nPru du 4 was strong. Patients #37 and 42 also demonstrated some discordance in their reactivity profiles between the dot blot assays and ELISAs.

To assess better the relative reactivity of the native and recombinant Pru du 4, an inhibition dot blot was performed by using serum from patient #34 (Fig 4), the serum most reactive with nPru du 4 by ELISA. Preadsorption with nPru du 4 nearly abolished IgE reactivity to both nPru du 4 and rPru du 4 on the dot blot. On the other hand, preadsorption with rPru du 4 inhibited IgE binding to rPru du 4, but, at best, only partially inhibited reactivity to nPru du 4 (Fig 4).

The presence of more than 1 isoform of almond profilin was detected by 2-DE immunoblot of almond extract probed with rabbit anti-maize profilin (ZMPPro5) polyclonal antibodies (Fig 5). The N-terminal amino acid sequence of reactive spots "Y" and "Z" revealed an identical sequence (MSWQQYVDDHLM) for both, matching that of rPru du 4.

The IgE cross-reactivity between almond and grass pollen profilin was demonstrated by inhibition dot blot (Fig 6) by using sera from 3 patients with almond allergy (patients #11, 41, and 34). nPru du 4 and nRGPP demonstrated complete reciprocal inhibition with sera from patients #11 and 41, whereas the binding of serum IgE to nRGPP from patient #34 showed only partial inhibition with nPru du 4.

DISCUSSION

The most common tree nut allergies in the United States are to walnut, cashew, and almond.³⁶ In a double-blind, placebo-controlled food challenge (DBPCFC) evaluation of 163 French children with asthma and food allergy, 1.5% showed sensitivity to almond.³⁷

Almond vicilins, legumins, and 2S albumins have been identified as allergens, primarily through immunoblotting and N-terminal amino acid sequencing.^{5,7,8,38} In an effort to characterize almond allergens better, we screened an almond cDNA library with human almond-allergic sera and identified profilin as an allergen.

Almond profilin has $\geq 90\%$ sequence identity with profilins from a variety of plant sources including apple, cherry, peach, orange, and melon.³⁹ Among the tree nuts, only almond belongs to the Rosaceae (synonym: Prunaceae) family. However, the Rosaceae family includes a large number of commonly consumed fruits such as strawberry, raspberry, plum, apricot, cherry, peach, apple, and pear. Apple Mal d 4 was the first profilin allergen to be cloned from this plant food family. Other cloned Rosaceae fruit profilins include cherry (Pru av 4), pear (Pyr c 4), and peach.^{18,39} Banana, pineapple, lychee, tomato, and bell pepper profilins have also been cloned.⁴⁰ As can be seen in this article's Table E2 in the Online Repository at www.jacionline.org, almond profilin shows a high degree of identity and similarity to profilins from other members of Rosaceae. Slightly lower, but still substantial,

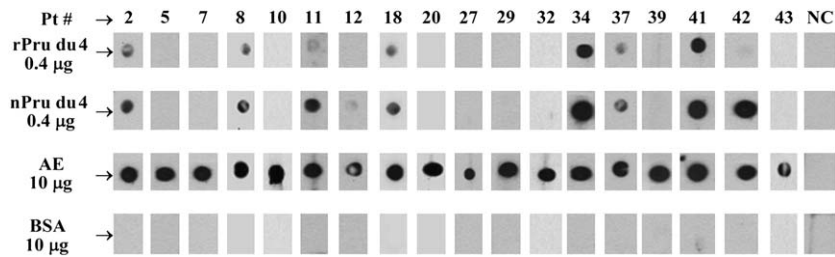


FIG 2. Dot-blot analysis of nPru du 4, rPru du 4, and almond extract (AE) with sera from patients with almond allergy. BSA was used as negative control. NC, Nonatopic control serum; Pt, patient.

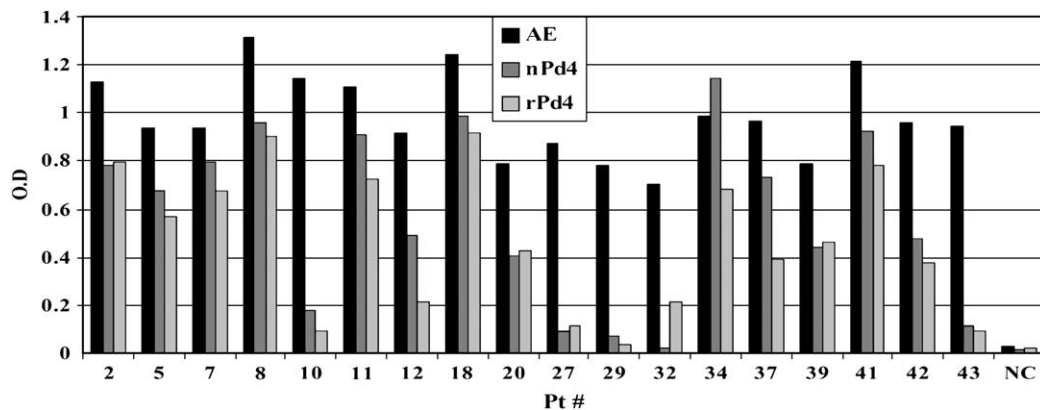


FIG 3. ELISA analysis of specific IgE reactivity of sera from patients with almond allergy to almond extract (AE) proteins and native (nPd4) and recombinant almond profilin (rPd4). NC, Nonatopic control serum; Pt, patient.

homology is seen with other plant profilins, which is a characteristic of this highly conserved ubiquitous protein.¹⁷⁻²⁰

Profilin has been found to be a minor but important allergen in a variety of plant foods, with reactivity ranging between 8% and 57%.^{15,21,41} Our results are in line with these and approach, at least in some assays, the rather arbitrary 50% threshold indicative of a major allergen. When the immunoreactivity of the recombinant almond profilin was compared with that of its native counterpart, we found that 44% of sera from subjects with almond allergy exhibited reactivity with recombinant profilin and 50% with native profilin. Inhibition studies also showed rPru du 4 to be less inhibitory of IgE–nPru du 4 interactions than nPru du 4. The reduced reactivity of the rPru du 4 is likely a result of the presence of multiple native profilin isoforms, each displaying a somewhat differing array of epitopes compared with the single recombinant isoform tested. In support of this interpretation, we identified 2 strongly staining, and several weakly staining, Pru du 4 spots on the 2-DE blots of almond extract, suggesting multiple isoforms. Others have also reported that recombinant profilins from various plant sources display a subset of epitopes compared with native counterparts.⁴²⁻⁴⁵ Even though the degree to which recombinant profilin displays the full panoply of native epitopes has been questioned,²⁵ the analysis of cherry profilin, for example, reveals that the

use of rPru av 4 in skin prick tests strongly correlates with patient symptoms,⁴⁶ indicating that the cloning of all isoforms in a given plant need not be a prerequisite for their useful application.

Two related manifestations of the conserved sequence of the various profilins are the observation that profilins from a wide variety of sources are allergenic and that the various profilins are cross-reactive in their interaction with patient IgE. We took advantage of this cross-reactivity by using rabbit anti-maize profilin antisera to identify the profilin band in 1-DE and 2-DE immunoblots of almond extract. The IgE cross-reactivity extends to sensitization of patients by pollen profilin causing reactivity to food profilins. For example, of 29 patients with peach allergy, the 15-patient subset that demonstrated reactivity to birch pollen profilin (Bet v 2) also reacted with recombinant peach profilin (Pru p 4.01).³⁹ In contrast, none of the 14 patients allergic to peach but not to Bet v 2 reacted with peach profilin, Pru p 4.01. A strong correlation between the reactivity to birch pollen profilin (Bet v 2) and sweet cherry (Pru av 4, 92%) and pear profilins (Pyr c 4, 88%) in patients allergic to the respective fruits has also been noted.¹⁸ Bet v 2 has been shown to inhibit completely the binding of patient IgE to a variety of fruit and vegetable profilins *in vitro*.²⁰ Clinical reactivity to melons, citrus, tomato, and banana have all been strongly correlated with profilin hypersensitivity and

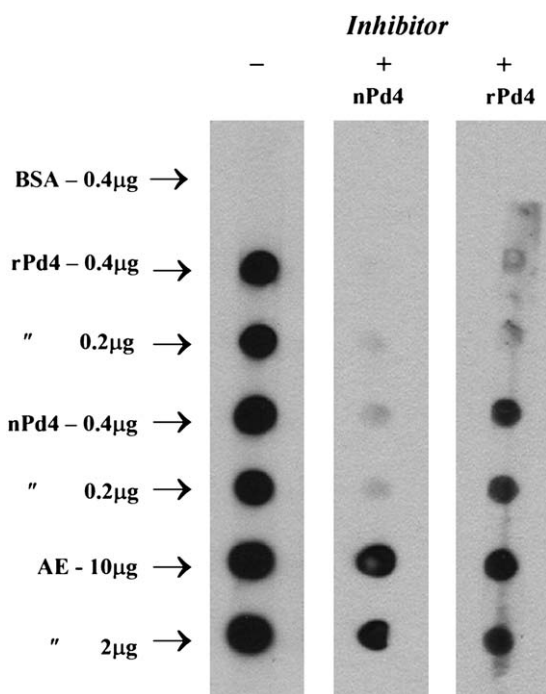


FIG 4. Inhibition dot-blot with patient #34 serum. Immunoreactivity of patient #34 serum with rPru du 4 (rPd4), nPru du 4 (nPd4), and almond extract (AE) showing inhibition by rPru du 4 and nPru du 4. BSA was used as a negative control.

also with Bet v 2 cross-reactivity.⁴⁷ In contrast, a study on German patients noted that only 16% of patients with cherry/birch pollen allergy were reactive with profilin, demonstrating that this association is not universal.¹⁸ Others have found that reactivity to grass profilin is more strongly correlated with fruit allergy than is birch profilin sensitivity.^{15,18,48,49} Our demonstration of IgE cross-reactivity between almond and grass pollen profilins in some of our reactive patients is in line with these studies and, because most of our patients reported pollinosis, suggests the possibility that the route of initial sensitization to Pru du 4 may have been via previous pollen exposure.

Despite the strong evidence of cross-reactivity between various profilins, in several studies there appears to be only a fair to poor correlation between profilin reactivity in *in vitro* tests and clinical food allergy in patients sensitive to both pollens and food.^{18,25} Some of this discordance may be traced to the observation that degranulating mast cells release chymase, an enzyme that lyses profilins and their IgE-reactive epitopes, thus potentially limiting the triggering effect of profilin-specific IgE.⁵⁰

Unlike many other food allergens, profilins display only moderate molecular stability.^{11,51,52} For example, roasting has been shown to decrease the allergenicity of native hazelnut Cor a 2 significantly.⁵³⁻⁵⁵ Similarly, melon profilin allergen is readily digested by simulated gastric fluid.⁵⁶ This degree of sensitivity to denaturation and digestion may explain the observation that profilin-sensitive patients with peanut allergy typically have milder

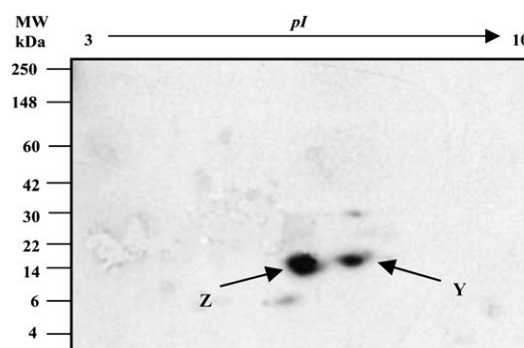


FIG 5. Detection of almond profilin isoforms with rabbit anti-mouse profilin polyclonal antibody (anti-ZMPro5) on 2-DE immunoblot of almond extract. N-terminal sequences were obtained for immunoreactive spots Y and Z. MW, Molecular weight.

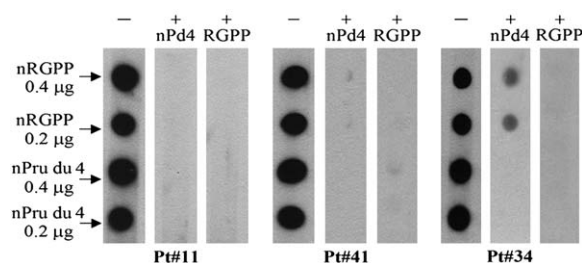


FIG 6. Inhibition dot blot demonstrating cross-reactivity between nPru du 4 and nRGPP. IgE cross-reactivity demonstrated by inhibiting reactivity of serum IgE (from patients #11, 41, and 34) to nRGPP and nPru du 4 with soluble nPru du 4 and nRGPP. (+) = With inhibitor; (-) = no inhibitor. Pt, Patient.

symptoms (oral allergy syndrome) than patients allergic to other constituent peanut allergens.⁵⁷ Our finding that higher percentages of patients react with Pru du 4 in those immunoassays in which the allergens are less subject to denaturing conditions (ie, dot blot and ELISA) is consistent with this observation and suggests that labile conformational epitopes may be primarily targeted by IgE in some patients. A primary role for conformational epitopes has previously been suggested for patient reactivity to other profilins.³⁵ The somewhat labile nature of the allergens coupled with the low levels found in almond nuts may explain why this allergen was not previously detected in immunoblot screens.^{8,58,59}

Overall from this study, we can conclude that almond profilin is an IgE-binding protein in 4 of 11 patients with a self-reported history of severe almond allergy, and in 5 of 7 of those with milder allergy (not confirmed by DBPCFC). The key history in common between these patients may be that 8 of the 9 with IgE reactive with profilin had a history of pollinosis. Indeed, preincubation of sera with ryegrass pollen extract was able to inhibit IgE binding to Pru du 4. Whether there is a clinical contribution to the food allergy by almond profilin, either mild or severe in nature, cannot be determined unless food challenges with purified profilin are performed, or unless profilin is the only IgE-binding protein detected in almond

extract in patients whose mild allergy is proven by DBPCFC. Our study did not include such patients.

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1 CTCCATCCATCTTCGCACCTTCCATTTTCGTTTCAGCTCTAGAACCTTCTC
52 CTCTCTCCCCAAAAGCACTCACCGCCAGAGCAGAAGATCAGCCAAAGCAA
103 GCAAGAAGAAGAAGAAATCAGAAGCAACGAAACGATGTCGTGGCAGCAGTA
1 M S W Q Q Y
154 CGTCGATGACCACTTGATGTGCGATATTGACGGCAACCGCCTCACCGGGC
7 V D D H L M C D I D G N R L T A A
205 GGCAATCCTCGGCCAAGACGGCAGCGTTTGGTCTCAGAGCGCCACCTTCC
24 A I L G Q D G S V W S Q S A T F P
256 TCGGTTTAAGCCTGAAGAGATAGCTGCCATTCTGAAAGATTTCGACCAACC
41 A F K P E E I A A I L K D F D Q P
307 CGGACGCTTGCCCCACTGGGTATTCCTTGGTGGGACAAAGTATATGGT
58 G T L A P T G L F L G G T K Y M V
358 GATCCAAGGTGAAGCCGGTGTGTCAATTCGAGGAAAGAAGGCTCTGGTGG
75 I Q G E A G A V I R G K K G S G G
409 CATTACTGTTAAGAAGACCAATCAGGCCCTCATCATTGGTATATATGATGA
92 I T V K K T N Q A L I I G I Y D E
460 GCCTTTGACTCCAGGGCAGTGCAACATGATTGTCGAAAGGCTTGGCGATTA
109 P L T P G Q C N M I V E R L G D Y
511 CCTTATCGAGCAGGGTCTGCAATTGCTCGAGTGACTATTTATTGGAAGT
126 L I E Q G L
562 TATGGTGAACCTGGTTTGAAAACAAAGTTGTTGCTTGCTGCTCGGGATGTG
613 ATGGGTATATGTATGAACATTTATCTTTGCATTCTGGGACCTCTGGATAT
664 GGATTGCTCTTGTGAATGATAGTGGTTTAATTTACAATTATTTTGCTTA
715 AGTTATTTTCGTCCTTTTGCTTGAAAACGCGTATGTAATTGGAGTTGTCAT
766 CGCTGGAGTGCCATGAATGAAACGGGTTATGTACTAAGTAGTTTTGTTCTA
817 TCAGAAAACGAGTTGGGTGAAAGACATTAAACATTTTGCTTACCTTCCT
868 TTCATTGAGAAAGCTTGTGGATTTTCGCAATTTAACTGAAAAA

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FIG E1. Nucleotide and deduced amino acid sequence (*bold*) of rPrU du 4, clone 1 (AY081850), showing start (*yellow*) and stop (*red*) codons. Also indicated are 2 codons (underlined) that differ (A310→T and A322→G, variant nucleotides highlighted in *purple*) in rPrU du 4, clone 2 (AY081852, not shown). The amino acids encoded by the codons with these silent mutations are highlighted in *green*.

TABLE E1. Clinical characteristics of subjects with almond allergy

Serum #	Sex/age (y)	Age at onset of tree nut allergy (y)	Other atopy	Reaction to almond	Other food allergy	<i>In vitro</i> IgE to almond* (kIU/L)	Positive dot blot to almond	Positive dot blot to profilin
2	F/55	53	AD, AR, As	U	Peanut, walnut (severe allergy, unlike to almond)	1.84	Yes	Yes
5	F/53	1	AD, AR, As	AE, oral/throat pruritus	Walnut, pecan, cashew	4.72	Yes	No
7	F/25	Child	AD, AR	Wheezing, AE	Peanut, walnut, other tree nuts	Class 2	Yes	No
8	F/38	1	AD, AR, As	Wheeze, V, AE	Peanut, walnut, cashew	Class 5	Yes	Yes
10	F/31	2	AR, As	Wheeze, stridor	Peanut, walnut	Class 2	Yes	No
11	M/50	1	AR, As	AE, throat swelling, V	Multiple tree nuts, mustard	0.64	Yes	Yes
12	F/26	3	AR, As	Wheeze, AE, throat swelling, V/D, U	Pistachio, cashew, hazelnut	<0.35	Yes	No
18	F/62	1	AR	Wheeze, U, oral AE	Peanut, walnut, cashew	1.15	Yes	Yes
20	F/48	4	AD, AR, As	Wheeze, AE, throat swelling, V/D, U, LOC	Peanut, walnut, pecan, hazelnut, cashew	<0.35 Class 4 modified RAST	Yes	No
27	M/38	2	AD, AR, As	Stridor, AE, U	Peanut, other tree nuts	Class 2	Yes	No
29	F/47	3	AD, AR, As	Wheeze, stridor, V/D, U, AE	Sesame, walnut	<0.35	Yes	No
32	M/38	1	As	Wheeze, AE, throat swelling, V, U	Walnut, pecan, cashew	<0.35	Yes	No
41	F/45	1	AD	Oral AE, oral pruritus	Walnut, pecan, sesame	<0.35	Yes	Yes
Subjects also with a fruit allergy								
34	M/33	child	AR	Oral pruritus, D to raw almond only, cooked is OK	Multiple fresh fruits, cooked are OK	<0.35	Yes	Yes
37	M/57	1	AD, AR	V/D, U	Peanut, walnut, avocado	<0.35	Yes	Yes
39	F/52	1	AD, As	AE, throat swelling	Peanut, other tree nuts, raw carrot, avocado, banana	0.49	Yes	No
42	F/16	10	AR	Oral pruritus to raw almond only, cooked is OK	Walnut, pecan, melon, banana	<0.35	Yes	Yes
43	F/48	30	AR	Oral AE, oral pruritus to raw almond only, cooked is OK	Raw walnut, pecan, pistachio, cherry, peach, apricot, avocado	<0.35 Positive prick/prick to raw foods	Yes	No

AD, Atopic dermatitis; AE, angioedema; AR, allergic rhinitis (pollinosis); As, asthma; D, diarrhea; F, female; M, male; U, urticaria; V, vomiting.

*Test performed was the Pharmacia ImmunoCAP-FEIA but was reported in classes that were related to percent of the Pharmacia reference value before the hospital's switch to the IU reporting system. Class 0: <60% of reference, Class 1: 70% to 110% of the reference, Class 2: 110% to 220% of the reference, Class 3: 220% to 600% of the reference, Class 4: 600% to 2000% of the reference, Class 5: 2000% to 6000% of the reference.

TABLE E2. Profilins displaying similarity to almond profilin

Organism (common name)	Accession #	Identity (%)	Similarity (%)	Allergen designation
<i>Prunus persica</i> (peach)	CAD37201	99	99	Pru p 4.01
<i>Prunus avium</i> (sweet cherry)	AAD29411	98	98	Pru av 4
<i>Cucumis melo</i> (muskmelon)	CAD 92666	97	98	Cuc m 2
<i>Citrus sinensis</i> (orange)	CAI23765	96	97	Cit s 2
<i>Malus x domestica</i> (apple)	AAD29414	93	98	Mal d 4
<i>Capsicum annuum</i> (bell pepper)	CAD10376	87	94	Cap a 2
<i>Lycopersicon esculentum</i> (tomato)	AAL 29690	87	93	Lyc e 1
<i>Pyrus communis</i> (pear)	AAD29410	85	93	Pyr c 4
<i>Hevea brasiliensis</i> (rubber tree)	AAF34341	85	94	Hev b 8.0201
<i>Cynodon dactylon</i> (Bermuda grass)	CAA69670	80	93	Cyn d 12
<i>Glycine max</i> (soybean)	CAA11756	80	90	Gly m 3
<i>Corylus avellana</i> (hazelnut)	AAK01236	79	91	Cor a 2
<i>Olea europaea</i> (olive)	CAA73039	79	90	Ole e 2
<i>Betula pendula</i> (birch)	AAA16522	75	88	Bet v 2