

Cloning, Expression and Patient IgE Reactivity of Recombinant Pru du 6, an 11S Globulin from Almond

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Key Words

Tree nut • Almond • Food allergy • Recombinant allergen • Amandin • Linear epitope • Solid-phase overlapping peptide analysis • IgE • 11S globulin

Abstract

Background: IgE-reactive proteins have been identified in almond; however, few have been cloned and tested for specific patient IgE reactivity. Here, we clone and express prunin 1 and prunin 2, isoforms of the major almond protein prunin, an 11S globulin, and assay each for IgE reactivity. **Methods:** Prunin isoforms were PCR-amplified from an almond cDNA library, sequenced, cloned and expressed in *Escherichia coli*. Reactivity to the recombinant (r) allergens, Pru du 6.01 and Pru du 6.02, was screened by dot blot and immunoblot assays using sera from almond-allergic patients and murine monoclonal antibodies (mAbs). Sequential IgE-binding epitopes were identified by solid-phase overlapping peptide analysis. Epitope stability was assessed by assaying denatured recombinant proteins by immunoblot. **Results:** IgE reactivity to rPru du 6.01 and rPru du 6.02 was found in 9 of 18 (50%) and 5 of 18 patients (28%), respectively. Four patients (22%) demonstrated reactivity to both isoforms. Murine anti-

almond IgG mAbs also showed greater reactivity to rPru du 6.01 than to rPru du 6.02. Both stable and labile epitopes were detected. Six IgE-binding sequential epitope-bearing peptide segments on Pru du 6.01 and 8 on Pru du 6.02 were detected using pooled almond-allergic sera. **Conclusions:** rPru du 6.01 is more widely recognized than rPru du 6.02 in our patient population. The identification of multiple sequential epitopes and the observation that treatment with denaturing agents had little effect on IgE-binding intensity in some patients suggests an important role for sequential epitopes on prunins.

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Introduction

Food allergy is a growing health concern, and tree nut allergy, in particular, affects 0.6% of the population [1, 2] and can be severe [3]. The 5 most commonly consumed tree nuts in the USA, in rank order, are almond, pecan, walnut, pistachio and cashew. Of these nuts, allergy to almond ranks third following walnut and cashew nut [2, 3]. Almonds (*Prunus dulcis*) are an important commercial crop, with the USA producing 65% of almonds

worldwide [4]. Four almond nut allergens have been identified, namely Pru du 4, a profilin, prunin, a seed storage protein, Pru du 5, a 60S ribosomal protein, and Pru du 3, a nonspecific lipid transfer protein [5–7] (www.IUIS.org).

Almond prunin (amandin) is an 11S globulin (a legumin-like protein) that is hexameric in form and composed of prunin polypeptides [8, 9]. Each monomeric subunit, like those of other 11S globulins, is comprised of a large 40- to 42-kDa acidic α -chain and a small 20-kDa basic β -chain that are linked by a disulfide bond [7–10]. Almond protein extract has been extensively studied by Sathe et al. [7] and Wolf and Sathe [11], who showed that prunin is a major component of the nut, accounting for up to 65% of the protein in a soluble aqueous extract. Immunoblotting assays using rabbit polyclonal and murine monoclonal anti-prunin antibodies reveal that a wide variety of commonly consumed almond cultivars have similarly high levels of prunin [12]. Immunoblot assays have also been performed using a pool of sera from almond-allergic patients to demonstrate high levels of IgE binding to presumptive prunin bands in aqueous almond extract [12, 13]. Two isoforms, prunin 1 and prunin 2, have been identified in almond nut by screening an almond cDNA library [9], and the N-terminal sequences of both have been reported [7], but the relative amounts and immunogenicity of the two prunins have not been compared.

The biochemical and structural features of prunin have recently been analyzed in detail. A circular dichroism spectroscopy study has shown that the native hexameric protein is quite resistant to thermal denaturation, retaining its secondary structure up to 90°C [14]. In another study, the crystal structure of native hexameric prunin was resolved to 2.4 Å to reveal an overall structure typical of the 11S globulin class of seed storage proteins [15]. Interestingly, an extra helix at the C-terminal end of the acidic domain was observed in half of the monomers comprising the hexameric prunin [15]. Structural alignment of prunin 1 with the 11S allergens from peanut (Ara h 3 [16]) and soybean (Gly m 6 [17]) revealed that the extra helix present in prunin corresponds to a presumptive flexible loop region in these legumes [15].

The studies described above have focused primarily on the structural and general immunological properties of prunin. We have now produced recombinant (r) prunin 1 and 2 polypeptides (allergen designations Pru du 6.0101 and Pru du 6.0201, herein abbreviated as Pru du 6.01 and 6.02, respectively), analyzed the degree of patient IgE reactivity and defined the sequential epitopes targeted.

Methods

Human Sera

Blood samples were obtained from patients with convincing histories of almond allergy, after informed consent was obtained, or were purchased from PlasmaLab International (Everett, Wash., USA). The study was approved by the human subjects review committee of University of California, Davis (Davis, Calif., USA). Sera were frozen at –80°C until use. The presence of almond-reactive IgE was confirmed by Pharmacia ImmunoCAP assay (Pharmacia Diagnostics, Uppsala, Sweden) or radioallergosorbent test. Clinical characteristics of the subjects are shown in table 1. Atopic control serum was obtained from a patient with a history of asthma and dust mite allergy but who was not food-allergic.

Construction of an Almond cDNA Library

Construction of an almond cDNA library was performed using mRNA derived from immature almond kernels using methods previously described in detail for cashew library generation [18, 19]. Briefly, developing nuts were chopped, frozen in liquid nitrogen and ground with a mortar and pestle. mRNA was isolated with a PolyAtract kit (Promega, Madison, Wisc., USA). Construction of the cDNA library was performed with the Uni-Zap XR Gigapack Cloning Kit (Stratagene Inc., Cedar Creek, Tex., USA). The double-stranded cDNAs with *Eco*RI- (using a 5' end adapter) and *Xho*I- (using a 3' end PCR primer) cohesive ends were cloned into the lambda Uni-ZAP XR expression vector. The cDNA was amplified in *Escherichia coli* strain XL1-Blue and used in subsequent PCR amplification.

PCR Amplification and DNA Sequencing

Gene-specific primers were designed for prunin 1 and prunin 2 based on the available National Center for Biotechnology Information (NCBI) sequences (accession numbers X78119 and X78120). Prunin 1 gene-specific primers (forward: 5'-AAATCTAGAGCACGCCAGTCCCAGT-3'; reverse: 5'-CGCAAGCTTTATACAAGTGGCCTC-3') and prunin 2 primers (forward: 5'-GGCAAGCTTCTAATAACCAAGGATG-3'; reverse: 5'-AGTCTAGATGCTTGCTTCTTCTTTTC-3') were designed. Platinum® PCR Supermix High Fidelity (Invitrogen, Carlsbad, Calif., USA) was used to amplify full-length prunin cDNAs, which were then TA cloned (TOPO TA Cloning Kit, Invitrogen). Three isolates for each prunin gene were sequenced on an ABI 3100 Genetic Analyzer (Applied Biosystems, Foster City, Calif., USA). The presumptive signal sequence on the prunin proteins was identified using the SignalP program (www.expasy.org, Swiss Institute of Bioinformatics, Basel, Switzerland). The segments of the cDNA sequences corresponding to the gene-specific primers were confirmed by PCR using primers designed within the 5' and 3' untranslated regions of the prunin sequences available in the NCBI database entries (prunin 1: forward 5'-CATCTATCTCCTTCCTATTCCTTGTC-3', reverse 5'-TTGGTAGCTAGACTCTCTCC-3'; prunin 2: lambda Uni-ZAP XR expression vector M13 primer 5'-GGAAACAGCTATGACCATG-3', reverse 5'-TCGTTACATGCAGAAGCCTCC-3'). The prunin cDNAs were amplified, TA cloned and sequenced as described above.

Table 1. Clinical characteristics of almond-allergic subjects

Patient No.	Sex/age years	Age at onset years	Other atopy	Other food allergy	ImmunoCAP or RAST to almond extract ¹	Positive dot blot to Pru du 6.01/6.02
4	M/27	5	As	walnut, Brazil nut, coconut, hazelnut, cashew	1.35	no/no
7	F/25	child	AD, AR	peanut, walnut, other tree nuts	class 2	no/no
8	F/43	1	AD, AR, As	peanut, tree nuts	class 5	yes/yes
9	F/34	1	AD, AR, As	walnut, cashew, pecan	class 3	yes/yes
11	M/50	1	AD, AR, As	tree nuts	0.64	no/no
13	F/39	1	AD as child, AR, As	peas, walnut, pistachio, peanut, Brazil nut	<0.35	no/no
14 ²	F/38	5	As as child	cashew, pistachio, Brazil nut, walnut	1.9	yes/no
18	F/62	1	AR	peanut, walnut, cashew	1.15	yes/no
29	F/49	3	AD, AR, As	peanut, sesame, tree nut	<0.35	yes/no
33	F/63	53	AD, AR, As	peanut, cashew, pistachio, fish, eggs	1.84	yes/no
44 ²	M/30	child	AD as child, AR, As	never eaten other nuts	2.88	yes/no
50	M/12	2	As	walnut	class 2	no/no
51	F/?	?	?	peanut, pistachio, walnut	10.8	yes/yes
52	M/25	child	U	chestnut, pecan, peanut, soybean, Brazil nut	16.4	no/no
53 ²	F/44	child	AD, U, AR, As	egg, soybean, crab, shrimp, tomato, beef, pork, carrot, potato, coconut, apple, milk, peach	15.8	no/yes
54	F/33	22	U	peanut, hazelnut, Brazil nut, pecan, cashew, pistachio, walnut	7.15	no/no
55 ²	M/39	10	U, AE	meat, egg, peanut, soybean, hazelnut, Brazil nut, fish, shellfish, carrot, orange, apple, corn, potato, coconut, rice	9.05	yes/yes
56	F/20	5	AR, As	tree nuts, peanut, soybean, apple, carrots, sprouts, celery	0.46	no/no

AD = Atopic dermatitis; AR = allergic rhinitis; As = asthma; AE = angioedema; U = urticaria; RAST = radioallergosorbent test.

¹ ImmunoCAP results are shown as kU/l, RAST as class.

² Patients whose sera were used for inhibition immunoblot assays.

Cloning, Expression and Purification of cDNA-Encoded Proteins

The prunin cDNA sequences, designated Pru du 6.01 and Pru du 6.02, were ligated into the fusion expression vector pMAL-c4X containing a factor Xa cleavage site (New England BioLabs Inc., Beverly, Mass., USA). The cloning, expression and purification of maltose-binding protein (MBP)-rPru du 6.01 and MBP-rPru du 6.02 fusion proteins were carried out as previously described (for cashew) [18]. Attempts to cleave the MBP tag from the fusion proteins using factor Xa resulted in nonspecific degradation of Pru du 6.01 and Pru du 6.02. Attempts were made to express the Pru du 6 proteins in expression vectors without the MBP tag; however,

the unfused proteins were insoluble and could not be purified from cell lysates. Consequently, subsequent experiments were performed using the recombinant fusion proteins MBP-rPru du 6.01 and MBP-rPru du 6.02. Recombinant proteins were stored at 4°C until use or, for long-term storage, frozen at -80°C.

Almond Protein Extracts

Almond protein extracts were obtained from defatted almond flour by extraction with buffered saline borate, pH 8.2 (0.1 M H₃BO₃, 0.025 M Na₂B₄O₇, 0.075 M NaCl) at room temperature (RT) for 1 h and stored at -20°C for later analysis as previously described [20].

Purification of Native Prunin

Native prunin was purified from aqueous almond extract by column chromatography as previously described [7]. Fractions containing prunin were collected, dialyzed against distilled water, lyophilized and stored at -20°C until further use.

Dot Blot Analysis

rPru du 6.01 and rPru du 6.02 fusion proteins, native prunin, MBP and almond protein extract were applied to nitrocellulose membranes using a 96-well Bio-Dot Microfiltration Apparatus (BioRad Laboratories) as previously described [5]. Briefly, recombinant proteins (0.6 μg per 2-mm dot), native prunin and almond extract (0.3 μg per 2-mm dot) and MBP (0.6 μg per 2-mm dot) were applied to nitrocellulose and blocked overnight at 4°C using phosphate-buffered saline-Tween 20 (PBS-T)/5% (v/v) nonfat dry milk. Dots were incubated with sera diluted 1:3 (v/v) overnight at 4°C , washed 3 times for 30 min at RT in PBS-T and incubated overnight at 4°C with ^{125}I -labeled anti-human IgE (Specific IgE Tracer, Hycor Biomedical Inc., Garden Grove, Calif., USA) diluted 1:10 in PBS-T/5% (w/v) nonfat dry milk. Membranes were washed again as above, and signal intensity was determined after a 10-day exposure to Kodak Biomax X-ray film (Kodak X-OMAT, Kodak Molecular Imaging, New Haven, Conn., USA) at -80°C .

Immunodot Blot Testing for IgE Epitope Conformational Sensitivity

Recombinant proteins (1 μg) were either boiled for 5 min in reducing sample buffer (70 mM Tris-HCl, pH 6.8, 10% glycerol, 2% SDS, 0.05% bromophenol blue and 5% β -mercaptoethanol) or were incubated at RT for 10 min in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) containing either 10% SDS or 6 M urea. Samples were then spotted onto nitrocellulose as described above. After spotting, dots were washed 3 times with 100 μl of TBS, cut into strips and used in IgE immunoblotting assays as described above.

PAGE and Protein Transfer

Almond extract (12–14 μg per 4-mm well width) was subjected to SDS-PAGE (12%). Samples were boiled for 5 min in reducing sample buffer containing β -mercaptoethanol, subjected to electrophoresis and either stained with Coomassie Brilliant Blue R (Sigma-Aldrich, St. Louis, Mo., USA) or transferred to nitrocellulose membranes as previously described [21].

IgE Immunoblotting and Inhibition

Nitrocellulose strips (4 mm wide) from gel transfers containing 12–14 μg of almond nut protein extract were probed as described above. Briefly, strips were blocked overnight then incubated with a pool of almond-allergic sera, diluted 1:8 (v/v), overnight at 4°C . The probed strips were washed and then incubated overnight with ^{125}I -labeled anti-human IgE diluted 1:10. Membranes were washed again and exposed to X-ray film.

For inhibition immunoblots, human sera at 1:8 dilution (50 μl in a total volume of 400 μl) were preincubated with 170 $\mu\text{g}/\text{ml}$ rPru du 6.01 or rPru du 6.02 or 60 $\mu\text{g}/\text{ml}$ native prunin inhibitor at 37°C for 1 h and used as described above. Controls included strips exposed to IgE without inhibitor and strips exposed to serum from an atopic individual without a history of tree nut allergies.

Solid-Phase Peptide Synthesis and Detection of IgE Binding

Two sets of overlapping 15-amino acid cellulose-derivatized peptides, each offset by 8 amino acids, corresponding to the entire deduced amino acid sequences of Pru du 6.01 and Pru du 6.02, were purchased from Sigma-Genosys (The Woodlands, Tex., USA). A spacer of 2 β -alanines was added between each of the peptides and cellulose membrane. The peptide purity was $>70\%$ by HPLC and mass spectroscopy according to the manufacturer. The peptide-containing cellulose membranes were probed as previously described [22].

Solid-Phase Peptide Inhibition Assay

The IgE-reactive peptides from Pru du 6.01 (numbers 15, 16, 19–21, 29, 36, 64, 65) and Pru du 6.02 (numbers 3, 14–16, 24, 27, 29, 36, 59) were purchased from Sigma-Genosys. The peptide-containing cellulose membranes were probed using pooled Pru du 6.01- (numbers 9, 14, 33, 44, 51, 55) and Pru du 6.02- (numbers 9, 51, 53, 55) reactive sera as previously described [22]. For IgE inhibition, solid-phase peptides were assayed with pooled Pru du 6-reactive sera that were first incubated with solid-phase native prunin spotted onto nitrocellulose (10 $\mu\text{g}/\text{spot}$), each spot treated separately with 6 M urea, 10% SDS or reducing sample buffer overnight at 4°C , followed by incubation with added soluble native prunin (60 $\mu\text{g}/\text{ml}$ final concentration) at 37°C for 1.5 h and then used as described above.

ELISA and Inhibition

Native prunin (20 $\mu\text{g}/\text{ml}$), rPru du 6.01 (34 $\mu\text{g}/\text{ml}$) and rPru du 6.02 (33 $\mu\text{g}/\text{ml}$) diluted in coating buffer (0.1 M carbonate-bicarbonate buffer, pH 9.6) were coated (60 $\mu\text{l}/\text{well}$) onto the wells of 96-microwell flat-bottom polystyrene microtiter ELISA plates (Costar, Cambridge, Mass., USA) at 37°C for 1 h. Plates were washed 3 times between steps with PBS-T. Blocking was performed using PBS-T/5% (w/v) nonfat dry milk at 37°C for 1 h. Serum from patient 8 was diluted 1:12 (v/v) in PBS-T/5% (w/v) nonfat dry milk then added to the coated wells (60 $\mu\text{l}/\text{well}$) and incubated at 37°C for 3 h. After washing, bound IgE was reacted with horseradish peroxidase-conjugated mouse antihuman IgE (Zymed Laboratories Inc.) at a dilution of 1:1,000 and incubated for 1 h at 37°C . IgE reactivity was detected by colorimetric reaction using *o*-phenylenediamine (Zymed Laboratories) and H_2O_2 as substrate. OD was measured in a KC4 v2.5 ELISA reader (Bio-Tek Instruments Inc., Winooski, Vt., USA) at 495 nm. All assays were performed in triplicate, and values below the cutoff value, $\text{OD}_{495} = 0.15$ (3 times the nonspecific baseline binding), were considered negative.

For IgE inhibition ELISA, solid-phase native prunin (120 and 12 $\mu\text{g}/\text{ml}$), rPru du 6.01 (206 and 20.6 $\mu\text{g}/\text{ml}$) or rPru du 6.02 (200 and 20 $\mu\text{g}/\text{ml}$) was assayed with serum from patient 8 preincubated with soluble inhibitors for 1 h at 37°C and used as described above. Controls included wells exposed to serum IgE without inhibitor and wells exposed to serum from an atopic individual without a history of tree nut allergies.

Sequence Analysis

Deduced amino acid sequences for the almond prunins were aligned with each other and with sequences of other 11S globulins using BLAST 2.0 (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>, NCBI, Bethesda, Md., USA). Sequential IgE-binding epitopes on each sequence were highlighted as previously described [23]. Solvent-

exposed residues (1.4-angstrom probe) on the monomer, trimer and hexamer of tree nut 11S globulins were determined based on the crystal structure of prunin 1 (PDB: 3EHK and 3FZ3) [15] and its analysis using the GETAREA program (<http://curie.utmb.edu/getarea.html>).

Monoclonal Anti-Almond Antibodies

Monoclonal antibodies (mAbs) against almond extract were raised in the Hybridoma Facility at Florida State University using standard techniques [24]. The guidelines for animal care and welfare described in the *Guide for the Care and Use of Laboratory Animals* [25] were followed. Briefly, pairs of mice were each immunized with 25 µg of almond extract in RIBI adjuvant (RIBI ImmunoChem Research Inc., Hamilton, Mont., USA) split between intraperitoneal and subcutaneous routes, boosted with 15 µg of almond extract in RIBI adjuvant, similarly divided, at 3-week intervals and given a final injection of 15 µg of extract in saline split equally between the intravenous and subcutaneous routes. Following fusion, the resultant hybridomas were screened and assayed for relative strength of reactivity to almond extract by direct-binding ELISA [26]. Clones recognizing almond extract were selected and further screened for protein specificity by Western blotting as previously described [12].

mAb Immunodot Blotting

Nitrocellulose strips/dots were prepared as described above and probed with the almond-specific mAbs, diluted 1:400 or 1:500 in TBS-T, at RT for 1 h. Strips were washed 3 times with TBS-T for 20 min each time and then incubated with horseradish peroxidase-labeled goat anti-mouse reagent (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa., USA) at a 1:5,000 (v/v) dilution in TBS-T for 1 h at RT and washed as above. Amersham ECL (GE Healthcare, Piscataway, N.J., USA) was used to detect reactivity upon exposure of dot strips to Kodak XAR film (Kodak Molecular Imaging).

Results

Gene Characterization

The prunin genes were amplified from the almond cDNA library by means of PCR with gene-specific primers. The resulting 1,656-bp PCR product (GenBank ID GU059260) for Pru du 6.01 encodes a 551-amino acid protein, and the 1,515-bp PCR product (GenBank ID GU059261) for Pru du 6.02 encodes a 512-amino acid protein. The SignalP program was used to identify a 20-amino acid presumptive signal sequence for Pru du 6.01 and a 19-amino acid presumptive signal sequence for Pru du 6.02 (in light-gray font, fig. 1).

Protein Sequence Homology and Recombinant Protein Expression

Comparison of the rPru du 6.01 and rPru du 6.02 amino acid sequences with the prunin 1 and prunin 2 sequences previously identified by Garcia-Mas et al. [9] re-

vealed that both recombinants share 99% sequence identity with their published prunin counterparts. Comparison of rPru du 6.01 with rPru du 6.02 revealed that they share 64% amino acid sequence identity and 77% similarity (fig. 1). Interestingly, the presumptive flexible loop region from amino acids 118 to 184, identified on the Pru du 6.01 atomic structure by Jin et al. [15], does not appear to be present on Pru du 6.02. The two prunins share the greatest degree of sequence homology in the basic chains, with alignment displaying 66% amino acid identity and 83% similarity (fig. 1). Comparison of the two amino acid sequences with the NCBI database using BLAST analysis identified homology with other members of the 11S globulin family of seed storage proteins, several of which are known food allergens, including 50 and 41% homology, respectively, between rPru du 6.01 and hazelnut Cor a 9 [27] and Brazil nut Ber e 2 [Beyer, unpubl. data], and 55 and 46% sequence identity, respectively, between rPru du 6.02 and English walnut Jug r 4 [28] and cashew Ana o 2 [19].

The entire Pru du 6.01 cDNA sequence, beginning at amino acid codon A21 following the presumptive signal peptide, and the entire Pru du 6.02 cDNA, beginning at amino acid codon C10, were cloned and expressed as approximately 103- and 100-kDa MBP fusion proteins, respectively. Efforts to enzymatically liberate the prunins from their fusion partners resulted in fragmentation of the prunins, and efforts to use other vectors yielded insoluble proteins. Consequently, the MBP fusion proteins were used in all assays.

Reactivity of Prunins with Patient IgE

Reactivity to rPru du 6.01 and rPru du 6.02 was screened using 18 patients with a history of almond allergy (patient numbers correspond to those used in our previous publications). IgE from 9 of the 18 patients tested (50%; patients 8, 9, 14, 18, 29, 33, 44, 51, 55) reacted with rPru du 6.01, and 5 (28%; patients 8, 9, 51, 53, 55) reacted with rPru du 6.02 in dot blot assays (fig. 2a). Of the 10 reactive patients, 5 (50%; patients 33, 14, 44, 18, 29) reacted only with rPru du 6.01, and 1 (patient 53) only with rPru du 6.02. To identify the Pru du 6.01 and Pru du 6.02 protein bands in Western blots of almond nut extract, inhibition immunoblotting was performed (fig. 2b). Native prunin and either rPru du 6.01 or rPru du 6.02 were assayed for their ability to inhibit the reaction between a pool of rPru du 6.01-reactive patient sera (patients 14, 44, 55) or the single rPru du 6.02 (-only)-strongly reactive patient serum sample (patient 53) and almond protein extract. Preincubation of the anti-Pru du 6.01 serum pool

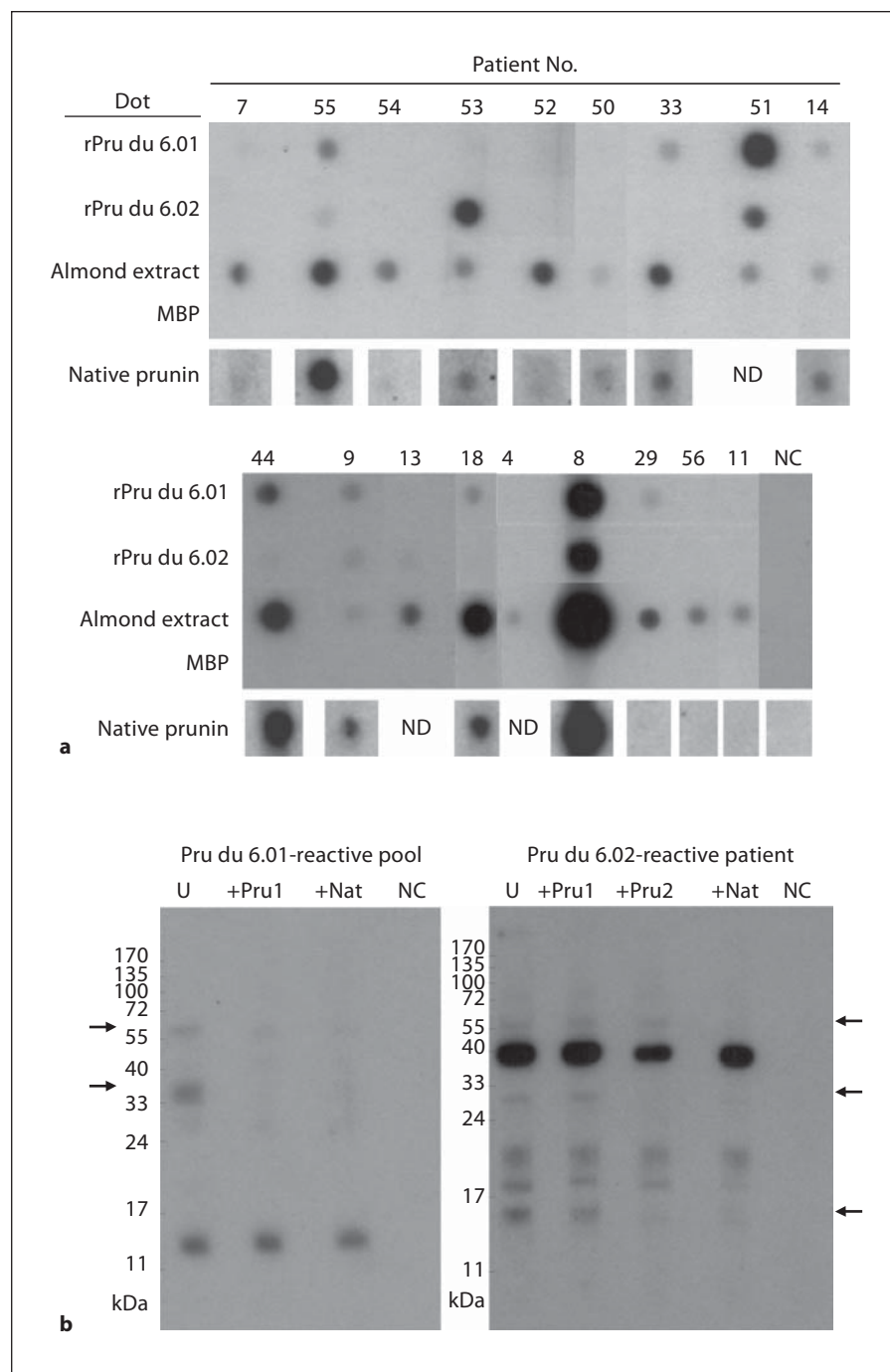


Fig. 2. Dot blot and inhibition immunoblots. **a** Dot blot of sera from almond-allergic patients showing IgE reactivity to rPru du 6.01 and rPru du 6.02 (compiled from two separate blots) and native prunin (from a third blot). Data from sera showing no signals are not shown. **b** Inhibition immunoblot of almond extract probed with a pool of sera from rPru du 6.01-reactive patients (patients 14, 44, 55) and the rPru du 6.02-reactive patient (patient 53), either unadsorbed (U) or preincubated (adsorbed) with rPru du 6.01 (+Pru1), rPru du 6.02 (+Pru2) or native prunin (+Nat). Arrows indicate inhibited bands. Almond extract = Aqueous almond extract; NC = negative control (atopic serum); ND = not determined due to serum limitation.

partial refolding of the recombinant protein during the subsequent washing steps used in the dot blot assay. The observed similar IgE binding intensity to rPru du 6 under all treatment conditions for patients 14 and 55 indicates that IgE from these patients primarily targets sequential epitopes on both Pru du 6.01 and Pru du 6.02.

Identification of IgE-Reactive Sequential Epitopes on rPru du 6.01 and rPru du 6.02

To identify sequential IgE-binding epitopes, a series of 68 overlapping solid-phase synthetic peptides representing the entire amino acid length of Pru du 6.01 was probed with a pool of available sera from 6 Pru du 6.01-reactive

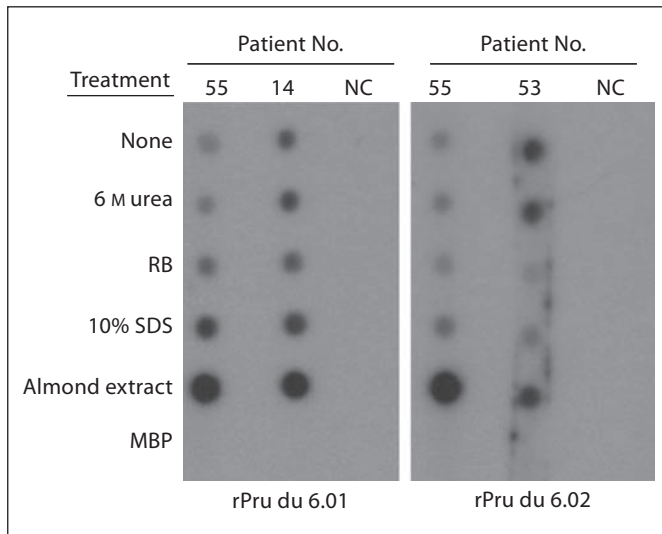


Fig. 3. Dot blot of rPru du 6.01 and rPru du 6.02 proteins treated with denaturing reagents and probed with individual rPru du 6.01- or rPru du 6.02-reactive serum. Recombinant proteins were either boiled for 5 min in reducing sample buffer (RB) or incubated at RT for 10 min in TBS containing either 10% SDS or 6 M urea, and then applied to nitrocellulose. NC = Negative control (atopic serum).

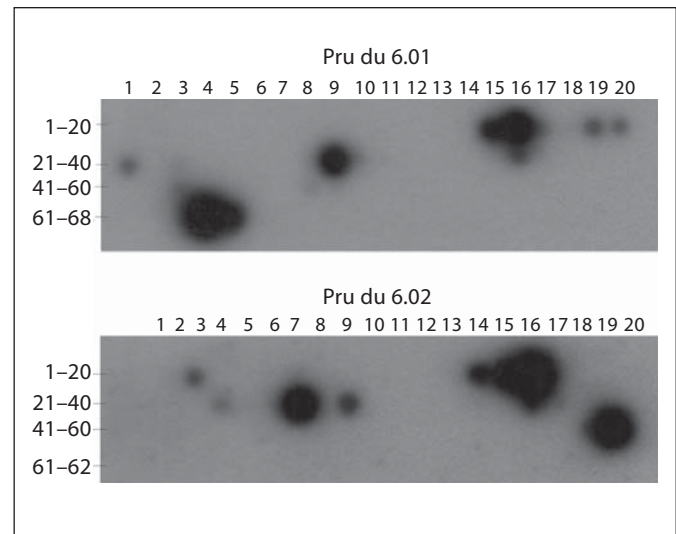


Fig. 4. SPOTs assay identifying sequential IgE-binding peptides in Pru du 6.01 and Pru du 6.02 using a pool of sera from rPru du 6.01-reactive patients (patients 9, 14, 33, 44, 51, 55) and rPru du 6.02-reactive patients (patients 9, 51, 53, 55).

Table 2. Sequences of epitopes identified on Pru du 6.01 and Pru du 6.02

Pru du 6.01 peptide No.	Amino acid sequence	Prunin 1 position	Pool reactivity	Pru du 6.01 epitope	Amino acid sequence
15	EESQQSSQQGRQQEQ	113–127	++	1	118 SSQQGRQQEQEQERQ 132
16	QGRQQEQEQERQQQQ	121–135	+++	2	145 QQEQQQERQGRQQGR 159
19	QQEQQQERQGRQQGR	145–159	+	3	161 QQEEGRQQEQQQGQQ 175
20	QGRQQGRQQQEEGRQ	153–167	+	4	225 LFHVSSDHNQLDQNP 239
21	QQEEGRQQEQQQGQQ	161–175	+	5	281 QQEQQGSGNNVFSGF 295
29	LFHVSSDHNQLDQNP	225–239	++	6	510 RALPDEVLANAYQIS 524
36	QQEQQGSGNNVFSGF	281–295	+		
64	RTSFLRALPDEVLAN	505–519	+++		
65	PDEVLANAYQISREQ	513–527	++		
Pru du 6.02 peptide No.	Amino acid sequence	Prunin 2 position	Pool reactivity	Pru du 6.02 epitope	Amino acid sequence
3	FGQNKEWQLNQLEAR	25–39	+	1	25 FGQNKEWQLNQLEAR 39
14	DSQPQQFQQQQQQQQ	113–127	++	2	113 DSQPQQFQQQQQQQQ 127
15	QQQQQQQFRPSRQEG	121–135	+++	3	129 RPSRQEGGQGGQQQFQ 143
16	RPSRQEGGQGGQQQFQ	129–143	+++	4	193 QNQLDQVPRRFYLAG 207
24	QNQLDQVPRRFYLAG	193–207	+	5	217 QQGRQQQQQQGQQG 231
27	QQGRQQQQQQGQQG	217–231	+++	6	233 GNNIFSGFDTQLLAQ 247
29	GNNIFSGFDTQLLAQ	233–247	++	7	289 RGDQERQQEEQQSQR 303
36	RGDQERQQEEQQSQR	289–303	++	8	473 QNAFRISRQEARNLK 487
59	QNAFRISRQEARNLK	473–487	+++		

+++ = Strong binding; ++ = moderate binding; + = weak binding.

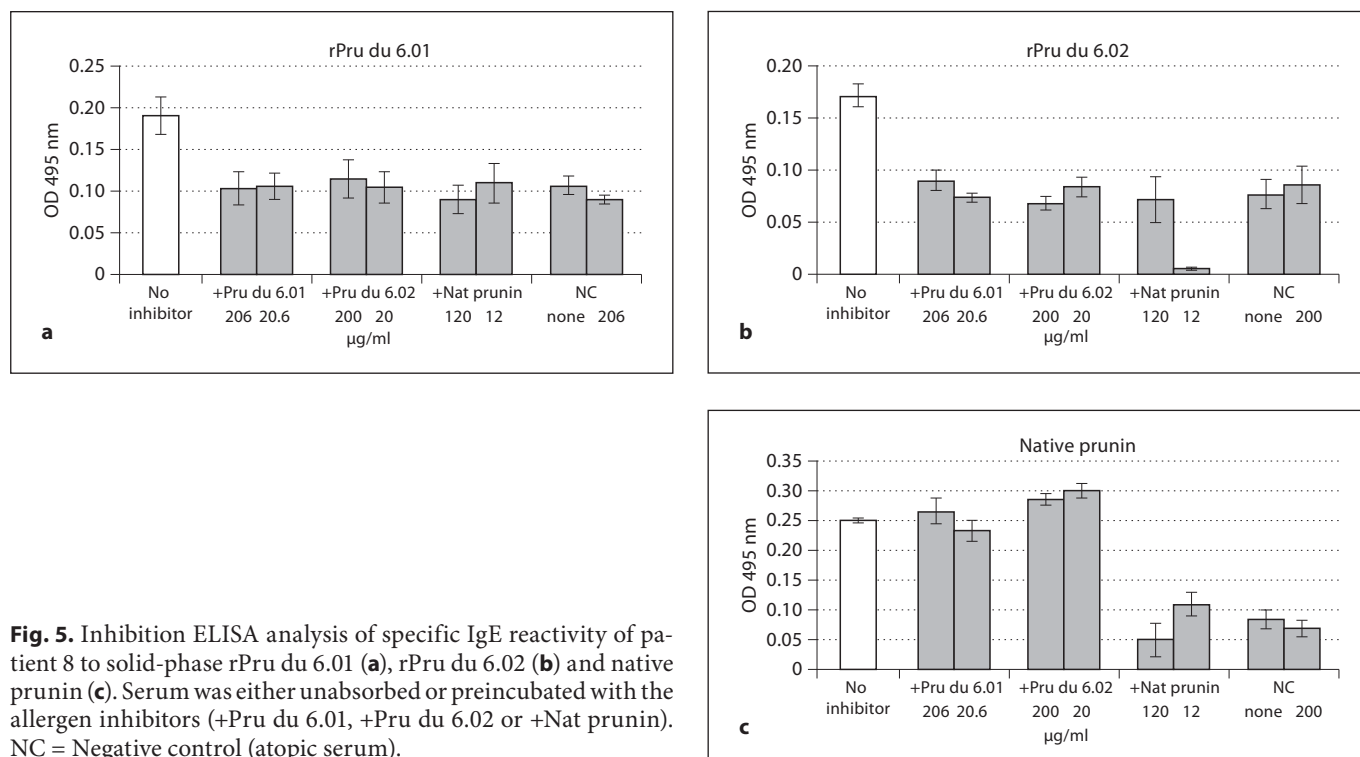


Fig. 5. Inhibition ELISA analysis of specific IgE reactivity of patient 8 to solid-phase rPru du 6.01 (**a**), rPru du 6.02 (**b**) and native prunin (**c**). Serum was either unabsorbed or preincubated with the allergen inhibitors (+Pru du 6.01, +Pru du 6.02 or +Nat prunin). NC = Negative control (atopic serum).

patients (patients 9, 14, 33, 44, 51, 55). The pool reacted weakly with 4 peptides (numbers 19, 20, 21 and 36), moderately with 3 peptides (numbers 15, 29 and 65) and strongly with 2 peptides (numbers 16 and 64) (fig. 4; table 2). Because of an 8-amino acid overlap between adjacent peptides, it is likely that IgE recognition of neighboring solid-phase overlapping peptide assays (SPOTs) represents recognition of the shared amino acid sequence and thus a single epitope. Two such peptide pairs are found in Pru du 6.01 (peptides 15 and 16 and 64 and 65). Assuming that the amino acid segment covered by peptides 19–21 represents at least 2 distinct epitopes based on similar reasoning (i.e. peptides 19 and 21 do not overlap), there are at least 6 IgE-reactive Pru du 6.01 epitopes detected (table 2). These epitopes are distributed throughout the length of the protein, with 5 residing on the acidic chain and 1 on the basic chain (table 2). Half of the epitopes (3 of 6) identified on Pru du 6.01 are located between amino acids 113 and 175, a region rich in glutamine residues (fig. 1).

Sixty-two overlapping solid-phase synthetic peptides corresponding to the Pru du 6.02 sequence were similarly analyzed using a pool of sera from 4 reactive patients (patients 9, 51, 53, 55). The pool reacted weakly with 2

peptides (numbers 3, 24), moderately with 3 peptides (numbers 14, 29, 36) and strongly with 4 peptides (numbers 15, 16, 27, 59) (fig. 4; table 2). Assuming peptides 14–16 represent 2 epitopes, a total of 8 IgE-reactive epitopes are present on Pru du 6.02, 7 of which reside on the acidic chain and 1 of which resides on the basic chain (table 2). As with Pru du 6.01, several of the Pru du 6.02-reactive peptides are glutamine rich. To compare the IgE-binding peptides, the Pru du 6.01 and Pru du 6.02 sequences were aligned and the identified epitopes highlighted (fig. 1). Analysis revealed that the majority of epitopes identified (5 of 8) are located in homologous regions on the Pru du 6 proteins (i.e. they overlap in position by >40% of their residues; fig. 1).

Epitope Analysis by Inhibition ELISA

The observation that some patients react with both prunin isoforms coupled with the overlapping positions of some cognate peptides in the 2 isoforms suggested that there might be cross-reactivity between the isoforms. To test for this, inhibition ELISA assays were performed using serum from patient 8. This serum sample was selected based on availability and the fact that it recognized both Pru du 6 isoforms by dot blot assay (fig. 2a). Native

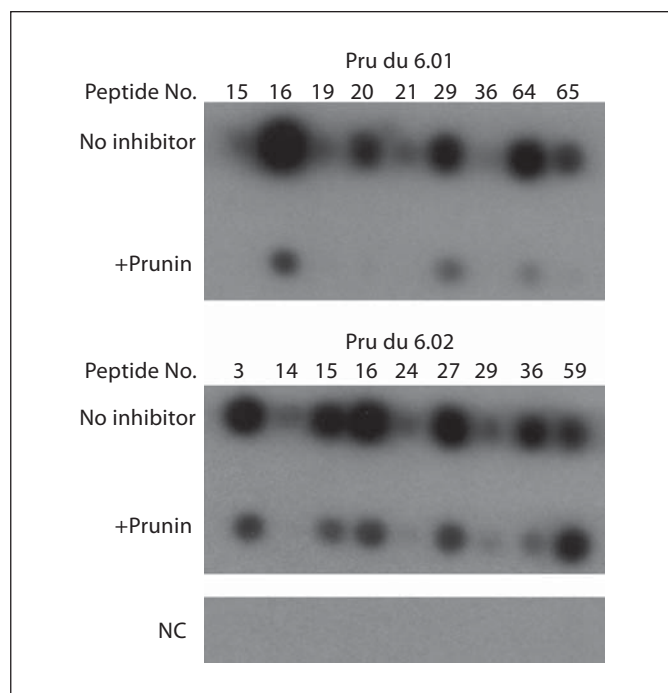


Fig. 6. SPOTs inhibition assay using pooled Pru du 6-reactive sera either unabsorbed or preincubated with denatured and soluble native prunin (+prunin). NC = Negative control (atopic serum).

prunin, rPru du 6.01 and rPru du 6.02 were assayed for their ability to inhibit the binding of IgE to the corresponding solid-phase antigen (fig. 5). Incubation with rPru du 6.01, rPru du 6.02 or native prunin resulted in a loss of IgE reactivity to both rPru du 6.01 and rPru du 6.02 (fig. 5a, b), suggesting the recognition of similar (cross-reactive) epitopes on the rPru du 6 isoforms by this patient. Because both isoforms completely inhibited one another, neither could be identified as the initial sensitizing agent. It is likely that cosensitization to both prunin proteins occurred. In contrast to these results, incubation of the serum with either of the rPru du 6 isoforms had little effect on IgE reactivity to native prunin (fig. 5c). This somewhat surprising result suggests that a significant part of the recognition of the native protein is dependent on the overall structure of the native protein. A likely source for the difference is the fact that most of the native prunin is in the form of mature cleaved hexamers, whereas the recombinant proteins are uncleaved proproteins, presumably in the trimeric form. In contrast to immunoblotting assays, which denature proteins and detect sequential epitopes, ELISA assays are performed under nondenaturing conditions.

Solid-Phase Peptide Inhibition Assay

In order to confirm the presence of sequential IgE-binding epitopes on Pru du 6, peptide inhibition assays were performed. Native prunin was assayed for its ability to inhibit the binding of pooled Pru du 6.01- (numbers 9, 14, 33, 44, 51, 55) and Pru du 6.02- (numbers 9, 51, 53, 55) reactive sera to the solid-phase peptides identified by the SPOTs assay (fig. 4; table 2). Sera were first incubated with solid-phase denatured native prunin to allow IgE binding to epitopes that may not be accessible on the protein surface, followed by incubation with soluble native prunin. Upon inhibition, IgE binding to the 18 identified Pru du 6 peptides was significantly reduced or completely eliminated as compared to the uninhibited controls for all peptides except Pru du 6.02 peptide 59, thus demonstrating the specificity of IgE binding to the Pru du 6 epitopes (fig. 6).

Comparison of Prunin Sequential Epitopes to Those on Other 11S Globulin Allergens

Sequential IgE-binding epitopes have been identified on a number of 11S globulins, including walnut Jug r 1, peanut Ara h 3, soybean Gly m 6, cashew Ana o 2 and hazelnut Cor a 9 [19, 22, 23, 29–32]. To compare these epitopes with those of almond Pru du 6.01 and 6.02, we modified a recently published figure (fig. 1 in Robotham et al. [23]) to include the Pru du 6 epitopes (fig. 7). Four allergen ‘hot spots’ (numbers 1–4), which represent regions in which a majority of the compared sequences display sequential epitopes, have been identified previously [23]. Surprisingly, only 2 of the hot spots (numbers 2 and 4) displayed Pru du 6 epitopes (fig. 7). On the other hand, all of the newly identified Pru du 6.01 and 6.02 epitope-containing peptides demonstrate significant positional overlap with sequential epitope-bearing peptides in one or more of the other compared allergen sequences (fig. 7). Further analysis of the sequential epitope locations identified a new hot spot (number 5) in the allergen sequences. In contrast, the region previously identified as hot spot 1 [23] no longer appears to be epitope rich. Clearly, the sequence segments designated as epitope ‘hot spots’ will have to be modified as new data are considered.

Reactivity of Almond mAbs with Recombinant Proteins

A panel of randomly selected murine mAbs raised against aqueous almond extract was used to determine if murine IgG is similarly more reactive to rPru du 6.01 as compared to rPru du 6.02. Of the 8 almond-specific

Fig. 7. Amino acid sequence and sequential epitope comparison of cashew (Ana o 2), almond (Pru du 6.01 and Pru du 6.02), walnut (Jug r 4), hazelnut (Cor a 9), soybean (Gly m 6.1 = Gly m 6.0101, and Gly m 6.2 = Gly m 6.0201) and peanut (Ara h 3) 11S globulins. Segments corresponding to peptides testing positive in IgE SPOTs assays are highlighted. Biochemically similar residues are indicated by a vertical line, identical amino acids by a dot, positions where a space was added to enhance alignment by a hyphen and sequences deleted to maximize alignment by a slash. Asterisks indicate an additional loop region present in Pru du 6.01. Numbered boxes with a dotted outline represent previously described (numbers 1–4) [23] and newly identified (number 5) allergen ‘hot spots’ (HS). Those residues solvent-exposed in the monomer, trimer and hexamer (≡), the monomer and trimer but occluded in the hexamer (=) and those only exposed in the monomer (–) of the modeled core structure of the prunin 1 hexamer (PDB 3FZ3) [15] are indicated in the ‘Solv Exposed’ line. Residues not exposed in the monomer (i.e. buried) have no symbol. Those residues for which there are no atomic coordinates are also indicated (^). Modified from Robotham et al. [23].

Ana o 2:	15	SREWQOOD--ECQIDR	DALEPDNRVEYEAGTVEAWDPNHEQFCAGVALVRHTIQP	70
Pru du 6.01:	21	...LSP...--...Q-R...	A...Q...T...F...QGD...AS...I...R	77
Pru du 6.02:	11	...G-NK--W...R...	S...VT...DP...L...R...	68
Jug r 4:	24	QSGGR...QFG...	...T...A...V...N...R...	81
Cor a 9:	28	R...QYFG--N...	...T...A...CO...H...D...	83
Gly m 6.2:	19	L...A...--...Q...	...S...G...F...T...P...S...C...NR	73
Gly m 6.1:	21	S...QP...--...Q...	...S...G...L...T...P...S...C...NR	76
Ara h 3.1:	2	F...QP...--A...FQ...	QR...S...G...Y...T...E...S...LV	57
Ara h 3*:	22	F...QP...--A...FQ...	QR...S...G...Y...T...E...S...LV	77
Solv Exposed		=====	=====	

HS #1

HS #5

Ana o 2:

Pru du 6.01:

Pru du 6.02:

Jug r 4:

Cor a 9:

Gly m 6.2:

Gly m 6.1:

Ara h 3.1:

Ara h 3*:

Solv Exposed

136

202

155

148

149

139

142

136

156

Ana o 2:	137	AIPAGVAHWVCYNEGN	SPVVTVLLDVNSQNO	LDRTPRKPHLAGNPKQ	VF-Q-QQ-QQHQR-GRN	198
Pru du 6.01:	203	...S... S... QE	A-N-FH-DH...	N...	E-N/...Q-GS-N	290
Pru du 6.02:	156	...S...N...EQ	A...	D...V... E...	E-N/...GQGN-N	227
Jug r 4:	149	F...T...S...	...A...	T...A...N...N...	D-E- ...R-Q/...	232
Cor a 9:	150	...HT...YA...	EN-H...	D-EH- ...GE-N		239
Gly m 6.2:	140	T...W-M-NE	A...	T...L...M...	Q-QE-/...E-S	215
Gly m 6.1:	143	T...W-M-NE	A...	T...L...M...	Q-QE-/...E-S	219
Ara h 3.1:	137	T...F-L-H	D-A-T-T	D...F...	Q-E/Y...SR- G	241
Ara h 3*:	157	T...F-M-H	D-A-T-T	D...F...	Q-E/Y...SR- G	261
Solv Exposed		=====	=====	=====	=====	

HS #2

HS #3

Ana o 2:

Pru du 6.01:

Pru du 6.02:

Jug r 4:

Cor a 9:

Gly m 6.2:

Gly m 6.1:

Ara h 3.1:

Ara h 3*:

Solv Exposed

256

358

286

297

303

276

280

306

346

Ana o 2:	257	EES-EDE-K-R-RWGQ-RD	GIETICTMLKENIND	PARADIYTPVGRGLTINS	-307
Pru du 6.01:	359	...E-L- E-QQ-GG-	...F... G-E... RA...		403
Pru du 6.02:	287	...E-Q S ER/E-	F-A-S G- V-R		351
Jug r 4:	298	R-S- S- G- D-	...G- E-A...		351
Cor a 9:	304	R-R- S- G- V-F	...CTR...		356
Gly m 6.2:	277	Q/T- S- SK-SR	...GQN SP...N-A-S	AT-	336
Gly m 6.1:	281	E- E- P-CRG-	...H-GQT SP...N-A-S	AT-	346
Ara h 3.1:	307	Y-Y- D- R- G-	...ASA...GRNRS	P-N-A-LSK-A-D	360
Ara h 3*:	347	Y-Y- D- R- SG-	...ASF-GRNRS	P-N-A-LSK-A-E/	390
Solv Exposed		=====	=====	=====	

← Acidic Subunit ↓ Basic Subunit →

Ana o 2:

Pru du 6.01:

Pru du 6.02:

Jug r 4:

Cor a 9:

Gly m 6.2:

Gly m 6.1:

Ara h 3.1:

Ara h 3*:

Solv Exposed

308

468

415

406

426

454

Ana o 2:	308	LNLPILKWLQLSVEKGVLYKN	ALVLPWNLNHSIY	GCKRGKQVQVVDNFGNRVFDGEVREGQM	372
Pru du 6.01:	404	H... A...FF...GYS	...VI-NA...	EN-ALQ...	468
Pru du 6.02:	352	N... A...N-YT...	T...PI-NR...	EN-PLD...	415
Gly m 6.1:	347	F-A-S...A-F-S-R	...F...ALN-AL...	CN-E...	406
Ara h 3.1:	361	L...G-A-Y-N...	F-A-T...RL-AH...	N...E...H	426
Ara h 3*:	391	L...G-A-Y-N...	F-A-T...AL-AH...	N...E...H	454
Solv Exposed		=====	=====	=====	

HS #4

Ana o 2:

Pru du 6.01:

Pru du 6.02:

Jug r 4:

Cor a 9:

Gly m 6.2:

Gly m 6.1:

Ara h 3.1:

Ara h 3*:

Solv Exposed

442

538

485

491

470

478

493

493

521

mAbs tested, 5 recognized rPru du 6.01 and none recognized rPru du 6.02 (fig. 8). Thus, with respect to murine IgG and patient IgE, reactivity to rPru du 6.01 was greater than to rPru du 6.02 as assessed by dot blot assay.

Discussion

Allergies to tree nuts are common and can be severe [1–3, 33–35]. Numerous studies have been directed toward the identification and immunological characterization of tree nut allergens [5, 27, 28, 36–42]. An important approach in the analysis of allergens is the use of recombinant proteins, which have repeatedly shown their value in basic studies, diagnosis and the development of potential immunotherapeutic agents [for reviews, see 43–47].

The 11S globulins are important nut and seed allergens. They constitute a large portion of the total seed protein content, and it is not uncommon for multiple 11S globulin isoforms to be expressed. Isoform sequences can be divergent, and not all isoforms in a given variety of plant seed are allergenic [48–51]. To date, two 11S isoforms have been identified in almond, prunin 1 and prunin 2 [9]. Here, we have cloned and expressed both in an effort to determine the degree to which patient IgE recognizes and distinguishes between the two. In this study, we found that of the 18 almond-allergic patients, 10 (56%) reacted to prunin (Pru du 6; fig. 2a). Interestingly, 5 of the patients tested (28%) reacted exclusively to prunin 1 (Pru du 6.01), and 1 patient, patient 53, recognized prunin 2 (Pru du 6.02) exclusively, indicating that some patients' IgE could distinguish between the isoforms to the exclusion of reactivity to one or the other, despite their degree of homology (78% amino acid similarity and 64% identity). This observation may reflect the fact that the protein core (internal) structures are most conserved, whereas the solvent-accessible surface is more variable and is the portion most likely to express epitopes. Based on our IgE inhibition immunoblots, native and recombinant Pru du 6.01 appear antigenically equivalent with respect to the tested patients' IgE. With regard to Pru du 6.02, the native counterpart found in the nut extract demonstrated a somewhat higher degree of inhibition than rPru du 6.02 in our tested patient (fig. 2b). This observation may be due, in part, to structural differences between the enzymatically cleaved (into acidic and basic chains), hexameric structure of the mature native form and the uncleaved, likely trimeric structure, of our pro-protein recombinant [48, 52, 53]. The native hexameric form, which predominates in the nut, is the likely sensi-

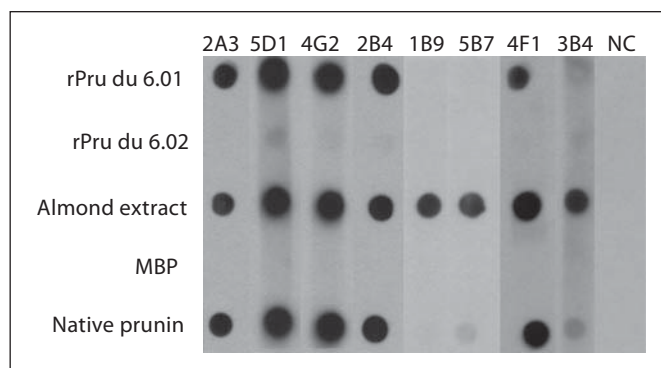


Fig. 8. Dot blot assay demonstrating anti-almond mAb reactivity to rPru du 6.01 and rPru du 6.02. The negative control (NC) contained no primary antibody.

tizing agent in our prunin-allergic patients. A conformational epitope sensitivity assay (fig. 3) indicated a predominance of reactivity to sequential (linear) IgE-binding epitopes. In support of this observation, probing of overlapping peptides by SPOTs analysis identified 6 IgE-binding peptides in Pru du 6.01 and 8 peptides in Pru du 6.02, which were distributed throughout the sequence. Serum IgE reactivity to all but one of these peptides was inhibited by a mix of native and denatured prunin, demonstrating relevance for the identified sequential epitope-bearing peptides.

An analysis of the amino acid sequences of the Pru du 6 epitopes revealed two distinctive features; firstly, more than half are located in regions rich in glutamine residues (Pru du 6.01 peptides 15 and 16 E113–Q135, 19–21 Q145–Q175 and 36 Q281–F295; Pru du 6.02 peptides 14–16 D113–Q143, 27 Q217–G231 and 36 R289–R303). Additionally, these epitopes display a preponderance of hydrophilic/charged residues (Pru du 6.01 epitopes 1–5 and Pru du 6.02 epitopes 2, 3, 5 and 7; table 2). Interestingly, most of these epitopes are located in one of the two regions unresolved in the recently reported crystal structure of prunin 1 (amino acids 118–184) [15]. The likely reasons for these characteristics are high degrees of peptide flexibility and solvent exposure. Consistent with this interpretation is the observation that glutamine-rich regions are inherently flexible and can adopt multiple conformations, including random coil, α -helix and extended loop structures [54]. The presence of exposed highly flexible regions on prunins, as well as other legumins, may provide convenient plant protease cleavage sites which are necessary to facilitate their rapid digestion and nutrient release in the germinating seed [48, 53]. In contrast, dur-

ing digestion within the human gut, proteins encounter a different series of proteases which facilitate the breakdown and absorption of food proteins. To analyze the susceptibility of Pru du 6.01 to proteases commonly found in the digestive tract, the protein sequence was analyzed for pepsin, trypsin and chymotrypsin cleavage sites using the automated program PeptideCutter (<http://www.expasy.org>). Interestingly, both pepsin and chymotrypsin cleavage sites were absent from the glutamine-rich loop on Pru du 6.01, and only a few trypsin cleavage sites were identified in this region. Based on these results, it appears that the glutamine-rich sequence located in this flexible loop region may help prevent excessive proteolytic degradation during the digestion process. In summary, it is likely that our identification of sequential Pru du 6.01 epitopes in these presumptive flexible loop regions is due to their enhanced solvent (and thus IgE antibody) accessibility and perhaps an enhanced facility for induced fitting to antibody complementarity-determining regions [55–57]. Moreover, a lack of rigidity in these regions might favor the generation of IgE against sequential epitopes over conformational epitopes. Additional studies will be undertaken to address these issues.

B cell epitopes are typically categorized as being either sequential (linear, continuous) or conformational (discontinuous) [35, 58–61]. Recently, there has been considerable debate over the clinical importance and relevance of the two epitope forms, and several studies have shown conflicting results. In a study by Sordet et al. [39], 4 IgE-binding regions were identified on the walnut allergen Jug r 1. In an inhibition ELISA in which soluble peptides corresponding to 2 of the identified epitopes served as inhibitors, a significant inhibition of IgE binding to both the native and recombinant walnut allergen was demonstrated, suggesting a preponderance of sequential epitope recognition. In another study, Schulmeister et al. [62] used soluble peptides to identify several sequential epitopes spanning the sequence of α S1-casein, an important allergen in milk. They found that preincubation of patients' IgE with the soluble peptide mixture significantly

inhibited IgE binding to α S1-casein [62]. In contrast, Albrecht et al. [63] identified several sequential epitopes on the shrimp tropomyosin Pen a 1 and the peanut allergen Ara h 2, but failed to find significant inhibition of IgE binding to the allergens using peptide inhibitors. Disruption of the secondary structure did, however, prevent IgE binding, thereby demonstrating the importance of conformational epitopes on these allergens [63]. Similarly, an analysis of the Brazil nut 2S albumin, Ber e 1, showed that IgE is predominantly directed against longer conformational epitopes [64].

From the above-described studies, it is now clear that generalities concerning the relative importance of sequential versus conformational epitopes cannot be drawn and that the spectrum of epitope types varies depending upon the allergen and perhaps even upon differential patient reactivity. Additionally, the long-standing definition of sequential and conformational epitopes may be too rigid, as short stretches of continuous residues may be part of more complex conformational epitopes [55, 59, 65, 66]. Though our studies do not resolve these issues with respect to almond Pru du 6, by identifying sequential IgE binding epitopes we lay the foundation for additional studies to analyze the IgE-binding profile of almond-allergic individuals to assess their relative reactivity to sequential versus conformational epitopes. It is apparent from our results that conformational epitopes may play a role in IgE recognition, as one of the tested patients appears to recognize labile epitopes of Pru du 6. We are currently developing assays to assess the relative contributions of conformational and sequential epitopes on almond Pru du 6 to recognition by patient IgE.

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