

Ana o 2, a Major Cashew (*Anacardium occidentale* L.) Nut Allergen of the Legumin Family

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Key Words

Ana o 2 · Food allergy · Cashew allergen · Tree nut
allergy · Legumin-like protein · 11S globulin · Seed
storage proteins

Abstract

Background: We recently cloned and described a vicilin and showed it to be a major cashew allergen. Additional IgE-reactive cashew peptides of the legumin group and 2S albumin families have also been reported. Here, we attempt to clone, express and characterize a second major cashew allergen. **Methods:** A cashew cDNA library was screened with human IgE and rabbit IgG anti-cashew extract antisera, and a reactive nonvicilin clone was sequenced and expressed as a fusion protein in *Escherichia coli*. Immunoblotting was used to screen for reactivity with patients' sera, and inhibition of immunoblotting was used to identify the corresponding native peptides in cashew nut extract. The identified allergen was subjected to linear epitope mapping using SPOTs solid-phase synthetic peptide technology. **Results:** Sequence analysis showed the selected clone, designated

Ana o 2, to encode for a member of the legumin family (an 11S globulin) of seed storage proteins. By IgE immunoblotting, 13 of 21 sera (62%) from cashew-allergic patients were reactive. Immunoblot inhibition data showed that the native Ana o 2 constitutes a major band at approximately 33 kD and a minor band at approximately 53 kD. Probing of overlapping synthetic peptides with pooled human cashew-allergic sera identified 22 reactive peptides, 7 of which gave strong signals. Several Ana o 2 epitopes were shown to overlap those of the peanut legumin group allergen, Ara h 3, in position but with little sequence similarity. Greater positional overlap and identity was observed between Ana o 2 and soybean glycinin epitopes. **Conclusions:** We conclude that this legumin-like protein is a major allergen in cashew nut.

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Introduction

Allergic reactions to cashew nuts, though relatively infrequent, are varied and can even be life threatening. Reactions include contact or systemic dermatitis (to cardol and anacardic acid found in the cashew nut shell oil) [1], flares of atopic dermatitis and IgE-mediated systemic allergic reactions [2–4]. Pistachios and mangos are other

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Table 1. Clinical characteristics of cashew-allergic subjects

No.	Sex	Age years	Age at onset of cashew allergy, years	Other atopy history	Other food allergy	ImmunoCAP or positive IgE immunoblot
1	M	25	3	asthma	walnut, pecan, hazel	5.66
3	F	26	2	AD, AR, asthma	peanut, walnut, pistachio	9.51
4	M	27	5	asthma	walnut, brazil, coconut, hazelnut	6.95
5	F	54	10	AR, asthma	walnut, pecan, hazelnut	1.62
7	F	30	10	AD, AR, asthma	peanut, walnut, hazelnut	4.04
8	F	43	1	AD, AR, asthma	peanut, tree nuts	41.9
9	F	35	2	AD, AR, asthma	walnut, pecan, almond	35.1
10	F	31	2	AR, asthma	walnut, sunflower	4.42
11	M	50	1	AD, AR, asthma	tree nuts	5.19
12	F	26	3	AR, asthma	peanut, tree nuts	2.41
13	F	39	1	AD, AR, asthma	peanut, tree nuts	9.53
14	F	39	5	asthma	pistachio, tree nuts	94.7
15	M	50	2	AR, asthma	tree nuts	1.87
16	F	44	1	AD, AR, asthma	peanut	+blot
17	F	39	6	AR, asthma	walnut, pine nut	+blot
18	F	62	1	AR	peanut, walnut, almond	+blot
20	F	48	1	AD, AR, asthma	peanut, walnut, hazelnut, chestnut	+blot
29	F	49	3	AD, AR, asthma	peanut, sesame, tree nuts	0.52
30	F	53	15	AD, AR, asthma	tree nuts except almond	+blot
31	F	44	1	AD, AR, asthma	walnut, almond, sesame	+blot
32	M	38	1	AD, asthma	walnut, pecan, hazelnut, pistachio	+blot

ImmunoCAP results are shown as kU/l and positive IgE immunoblot as +blot. AD = Atopic dermatitis; AR = allergic rhinitis.

edible members of the Anacardiaceae family and are also allergenic, with pistachios showing extensive in vitro and possible clinical cross-reactivity with cashews [5–7]. Cashew nuts are widely used in snack foods and as an ingredient in a variety of processed foods such as ‘butters’ and bakery and confectionery products.

We recently demonstrated that the major IgE-reactive proteins in extracts of cashew nut are legumin-like proteins and 2S albumins, as assessed by N-terminal and enzymatic fragment sequencing of native proteins [8]. We have also constructed a cashew nut cDNA library and, upon initial screening, cloned and sequenced an allergen in the 7S superfamily, which includes vicilin-like and sucrose-binding proteins [9]. Here, we present the sequence and characteristics of a second cDNA encoding a protein, designated Ana o 2, which is a member of the legumin (11S globulin) family of seed storage proteins. We also describe its ability to bind IgE from sera of cashew-allergic patients and map its linear epitopes.

Materials and Methods

Human Sera

Blood samples were drawn after obtaining informed consent from patients with life-threatening systemic reactions to cashew nut, and the sera were frozen at -70°C until use. The study was approved by the institutional review board of the University of California at Davis. The presence of cashew-reactive IgE was confirmed by Pharmacia ImmunoCAP assay (Pharmacia Diagnostics, Uppsala, Sweden) or by Western immunoblotting. Control sera were obtained from patients with a history of anaphylaxis to walnut, pistachio or hazelnut who reported tolerance to cashews, and additional sera were used from patients who had never eaten cashew but had life-threatening allergy to other tree nuts. The characteristics of the different groups of subjects are shown in tables 1–3.

Cashew Protein Extract

Soluble protein extract was prepared from defatted cashew nut flour as previously described [10]. Protein concentrations were measured by use of the Bradford protein assay (BioRad Laboratories Inc., Hercules, Calif., USA) using bovine serum albumin as the standard protein.

Table 2. Clinical characteristics of cashew-tolerant subjects with life-threatening food allergy to another nut/seed

No.	Sex	Age years	Age at onset of other nut allergy, years	Other atopy history	Food allergy	ImmunoCAP or RAST to cashew
22	M	27	7	asthma	walnut, pecan, brazil nut	negative skin test
23	F	27	'child'	AR	walnut (class 3), macadamia (only throat 'irritation' to cashew)	0.43
27	M	43	2	AR, AD asthma	peanut (class 3), walnut (class 3), pecan	class 0

ImmunoCAP results are shown as kU/l, RAST as class. AD = Atopic dermatitis; AR = allergic rhinitis.

Table 3. Clinical characteristics of subjects with life-threatening food allergy to other tree nuts who have never eaten cashew

No.	Sex	Age years	Age at onset of other nut allergy, years	Other atopy	Food allergy (ImmunoCAP or RAST value)	RAST or ImmunoCAP to cashew
33	F	42	1	AR, asthma	peanut (mild), walnut (2.27), tolerates pistachios	<0.35
34	M	33	1	AR, asthma	peanut, walnut, almond, hazelnut	1.42
35	F	54	2	AD, AR, asthma	walnut (class 5), pecan (class 4), hazelnut (class 3)	class 3
36	M	43	2	AD, AR, asthma	walnut (class 3)	class 0
37	M	57	1	AD, AR, asthma	peanut, almond, tolerates pistachio	not done
38	F	50	2	AD, AR, asthma	walnut, pecan, pine nut	12.6
39	F	52	1	AD, asthma	walnut, brazil nut	3.56

ImmunoCAP results are shown as kU/l, RAST as class. AD = Atopic dermatitis; AR = allergic rhinitis.

Production of Rabbit Polyclonal Antiserum

A rabbit was immunized with 5 mg of cashew extract in Freund's complete adjuvant and boosted 4 weeks later with 5 mg of cashew extract in incomplete Freund's adjuvant. The rabbit was subsequently bled and the serum was stored at -20°C. Guidelines for animal care and welfare as set down in the 'Guide for the Care and Use of Laboratory Animals' prepared by the Institute of Laboratory Animal Resources (National Research Council, National Academy Press, revised 1996) were followed.

Construction and Immunoscreening of the Cashew cDNA Library

Library construction and immunoscreening have previously been described in detail [9]. Briefly, cashew nuts in late maturation were chopped, frozen and ground. Total RNA was extracted in TRIzol (GIBCO BRL Life Technologies Inc., Rockville, Md., USA) and

mRNA was isolated using a PolyATtract kit (Promega, Madison, Wisc., USA). The cDNA library was constructed using the Uni-ZAP XR Gigapack Cloning Kit (Stratagene Inc., Cedar Creek, Tex., USA) and cloned into the lambda Uni-ZAP XR expression vector. The library was amplified in *Escherichia coli* strain XL1-Blue and screened with rabbit (IgG) and human (IgE) antisera. The immunopositive clones were picked, plaque purified and stored in SM buffer (100 mM NaCl, 8 mM MgSO₄·7H₂O, 50 mM Tris-HCl, pH 7.5, 0.01% gelatin) supplemented with 2% chloroform at 4°C.

Sequencing and Corresponding Analysis of Selected Genes

Inserts from the selected phage clones were amplified with M13 forward and reverse primers by PCR. Both strands of the PCR products were then sequenced on an ABI 3100 Genetic Analyzer (Foster City, Calif., USA) using capillary electrophoresis and Version 2 Big Dye Terminators as described by the manufacturer. Similarity

searches and alignments of deduced amino acid sequences were performed on Genetics Computer Group software (Accelrys Inc. San Diego, Calif., USA) using the BLAST 2.0 program (www.ncbi.nlm.nih.gov/BLAST/). Leader peptide prediction was performed using the SignalP V1.1 World Wide Web Prediction Server (www.cbs.dtu.dk/services/SignalP/).

Cloning, Expression and Purification of cDNA-Encoded Proteins

As previously described in detail [9], cDNA coding sequences were modified by the addition of a *Sal* I site at the 5' end and a *Pst* I site at the 3' end by PCR using *PfuTurbo* DNA polymerase (Stratagene Inc., Cedar Creek, Tex., USA) followed by digestion with *Sal* I and *Pst* I and ligation to maltose-binding protein (MBP) fusion expression vector pMAL-c2 (New England BioLabs Inc., Beverly, Mass., USA), 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 prior to the stop codon using the primers TCTAGAGTCGACCGCCAGGAATGGCAAC (forward) and GCTTGCCTGCAGTTAAGCATCATCCCTCATG (reverse).

For expression, competent *E. coli* BL21 (DE3) cells (Novagen Inc., Madison, Wisc., USA) were transformed. Positive clones were identified by PCR screening using cDNA sequence-specific primers. Single colonies were grown and induced with isopropyl-*D*-thiogalactopyranoside. The cells were harvested, lysed with mild sonication and centrifuged at 12,000 *g*. The lysate supernatant was passed over an amylose affinity column and the fusion protein was eluted with 10 mM maltose. A yield of 15–20 mg of soluble fusion protein per liter of cultured cells was routinely recovered after column or batch purification. In an attempt to avoid protein aggregation upon cleavage, the eluted fusion protein was dialyzed overnight in borate saline buffer (100 mM boric acid, 25 mM sodium borate, 75 mM NaCl, pH 8.2), while changing the dialysis buffer every 4 h, prior to a 12-hour cleavage with 0.1 U of thrombin (Sigma, St. Louis, Mo., USA) per mg of fusion protein. The liberated recombinant protein was purified on a Superdex 200 column (Amersham Pharmacia, Piscataway, N.J., USA) by high-performance liquid chromatography. Cleaved and uncleaved recombinant proteins were concentrated and either stored (briefly) at 4°C until use or frozen at –70°C.

Gel Electrophoresis (SDS-PAGE), Immunoblotting and Inhibition

Recombinant (r) Ana o 2 (at 0.5 µg/4 mm width of nitrocellulose; NC) or total cashew extract (10–15 µg/4 mm width) samples were boiled in reducing sample buffer and subjected to electrophoresis as previously described [9]. Immunoblots were rinsed for 2 min in Tris-buffered saline (TBS; 20 mM Tris, 137 mM NaCl, pH 7.6) containing 0.2% Tween-20 (TBS-T), blocked for 1 h at room temperature in TBS-T containing 2% bovine serum albumin (Sigma) and then incubated overnight at 4°C with diluted patient sera (1:5 to 1:20, v/v, in TBS-T). Membranes were then washed once for 15 min, and 3 times for 5 min in TBS-T prior to being incubated overnight at 4°C with ¹²⁵I-labeled anti-human IgE (Hycor Biomedical Inc., Garden Grove, Calif., USA) diluted 1:10 in a mixture of PBS, 5% nonfat dry milk and 0.05% Tween-20. Three final 10-min washes in PBS-0.05% Tween-20 were performed, and IgE Ana o 2 reactivity was identified after a 2-day or 1-week exposure at –70°C to Kodak Biomax X-ray film (Kodak, Rochester, N.Y., USA). For inhibition experiments, 10 µg of rAna o 2, rAna o 1 and/or MBP were preincubated with

human antiserum (from patient No. 9 at 1:20 dilution) for 1 h at 37°C and then incubated with NC strips containing blotted cashew extract overnight at 4°C. The strips were then incubated with the appropriate labeled second antibody, as described above, and exposed to X-ray film.

Solid-Phase Peptide (SPOTs) Synthesis and Binding to IgE

Based on the derived amino acid sequence of the 457-amino acid Ana o 2 protein (including the presumptive leader sequence, amino acids 1–14), 57 overlapping 15-amino acid peptides, each offset by 8 amino acids, were synthesized. An additional peptide, corresponding to presumptive N-terminal amino acids 15–29, was also produced. Peptides were synthesized on derivatized cellulose sheets using 9-fluorenylmethoxy carbonyl-derived amino acids (Genosys Biotechnologies Inc., The Woodlands, Tex., USA) and probed as previously described [9]. Briefly, the peptide-containing membranes were washed in TBS and incubated overnight at room temperature in blocking solution, washed in TBS-T and incubated overnight at 4°C with pooled patients' sera diluted 1:5 (v:v). This incubation was followed by three 5-min washes in TBS-T and an overnight (4°C) incubation with ¹²⁵I-anti-human IgE which had been prepared as described above. After additional washes, IgE peptide reactivity was identified after a 1-week exposure at –70°C to X-ray film or a 17-hour incubation with a Molecular Dynamics phosphorimaging screen (Kodak). Phosphorimaging data were quantified using a Storm 860 scanner (Molecular Dynamics Inc., Sunnyvale, Calif., USA) and its accompanying software.

Results

cDNA Library Screening and Gene Characterization

The cDNA library was initially screened for reactivity with both cashew extract-specific rabbit IgG and cashew-allergic human IgE. As previously reported, a total of 50 clones displaying dual reactivity were selected for further analysis [9]. The first four clones selected for sequencing all proved to encode one of two vicilin variants and were designated Ana o 1.0101 and Ana o 1.0102. A further analysis of the remaining 46 clones by PCR using Ana o 1-specific primers showed that 45 of the 46 were positive for Ana o 1. The remaining clone, upon sequencing and comparison with GenBank, proved to be homologous with genes encoding the legumin family of seed storage proteins (fig. 1A) and was designated Ana o 2. Table 4 lists the 11 proteins having the highest degrees of identity (45–58%) and similarity (63–74%) to Ana o 2 and includes proteins described as legumin-like, legumin precursor, legumin A precursor, 11S globulin and 11S globulin β-subunit precursor, representing a diversity of tree and plant species (table 4). Also included are two allergens from the peanut, Ara h 3 (43% identity and 59% similarity) and Ara h 4 (42% identity and 58% similarity).

Protein Sequence Characterization

Analysis of the nucleotide (fig. 1A) and deduced amino acid sequences (fig. 1B) revealed a 457-amino acid open reading frame with a predicted leader peptide from 1 to 15. This 15-amino acid segment is homologous to leader peptides identified in legumins of at least one other species [11]. Comparison of the 11 most similar sequences and the two above-mentioned allergens (listed in table 4) with the Ana o 2 amino acid sequence shows clustered sites of insertions (italicized segments, fig. 1B) and deletions (bold segments, fig. 1B). Figure 1C shows the sites of insertions (vertical lines) and deletions (over- and underlined) of three or more amino acids in the 11 compared sequences with respect to the Ana o 2 sequence.

Multiple insertions are seen between amino acids 14 and 24, 105 and 121, 180 and 198, and 223 and 236, with additional scattered insertions between 245 and 271. In contrast to the 30 insertions, only four deletions are present, all of which are clustered in the 245–270 region.

We have previously reported N-terminal and tryptic peptide sequences for several native IgE-reactive cashew proteins [8]. Four of these were found to have similarity to the cDNA sequence of Ana o 2 (fig. 1D and underlined sequence segments in fig. 1B), though none was identical, suggesting that Ana o 2 is a member of a larger gene family or that there is extensive polymorphism at the relevant loci.

Reactivity of the Recombinant Protein with Human IgE and Rabbit IgG

For immunological characterization, we cloned and expressed Ana o 2 from nucleotide 47 (after the presumptive leader peptide) through to nucleotide 1375. The DNA segment was ligated into an expression vector designed to allow for purification of the recombinant molecule via an MBP fusion domain in conjunction with an amylose affinity column and a thrombin-specific cleavage site. The resulting approximately 93-kD Ana o 2 fusion protein was affinity purified as previously described and digested with thrombin to yield a peptide of approximately 52 kD as well as the 43-kD MBP (data not shown). Both cleaved and uncleaved peptides were reactive with specific human IgE and rabbit IgG (data not shown).

Recognition of Ana o 2 as an Allergen

The prevalence of reactivity to rAna o 2 among cashew-allergic patients by Western immunoblotting is shown in table 5. IgE from 13 of 21 sera (62%) from patients with a history of life-threatening reactions to cashews bound the recombinant protein. In six cases, the intensity of the signal was strong, but in seven cases it was

weak. None of the three sera from patients tolerant of cashew but with clinically life-threatening reactions to other tree nuts reacted with the rAna o 2, while 1 of 7 sera from subjects who had never eaten cashew but had other tree nut allergy were positive for IgE to the recombinant.

Identification of Native Ana o 2 by SDS-PAGE

Immunoblotting

Having established that Ana o 2 is a major cashew allergen, we next sought to identify the band or bands in a typical total cashew immunoblot that correspond(s) to the cloned polypeptide storage protein precursor. Figure 2A shows NC strips in which the total soluble cashew extract has been blotted and probed with serum from allergic patient No. 9. The first strip (left lane) shows this serum reacting with three major bands at approximately 53, 36 and 33 kD. The second through fourth lanes were incubated with the same human sera preincubated with rAna o 2, rAna o 1 plus rAna o 2, and the fusion protein, MBP, respectively. Note that Ana o 2 inhibited the strong band at approximately 33 kD and weakened the band at approximately 53 kD, suggesting that there were two polypeptides at this higher molecular weight – both the vicilin and a small quantity of legumin group protein (most likely an intact precursor protein). The addition of Ana o 1 inhibited the residual signal at approximately 53 kD. MBP had no inhibitory effect, as expected. The remaining band at approximately 36 kD is as yet unidentified. The last lane (right) shows no reactivity with sera from a patient with allergies to agents other than cashew.

Figure 2B shows reactivity of the human sera with rAna o 2 (approximately 53 kD) and its inhibition with purified rAna o 2 but not rAna o 1 nor MBP. The band at approximately 120 kD is presumed to be a dimer of rAna o 2.

Identification and Recognition of IgE-Reactive Linear Epitopes on Ana o 2 and Comparison to Peanut and Soybean Legumin Epitopes

The entire amino acid length of Ana o 2 was studied by probing 58 overlapping solid-phase synthetic peptides with sera from 12 patients assigned to three pools. Each pool consisted of sera from 4 patients and included sera from at least 1 patient that reacted strongly to the rAna o 2 in our Western blots and from 3 other patients with varying degrees of reactivity. Collectively, the three pools reacted weakly (phosphoimaging score from 2.0 to 3.9×10^{-3}) with 12, moderately (4.0 – 6.9×10^{-3}) with 3 and strongly ($\geq 7.0 \times 10^{-3}$) with 7 linear IgE-binding epitopes. The 22 reactive epitopes were distributed through-

A

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1  TCTTTCTGTTTGCTTTTAAATCTCTTTTCATGGTTGCCTAGCTTCTCGCCAGGAATGGCAACAACAAGATGAGTGCCAAA
81  TCGATAGGCTGGATGCCCTTGAACCCGATAACCGAGTTGAGTATGAAGCCGGTACGGTGGAAGCCTGGGATCCTAACCAT
161 GAGCAATTCGGATGCGCTGGTGTTCCTTGGTTAGGCATACCATCCAACCTAATGGCCTTCTCTTGCCTCAATATTCTAA
241 TGCTCCTCAACTTATTTACGTTGTCCAGGGTGAGGGTATGACAGGAATATCATATCCAGGATGCCAGAACTTACCAAG
321 CGCCCCAACAGGGACGACAACAGGGACAGAGTGGTAGGTTCCAGGACCGGCATCAAAGATTTCGACGCTTCCGTCGAGGC
401 GATATCATCGCAATCCCCGCCGGAGTAGCACACTGGTGTACAACGAGGGCAATTCCCCGGTCGTCACTGTTACTCTTCT
481 AGACGTCTCAAACAGTCAAAATCAGCTTGATAGGACCCACGAAAATTCATCTGGCTGGTAACCCAAAAGATGTGTTCC
561 AGCAGCAGCAACAACACCAATCTCGCGGGCGTAACCTTTTTTCTGGCTTCGATACAGAGTTATTGGCTGAGGCTTTCCAA
641 GTGGACGAACGTCTCATAAAGCAGCTCAAAAGCGAGGACAACAGGGGTGGCATTGTTAAGGTGAAGGATGACGAACCTTCG
721 GGTGATCCGCCCATCAAGGAGTCAGAGCGAGCGTGGAAGTGAGAGTGAAGAGGAAAGTGAGGATGAAAAACGCCGATGGG
801 GACAGCGTGACAATGGGATTGAAGAGACCATTTCACACTATGAGACTCAAAGAGAATATCAATGATCCTGCTCGCGCTGAC
881 ATTTACACCCAGAAGTCGGTCGTCTTACCACACTCAACAGCCTCAACCTCCCAATCCTCAAATGGCTTCAACTCAGTGT
961 TGAAAAGGGTGTGCTATACAAAAATGCTCTAGTGCTGCCACACTGGAACCTCAACTCGCACAGCATAATATACGGGTGCA
1041 AGGGTAAAGGCCAGGTTCAAGTAGTAGACAACCTCGGCAACAGAGTGTTTCGACGGCGAAGTCCGCGAGGGACAGATGTTG
1121 GTGGTGCCACAAAACCTTTCAGTAGTGAAACGTGCAAGAGAGGAAAGATTGGAATGGATTTCTTTCAAGACCAATGATCG
1201 GGCCATGACGAGTCTCTCGCTGGACGCACCTCGGTGCTTGGTGGCATGCCAGAGGAAAGTGTTAGCCAATGCGTTCAGA
1281 TCTCAAGAGAAGATGCTAGGAAGATCAAGTTCAACAATCAGCAGACAACCTTTGACAAGTGGAGAGTCAAGCCACCACATG
1361 AGGGATGATGCTTAAATTTTAAAGTAATTTGAGCTGAGCTAGTGGTGATTTAAAGCCGAATGCATGTGGTGTACGTACTAT
1440 GTTTTTTGTGTTTGTGTTTGAAGGGGGATAGGTAATGAATAATAAAGGAGAGCTTGGATAGTCTCTGCTGTGAGAGGGGAG
1521 AAGAAAGCAGGGAGCAGAGAGCAGAGAGCTTGTATGTAGTTAAGTTAATATTACTACTACTACTACTACGATGTGAATGA
1601 ACTCTTGATGAGTTCTGTCCAATAAAAAACTACTTTTCTACTCAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA

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B

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1  LSVCFILIFH  GCLASRQEWQ  QQDECQIDRL  DALEPDNRVE  YEAGTVEAWD  PNHEQFRCAG
61  VALVRHTIQP  NGLLLPQYSN  APQLIYVVQG  EGMTGISYPG  CPETYQAPQQ  GRQQGQSGRF
121 QDRHQKIRRF  RRGDIIPA  GVAHWYNEL  NSPVVTVTL  DVSNSQNQLD  RTPRKFLHAG
181 NPKDVFQQQ  QHQSRRNLF  SGFDTELLAE  AFQVDERLIK  QLKSEDNRGG  IVKVKDDELRL
241 VIRPSRSQSE  RGSESEEESE  DEKRRWGQRD  NGIEETICTM  RLKENINDPA  RADIYTPEVG
301 RLTTLNSLNL  PILKWLQLSV  EKGVLKYNAL  VLPHWNLNSH  SIIYGCKGKG  QVQVVDNFGN
361 RVFDGEVREG  QMLVVPQNFA  VVKRAREERF  EWISFKTNR  AMTSPLAGRT  SVLGGMPPEEV
421 LANAFQISRE  DARKIKFNNQ  QTTLTSGESS  HMRDDA 457

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Fig. 1. Nucleotide and derived amino acid sequence analysis of Ana o 2 cDNA clone Ana o 2. **A** Nucleotide sequence with the stop codon underlined and in bold. **B** The derived amino acid sequence of the Ana o 2 coding region, with sites of sequence insertions (italics) and deletions (bold) in the 11 most similar sequences in GenBank (see table 4) as compared to Ana o 2 indicated. Sequences similar to previously reported cashew legumin group proteins derived by amino acid sequencing [8] are underlined. **C** Details of the sites of insertion and deletion as described in **B**. The vertical line between residues indicates the sites of insertions in homologous proteins with the size (number of amino acids) and plant species (two- or three-letter code) shown above the insertion site. Deletions are under- and/or

overlined. Only deletions and insertions of three or more residues are included. Rc = *Ricinus communis*; Cav = *Corylus avellana*; Qr = *Quercus robur*; Ahy = *Amaranthus hypochondriacus*; Si = *Sesamum indicum*; Ms = *Magnolia salicifolia*; Vs = *Vicia sativa*; Cp = *Cucurbita pepo*; Car = *Coffea arabica*; G1 = *Glycine max* (G1 subunit); G2 = *Glycine max* (G2 subunit); Ah3 = *Arachis hypogaea* (Ara h 3); Ah4 = *Arachis hypogaea* (Ara h 4). **D** Comparison of cDNA-derived Ana o 2 sequences with N-terminal and tryptic peptide fragments from cashew legumin proteins previously shown to be reactive to patient IgE [8]. Variant residues are underlined and the residue numbers are adjacent to the sequence. Accession number for Ana o 2 is AF453947.

C

Cav Ms Car Cp Cp
 5 14 4 11 5
 14 A|S|RQ|E|WQQQD|E 24

 Car Ah3,4-Ms Ahv Ahv Cav
 17 12-4 6 9 7
 105 Y|QAPQQGR|QQG|QSG|RF|Q 121

 Mg Cav G2 G1 Rc Qr Si G2 V Ah3,4 Cp-Rc-Ahv
 5 21 3 15 7 8 6 12 7 41 9-10-7
 180 G|NPKD|V| F |Q|Q|Q|QQ|HQ|SR|G R | N 198

 G2 Ah3-4 Qr
 3 3-4 7
 223 K|S E | DNRGGIVKVK|D 236

 Ah3 Vs G2 G2 Ah3,4 Cav-Vs-G1
 4 Ms 19 4 3 Cp 4 8-7-15
 245 SRS|QSERGSES|EEES|EDEK|RRWGQ| R D | N 271
 Ahv Si

D

cDNA segment 30 **LDALPDNR** 38
 33 kD Tryptic fragment **LDALPDNK**

 cDNA segment 72 **GLLLPQYSNAPQLIYVVQG** 91
 47 kD Tryptic fragment **GLLVPSYNN-PELVYVVQG**
 30 kD Tryptic fragment **GLLVPSYNN-PELVYVVQ**

 cDNA segment 272 **GIEETICTMR** 281
 28 kD N-terminal fragment **GLEETICTMT**
M

Table 4. Proteins showing identity and similarity to Ana o 2

Protein description	Organism	Accession No.	AA overlap ¹	Identity %	Similarity %	Report
Legumin-like protein	<i>Ricinus communis</i> (castor bean)	AAF73007	1–446	58	74	Shearer et al. [unpubl. results]
11S globulin-like protein	<i>Corylus avellana</i> (hazelnut)	AF449424	2–444	55	71	Beyer et al., 2002 [28]
Legumin precursor	<i>Quercus robur</i> (English oak)	CAA67879	2–445	55	72	Fischer [unpubl. results]
11S globulin	<i>Sesamum indicum</i> (sesame)	AAK15087	20–451	51	69	Tai and Tzen [unpubl. results]
Legumin precursor	<i>Magnolia salicifolia</i>	S54206	1–454	50	68	Fischer [unpubl. results]
		S54208	1–441	50	68	
11S globulin	<i>Amaranthus hypochondriacus</i> (grain amaranth)	S49422	1–454	48	68	Barba de la Rose et al. [unpubl. results]
Glycinin G2 subunit	Glycine max (soybean)	AAB23210	1–450	47	66	Nielsen et al., 1989 [35]
Glycinin G1 subunit	Glycine max (soybean)	AAB23209	2–439	47	65	Nielsen et al., 1989 [35]
11S storage globulin	<i>Coffea arabica</i> (coffee)	AAC61881	1–457	46	64	Acuna et al., 1999 [36]
Legumin A precursor	<i>Vicia sativa</i> (spring vetch)	S44294	1–439	45	65	Nong et al. [unpubl. results]
11S globulin β-subunit precursor	<i>Cucurbita pepo</i> (winter squash)	AAA33110	3–455	45	64	Hayashi et al., 1988 [11]
Glucinin Ara h 3	<i>Arachis hypogaea</i> (peanut)	AF093541	21–439	43	59	Rabjohn et al., 1999 [12]
Glucinin Ara h 4	<i>Arachis hypogaea</i> (peanut)	AF086821	4–439	42	58	Kleber-Jankeet al., 1999 [25]

AA = Amino acid. ¹Ana o 2 residue numbers.

Fig. 2. Identification of native Ana o 2 peptide in cashew extract by means of Western blotting. NC blots of total cashew extract (**A**) and rAna o 2 (**B**) were probed with serum from a cashew-allergic patient (No. 9) and tagged with ¹²⁵I-labeled anti human IgE. The first (left) lane in each blot was incubated without inhibition. The second through fourth lanes were incubated with rAna o 2, rAna o 1 and/or MBP as indicated. The right lanes were incubated with control human sera. Arrowheads indicate locations of native Ana o 1 (top), Ana o 2 (acidic subunit; bottom) and an, as yet, unidentified band (middle). The lower and upper bands in **B** represent the uncleaved (precursor) form of rAna o 2 in its monomer and presumptive dimer configurations, respectively. M = Molecular mass markers.

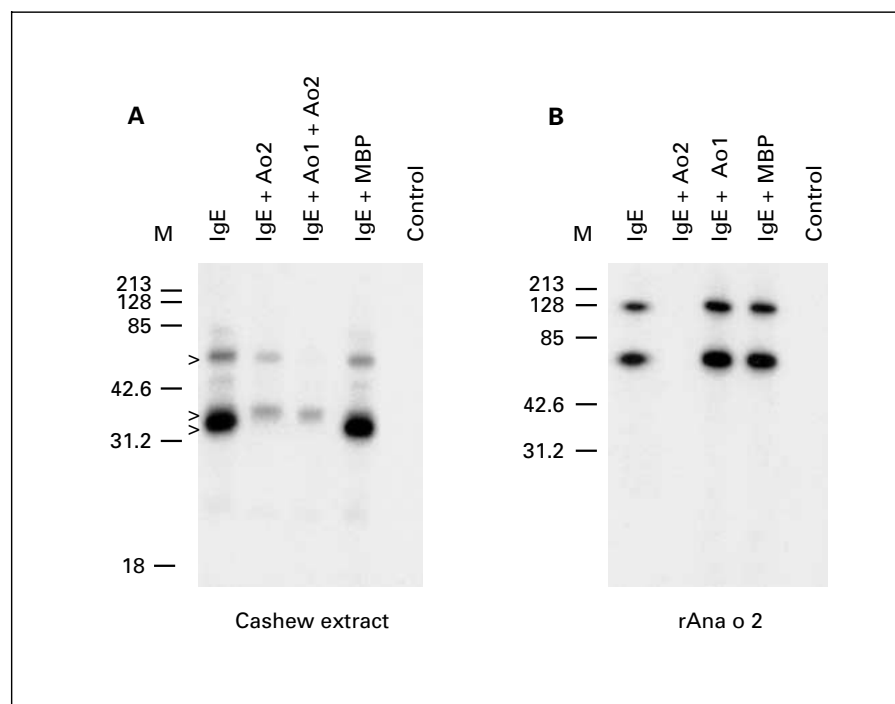


Table 5. IgE Western blot reactivity with rAna o 2

Cashew-allergic subjects	Reactivity	Cashew-tolerant subjects	Reactivity	Unexposed subjects	Reactivity
1	–	22	–	33	–
3	–	23	–	34	–
4	+++	27	–	35	–
5	+++			36	–
7	+			37	–
8	+++			38	+++
9	+++			39	–
10	+++				
11	+				
12	–				
13	+				
14	+++				
15	–				
16	–				
17	–				
18	+				
20	+				
29	+				
30	+				
31	–				
32	–				

Subjects numbered 1–27 correspond to those described in reactivity studies with rAna o 1 [9]. Unexposed subjects are those who reported that they had never eaten cashew nuts.

out the length of the protein (table 6), with 68% (15/22) of all epitopes residing on the acidic chain region of the protein, including 86% (6/7) of those epitopes that were found to react strongly with pooled patient IgE. Only two of the identified epitopes were bound by patients' sera from all three pools. Epitope No. 6 was bound strongly by pools 1 and 3, and moderately by pool 2, whereas epitope No. 3 was bound strongly by pool 1, and moderately by pools 2 and 3.

To compare the linear epitopes of cashew legumin (Ana o 2) to those of peanut (Ara h 3) [12] and the soybean legumins (G2 and G1 glycinin) [13, 14], we aligned the sequences using the BLAST 2.0 program and highlighted the corresponding linear epitopes (fig. 3). Of the regions included in the BLAST alignment, only 2 of the 22 Ana o 2 linear epitope-bearing peptides (No. 13 and 15) showed significant (>7 amino acids) positional overlap with any of the four previously identified peanut Ara h 3 epitope-bearing peptides (fig. 3) [12]. Even among these two overlapping regions, there was little identity or similarity between the Ana o 2 and Ara h 3 epitopes [12]. A much greater degree of correspondence was observed when the sequences of Ana o 2 and G2 glycinin were aligned; 9 of the 11 previously identified G2 glycinin epitopes (82%) showed significant positional overlap with the Ana o 2 epitopes. All 9 showed a significant degree of similarity ($\geq 60\%$) and two were quite similar in sequence to Ana o

Table 6. Linear Ana o 2 epitopes and IgE binding intensity

Epitope	aa position	aa sequence	Phosphoimaging values, $\times 10^{-3}$		
			pool 1	pool 2	pool 3
1	1–15	LSVCFILFHGCLAS	2.1	<	<
2	9–23	FHGCLASRQEWQQQD	<	<	2.4
3	15–29	<u>SRQEWQQQDECQIDR</u>	38.5	2.1	3.8
4	17–31	QEWQQQDECQIDRLD	2.7	<	<
5	89–103	QGEGMTGISYPGCPE	3.3	<	<
6	105–119	<u>YQAPQQGRQQGQSGR</u>	39.5	4.1	16.2
7	113–127	QQGQSGRFQDRHQKI	3.1	<	2.5
8	121–135	QDRHQKIRRFRRGDI	3.3	<	<
9	137–151	AIPAGVAHWCYNEGN	<	<	5.6
10	169–183	LDRTPRKFHLAGNPK	<	6.3	<
11	185–199	<u>VFQQQQQHQSRRNL</u>	10.0	<	<
12	217–231	RLIKQLKSEDNRGGI	4.6	2.9	<
13	233–247	<u>KVKDDELVRVPSRS</u>	2.2	11.6	<
14	241–255	<u>VIRPSRSQSERGES</u>	17.6	<	<
15	257–271	<u>EESEDEKRRWGQRDN</u>	8.6	51.4	<
16	313–327	LKWLQLSVEKGVLYK	3.0	<	<
17	329–343	ALVLPHWNLNSHSII	<	<	2.8
18	337–351	LNSHSIIYGCKGKGQ	2.3	<	<
19	377–391	QNFAVVKRAREERFE	2.6	<	2.0
20	385–399	AREERFEWISFKTND	3.0	<	<
21	417–431	PEEVLANAFQISRED	2.4	3.6	<
22	425–439	<u>FQISREDARKIKFNN</u>	<	9.3	<

Phosphoimaging data were obtained from SPOTs probed with the three random pools of patient sera. A score from 2.0 to 3.9 was considered weak, from 4.0 to 6.9 moderate, and ≥ 7.0 strong. Scores of < 2.0 are indicated as '<'. Bold print denotes strongly reacting epitopes, and underlining denotes those epitopes bound by sera from all three serum pools. aa = Amino acid.

2 epitopes No. 1 (77%) and the adjacent pair, No. 10 and 11 (80%) (fig. 3). Two IgE-binding linear epitopes have been identified on G1 glycinin [14], both on the acidic chain. Sequence alignment revealed that only the second glycinin epitope displayed significant positional overlap and no significant identity with a cashew legumin epitope (No. 13). Interestingly, this epitope (No. 13) is the only one identified that displays positional overlap with an epitope in each of the other three 11S globulins compared (fig. 3), suggesting the possibility of a shared structural motif favoring immunogenicity.

Comparison of rAna o 2 to Native Peptides

We recently reported the N-terminal and tryptic peptide sequences of four native legumin protein bands [8]. In comparing these sequences to those of rAna o 2, we note that none are identical in sequence (fig. 1D). One tryptic peptide from a 33-kD protein matched at 8 of 9 positions. Two other nearly identical tryptic peptides matched rAna o 2 at only 13 of 19 positions (from a 47-kD

protein) and 12 of 18 positions (from a 30-kD protein). An N-terminal peptide matched at 7 or 8 of 10 positions beginning at residue 272 of rAna o2. Together, these data suggest that the legumins represent a complex family of differentially processed proteins.

Discussion

Approximately 0.5% of the US population is believed to be allergic to tree nuts [15], and the data from a voluntary registry of peanut- and tree nut-allergic US patients shows that 20% of those reporting allergy to tree nuts list sensitivity to cashews, the second highest percentage for any tree nut following walnut [16]. In another study restricted to pediatric patients (n = 54) at Johns Hopkins (Baltimore, Md., USA), cashew reactivity ranked fourth (11%) behind walnut (26%), almond (13%) and pecan (13%), perhaps reflecting a delayed dietary introduction of cashews relative to other nuts [17].

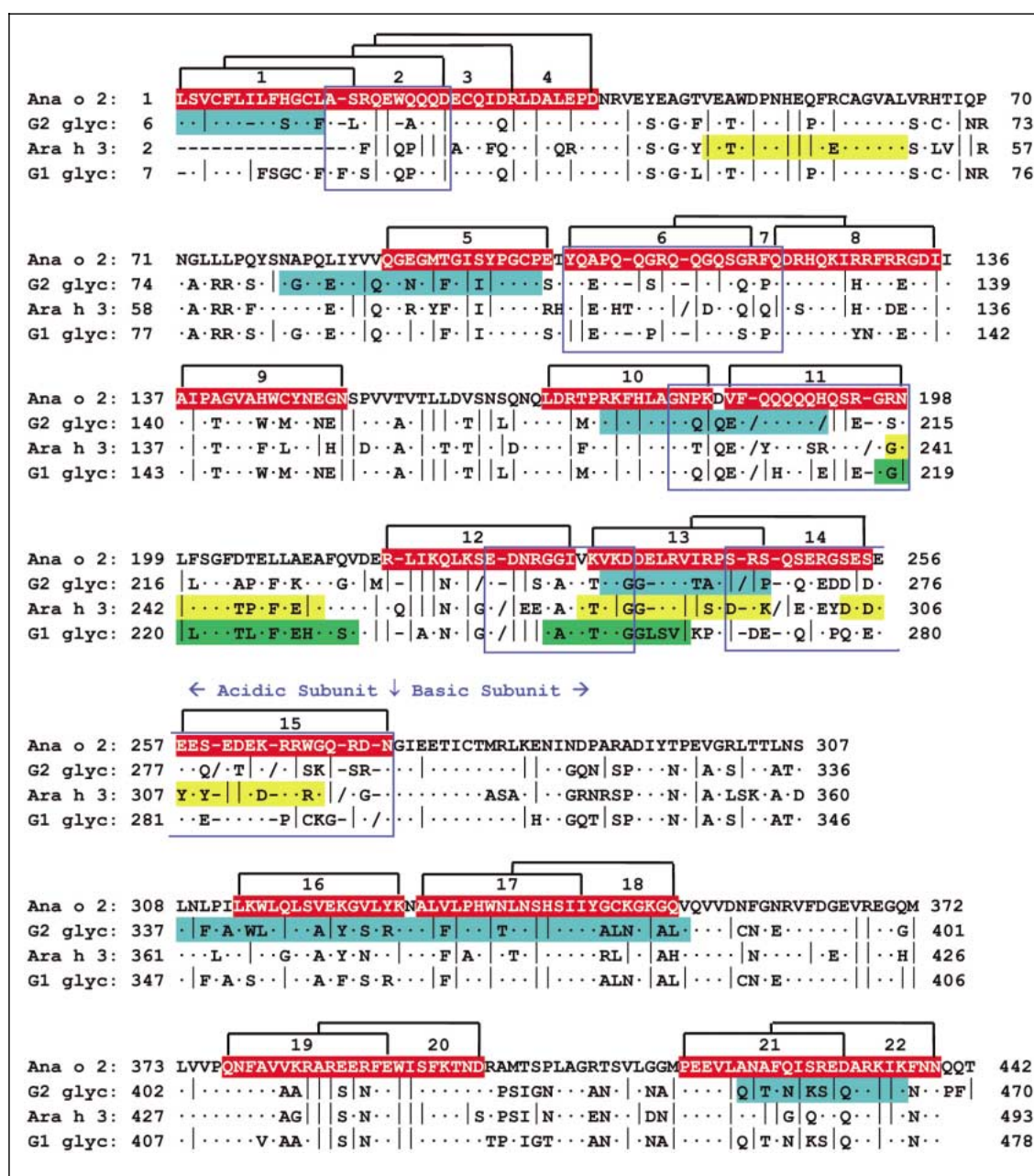


Fig. 3. Comparison of the IgE-binding epitopes of various 11S globulins with those of the cashew legumin, Ana o 2. The bracketed and numbered regions indicate the locations of the reactive peptides in Ana o 2 (also listed in table 6). Colored segments indicate regions expressing linear epitopes on the different allergens. Identical amino acids are indicated with a dot (.), while similar residues are depicted with a vertical line (|). A slash (/) shows where a portion of a sequence was deleted and a long dash (-) represents where a space was added

in the sequence to maximize alignment. The presumed cleavage site between the acidic and basic subunits (between amino acids 271 and 272) is shown in blue text above the sequence. The blue boxes denote regions where numerous insertions and deletions are found when comparing multiple legumin sequences (fig. 1C). Soybean G1 and G2 glycinin and peanut Ara h 3 epitopes have been described previously [12–14].

A number of plant, animal and fungal allergenic proteins have been identified by biochemical and molecular genetic means in recent years, reflecting the hope that the detailed information provided by such studies will lead to the development of hypoallergenic foods, new therapeutic and diagnostic tools and an increased understanding of the properties that render some proteins more allergenic than others [18–22].

Our previous screening of a cashew nut cDNA library with human cashew-allergic sera primarily yielded clones encoding an IgE-reactive vicilin-like protein, designated Ana o 1 [9]. Here, we describe the product of the single identified clone encoding a legumin family (11S globulin) seed storage protein, designated Ana o 2.

The legumins are large (300–450 kD), generally hexameric, nonglycosylated members of a complex family of proteins. Individual subunits are found both as intact precursor proteins (50–60 kD) and as processed acidic (30–40 kD) and basic (20 kD) subunits that generally remain covalently associated in seeds [23]. Legumins represent the major storage protein in cashew, accounting for approximately 50% of the total seed protein [10, 24]. In addition to Ana o 2, several other legumins have been identified as food allergens. These include Ara h 3 [12] and Ara h 4 [25] of the peanut, the soybean G1 and G2 glycinins [26, 27], Cor a 9 of the hazelnut [28] and possibly a legumin from buckwheat [29, 30]. The legumins are related to each other structurally and are believed to share ancestors in common with vicilin-like 7S proteins. The vicilins, or 7S globulins, are also multimeric, digestion resistant, seed storage molecules found in many edible plants and are frequently allergenic.

In the present study, more than 50% of the sera from cashew-allergic patients (13 of 21, 62%) reacted with rAna o 2. Six of the sera (29%) reacted strongly. Because of the known complexity of the legumin family and the differences observed between the single recombinant cashew legumin cDNA-derived sequence reported here and the sequenced cashew peptide fragments from IgE-reactive proteins previously reported [8], it would not be unexpected if additional reactivity to legumin epitopes not represented on our cloned and expressed version were yet to be discovered. Also, we compared a recombinant precursor protein to the native protein that has been cleaved into acidic and basic chains, a process that could potentially affect epitope complexity. Nevertheless, at least for the patients' sera tested, it appears that the recombinant protein fully inhibited reactivity to the native extracted protein, suggesting that most or all members of the cashew legumin family share common allergenic epitopes with

each other and with the recombinant form used in this study. However, further investigations comparing additional cloned versions of the legumins to each other and to highly purified native molecules for reactivity to human IgE will be needed to adequately determine if this Ana o 2 clone expresses the full range of cashew legumin allergenic epitopes. The unique IgE-binding patterns of each pool of sera to the Ana o 2 linear epitopes suggests a heterogeneity of recognition patterns between patients, though some peptides were recognized by members of two or all three pools.

Linear epitope mapping has shown that the soybean G1 glycinin acidic chain may share cross-reactive epitopes with the peanut Ara h 3 [14]. Both mapped epitopes of the G1 acidic chain show a considerable degree of identity (60 and 70%) with the overlapping region of the homologous Ara h 3 epitopes, though differences were noted in the critical amino acids [14]. It has been shown that the IgE-binding regions of the soybean G2 glycinin also bind IgE from peanut-sensitive patients [13]. Such findings are not surprising when one considers that both are members of the legume family and patients allergic to one sometimes have serum IgE antibodies that immunologically cross-react with other legumes [31]. While no such direct analysis has been performed to determine if there is cross-reactivity between our cashew legumin (Ana o 2) and that of peanut (Ara h 3) and soybean (G1 and G2 glycinin), the BLAST alignment of these protein sequences and subsequent analysis of the positional relationship and degree of homology between linear epitopes on each protein have been informative. The major findings are that there is considerable positional overlap and some shared sequence identity between Ana o 2 and soybean G2 glycinin but little in common between Ana o 2 and peanut Ara h 3 linear epitopes. We have shown that the majority of Ana o 2 linear epitopes (68% overall and 83% of the most strongly reactive) are on the acidic chain of the protein. Previous studies have also observed stronger reactivity with the acidic chain than with the basic chain of allergenic legumins [12–14, 32, 33].

It is worth noting that the majority of Ana o 2 epitopes are located in the same areas of the protein sequence that is evolutionarily least conserved with respect to insertions and deletions among a variety of legumins (compare fig. 1C with the acidic chain of Ana o 2 in fig. 3), features that would further minimize cross-reactivity between plant legumins.

It should be borne in mind though that these various results represent only those reactions between a subset of legumin sequences in each of the compared plant seeds

and the IgE from a limited set of patients. The inclusion of more versions of the legumins and a broader pool of patient sera in future analyses could change our views of these various relationships.

Clinical information regarding cross-reactivity between cashews and legumes has not been well documented, but based on our findings, we can predict that while there may be a basis for cross-reactivity between cashew and soybean, it is unlikely that such cross-reactivity exists between cashew and peanut legumins, at least with regard to linear epitopes. Interestingly, none of our subjects were allergic to soy, while 9 of 21 were allergic to peanuts. Similarly, we previously reported that cross-reactivity between the vicilin linear epitopes of cashew (Ana o 1) and peanut (Ara h 1) would also be unlikely [9], observations which, together, further explain the lack of cross-reactivity between tree nut- and peanut-reactive patient sera [16].

To date, no common structural characteristics of linear IgE epitopes have been identified, but our finding that all four legumins analyzed share an epitope with positional overlap suggests that epitope mapping can provide important clues to the nature of allergenicity [34]. Three-dimensional modeling of our cashew legumin is under way, and when compared to the models of G2 glycinin [13] and other homologous allergens, may provide additional insights.

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References

- Marks JJ, DeMelfi T, McCarthy MA, Witte EJ, Castagnoli N, Epstein WL, Aber RC: Dermatitis from cashew nuts. *J Am Acad Dermatol* 1984;10:627-631.
- Burks AW, James JM, Hiegel A, Wilson G, Wheeler JG, Jones SM, Zuerlein N: Atopic dermatitis and food hypersensitivity reactions. *J Pediatr* 1998;132:132-136.
- Tariq SM, Stevens M, Matthews S, Ridout S, Twiselton R, Hide DW: Cohort study of peanut and tree nut sensitization by age of 4 years. *BMJ* 1996;313:514-517.
- Garcia F, Moneo I, Fernandez B, Garcia-Menaya JM, Blanco J, Juste S, Gonzalo J: Allergy to *Anacardiaceae*: Description of cashew and pistachio nut allergens. *J Invest Allergol Clin Immunol* 2000;10:173-177.
- Fernandez C, Fiandor A, Martinez-Garate A, Martinez QJ: Allergy to pistachio: Cross-reactivity between pistachio nut and other *Anacardiaceae*. *Clin Exp Allergy* 1995;25:1254-1259.
- Parra FM, Cuevas M, Lezaun A, Alonso MD, Beristain AM, Losada E: Pistachio nut hypersensitivity: Identification of pistachio nut allergens. *Clin Exp Allergy* 1993;23:996-1001.
- Quercia O, Rafanelli S, Marsigli L, Foschi FG, Stefanini GF: Unexpected anaphylaxis to cashew nut. *Allergy* 1999;54:895-897.
- Teuber SS, Sathe SK, Peterson WR, Roux KH: Characterization of the soluble allergenic proteins of cashew nut (*Anacardium occidentale* L.). *J Agric Food Chem* 2002;50:6543-6549.
- Wang F, Robotham JM, Teuber SS, Tawde P, Sathe SK, Roux KH: Ana o 1, a cashew (*Anacardium occidentale*) allergen of the vicilin seed storage protein family. *J Allergy Clin Immunol* 2002;110:160-166.
- Sathe SK, Sze-Tao KWC, Wolf WJ, Hamaker BR: Biochemical characterization and in vitro digestibility of the major globulin in cashew nut (*Anacardium occidentale*). *J Agric Food Chem* 1997;45:2854-2860.
- Hayashi M, Mori H, Nishimura M, Akazawa T, Hara-Nishimura I: Nucleotide sequence of cloned cDNA coding for pumpkin 11S globulin beta subunit. *Eur J Biochem* 1988;172:627-632.
- Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, Bannon GA: Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 1999;103:535-542.
- Helm RM, Cockrell G, Connaughton C, Sampson HA, Bannon GA, Beilinson V, Nielsen NC, Burks AW: A soybean G2 glycinin allergen. 2. Epitope mapping and three-dimensional modeling. *Int Arch Allergy Immunol* 2000;123:213-219.
- Beardslee TA, Zeece MG, Sarath G, Markwell JP: Soybean glycinin G1 acidic chain shares IgE epitopes with peanut allergen Ara h 3. *Int Arch Allergy Immunol* 2000;123:299-307.
- Sicherer SH, Munoz-Furlong A, Burks AW, Sampson HA: Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J Allergy Clin Immunol* 1999;103:559-562.
- Sicherer SH, Furlong TJ, Munoz-Furlong A, Burks AW, Sampson HA: A voluntary registry for peanut and tree nut allergy: Characteristics of the first 5,149 registrants. *J Allergy Clin Immunol* 2001;108:128-132.
- Sicherer SH, Burks AW, Sampson HA: Clinical features of acute allergic reactions to peanut and tree nuts in children. *Pediatrics* 1998;102:1-6.
- Heiss S, Mahler V, Steiner R, Spitzauer S, Schweiger C, Kraft D, Valenta R: Component-resolved diagnosis (CRD) of type I allergy with recombinant grass and tree pollen allergens by skin testing. *J Invest Dermatol* 1999;113:830-837.
- Valenta R, Vrtala S, Focke-Tejkl M, Bugajska-Schretter A, Ball T, Twardosz A, Spitzauer S, Gronlund H, Kraft D: Genetically engineered and synthetic allergen derivatives: Candidates for vaccination against type I allergy. *Biol Chem* 1999;380:815-824.
- Bannon GA, Cockrell G, Connaughton C, West CM, Helm RM, Stanley JS, King N, Rabjohn P, Sampson HA, Burks AW: Engineering, characterization and in vitro efficacy of the major peanut allergens for use in immunotherapy. *Int Arch Allergy Immunol* 2001;124:70-72.
- Niederberger V, Stubner P, Spitzauer S, Kraft D, Valenta R, Ehrenberger K, Horak F: Skin test results but not serology reflect immediate type respiratory sensitivity: A study performed with recombinant allergen molecules. *J Invest Dermatol* 2001;117:1-5.
- Bhalla PL, Swoboda I, Singh MB: Reduction in allergenicity of grass pollen by genetic engineering. *Int Arch Allergy Immunol* 2001;124:51-54.
- Casey R: Distribution and some properties of seed globulins; in Shewry PR (ed): *Seed Proteins*. Boston, Kluwer Academic, 1999, pp 159-169.

- 24 Ventura MM, Filho JX: Physical and chemical characteristics of the major globulin from the cashew nut (*Anacardium occidentale* L.). *An Acad Bras Cienc* 1964;36:21–27.
- 25 Kleber-Janke T, Cramer R, Appenzeller U, Schlaak M, Becker WM: Selective cloning of peanut allergens, including profilin and 2S albumins, by phage display technology. *Int Arch Allergy Immunol* 1999;119:265–274.
- 26 Zeece MG, Beardslee TA, Markwell JJP, Sarath G: Identification of an IgE-binding region in soybean acidic glycinin G1. *Food Agric Immunol* 1999;11:83–90.
- 27 Helm RM, Cockrell G, Connaughton C, Sampson HA, Bannon GA, Beilinson V, Livingstone D, Nielsen NC, Burks AW: A soybean G2 glycinin allergen. 1. Identification and characterization. *Int Arch Allergy Immunol* 2000;123:205–212.
- 28 Beyer K, Grishina G, Bardina L, Grishin A, Sampson HA: Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *J Allergy Clin Immunol* 2002;110:517–523.
- 29 Fujino K, Funatsuki H, Inada M, Shimono Y, Kikuta Y: Expression, cloning, and immunological analysis of buckwheat (*Fagopyrum esculentum* Moench) seed storage proteins. *J Agric Food Chem* 2001;49:1825–1829.
- 30 Yamada K, Urisu A, Morita Y, Kondo Y, Wada E, Komada H, Yamada M, Inagaki Y, Torii S: Immediate hypersensitive reactions to buckwheat ingestion and cross allergenicity between buckwheat and rice antigens in subjects with high levels of IgE antibodies to buckwheat. *Ann Allergy Asthma Immunol* 1995;75:56–61.
- 31 Eigenmann PA, Burks AW, Bannon GA, Sampson HA: Identification of unique peanut and soy allergens in sera adsorbed with cross-reacting antibodies. *Allergy Clin Immunol* 1996;98:969–978.
- 32 Pedersen HS, Djurtoft R: Antigenic and allergenic properties of acidic and basic peptide chains from glycinin. *Food Agric Immunol* 1989;1:101–109.
- 33 Herian AM, Taylor SL, Bush RK: Identification of soybean allergens by immunoblotting with sera from soy-allergic adults. *Int Arch Allergy Appl Immunol* 1990;92:193–198.
- 34 Bredehorst R, David K: What establishes a protein as an allergen? *J Chromatogr B Biomed Sci Appl* 2001;756:33–40.
- 35 Nielsen NC, Dickinson CD, Cho TJ, Thanh VH, Scallion BJ, Fischer RL, Sims TL, Drews GN, Goldberg RB: Characterization of the glycinin gene family in soybean. *Plant Cell* 1989;1:313–328.
- 36 Acuna R, Bassuner R, Beilinson V, Cortina H, Cadena-Gomez G, Montes V, Nielsen NC: Coffee seeds contain 11S storage proteins. *Physiol Plant* 1999;105:122–131.