Ana o 1, a cashew (*Anacardium occidental*) allergen of the vicilin seed storage protein family

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Background: The allergens responsible for cashew food allergy have not been well characterized.
Objectives: We initiated a study to clone cDNAs encoding cashew food allergens.
Methods: A cashew cDNA library was screened with human serum for IgE-reactive clones and rabbit IgG anti-cashew extract antisera. Reactive clones were sequenced and expressed, and linear epitopes were identified by means of solid-phase overlapping peptide analysis. Immunoblot inhibition was used to identify the native peptide in cashew extract.
Results: Four closely related clones reactive with both human and rabbit antisera were sequenced. Sequence analysis showed that these encode members of the vicilin/sucrose-binding protein family of plant seed storage proteins. Screening of the recombinant protein with sera from 20 patients with cashew allergy and 8 cashew-tolerant patients with allergies to other tree nuts showed that 50% and 25% of sera from patients with cashew allergy and cashew-tolerant subjects, respectively, bound the recombinant protein. The corresponding native allergen protein, designated Ana o 1, was located at approximately 50 kd. Epitope mapping revealed 11 linear IgE-binding epitopes, of which 3 appear to be immunodominant. None of the epitopes were shared in common with those of the peanut vicilin allergen Ara h 1.
Conclusion: Ana o 1, a vicilin-like protein, is a major food allergen in cashews. Cashew and peanut vicilins do not share linear epitopes. (J Allergy Clin Immunol 2002;110:160-6.)

Key words: *Ana o 1,* Anacardium occidental*, food allergy, cashew allergy, tree nut allergy, vicilin, 7S globulin, sucrose-binding protein, seed storage proteins, linear epitope, epitope map*

Cashew nuts have been associated with contact or systemic dermatitis (to cardol and anacardic acid found in the cashew nut shell oil[1]), atopic dermatitis, and IgE-mediated systemic allergic reactions.[2-4] Pistachios are another allergenic member of the Anacardiaceae family and show extensive in vitro cross-reactivity with cashews.[5,6] Cashew nuts are widely used in snack foods and as an ingredient in a variety of processed foods, such as bakery and confectionery products.

We have recently found that the major allergenic proteins in cashew nut are legumin-like proteins and 2S albumins by sequencing of the N-termini and selected internal digests (unpublished data). We also identified an allergen in the 7S superfamily, which includes vicilin-like and sucrose-binding proteins (unpublished data). Here we present the sequence of a cDNA encoding this protein, designated Ana o 1, and describe the characterization of the expressed recombinant protein. In addition, we identify several linear epitopes and compare these with the allergenic peanut vicilin Ara h 1.[7]

Abbreviations used
HRP: Horseradish peroxidase
RT: Room temperature

METHODS

Human sera

Blood samples were drawn after 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 human subjects review committee of the University of California at Davis. The presence of cashew-reactive IgE was confirmed by means of Pharmacia ImmunoCAP assay or Western immunoblotting, as described below. Control sera were obtained from patients with histories of anaphylaxis to walnut, pistachio, or hazelnut who reported tolerance of cashews.

Cashew protein extract

Cashew protein extract was obtained from defatted cashew flour by means of extraction with 0.1 mol/L Tris-HCl, pH 8.1, and stored at –20°C for later analysis. Protein concentrations were measured by use of the Bradford protein assay (BioRad Laboratories, Inc, Hercules, Calif).

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.

Construction and IgE immunoscreening of cashew cDNA library

Four cashew nuts in late maturation were chopped, frozen in liquid nitrogen, and ground with a mortar and pestle. Total RNA was

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extracted in TRIzol (GIBCO BRL Life Technologies Inc, Rockville, Md), as previously described,[8] and mRNA was isolated with a PolyATtract kit (Promega, Madison, Wis), according to the manufacturer's instructions. The construction of the cDNA library was performed with the Uni-ZAP XR Gigapack Cloning Kit (Stratagene Inc, Cedar Creek, Tex), according to the manufacturer's instructions. 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 library was amplified on *Escherichia coli* strain XL1-Blue. The amplified library was initially screened with rabbit anti-cashew serum at 1:5000 dilution. Bound IgG was detected with horseradish peroxidase (HRP)–conjugated goat anti-rabbit IgG antibody (Sigma, St Louis, Mo) at 1:50,000 dilution and developed with the ECL Plus chemiluminescent kit (Amersham Pharmacia Biotech, Piscataway, NJ). Clones were subsequently screened with antiserum from patients with cashew allergy (at 1:20 dilution) and detected with HRP-conjugated goat anti-human IgE antibody (Biosource International, Camarillo, Calif) at 1:2000 dilution and developed with ECL. The immunopositive clones were picked, plaque purified, and stored in SM buffer 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 means of PCR. Both strands of the PCR products were then sequenced on an ABI 3100 Genetic Analyzer (Foster City, Calif) by 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) by using the basic local alignment search tool program.

Cloning, expression, and purification of cDNA-encoded proteins

cDNA-coding sequences were modified by the addition of an *Xba*I site at the 5′ end and a *Pst*I site at the 3′ end by using PCR with PfuTurbo DNA polymerase (Stratagene Inc) followed by digestion and ligated to their respective sites of the maltose-binding protein (MBP) fusion expression vector pMAL-c2 (New England BioLabs Inc, Beverly, Mass), containing an added thrombin cleavage site.

Competent *E coli* BL21 (DE3) cells (Novagen Inc, Madison, Wis) were transformed with cDNA/pMAL-c2 plasmids, and single colonies were grown at 37°C to an OD_{600} of 0.5, followed by induction with isopropyl-D-thiogalactopyranoside. The cells were harvested, resuspended in amylose resin column buffer (20 mmol/L Tris-HCl [pH 7.4], 200 mmol/L NaCl, 10 mmol/L β-mercaptoethanol, and 1 mmol/L EDTA), lysed with mild sonication, centrifuged at 12,000*g*, and passed over an amylose affinity column, and the fusion protein was eluted with column buffer containing 10 mmol/L maltose. Fusion proteins were cleaved with thrombin (Sigma) at 1 mg of fusion protein per unit of thrombin in column buffer (3 hours at room temperature [RT]). The liberated recombinant protein was purified by means of HPLC on a Superdex 200 column (Amersham Pharmacia), concentrated, and either stored (briefly) at 4°C until use or frozen at –70°C.

Polyacrylamide gel electrophoresis and protein transfer

Recombinant protein or cashew extract samples were subjected to SDS-PAGE (12%) with 0.5 μg of recombinant protein or 11 to 17 μg of total cashew extract per 4-mm well width and used for immunoblotting, as previously described.[9]

IgE Western immunoblotting and inhibition

Strips (3- to 4-mm wide) of blotted nitrocellulose were blocked for 1 hour at RT in PBS/3% nonfat dry milk/0.2% Triton X-100. Diluted sera (1:5 vol/vol in the blocking buffer but 1:20 vol/vol for highly reactive sera) were added to the strips and incubated overnight at RT. The strips were then washed for 20 minutes 3 times in PBS/0.01% Triton X-100 and incubated overnight at RT with equine polyclonal iodine 125 [^{125}I]–labeled anti-human IgE (Hycor Biomedical Inc, Garden Grove, Calif) diluted 1:5 in the nonfat milk buffer. The strips were washed as above and exposed to x-ray film (Kodak X-OMAT).

For inhibition experiments, 10 μg of recombinant Ana o 1 (with and without associated MBP) was preincubated with human or rabbit antisera. Human antiserum (from patient 9 at 1:20 dilution) or rabbit antiserum (at 1:5000 dilution) were incubated with Ana o 1 for 1 hour at 37°C and then incubated with nitrocellulose strips containing blotted cashew extract overnight at 4°C. Controls included strips incubated with human or rabbit antisera without added Ana o 1 and with normal human and rabbit serum. The strips were then washed for 15 minutes, followed by three 5-minute washes in Tris-buffered saline–Tween and incubated with goat anti-rabbit IgG-HRP (Sigma) at 1:50,000 in Tris-buffered saline–Tween for 1 hour at RT or ^{125}I-labeled anti-human IgE at 1:10 in PBS-Tween with 5% dried milk overnight at 4°C. The strips were washed as above, and for human antibody, the reactive bands were identified by means of exposure to x-ray film (Kodak X-OMAT) directly or, for rabbit antibody, developed by using the ECL-Plus chemiluminescence kit (Amersham Pharmacia Biotech), as described by the manufacturer, followed by exposure to x-ray film (Kodak X-OMAT).

Amino acid sequencing

SDS-PAGE was carried out as above on reduced cashew polypeptides by using a 10% acrylamide gel, and proteins were transferred to Trans-Blot PVDF membrane (0.2 μm, BioRad). The N-terminal sequence of the approximately 50-kd protein was determined by using blotted protein on an ABI 477A sequencer with an online 120A HPLC system (Applied Biosystems, Inc, Foster City, Calif). Internal tryptic digests after carboxymethylation were performed on the bands from a wet acrylamide gel, separated by means of ABI 173 Microbore HPLC, and then sequenced as above. Sequence data were collected with ABI Procise software (Applied Biosystems, Inc) and analyzed with FASTA programming (European Bioinformatics Institute, http://www2.ebi.ac.uk/fasta3/).

Solid-phase peptide synthesis and binding to IgE

On the basis of the derived amino acid sequence of the 540-amino-acid Ana o 1 protein, 66 overlapping 15-amino-acid peptides, each offset by 8 amino acids, were synthesized and probed with pooled sera diluted 1:5 (vol/vol) in Genosys blocking buffer, followed by washing, incubation with ^{125}I-labeled anti-human IgE (Hycor Biomedical Inc), and 48 hours' exposure at –70°C to Kodak Biomax x-ray film, as previously described.[10]

RESULTS

Library screening and gene characterization

The initial screening included separate probings with human serum (IgE immunodetection) and rabbit antisera (IgG immunodetection). Clones that gave positive signals for both probes were selected because one of our future goals is to determine the degree of epitope overlap between these 2 species. A total of 50 clones were selected, of which 4 were purified and sequenced. The 4 clones varied in

A

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Ao1.1    GCCATAATGGGTCCGCCTACAAAGTTTTCTTTTTCTCTTTTTCTCGTTTCTGTTTTGGTCCTGTGTTTAGGTTTTGCTTT   80
Ao1.2              ************************************************************************

Ao1.1,2 GGCTAAAATAGACCCGGAGCTGAAACAGTGCAAGCACCAGTGCAAAGTCCAGAGGCAGTATGACGAGCAACAGAAGGAGC  160
Ao1.1,2 AGTGTGTGAAAGAGTGTGAAAAGTACTACAAAGAGAAGAAAGGACGGGAACGAGAGCATGAGGAGGAAGAAGAAGAATGG  240
Ao1.1,2 GGAACTGGTGGCGTTGATGAACCCAGCACTCATGAACCAGCTGAAAAGCATCTCAGTCAGTGCATGAGGCAGTGCGAGAG  320
Ao1.1,2 ACAAGAAGGAGGACAACAAAAGCAACTATGCCGCTTTAGGTGTCAGGAGAGGTATAAGAAAGAGAGAGGACAACATAATT  400
Ao1.1,2 ACAAGAGAGAAGACGATGAAGACGAAGACGAAGACGAAGCCGAGGAAGAAGATGAGAATCCCTATGTATTCGAAGACGAA  480
Ao1.1,2 GATTTCACCACCAAAGTCAAGACTGAGCAAGGAAAAGTTGTTCTTCTTCCCAAGTTCACTCAAAAATCGAAGCTTCTTCA  560
Ao1.1,2 TGCCCTGGAGAAATACCGTCTAGCCGTTCTCGTTGCGAATCCTCAGGCTTTTGTAGTTCCAAGCCACATGGATGCTGACA  640
Ao1.1,2 GTATTTTCTTCGTTTCTTGGGGACGAGGAACGATCACCAAGATCCTTGAGAACAAACGAGAGAGCATTAATGTCAGACAG  720
Ao1.1,2 GGAGACATCGTCAGCATTAGTTCTGGTACTCCTTTTTATATCGCCAATAACGACGAAAACGAGAAGCTTTACCTCGTCCA  800
Ao1.1,2 ATTCCTCCGACCAGTCAATCTTCCAGGGCATTTCGAAGTGTTTCATGGACCAGGCGGTGAAAATCCAGAGTCTTTCTACA  880
Ao1.1,2 GAGCTTTCAGCTGGGAAATACTAGAAGCCGCACTGAAGACCTCAAAGGACACACTTGAGAAACTTTTCGAGAAACAGGAC  960

Ao1.1    CAAGGAACTATCATGAAAGCCTCCAAAGAACAAATTCGGGCTATGAGCCGGAGAGGCGAAGGCCCTAAAATTTGGCCATT 1040
Ao1.2    **********************************G********************************************

Ao1.1,2 TACAGAGGAATCAACGGGATCATTCAAACTTTTCAAAAAGGATCCCTCTCAATCCAATAAATACGGCCAACTCTTTGAAG 1120
Ao1.1,2 CTGAACGTATAGATTATCCGCCGCTTGAAAAGCTTGACATGGTTGTCTCCTACGCGAACATCACCAAGGGAGGAATGTCT 1200
Ao1.1,2 GTTCCATTCTACAACTCACGGGCAACGAAAATAGCCATTGTTGTTTCAGGAGAAGGATGCGTTGAAATAGCGTGTCCTCA 1280
Ao1.1,2 TCTATCCTCTTCGAAAAGCTCACACCCAAGTTACAAGAAATTGAGGGCACGGATAAGAAAGGACACAGTGTTCATTGTCC 1360
Ao1.1,2 CGGCGGGTCACCCTTTCGCGACTGTTGCTTCGGGAAATGAAAACTTGGAAATCGTGTGCTTTGAAGTAAACGCAGAAGGC 1440
Ao1.1,2 AACATAAGGTACACACTTGCGGGGAAGAAGAACATTATAAAGGTCATGGAGAAGGAAGCGAAAGAGTTGGCATTCAAAAT 1520
Ao1.1,2 GGAAGGAGAAGAAGTGGACAAAGTGTTTGGAAAACAAGATGAAGAGTTTTTCTTCCAGGGGCCGGAATGGCGAAAGGAAA 1600

Ao1.1    AAGAAGGGCGTGCTGATGAATGAGAAGAAATGGGAAGGTTGTTTGGGGTCTGAGAAAGGCTGAGCTACTGACTAGTGAAC 1680
Ao1.2    ************************************************************************

Ao1.1    GTTATATATGGATAACGTATATATGTATGTAAATGTGAGCAGCGGACATCATCTTCCCAACTGCATTAAGCAAAACTAAA 1760

Ao1.1    TAAAAAGAAAAGGCTTTAGCCAAAAAAAAAAAAAAAAAAAAAAAAAAAA                               1810
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B

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                                    ^Ana o 1a
Ao1.1    AIMGPPTKFSFSLFLVSVLVLCLGFALAKIDPELKQCKHQCKVQRQYDEQQKEQCVKECEKYYKEKKGREREHEEEEEEW  80
Ao1.2        ***************************************************************************

                               ^Ana o 1b
Ao1.1,2 GTGGVDEPSTHEPAEKHLSQCMRQCERQEGGQQKQLCRFRCQERYKKERGQHNYKREDDEDEDEDEAEEEDENPYVFEDE 160
Ao1.1,2 DFTTKVKTEQGKVVLLPKFTQKSKLLHALEKYRLAVLVANPQAFVVPSHMDADSIFFVSWGRGTITKILENKRESINVRQ 240
Ao1.1,2 GDIVSISSGTPFYIANNDENEKLYLVQFLRPVNLPGHFEVFHGPGGENPESFYRAFSWEILEAALKTSKDTLEKLFEKQD 320

Ao1.1    QGTIMKASKEQIRAMSRRGEGPKIWPFTEESTGSFKLFKKDPSQSNKYGQLFEAERIDYPPLEKLDMVVSYANITKGGMS 400
Ao1.2    ***********V************************************************************************

Ao1.1,2 VPFYNSRATKIAIVVSGEGCVEIACPHLSSSKSSHPSYKKLRARIRKDTVFIVPAGHPFATVASGNENLEIVCFEVNAEG 480
Ao1.1,2 NIRYTLAGKKNIIKVMEKEAKELAFKMEGEEVDKVFGKQDEEFFFQGPEWRKEKEGRADE                     540
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FIG 1. Nucleotide **(A)** and derived amino acid sequence **(B)** of the Ana o 1 cDNA clones *Ana o 1.0101 (Ao1.1)* and *Ana o 1.0102 (Ao1.2)*. The N-terminal amino acid of the recombinant peptides, Ana o 1a and Ana o 1b, are indicated *(^)*. The presumed allelic difference between the 2 genes is indicated in bold type. The presumed start and stop codons are underlined. *Asterisks* denote identity. GenBank accession numbers: AF395893 and AF395894.

length from 1699 to 1781nt, excluding the poly-A tails. Comparison of the sequences revealed that 3 differed only in the length of their 3′ ends. A fourth shared a common 5′ end, but its 3′ end was truncated and fused through an adapter sequence, with another apparently unrelated cDNA sequence showing homology with plant ABA-responsive protein (data not shown). *Ana o1.0101* (*Ao1.1*; Fig 1, *A*) represents the longest version of the cDNAs, and *Ana o1.0102 (Ao1.2)* is the truncated version. Note that only a single nucleotide substitution (A for G) at residue 994 distinguishes *Ao1.1* and *Ao1.2* in their region of common overlap, suggesting that this represents an allelic difference.

Protein sequence characterization

Analysis of the deduced amino acid sequence revealed a 540-amino-acid open-reading frame, a possible start codon at position 3, and possible leader peptide from 3 to 28 (Fig 1, *B*). Probing of the selected clones by means of PCR with an internal (*Ao1*–specific) primer paired with a vector primer to amplify the 3′ ends of the cDNA inserts revealed that all but 2 of the remaining 46 plaques were *Ao1* like, and each produced an amplicon similar in size to *Ao1* (data not shown).

A search of Genbank revealed that *Ao1.1* and *Ao1.2* encode members of the 7S (vicilin) superfamily of pro-

TABLE I. Proteins showing identity and similarity to Ana o1

Protein	Organism	Accession no.	Amino acid overlap	Identity	Similarity
7S globulin	*Elaeis guineensis* (African oil palm)	AAK28402	6-524	40%	59%
Vicilin precursor[11]	*Macadamia intergrifolia* (macadamia nut)	AAD54246	35-538	37%	57%
Sucrose-binding protein homolog	*Pisum sativum* (garden pea)	TO6459	95-538	43%	62%
Sucrose-binding protein precursor[12]	*Glycine max* (soybean)	Q04672	3-539	36%	52%
Vicilin-like protein precursor[13]	*Juglans regia* (English walnut)	AF066055	33-537	33%	54%

teins. Sequences with the highest amino acid identity and similarity were in the 33% to 43% and 52% to 62% range, respectively, and were variously described as vicilins and sucrose-binding proteins and their precursors and 7S globulins from nut and seed crops (Table I).[11-13]

Reactivity of the recombinant protein with human IgE and rabbit IgG

For immunologic characterization, we cloned a long version of the *Ao1.1* cDNA (designated *Ao1.1a*) beginning (at K29) after the presumptive leader peptide and a short version *(Ao1.1b)* beginning with the methionine residue 102 (M102; Fig 1, *B*). The DNA segments were ligated into an expression vector designed to allow for purification of the recombinant molecules by way of a maltose-binding protein fusion domain in conjunction with an amylose affinity column and a thrombin-specific cleavage site. The resulting approximately 105- and 93-kd fusion proteins Ana o 1a and Ana o 1b, respectively, were affinity purified and digested with thrombin to yield approximately 65- and 55-kd peptides, respectively, as well as the 43-kd MBP. Both cleaved and uncleaved peptides were reactive with specific human IgE and rabbit IgG (data not shown).

TABLE II. IgE Western blot reactivity with rAna o 1

Cashew allergy		Cashew tolerance	
Subject no.	Reactivity	Subject no.	Reactivity
1	–	21	+++
2	–	22	+
3	+++	23	–
4	–	24	–
5	–	25	–
6	+	26	–
7	+	27	–
8	–	28	–
9	+++		
10	–		
11	+		
12	–		
13	–		
14	+++		
15	+		
16	+		
17	+		
18	+		
19	–		
20	–		

Recognition of Ana o 1 as an allergen

The prevalence of reactivity to rAna o 1 among patients with cashew allergy by means of Western immunoblotting is shown in Table II. IgE from 10 of 20 sera from patients with a history of life-threatening reactions to cashews bound the recombinant. In 3 cases the intensity of the signal was strong, but it was weak in 7 cases, implying, although not proving, variable titers of antibody directed to this protein. In contrast, 2 of 8 sera from patients tolerant of cashew but clinically with life-threatening reactions to other tree nuts reacted with the rAna o 1. One of these (sera 22) showed weak binding (the patient self-reported mild throat scratchiness with cashew), whereas the other (sera 21) showed strong binding, yet the patient reconfirmed no symptoms on cashew ingestion but has experienced 4 emergency department visits after accidental walnut or pecan ingestion and recent strong wheal-and-flare reactions to walnut and cashew on skin prick testing.

Identification of native Ana o 1 by means of SDS-PAGE immunoblotting

To identify the band or bands in a typical total cashew immunoblot that correspond to the cloned polypeptide storage protein precursor, we attempted to inhibit the binding of sera from a cashew extract–immunized rabbit and allergic patient 9 to nitrocellulose strips blotted with total soluble cashew extract by using purified rAna o 1 as the inhibitor (Fig 2). No inhibitor was added to the antisera used to probe the left strip in each pair (Fig 2, *lanes 1, 3,* and *5*), whereas the antisera used to probe the right strip in each pair (Fig 2, *lanes 2* and *4*) was preincubated and coincubated with 5 μg of purified rAna o 1 protein. Serum used in lane six was preincubated and coincubated with 5 μg of recombinant MBP. It can be seen that recombinant protein inhibits IgE binding to a band of molecular mass of approximately 50 kd. Undigested rAna o 1/MBP inhibitor yielded similar results (data not shown). Thus the native precursor, corresponding to our approximately 65-kd recombinant protein, appears to undergo further cleavage as in other vicilin group proteins.

Identification of native Ana o 1 by sequencing

N-terminal sequencing of the band identifying the immunoblot inhibition was uninformative; however, one of the internal tryptic digest peptides showed 100% homology with the translated amino acid sequence of Ana o 1 from position 295 to 306, AFSWEILEAALK.

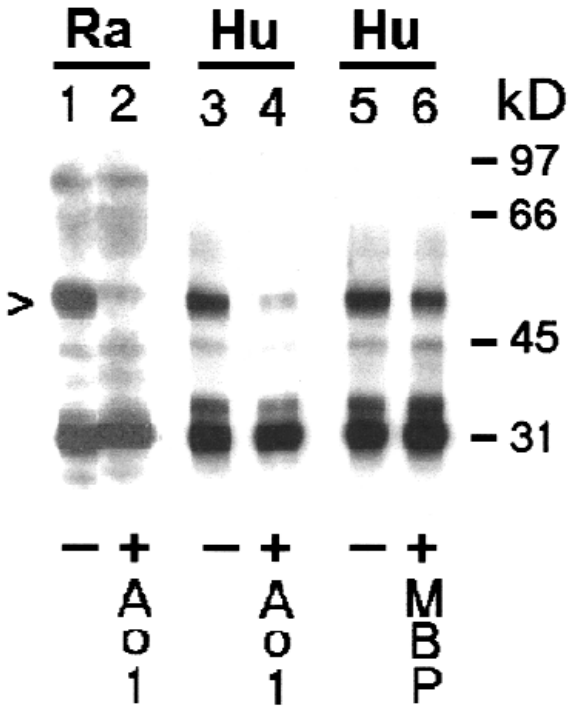

FIG 2. Identification of native Ana o 1 peptide in cashew extract by means of Western blotting. Nitrocellulose blots of total cashew extract probed with rabbit anti-cashew extract antiserum *(Ra)* and human serum from a patient with cashew allergy (no. 9, *Hu)* are shown. *Lanes 1, 3,* and *5,* No inhibitor added (–); *lanes 2* and *4,* 5 μg of purified rAna o 1 preincubated and coincubated with anti-sera; *lane 6,* 5 μg of recombinant MBP preincubated and coincubated with antisera. *Arrow* indicates inhibited band in lanes 2 and 4.

Identification and recognition of IgE-reactive linear epitopes on Ana o 1 and comparison with peanut vicilin linear epitopes

The entire amino acid length of Ana o 1 was studied by probing overlapping solid-phase synthetic peptides with rAna o 1–reactive sera from 12 patients randomly assigned to 3 pools. Collectively, the 3 pools reacted with 11 linear IgE-binding epitopes, which were distributed throughout the length of the protein (Table III). Three of the identified epitopes were bound by patient sera from all 3 pools. Epitope 4 was bound strongly by pools 1 and 2 and moderately by pool 3. Epitope 11 was bound strongly by pool 2 but only moderately by pools 1 and 3. The third epitope, epitope 1, was bound moderately by all 3 pools. Some epitopes gave moderate (epitopes 2, 3, 7, 8, 9, and 10) or strong (epitope 6) signals only when probed with pool 1. Similarly, epitope 5 was moderately recognized only by pool 2.

To compare the recognized linear epitopes of cashew vicilin (Ana o 1) with those of peanut vicilin (Ara h 1),[7] we aligned the 2 sequences with the BLAST program and highlighted the corresponding linear epitopes (Fig 3). Only 4 of the 11 Ana o 1 and 23 Ara h 1 linear epitopes showed significant (≥7 amino acids) positional overlap. Even among the overlapping epitopes there was no significant homology or similarity between the Ana o 1 and Ara h 1 sequences especially when considering that a single amino acid substitution usually eliminates reactivity in allergens.[7,10,14-17]

DISCUSSION

There has been considerable interest in recent years in precise biochemical and immunologic characterization of allergens for possible application to immunotherapy, clinical screening, and immunoassay development.[18-21] Moreover, new advances in plant genetic engineering may allow for the alteration of plant proteins to generate hypoallergenic cultivars.[22] Not only is it important to identify allergens for possible modification in host plants, but there is a need for information on the potential for allergenicity of the products of genes that are to be transferred to unrelated hosts for nutritional, processing, or management purposes, as witnessed by the ill-fated attempt to transfer Brazil nut 2S albumin genes to certain crops.[23] It is thus important that as many food allergens as possible, both major and minor, be identified to add to this database of information.

An invaluable method for allergen identification relies on the screening of cDNA libraries produced from the offending tissue and screened with allergen-specific antibody. We have applied this approach to the identification of the precursor of a cashew allergen, designated Ana o 1, another allergen of the vicilin-like protein family. The 7S globulins from legumes are referred to as vicilins and are seed storage proteins that often exist as large trimeric oligomers in the seed protein bodies, with individual subunits usually 40 to 70 kd in size. Peanut vicilin, Ara h 1, has been shown to resist proteolysis when in a trimeric configuration, a property that may contribute to its allergenicity.[24] Globulin proteins from several nonleguminous plant seeds have been found to have significant sequence homology to the vicilins and can thus be designated as part of the vicilin group of proteins (vicilin-like), such as the previously described walnut *(Juglans regia)* tree nut allergen Jug r 2.[13]

The unique IgE-binding patterns of each pool of sera to the Ana o 1 epitopes suggests a heterogeneity of recognition patterns between patients, although some peptides were recognized by members of 2 or all 3 pools. Perhaps significantly, the most strongly reacting peptides were the 3 recognized by the sera in all 3 pools, with the exception of epitope 6, which was strongly recognized only by pool 1.

Multiple linear IgE-binding epitopes residing on one allergenic peptide have been observed in many other cases of foods known to cause immediate hypersensitivity reactions,[25-31] although we recently reported finding only a single linear epitope on the walnut 2S albumin, Jug r 1.[10] The fact that most allergens have multiple IgE-binding sites is at least partly the result of the polyclonal nature of the immune response to these allergens.[16]

When considered together, conformational and linear epitopes may cover the entire surface of some allergens

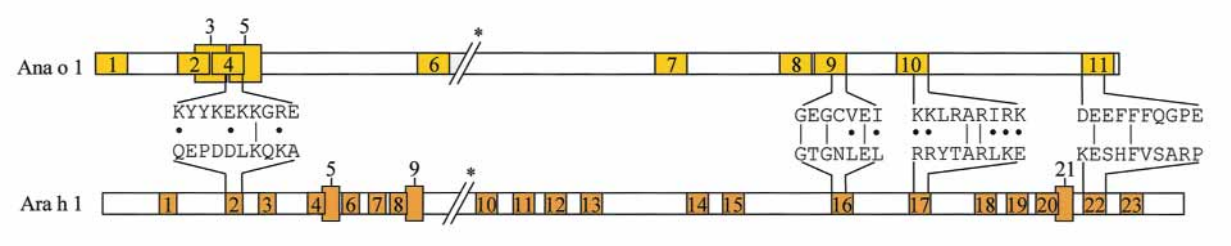

FIG 3. Comparison of the linear epitope maps of cashew Ana o 1 and peanut Ara h 1.[7] Epitopes are numbered, and larger boxes depict epitopes that overlap within a sequence. Sequences of epitopes showing positional overlap between Ana o 1 and Ara h 1 are shown for comparison. *Asterisks* indicate breaks in sequence bars corresponding to 132-amino-acid epitope-free regions in each sequence. |, Identical amino acids; •, similar amino acids.

TABLE III. Ana o 1 IgE-binding epitopes

Epitope no.	Amino acid sequence*	Ana o 1 position	Pool reactivity†		
			Pool 1	Pool 2	Pool 3
1	**AIMGPPTKFSFSLFL**	1-15	++	++	+
2	CKVQRQYDEQQKEQC	41-55	+	–	–
3	EQQKEQCVKECEKYY	49-53	+	–	–
4	**KECEKYYKEKKGRER**	57-71	+++	+++	++
5	EKKGREREHEEEEEE	65-79	–	++	–
6	DEAEEEDENPYVFED	145-159	+++	–	–
7	RRGEGPKIWPFTEES	337-351	++	–	–
8	NITKGGMSVPFYNSR	393-407	+	–	–
9	TKIAIVVSGEGCVEI	409-423	+	–	–
10	SSHPSYKKLRARIRK	433-447	+	–	–
11	**EEFFFQGPEWRKEKE**	521-535	+	+++	+

+++, strong binding; ++, moderate binding; +, weak binding.
*Peptides believed to be immunodominant epitopes are shown in bold.
†Pool 1 was composed of patients 3, 7, 9, and 23; pool 2 was composed of patients 4, 11, 14, 15, and 18; and pool 3 was composed of patients 6, 17, and 21.

(ie, Hol l 5 grass allergen).[32] Interestingly, the linear epitopes, rather than the conformational epitopes, have been reported to be better predictors of allergy persistence, as in cows' milk allergy.[33]

To date, no common structural characteristics of linear IgE epitopes have been identified,[34] but this could change as more epitope-mapping studies are completed. Cashew Ana o 1 and the major allergen in peanuts, Ara h 1, are both vicilins and both express multiple epitopes; in fact, 23 linear epitopes have been described for Ara h 1.[7] In parallel with the observations on Ara h 1, we did not observe any obvious sequence motifs that are shared by any 2 linear epitopes within Ana o 1. Surprisingly, even though these 2 proteins share 27% identity and 45% similarity in amino acid sequence and presumably are similar in overall structure, there was no significant sequence conservation between epitopes of the 2 allergens. Moreover, even though 4 epitope regions showed positional overlap between Ana o 1 and Ara h 1, most did not (Fig 3). Although it is still possible that conformational (discontinuous) epitopes could be shared in common, these remarkable observations may help explain the lack of cross-reactivity between tree nut– and peanut-reactive patients' sera.[35]

On the basis of our previous immunoblotting of cashew proteins, it is known that major allergens are located in the 2S albumin protein family and the legumin family. Interestingly, our cDNA library constructed late in maturation appears to have overwhelmingly contained mRNA for the vicilin-like storage protein. This protein is a major allergen by definition (bound by ≥50% of allergic patients). Because several studies have shown that there is no direct correlation between binding strength in in vitro assays and in vivo reactivity,[21,36-38] the relative importance of this protein among the various cashew proteins responsible for the allergic reaction in sensitive patients is yet to be determined. Efforts are underway to clone cDNAs encoding the additional allergenic cashew seed storage proteins.

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