

Ana o 3, an important cashew nut (*Anacardium occidentale* L.) allergen of the 2S albumin family

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Background: Cashew nut allergy is the second most commonly reported tree nut allergy in the United States. We have previously cloned and characterized major cashew allergens belonging to the vicilin and legumin families of seed storage proteins.

Objective: Here we set out to describe a third major cashew allergen, a 2S albumin.

Methods: The recombinant cashew 2S albumin was amplified from a cDNA library by means of PCR, sequenced, and expressed in *Escherichia coli*. Immunoblotting was used to screen for reactivity with patients' sera, and inhibition immunoblotting was used to identify the corresponding native cashew nut proteins. The mass of affinity-purified native allergen was determined by means of matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectroscopy. Patients' sera were used to probe solid-phase 2S albumin peptides to identify linear epitopes.

Results: The cloned allergen, designated Ana o 3, was identified as 2S albumin. MALDI-TOF mass spectroscopy of native Ana o 3 yielded a molecular mass of 12,598 d. Immunoblot analysis showed 21 (81%) of 26 sera from patients with cashew allergy were reactive. Three native Ana o 3 large-subunit isoforms with molecular weights ranging from approximately 6 to 10 kd were identified. Probing of overlapping synthetic Ana o 3 peptides with patients' sera identified 16 reactive peptides, 4 of which gave strong signals and one of which positionally overlaps linear epitopes in mustard and walnut allergenic 2S

albumins. The overlapping cashew and walnut epitopes also share considerable homology.

Conclusions: We conclude that this 2S albumin protein is a major allergen in cashew nut and demonstrates a possible basis for cross-reactivity with walnut 2S albumin. (J Allergy Clin Immunol 2005;115:1284-90.)

Key words: Ana o 3, food allergy, cashew allergen, tree nut allergy, 2S albumin, epitope mapping, seed storage proteins

Cashew nuts are associated with IgE-mediated anaphylaxis, and cashew nut allergy is the second most commonly reported tree nut allergy after walnut allergy in the United States.¹ A recent random-digit-dial telephone survey found that as many as 41% of individuals with tree nut hypersensitivity self-reported allergy to cashew.² Pistachio and mango are other edible members of the Anacardiaceae family and are also allergenic, with pistachio showing extensive *in vitro* and possible clinical cross-reactivity with cashews.³⁻⁵ 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.

2S albumins from a variety of seeds and nuts have been shown to bind IgE from allergic patients' sera.⁶⁻¹⁸ Garcia et al¹⁹ reported 3 cases of anaphylactic reactions to cashew nuts in which the strongest binding proteins in immunoblots were 15, 30, and 60 kd. The authors suggested that the 15-kd polypeptide was a member of the 2S albumin family, but no confirmatory data were produced. We have recently demonstrated that the major IgE-reactive proteins in seed protein extracts of cashew nut are legumin-like proteins and 2S albumins, as assessed by means of N-terminal and enzymatic fragment sequencing of native proteins.¹⁸ We have recently cloned and expressed a 55-kd legumin-like protein and a similarly sized 7S globulin or vicilin-like protein.^{20,21} Here we present the sequence and characteristics of a third cDNA encoding an IgE-reactive proprotein designated Ana o 3, a member of the 2S albumin family of seed storage proteins, and map its IgE-reactive linear epitopes.

METHODS

Human sera

Blood samples were drawn after obtaining informed consent from patients with self-reported reactions to cashew nut, and the sera were

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Supported in part by USDA grant no. 2003-01212 (K.H.R. and S.K.S.) and National Institutes of Health grant K23 A101604-01 (S.S.T.).

Disclosure of potential conflict of interest: J. M. Robotham—none disclosed. F. Wang—none disclosed. V. Seamon—none disclosed. S. Teuber—none disclosed. S. Sathe—none disclosed. H. Sampson serves as a consultant to Seer, Inc, and Genentell. K. Beyer—none disclosed. M. Seavey—none disclosed. K. H. Roux—none disclosed.

Received for publication November 10, 2004; revised February 8, 2005; accepted for publication February 22, 2005.

Available online April 25, 2005.

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0091-6749/\$30.00

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doi:10.1016/j.jaci.2005.02.028

Abbreviations used

MALDI-TOF: Matrix-assisted laser desorption
ionization–time of flight
MBP: Maltose-binding protein
MS: Mass spectroscopy

stored at -70°C until use. The study was approved by the relevant institutional review boards. The presence of cashew-reactive IgE was confirmed with the ImmunoCAP assay (Pharmacia Diagnostics, Uppsala, Sweden) or by means of immunoblotting. Clinical characteristics of the subjects are shown in Table I.^{19,20,22} Control sera were obtained from patients with a history of pollinosis to weeds, trees, or grasses but no food allergy.

Cashew protein extract

Soluble protein extract was prepared from defatted cashew nut flour, as previously described.²³ Protein concentrations were measured by using the Bradford protein assay (BioRad Laboratories, Inc, Hercules, Calif), with BSA as the standard protein.

Construction of cashew cDNA library and identification of the 2S albumin gene

Library construction has previously been described in detail.²⁰ A 2S albumin gene from the cashew cDNA library was amplified by means of PCR with the aid of a degenerate forward primer, 5'-CGTCAAGAGTCTYTTCGTGARTGYTGTCAR-3' (Y = C/T, R = A/G), deduced from the previously published cashew 2S albumin N-terminal amino acid sequence data¹⁸ and a lock-dock reverse primer, 5'-TTTTTTTTTTTTTTTTTNN-3' (N = A, C, G, and T; Qiagen, Inc, Valencia, Calif). Both strands of the PCR product were sequenced on an ABI 3100 Genetic Analyzer (Foster City, Calif). Similarity searches of deduced amino acid sequences were performed on Genetics Computer Group software (Accelrys, Inc, San Diego, Calif) with the BLAST 2.0 program (www.ncbi.nlm.nih.gov/BLAST/). Leader peptide prediction was performed with the SignalP V.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,²⁰ after the gene was sequenced, a pair of primers was designed for expression by incorporating a *Bam*HI site (underlined) at the 5' end of the forward primer, 5'-GTCTGGATCCATGGCAAAGTCTTACTCC-3', and a *Sal*I site (underlined) in the reverse primer, 5'-TCTAGAGTCGACTTAC-TAATAAGATGACTGAAGT-3'. The insert was PCR amplified, digested with *Bam*HI and *Sal*I, and ligated into a maltose-binding protein (MBP) fusion expression vector, pMAL-c2 (New England BioLabs Inc, Beverly, Mass), containing a thrombin cleavage site.

Positive clones were identified by means of PCR screening and expressed, and the protein was purified as previously described.²⁰ A yield of 1 to 3 mg of soluble fusion protein per liter of cultured cells was routinely recovered.

Gel electrophoresis (SDS-PAGE), immunoblotting, inhibition, and N-terminal sequencing

Electrophoresis and immunoblotting to test for reactivity in patients with cashew allergy to rAna o 3 were carried out as previously described.²⁰ Alternatively, 2.0 μg of the recombinant

fusion protein in 2.0 μL of ddH₂O were dotted onto nitrocellulose for use in a less denaturing dot-blot assay, as previously described.²⁴

Total cashew extract samples (at 200 $\mu\text{g}/5\text{-mm}$ well) were boiled in reducing sample buffer and electrophoresed on large-format (160 $\times$ 180 mm) 18% Laemmli gels in a Hofer SE 600 Series standard dual-cooled gel electrophoresis unit (Hoefer Scientific Instruments, San Francisco, Calif) to allow for optimal separation of low-molecular-weight bands and enhanced resolution for SDS-PAGE, sequencing, and inhibition experiments. The gel was either stained with colloidal Coomassie stain (Sigma-Aldrich, St Louis, Mo) or transferred to ProBlot PVDF membrane (Applied Biosystems, Foster City, Calif) for sequencing or to nitrocellulose for blotting in inhibition assays. N-terminal sequence analysis was performed on a Procise 492 cLC Protein Sequencing System (Applied Biosystems). For inhibition experiments, 40 μg of rAna o 3–MBP fusion protein or MBP alone (negative control) was preincubated with human antiserum from patient 31 at 1:5 dilution for 3 hours at room temperature and then incubated with nitrocellulose strips containing the blotted whole cashew extract overnight at 4°C . The strips were subsequently incubated with the appropriately labeled second antibody and exposed to x-ray film, as previously described.

Affinity column purification of native Ana o 3

Recombinant Ana o 3–MBP fusion protein, covalently attached to cyanogen bromide-activated Sepharose 4B (Sigma-Aldrich), was used to purify goat anti-cashew antibody previously obtained by immunizing with an aqueous cashew nut extract in Freund's adjuvant. A second affinity column coupled with purified goat anti-Ana o 3 antibody was used to purify the native homologue from total cashew nut protein extract.

Matrix-assisted laser desorption ionization–time of flight mass spectroscopic analysis of affinity-purified native Ana o 3

For precise mass determination of native Ana o 3, matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) mass spectroscopy (MS) was performed on a Bruker Biflex III mass spectrometer (Bruker Daltonics, Billerica, Mass) equipped with a nitrogen laser (337 nm), Scout 384 target-ion source and video system, pulsed ion extraction, and dual-channel plate detector. Sinapinic acid 3,5 dimethoxy-4-hydroxycinnamic acid (Sigma-Aldrich) was used as the MALDI matrix. Briefly, 1 μL of native Ana o 3 (0.2 $\mu\text{g}/\mu\text{L}$) was mixed with 9.0 μL of sinapinic acid (10 mg/mL in acetonitrile/0.1% trifluoroacetic acid, 1:2, vol/vol). One microliter of this mixture was spotted onto the MALDI target, which had been precoated with 1 μL of the matrix solution. The sample was allowed to dry under ambient conditions.

Solid-phase peptide synthesis and binding to IgE

On the basis of the cDNA-derived amino acid sequence of the 138-amino-acid Ana o 3 protein (excluding the presumptive leader sequence, amino acids 1–20), 36 overlapping 12-amino-acid peptides, each offset by 3 amino acids, were synthesized. Peptides were synthesized on derivatized cellulose sheets by using 9-fluorenylmethoxycarbonyl-derived amino acids (Genosys Biotechnologies, Inc, The Woodlands, Tex) and probed as previously described.²⁰

RESULTS

Identifying, sequencing, and characterizing the Ana o 3 gene

The 2S albumin gene was amplified from the cashew cDNA library by means of PCR with a degenerate forward

TABLE I. Clinical characteristics and reactivity scores of patients with self-reported cashew allergy

No.*	Sex	Age	Age of onset of cashew IgE allergy	Severity grade†	Other atopy history	Other food allergy	Western blot reactivity with rAna o 3‡	Cashew ImmunoCAP (kU/L) or positive immunoblot (+ blot)
1	M	25	3	4	As	wal, pec, haz	++	5.66
3	F	26	2	4	As, AD, AR	PN, wal, pis, haz	+/-	9.51
4	M	27	5	4	As	wal, bra, coc, haz, alm	+++	6.95
5	F	54	10	4	As, AR	wal, pec, haz, alm	-	1.62
7	F	30	10	4	AD, AR	PN, wal, haz, alm	+/-	4.04
8	F	43	1	4	As, AD, AR	PN, wal	-	41.9
9	F	35	2	4	As, AD, AR	wal, pec, alm	+++	35.1
10	F	31	2	4	As, AR	wal, sun	+++	4.42
11	M	50	1	4	As, AD, AR	TN and mus	+	5.19
12	F	26	3	5	AR, asthma	PN, TN, NE pis, bra	+	2.82
13	F	39	1	4	As, AD, AR	PN, TN, NE pec, mac	++	9.53
14	F	39	5	5	As	Multiple TN	+++	94.7
15	M	50	2	4	As, AR	Multiple TN	+	1.87
20	F	48	1	4	As, AD, AR	PN, wal, haz, ches	-	+ blot
29	F	49	3	4	As, AD, AR	PN, wal, ses, TN, NE bra	-	0.52
30	F	53	15	3	As, AD, AR	TN except alm	+/-	+ blot
31	F	44	1	4	As, AD, AR	wal, alm, ses, mus	++	2.15
32	M	38	1	4	As, AD wal, pis, pec, haz		+	1.64
33	F	63	53	3	As, AD, AR	PN, alm, fish, eggs, NE other TN	+++	90.4
34	F	15	2	5	AD	NE other TN	++	6.78
35	M	13	1	4	As, AD, AR	NE other TN	-	2.82
36	F	3	1	2	AD	Tolerates pec, NE other TN	+	3.52
37	M	3	2	4	As	PN, tolerates alm, NE other TN	+	4.67
38	M	4	2	2	As tolerates alm, NE other TN		++	2.29
39	M	10	2	2	As, AD, AR	NE other TN	+++	16.4
40	F	2	2	2	AD	PN, wal, tolerates pis, NE other TN	+	5.97

As, Asthma; AD, allergic dermatitis; AR, allergic rhinitis; wal, walnut; pec, pecan; haz, hazelnut; PN, peanut; pis, pistachio; bra, brazil nut; coc, coconut; alm, almond; sun, sunflower; TN, tree nut; mus, mustard seed; NE, never eaten; mac, macadamia nut; ches, chestnut; ses, sesame.

*Patients 1 through 32 correspond to those in previous publications.^{19,20}

†Anaphylaxis grades assigned by Teuber on the basis of the scoring system suggested by Sampson²²: grade 1, localized skin findings; grade 2, generalized skin findings, nausea, and/or emesis $\times$ 1, nasal congestion and/or sneezing; grade 3, any skin symptoms, repetitive vomiting, sensation of throat pruritus or tightness, tachycardia, anxiety; grade 4, any skin symptoms, diarrhea, hoarseness, "barky" cough, difficulty swallowing, dyspnea, wheezing, cyanosis, mild hypotension, lightheadedness; grade 5, any skin symptoms, any gastrointestinal symptoms, respiratory arrest, severe bradycardia and/or hypotension or cardiac arrest, loss of consciousness.

‡The number of plus (+) signs represents the degree of IgE binding. Patients with a minus (-) sign did not have reactive IgE, and those with both a plus and a minus sign (+/-) reacted only to the recombinant protein in dot assays.

primer and a lock-dock reverse primer. The resulting 585-bp PCR product (GenBank ID AY081853; Fig 1, A) encodes a 138-amino-acid protein designated Ana o 3 (Fig 1, B). When compared with the GenBank database, the sequence proved to be homologous to other members of the 2S albumin family of seed storage proteins. Table E1 (available in the Journal's Online Repository at www.mosby.com/jaci) lists 8 proteins with the greatest similarity to Ana o 3 and includes 2S albumins and albumin seed storage protein precursors. The homologous 2S albumins from castor bean (*Ricinus communis*, Ric c 1),⁶ black walnut (*Juglans nigra*, Jug n 1),¹⁶ English walnut (*Juglans regia*, Jug r 1),¹⁵ pecan (*Carya illinoensis*, Car i 1, accession no. AAO32314), and sesame (*Sesamum indicum*, Ses i 2)¹³ have all been

identified as allergens. Four other known allergenic 2S albumins (the mustards Sin a 1 and Bra j 1, sesame seed [Ses i 1], and Brazil nut [Ber e 1])^{7,8,12,14} with lesser sequence similarity (55%, 53%, 53%, and 51%, respectively) are also included (Table E1 available in the Journal's Online Repository at www.mosby.com/jaci).

Reactivity of the recombinant protein with human IgE

The approximately 57-kd rAna o 3-MBP fusion protein was affinity purified, as previously described,²⁰ and used to test for reactivity with IgE from sera of patients with cashew allergy (Table I). We note a lack of correlation between the degree of disease severity and ImmunoCAP

(A)

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1  gaataacagc aatggcaaag ttcttactcc tectatctgc cttcgcagtc ctcctcctgg
61  tggctaacgc ctccatttac cgagccattg tggaggttga agaagactcg ggcctgagc
121 agagttgcca acggcagttc gaagagcagc agcgattccg gaactgtcaa aggtacgtga
181 agcaggaggt ccagagggga ggacgtata accagagaca agaaagcttg agggaatgct
241 gccaggagtt gcaggaagta gacagaaggt gccgctgcca gaacctagag caaatggtga
301 ggcagctgca gcaacaggaa caaataaagg gtgaggaggt tcgggaactt tatgaaacag
361 ccagtgaatt gcctcgcat tgcagtattt caccagacca gggctgtcag tttcagtcac
421 cttattagat taattcactg gactgccatg aagtgaagca gtagtagaag aagtaataag
481 cttcacaact ctggcggtta agctggagat taaaggtgaa taaagagtag tagaatcagc
541 acagagtctt gtgtgtcagt gattgaggct atgtaatcga tcgag

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(B)

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1  MAKFLLLLSAFAVLLLVANASIYRAIVEVEEDSGREQSCQRQFEEQQRFRNCQRYVKQE
60  VQRGGRYNQRESLECCQELQEVDRRCRCQNLEQMVRQLQQEQEIKGEEVRELYETAS
119 ELPRICISISFSQGCQFQSSY

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FIG 1. Nucleotide and derived amino acid sequence of Ana o 3 cDNA. **A**, Nucleotide sequence (GenBank accession no. AY081853). **B**, Amino acid sequence of the Ana o 3 coding region. The predicted leader peptide is underlined, and amino acids differing among the identified isoforms, as determined by N-terminal sequencing, are highlighted in gray.

scores, a finding consistent with those of other authors.²⁵ IgE from 21 (81%) of 26 sera from patients with self-reported reactions to cashews bound to the recombinant protein. In 6 cases the intensities of the immunoblot signal were strong, in 5 they were moderate, and in 7 they were weak (Table I). Sera from 3 (14%) of the 21 reactive patients did not bind the recombinant protein in immunoblotting but did show weak reactivity when subjected to a dot-blot assay with rAna o 3 (data not shown).

Affinity purification and MALDI-TOF MS analysis of native Ana o 3

Affinity-purified native, nonreduced Ana o 3 2S albumin derived from a total cashew extract has a molecular mass of 12,598 d, as determined by means of MALDI-TOF MS, compared with a predicted mass for Ana o 3 of 16,335 d (EXPASY Molecular Biology Server, <http://us.expasy.org/>). The mass difference can probably be attributed to the absence of posttranslational modifications, including the removal of excised peptides, as is typical of other 2S albumins,²⁶ on the bacterially expressed rAna o 3.

Comparison of rAna o 3 with native peptides

We have recently reported N-terminal and tryptic peptide sequences for several native IgE-reactive cashew proteins, including 2S albumin.⁸ Here we confirm the N-terminal sequence of 3 apparent isoforms of the large subunit of cashew 2S albumin (6-10 kd; Fig 2, A), each differing from the others by 1 or 2 amino acids over the sequenced 10 amino acids. The deduced N-terminal sequence of rAna o 3 is identical to one of the sequenced isoforms.

Patient 31 recognized all 3 Ana o 3 isoforms of the presumptive large subunit, and reactivity to each is inhibited by rAna o 3 (Fig 2, B). The left-hand strip in Fig 2, B, shows IgE reacting with major bands in the 8- and 10-kd range on a blot of total cashew extract in a reducing gel. Identical serum was preincubated with rAna o 3 before incubation with the center strip. The right-hand strip was

incubated with serum preincubated with the fusion protein (MPB) as a negative control. Inhibition assays with sera from 2 other patients similarly led to complete inhibition of the reactive bands (data not shown).

Ana o 3 linear epitopes

The amino acid sequence of rAna o 3 was screened for IgE-binding linear epitopes by probing 36 overlapping solid-phase synthetic peptides with sera from 17 of the 21 reactive patients; the 3 sera that only reacted in the dot-blot assay were not used, and our supply of one other patient's serum was exhausted. Limitation on the amount of available sera precluded analysis of individual patients' sera with the epitope arrays. However, to gain some sense of patient reaction variability, each reactive serum sample was assigned to one of 4 pools. Each pool was limited to 4 or in one case 5 patients' sera so as not to excessively dilute each individual sample and included at least one serum that strongly reacted to the rAna o 3 in our immunoblots and 3 other sera with varying degrees of reactivity. As presented in Table II, the 4 pools collectively reacted weakly with 9 peptides, moderately with 3 peptides, and strongly with 4 peptides, many of which overlapped with one another. Only 2 of the identified peptides (6 and 7) were bound by patients' sera from all 4 pools. Peptide 6 was bound moderately by pools 1 and 2 and weakly by pools 3 and 4, whereas peptide 7 was bound strongly by pools 1 and 2 and moderately by pools 3 and 4.

To compare the IgE-binding peptides of cashew 2S albumin (Ana o 3) with those of walnut (Jug r 1),²⁴ sesame,²⁷ and yellow mustard seed (Sin a 1)²⁸ 2S albumins, we aligned the sequences by using the BLAST 2.0 program and highlighted the corresponding IgE-binding regions (Fig 3). Because only a small portion of the yellow mustard Sin a 1 2S albumin was screened and found to bind patient IgE,²⁹ only this portion was included in our analysis. The lone linear IgE-binding epitope of the large subunit of Jug r 1 showed significant sequential overlap with one of the strongly IgE-binding Ana o 3 peptides (14, Table II) and, although differing in sequence,

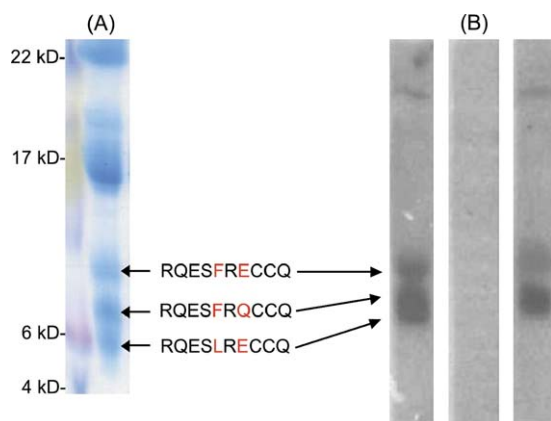


FIG 2. Identification of 3 Ana o 3 large-subunit isoforms and inhibition with recombinant Ana o 3. **A**, Coomassie stain of reduced whole cashew extract. N-terminal aa sequences are shown, with varying residues in red. **B**, Inhibition of IgE binding to large-subunit isoforms. Nitrocellulose probed with patient 31 without inhibition (*left*) and after inhibition with rAna o 3-MBP (*middle*) or MBP (*right*) is shown.

shared positional homology with epitopes in the yellow mustard 2S albumin Sin a 1 and the oriental mustard 2S albumin Bra j 1. Comparison of the Jug r 1 and Ana o 3 shared epitope showed that both contained the 4 amino acids found to be critical (Fig 3, shown in red), and the one residue found to be influential (Fig 3, shown in blue type) for IgE binding to Jug r 1 and shared 100% similarity and 80% identity overall. In contrast, the sequence similarity between the single mustard seed epitope and the corresponding epitope in Ana o 3 was much less (46%). Consistent with this observation is the finding that only 2 of the patients with cashew allergy reported concomitant mustard allergy, but all patients who listed specific tree nut allergies specified walnut as an allergen (Table I).

In comparing the Ana o 3 epitope map with that of the sesame seed 2S albumin, we found that an extended segment (residues 24-94, blue shading in Fig 3) showed considerable positional overlap with 3 of the 4 strongly reactive Ana o 3 epitopes (1, 7, and 10). Two of the cashew epitopes (1 and 7) displayed minimal homology with the corresponding sesame sequence, but the third, epitope 8, showed 75% similarity (58% identity).

DISCUSSION

Approximately 0.5% of the US population is believed to be allergic to tree nuts,¹ and up to 41% of those reporting allergy to tree nuts list sensitivity to cashews, the second highest percentage for any tree nut after walnut.²

Our previous screening of a cashew nut cDNA library with human sera from patients with cashew allergy yielded clones encoding an IgE-reactive vicilin-like protein designated Ana o 1²⁰ and a legumin-like protein designated Ana o 2.²¹ Here we describe the product of a PCR-amplified cDNA sequence encoding a 2S albumin family

seed storage protein designated Ana o 3. IgE from 21 of 26 patients with cashew allergy bound rAna o 3 in either immunoblot or dot-blot assays, leading us to define the 2S albumin as an important allergen.

Typical 2S albumins are small globular proteins that undergo proteolytic processing in the vacuoles of the plant cells, whereby the full-length precursor protein is usually cleaved into large and small subunits that stay associated through 2 disulphide bonds. During this posttranslational processing, a signal peptide, short linker, and flanking sequences are excised, resulting in a smaller mature product.²⁶ For example, in Brazil nuts the precursor protein is 15 kd, but the mature 2S albumin obtained from nut extract is 13 kd.³⁰ We have found, through affinity chromatography and subsequent MALDI-TOF MS analysis, that native intact Ana o 3 has a molecular mass of 12,598 d, similar to the masses reported for 2S albumins of Brazil nut (Ber e 1, 13 kd),³⁰ castor bean (Ric c 1, 12,032 d),³¹ and almond (12 kd).¹⁷

We have identified 3 isoforms of the cashew 2S albumin presumed large subunit, with molecular weights ranging from 6 to 10 kd, as estimated by means of SDS-PAGE. This finding is not unexpected because other 2S albumin allergens, such as sesame Ses i 2¹³ and hazelnut Cor a 1,³² share this characteristic, suggesting 2S albumins represent a complex gene family of differentially processed proteins. Depending on the source, the 2S albumins might or might not be glycosylated.³³ The ability of rAna o 3 to inhibit IgE binding to the large-subunit isoforms bound by a patient's sera in our inhibition assay confirmed the identity of these native subunits and the relevance of using the recombinant protein in our patient-screening assay and potential for use in diagnostic assays, despite lacking the posttranslational modifications that alter the final structure and size of the native protein.

Linear epitope mapping revealed 16 immunoreactive peptides, 4 of which produced strong signals. The peptides define at least 8 linear epitopes. One strongly reactive Ana o 3 epitope overlapped in amino acid position with the lone linear epitope of the large subunit of the English walnut allergenic 2S albumin, Jug r 1, with which it shares considerable homology (81% similarity). Significantly, all 5 amino acids found to be critical or influential for IgE binding to the Jug r 1 epitope²⁴ were conserved in Ana o 3 (100% similarity and 80% identity). This finding might partly explain why the majority of patients with cashew allergy in our test group were also allergic to walnut (15 of the Ana o 3 immunoblot-reactive patients had eaten walnuts in the past, and all but 2 were allergic to walnut by self-report).

Although the 2S albumins are common seed and tree nut allergens, few have been tested for cross-reactivity. Cross-reactivity has only been demonstrated between rapeseed and mustard seed¹⁰ and among almond, walnut, and hazelnut 2S albumins.¹⁷ In contrast to the possible shared epitope between cashew and walnut described above, comparable analyses of shared linear epitopes have not been performed to determine the molecular basis for cross-reactivity among the cashew, mustard seed, and

TABLE II. Linear Ana o 3 epitopes and relative IgE-binding intensity

Epitope	AA position	AA sequence	IgE-binding intensity			
			Pool 1‡	Pool 2	Pool 3	Pool 4
1*	33-44	SGREQSCQRQFE	+++		+	
2	39-50	CQRQFEEQQRFR		++	+	+
3	42-53	QFEEQQRFRNCQ		+	+	
4	45-56	EQQRFRNCQRYV		+	+	
5	48-59	RFRNCQRYVKQE		+	+	
6†	54-65	RYVKQEVQRGGGR	++	++	+	+
7	57-68	KQEVQRGGGRYNO	+++	+++	++	++
8	66-77	YNQRQESLRECC			+	
9	69-80	RQESLRECCQEL			+	
10	72-83	SLRECCQELQEV	+		+++	+++
11	75-86	ECCQELQEVDRR		+	+	+
12	78-89	QELQEVDRRCRC		+	+	+
13	99-110	LQQEQIKGEEV	++			
14	102-113	QEQIKGEEVREL	+++		+	
15	105-116	IKGEEVRELYET			+	
16	123-134	ICSISPSQGCQF			+	

*Bold print denotes strongly reacting epitopes.

†Underlining denotes those epitopes bound by sera from all 4 patient pools.

‡Pool 1 included patients 9, 12, 13, and 15; pool 2 included patients 1, 4, 31, and 33; pool 3 included patient 14, 10 32, and 34; and pool 4 included patients 36, 37, 38, 39, and 40.

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Ana o 3: 1 MAKFLLLSAFVLLLVANASIYRAIV---EVEEDSG---REQSCQRQFEQQR---FR 50
Jug r 1: 7 -----|F| | | |A|TT|TMM| | |IDNFR|G|G|E|I|R| |N---LN 50
Sesame : 1 | |R|T| | |VLFAAA| | | |A|T| |---TTSVAEE---G|EN| |GC|W|S|QCQM| 52

Ana o 3: 51 NCQRYVK-----QEVQRGG---RYNCRQ-EESLRECCQELQEVDRRCRCQNLQEMVRQ 98
Jug r 1: 51 | |Y| | |-----|S|S|YDED| | |H-F| | | |S| |E| | |G|R| | | 99
Sesame : 53 | |N| | |SMRGQYE-SFL|SA--EA| |G|F|HF| | |N| | |KSH| | |A|RC| | | 108

Ana o 3: 99 LQEQEQIKGEEVRELYETASELPKICISISPSQGCQFQSSY 138
Jug r 1: 100 Q| | |G| | | |E|W| |R| |NE|G| | |R| |I|R| | 138
Sesame : 109 | | |YG| | | |Q|NHQY| | |G| |YPTE| |M| 144
Sin a 1: 84 |E| |G|QGPHV|SR| | | | 103

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FIG 3. Amino acid sequence and linear epitope comparison of cashew (Ana o 3), walnut (Jug r 1), sesame, and mustard seed (Sin a 1, segment) 2S albumins. Epitope-containing regions are highlighted or shaded. Strongly reactive Ana o 3 epitopes are boxed. Residues known to differ among the 3 Ana o 3 isoforms are in green underlined type. Critical and influential Jug r 1 epitope residues are in red and blue underlined type, respectively. Symbols: |, identical residues; ·, similar residues; –, space added for alignment.

sesame 2S albumins. However, a BLAST alignment of these and the walnut 2S albumins has been informative. A 14-amino-acid IgE-binding region of the mustard seed (Sin a 1 and Bra j 1E) 2S albumins^{28,29} shows significant positional overlap with the potential cross-reactive epitope of Ana o 3 and Jug r 1 but shares little sequence homology. In addition, the His-58 residue of the mustard allergens believed to be critical for IgE reactivity in patients with mustard allergy²⁸ is lacking in the other compared 2S albumin allergens. Nevertheless, with the exception of sesame, the compared allergens have demonstrable IgE-reactive epitopes in this region, suggesting that it represents a hot spot for allergenicity.

Our findings that the cross-reactive 2S albumins in cashew (Ana o 3) and walnut (Jug r 1) share an epitope with high sequence identity and that several epitopes share positional overlap with the IgE-binding regions of mustard and sesame suggest that epitope mapping can be an important tool in defining the molecular nature of

allergenicity.³⁴ As the database for linear allergens grows, patterns of additional IgE-reactive hot spots are likely to emerge. With this information, studies involving the analysis of new allergens and the generation of engineering allergens for plant breeding and therapeutic agents can be better focused.

We thank Mrs Rani Dhanarajan (Florida State University Molecular Cloning Laboratory) for her technical assistance in the cashew cDNA library construction.

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