JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

ARTICLE

pubs.acs.org/JAFC

Cloning and Characterization of 2S Albumin, Car i 1, a Major Allergen in Pecan

Girdhari M. Sharma,[†,‡] Andre Irsigler,[§] Pushparani Dhanarajan,[§] Rosalia Ayuso,[∥] Luda Bardina,[∥] Hugh A. Sampson,[∥] Kenneth H. Roux,[§] and Shridhar K. Sathe[*,†]

[†]Department of Nutrition, Food and Exercise Sciences and [§]Department of Biological Science, The Florida State University, Tallahassee, Florida 32306, United States

[∥]Division of Pediatric Allergy and Immunology and the Jaffe Food Allergy Institute, The Mount Sinai School of Medicine, New York, New York 10029, United States

ABSTRACT: Although pecans are associated with IgE-mediated food allergies, the allergens responsible remain to be identified and characterized. The 2S albumin gene was amplified from the pecan cDNA library. Dot-blots were used to screen the recombinant protein with pecan allergic patients' serum. The affinity purified native protein was analyzed by Edman sequencing and mass spectrometry/mass spectrometry (MS/MS) analysis. Cross-reactivity with walnut was determined by inhibition enzyme-linked immunosorbent assay (ELISA). Sequential epitopes were determined by probing the overlapping peptides with three different patients' serum pool. The 3-dimensional homology model was generated, and the locations of the pecan epitopes were compared with those of known sequential epitopes on other allergenic tree nut homologues. Of 28 patients tested by dot-blot, 22 (79%) bound to 2S albumin, designated as Car i 1. Edman sequencing and the MS/MS sequencing of native 2S albumin confirmed the identity of recombinant (r) Car i 1. Both pecan and walnut protein extracts inhibited the IgE-binding to rCar i 1. Sequential epitope mapping indicated weak, moderate, and strong reactivity against 12, 7, and 5 peptides, respectively. Of the 11 peptides recognized by all serum pools, 5 peptides were strongly reactive and located in 3 discrete regions of the Car i 1 (amino acids 43−57, 67−78, and 106−120). Three-dimensional modeling revealed IgE-reactive epitopes to be solvent accessible and share significant homology with other tree nuts providing a possible basis for previously observed cross-reactivity.

KEYWORDS: food allergy, allergen, pecan, seed storage protein, 2S albumin, Car i 1, sequential epitope, homology model

INTRODUCTION

In recent years, incidences of food allergies have increased worldwide. IgE-mediated type I hypersensitivity to foods affect approximately 3−4% of the adult population and about 6% of children.[1] More than 90% of allergic reactions to foods are associated with the "Big 8" food groups including milk, egg, wheat, soy, peanut, tree nut, fish and shellfish. More than 75% of total food allergies in young children are attributed to milk (∼41%), egg (∼21%), and peanuts (∼13%) whereas shellfish (∼54%), peanuts (∼16%) and tree nuts (∼13%) account for about 85% of the total food allergies in adults.[2] Tree nut allergy affects up to 1% in the Western population and, along with peanut allergy, is responsible for more than 90% of fatal anaphylactic reactions to food.[3,4] Unlike egg, milk, wheat, and soy allergy, tree nut allergy is usually permanent with only 9% of the patients outgrowing tree nut allergy.[5] Tree nuts commonly reported to cause allergic reactions in the US include walnut (34%), cashew nut (20%), almond (15%), pecan (9%), and pistachio (7%).[6]

The US produces ∼70% of the world's pecans. In the US, excluding coconut (0.54 lb), pecan ranks third (0.49 lb), after almond (1.01 lb) and walnut (0.53 lb) in annual per capita consumption.[7] Pecans have a rich, buttery flavor and can be eaten fresh or used in bakery, confectionery, salads and as a snack. Pecan oil is used for flavoring in salads, dressings and pasta. Pecans have gained importance in recent years for their health benefits, especially their potential for reducing the risk of cardiovascular disease.[8,9] Pecans are a high calorie food (690 kcal/100 g) composed of about 9% protein, 72% fat, and 14% carbohydrate of which up to 10% may be fiber.[10,11]

Pecan (*Carya illinoinensis*) belongs to the family of Juglandaceae that also includes other commercially important tree nuts such as walnut and hickory. Most trees produce pecan nuts every alternate year, and the nut matures after 150 days post pollination, with harvest occurring around the months of October and November in North America.[12] The soluble proteins are synthesized during the cotyledon stage, while the storage protein synthesis starts from maturation stage until post abscission. The Osborne fractionation of pecan proteins has revealed the seed proteins to consist of about 60% alkali glutelins, 32% globulins, 3% prolamins, and 2% albumins.[13]

Allergic reactions to pecan nut[3] and *in vitro* cross-reactivity with walnut[14] have been reported. Venkatachalam et al.[15] observed multiple reactive bands by immunoblotting within the range of 7−66 kDa when pecan protein extract was probed with a pool of pecan allergic patients' sera. It was further shown that the major reactive bands were stable toward thermal processing. There have been reports on the development of neoallergens during the processing or storage of pecan nut.[16,17] However, there is no information on the synthesis of allergenic proteins in pecan nut at the nucleic acid level.

Received: November 7, 2010
Accepted: February 15, 2011
Revised: February 14, 2011

 © XXXX American Chemical Society

Typically, 2S seed storage albumin proteins are low molecular weight heterodimers that are composed of a large and a small subunit linked by disulfide bonds.[18] This class of proteins has been shown to be IgE-reactive in peanut[19] and several tree nuts including almond,[20] Brazil nut,[21] cashew,[22] pistachio[23] and walnut.[24] Though low molecular weight polypeptides (~6−15 kDa) have been shown to be recognized by human sera IgE,[15,16] the immunoreactivity of pecan 2S albumin against human serum IgE remains to be demonstrated. Here we report the cloning and sequencing of a 2S albumin, designated as Car i 1, from a pecan cDNA library, and demonstrate its immunoreactivity with allergic patients' serum IgE.

MATERIALS AND METHODS

Pecan Protein Extract. Shelled pecans were ground to homogeneous flour and defatted for 8 h using a Soxhlet apparatus and petroleum ether (boiling point range of 38.2−54.3 °C) as extraction solvent. After overnight drying in a fume hood, the powder was passed through a 40 mesh sieve and stored in screw-capped plastic vials at −20 °C until further use. Pecan protein extract was prepared by vortexing defatted pecan flour in borate saline buffer (BSB, 0.1 M H_3BO_3, 0.025 M $Na_2B_4O_7$, 0.075 M NaCl, pH 8.45) (flour/solvent = 1:10 w/v) for 1 h, followed by centrifugation at 16000*g* for 15 min at room temperature (RT, ~25 °C). The protein content of the supernatant was estimated as per the method of Bradford,[25] and the supernatant was stored at −20 °C until further use.

Production of Rabbit Polyclonal Antibody (pAb). New Zealand white female rabbits were immunized subcutaneously, each with BSB extracted pecan proteins (500 μg) in 0.5 mL of RIBI adjuvant as described by Acosta et al.[26] Three booster doses were administered in RIBI adjuvant each at 4 week intervals. Each rabbit was subsequently bled, and the serum was collected and stored at −20 °C until further use. Preimmune serum was collected to serve as control when determining the antibody titer.

Human Sera. Sera from patients with a convincing history of pecan allergy and/or detectable pecan specific IgE were from Mount Sinai school of medicine (New York, NY; patient numbers 8−28) following approval by the relevant institutional review boards. Additional pecan allergic human sera were purchased from PlasmaLab International (Everett, WA; patient numbers 1−7).The presence of pecan-reactive IgE was confirmed with the ImmunoCAP assay. The allergic and control (patients with no history of pecan hypersensitivity) sera were stored at −70 °C until further use.

Isolation of Total RNA and Pecan cDNA Library Construction. Maturing pecan nuts stored at −80 °C immediately after harvest were used to isolate the total RNA as described by Levi et al.[27] Total RNA was dissolved in 0.1% (v/v) diethyl pyrocarbonate (DEPC) treated water and stored at −80 °C until further use. The mRNA was separated from the total RNA using PolyATtract kit (Promega, Madison, WI) according to the manufacturer's protocol. The synthesis of cDNA library was performed with the ZAP-cDNA Gigapack III Gold cloning kit (Stratagene Inc., Cedar Creek, TX) according to the manufacturer's instruction. The double stranded cDNA was cloned directionally into the lambda Uni-ZAP XR expression vector, packaged *in vitro*, and transfected in *Escherichia coli* XL1-Blue MRF' strain to determine the titer of the cDNA library and for amplification of the cDNA library.

Identification of 2S Albumin Gene. 2S albumin gene was amplified from the cDNA library using a pair of primers designed for cloning by incorporating a *Bam*HI site (underlined) at the 5′ end of the forward primer CGCGGATCCATGGCACGAGTAGCAGCTCTC, and a *Hin*dIII site (underlined) at the 5′ end of the reverse primer CCCAAGCTTCTAGAACCAGCTTCTGCGAATTTC, based on the available sequence of pecan 2S albumin (accession no. AY192569; NCBI database: http://www.ncbi.nlm.nih.gov/protein/28207731). The amplicon was cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and the corresponding insert was sequenced.

Nucleotide Sequencing and Analysis of cDNA. The plasmid cDNAs were sequenced from both directions on an ABI 3100 Genetic Analyzer (Foster City, CA) by using capillary electrophoresis and Version 2 Big Dye Terminators, as described by the manufacturer. Similarity searches for deduced nucleotide and amino acid sequences were performed by using the BLAST program accessible at the Web site of the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST/). The signal peptide cleavage site was predicted by using SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/). The multiple sequence alignment for the nucleotide and amino acid sequences were performed using the ClustalW program accessible at the Web site of European Bioinformatics Institute (www.ebi.ac.uk/clustalw/).

Subcloning, Expression and Purification of Recombinant Proteins. The cDNA insert produced by PCR amplification with appropriate restriction sites at 5′ and 3′ end was subcloned into the maltose binding protein (MBP) expression vector pMAL-c4x (New England Biolabs Inc., Ipswich, MA) and transformed into the competent *E. coli* BL21 (DE3) cells (Novagen Inc., Madison, WI). Single colonies were grown in LB broth at 37 °C to an OD_{600} of 0.5−0.7 followed by induction with 1 mM IPTG for 4 h at 37 °C. The cells were harvested by centrifugation at 5000*g* for 15 min at 4 °C, resuspended in wash buffer (10 mM Tris-HCl, pH 7.1, 30 mM NaCl) and lysed using a microfluidizer (model M-110 L, Microfluidics, Newton, MA). The lysate supernatant was passed over an amylose affinity column (New England Biolabs Inc., Ipswich, MA), and the fusion protein was eluted with column elution buffer (10 mM Tris-HCl, pH 7.5, 10 mM maltose). The fusion protein was concentrated using membrane filter YM-10 (Millipore Corporation, Billerica, MA) and cleaved with factor Xa (New England Biolabs Inc., Ipswich, MA). The recombinant protein was frozen at −20 °C until further use.

SDS−PAGE. SDS−PAGE was done by using the method of Fling and Gregerson.[28] Protein extract or recombinant protein samples were boiled in sample buffer (50 mM Tris-HCl, pH 6.8, 1% SDS, 0.01% bromophenol blue, 30% glycerol, and 2% β-mercaptoethanol) for 10 min, and suitable aliquots were loaded on the gel along with the molecular weight markers. All gels were run at a constant current of 12 mA/gel until the tracking dye passed the gel edge. The gels were either used for protein transfer or stained with 0.25% Coomassie Brilliant Blue R (Sigma-Aldrich, St Louis, MO) in 50% methanol and 10% acetic acid and destained with 50% methanol containing 10% acetic acid.

Western Immunoblotting and Inhibition Immunoblotting. SDS−PAGE was carried out as described above, and proteins were transferred on to 0.22 μm nitrocellulose (NC) membrane (Schleicher & Schuell Bioscience, Inc., Keene, NH) as described by Towbin et al.[29] at 4 °C for 2−3 h. Blotted NC strips were blocked for 1 h at RT (overnight at 4 °C for human sera blots) using 5% nonfat dried milk (NFDM) in Tris-buffered saline (TBS-T; 10 mM Tris, 0.9% w/v NaCl, 0.05% v/v Tween 20, pH 7.6). Strips were incubated with diluted human sera (1:3 to 1:10 dilution depending on immunoreactivity) or rabbit anti-pecan pAb (1:10,000 dilution) overnight at 4 °C. The strips were then washed 3 times with TBS-T for 15 min each (30 min for human sera blots) and incubated with ^{125}I labeled anti-human IgE (1:10 dilution; Hycor Biomedical Inc., Garden Grove, CA) overnight at 4 °C or goat anti-rabbit IgG (1:40,000 dilution; Sigma Chemical Co., St. Louis, MO) for 1 h at RT. The strips were washed 3 times, 15 min each (30 min for human sera blots), in TBS-T, and developed on X-ray film (Eastman Kodak Co., Rochester, NY). The exposure time was generally 30 s for the blot using rabbit anti-sera as primary antibody, and 4−10 days at −80 °C for blot using human sera. For dot-blot

```
(A)
  1 atggcacgag tagcagctct ccttgtagcc cttttgttcg tagccaacgc cgctgccttc
 61 cgcaccacca taacaaccat ggagatcgac gaggacattg acaacccgag aaggcgaggc
121 gagagctgcc gggaacagat tcaacgccag caatacctca atcgctgcca ggactacctg
181 aggcagcaat gccggtcagg gggttatgat gaagacaacc agcggcagca tttcaggcag
241 tgctgccagc agctgagtca gatggaggag cagtgccagt gtgaggggtt gaggcaggcg
301 gtgaggcagc agcagcagga ggagggtatc cggggtgagg aaatggagga gatggtgcag
361 tgtgctagtg acttgccaaa agaatgcggc atcagcagcc ggagctgtga aattcgcaga
421 agctggttct ag

(B)
  1 MARVAALLVA LLFVANAAAF RTTITTMEID EDIDNPRRRG ESCREQIQRQ QYLNRCQDYL
 61 RQQCRSGGYD EDNQRQHFRQ CCQQLSQMEE QCQCEGLRQA VRQQQQEEGI RGEEMEEMVQ
121 CASDLPKECG ISSRSCEIRR SWF
```

Figure 1. Nucleotide (A) and deduced amino acid (B) sequence of pecan 2S albumin cDNA. The predicted leader peptide is underlined. MALDI-TOF matched peptides for the small and large subunit are indicated in blue and green color, respectively. The presumed cleavage site is marked by an arrow.

Table 1. Sequence Comparison of Car i 1 with Other 2S Albumin

source	accession no.	% identity	% similarity	allergen designation
Juglans regia (English walnut)	U66866	88	92	Jug r 1[24]
Juglans nigra (black walnut)	AY102930	88	93	ND[40,a]
Corylus avellana (hazelnut)	FJ358504	56	71	Cor a 14[41,b]
Ricinus communis (castor bean)	EQ973796	40	59	Ric c 1[42]
Anacardium occidentale (cashew nut)	AY081853	41	63	Ana o 3[22]
Bertholletia excelsa (Brazil nut)	X54491	41	62	Ber e 1[21]
Pistacia vera (pistachio)	DQ631675	35	63	Pis v 1[23]

[a] ND: not designated allergen by International Union of Immunological Societies, Allergen Nomenclature Sub-committee, http://www.allergen.org/Allergen.aspx. [b] Direct submission to NCBI.

experiments, appropriate amounts of protein (~1 μg) were dotted on the NC membrane and allowed to air-dry. The membrane was then blocked and treated as described above for Western blotting.

For inhibition immunoblotting, 10 μg of native or recombinant pecan 2S albumin was preincubated for 2 h at RT with rabbit or human anti-sera at an appropriate dilution determined by optimal signal upon visual inspection of reactive polypeptides. The pecan protein blotted and blocked NC strips were incubated overnight at 4 °C with the preincubated sera. The strips were then treated as described above for Western blotting.

Inhibition ELISA. Ninety-six well polyvinyl microtiter ELISA plates were coated with 50 μL/well of 20 μg/mL rCar i 1 prepared in coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6) and incubated for 1 h at 37 °C. The plates were washed thrice with phosphate buffered saline (EMD Chemicals, Gibbstown, NJ) containing 0.2% v/v Tween 20 (PBS-T, pH 7.3) and blocked for 1 h at 37 °C with 100 μL/well of 5% NFDM in PBS-T. The plates were then washed thrice with PBS-T and incubated overnight at 4 °C with 50 μL of human sera (3× diluted in blocking buffer) that was preincubated with appropriate inhibitor (100 μg) for 3 h at 37 °C. The plate was again washed thrice with PBS-T, and incubated with HRP conjugated mouse anti-human IgE (Zymed Lab Inc., San Francisco, CA) at a dilution of 1:1000 made in the blocking buffer for 1 h at 37 °C. After three subsequent washings with PBS-T, the bound specific IgE was detected by colorimetric reaction using *o*-phenylenediamine (OPD, Zymed Lab Inc., San Francisco, CA) and H_2O_2 as substrate. The reaction was stopped after 20 min by adding 50 μL/well of 2N H_2SO_4, and optical density (OD) was measured at 495 nm. Inhibition was calculated using the equation

$$\% \text{ inhibition} = 100 - (\text{OD}_{495} \text{ with inhibitor}/\text{OD}_{495} \text{ without inhibitor}) \times 100$$

Purification of Native Protein. The native protein was purified by affinity chromatography using rabbit pAb raised against pecan whole extract. Cyanogen bromide (CNBr) activated Sepharose resin (0.2 g; Sigma Chemical Co., St. Louis, MO) was washed and swollen in cold 1 mM HCl for 30 min, followed by washing with distilled water and BSB. The recombinant protein (~4 mg in 3 mL of 10 mM Tris-HCl, pH 7.5) was covalently immobilized on CNBr activated resin by incubating for 2 h at RT. The resin was then washed with BSB followed by blocking with 0.2 M glycine (blocking buffer) in BSB for 2 h at RT. The resin was alternatively washed with BSB and 0.1 M acetic acid containing 0.5 M NaCl, pH 4.0 about 4−5 times. This recombinant protein attached CNBr resin was incubated with 1.5 mL of rabbit anti-pecan pAb (39 mg protein/ml) overnight at 4 °C. The slurry was transferred to a disposable column and washed extensively with PBS until absorbance at 280 nm reached baseline. The affinity purified rabbit pAb reacting with the recombinant protein was eluted using 0.2 M glycine sulfate (pH 2.3), neutralized and used for immobilization on CNBr activated resin as described above. The CNBr-pAb resin was incubated with 1.5 mL of pecan extract in BSB overnight at 4 °C. The slurry was transferred to a disposable column, and the native proteins reactive to immobilized pAbs were eluted using 0.2 M glycine sulfate (pH 2.3).

N-Terminal Amino Acid Sequencing. SDS−PAGE of pecan protein extract was carried out as described above. The proteins were transferred to the 0.2 μm polyvinylidene fluoride (PVDF) membrane (Whatman Inc., Piscataway, NJ) presoaked in 100% methanol. The protein band of interest was sequenced from the N-terminus using an ABI 477A sequencer with an online 120A HPLC system (Applied Biosystems, Foster City, CA).

Matrix-Assisted Laser Desorption Ionization Time-of-Flight (MALDI-TOF) and Mass Spectrometry (MS)/MS Peptide Sequence Analysis. MALDI-TOF and MS/MS sequencing was performed by Alphalyse Inc. (Palo Alto, CA). Protein bands were excised from SDS−PAGE gels and washed with 50 mM NH_4HCO_3/acetonitrile (1:1), followed by dehydration with acetonitrile. The proteins were reduced in 10 mM dithiothreitol/50 mM NH_4HCO_3 for 1 h at 56 °C and alkylated in 55 mM iodoacetamide/50 mM NH_4HCO_3 for 2 h at RT. The gel pieces were washed several

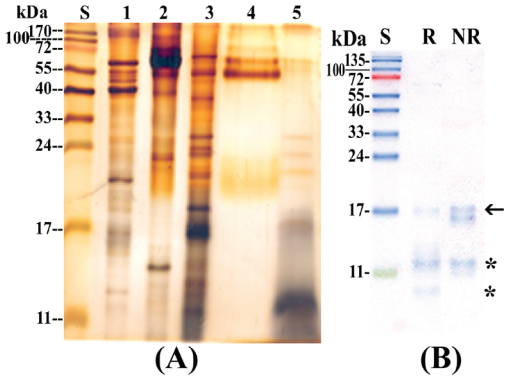

Figure 2. Silver staining (A) and coomassie stain (B) of affinity purified nCar i 1: S, protein standard; 1, rCar i 1 fusion protein; 2, rabbit anti-pecan serum; 3, pecan extract; 4, affinity purified rabbit anti-rCar i 1; 5, affinity purified nCar i 1. R and NR represent nCar i 1 under reducing and nonreducing conditions, respectively. Protein load in each lane was 2 μg. "←" represents the precursor or unreduced nCar i 1, while "*" represents the large and small subunits of nCar i 1.

times in 50 mM NH_4HCO_3, followed by dehydration with acetonitrile. The proteins were digested overnight with trypsin (Promega, modified trypsin) at 37 °C; the resulting peptide mixtures were analyzed by MALDI-TOF peptide mass fingerprint, and 5−10 peptides were selected for MALDI TOF/TOF tandem mass spectrometry sequencing (Autoflex III, Bruker Daltonics, Bremen, Germany). The peptide masses and peptide sequencing data were combined and used to query the nonredundant sequence database (NRDB-NCBI) for protein identification using the Mascot (version 1.9.03) search program (Matrix Science, U.K.). Database search parameters considered were (1) carbamidomethyl cysteine and oxidation methionine variable modifications, (2) up to 1 missed cleavage permitted, and (3) peptide tolerance set at 60 ppm.

Solid-Phase Peptide Synthesis. Synthetic overlapping 12-amino acid long peptides (Sigma Genosys, JPT Peptide Technologies, Acton, MA), offset by 3 amino acids, synthesized based on the deduced amino acid sequences of pecan 2S albumin, were used for immunoblot, as per the manufacturer's instructions. Briefly, the membrane containing the peptides was rinsed with methanol for 5 min, followed by washing 3 times with TBS-T for 10 min each. The membrane was blocked overnight at RT with the blocking buffer (10% casein (w/v) containing 100 mM maleic acid, 150 mM NaCl, and 2.4 mM sodium azide, pH 7.5). The membrane was washed once with TBS-T for 10 min. The steps from incubation with human serum IgE to development were the same as described for immunoblotting.

The membrane was regenerated in a polypropylene box before reusing. Briefly, to remove bound IgE the membrane was washed twice with Milli-Q water for 10 min, followed by incubation with *N*,*N*-dimethylformamide (DMF) twice for 10 min each. The membrane was again washed with Milli-Q water for 10 min. The membrane was washed twice with regeneration buffer A (8 M urea, 1% SDS, 0.1% β-mercaptoethanol) for 10 min each, followed by washing twice with regeneration buffer B (50% ethanol, 10% acetic acid) for 10 min each. Finally the membrane was washed in 100% methanol for 10 min. The regeneration efficiency was tested by incubating the membrane with secondary antibody alone and the membrane was further regenerated if the peptides showed signal upon development.

Molecular Modeling. Homology modeling of Car i 1 was carried out under the automated mode of protein structure homology-modeling server SWISS-MODEL[30] by submitting the amino acid sequence of 2S albumin and comparing with the rape seed 2S albumin NMR structure (RCSB protein data bank code 1sm7A). The ribbon diagram and electrostatic potential model of the templated regions were generated on the Swiss-PdbViewer v4.0.1 (http://www.expasy.org/spdbv/). Amino acids with ≥30% accessible surface were used for computing the molecular surface model.

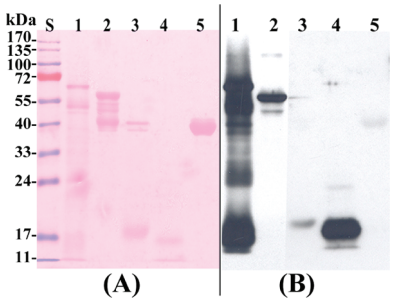

Figure 3. Ponceau S stain (A) and Western blot (B) of pecan 2S albumin using rabbit anti-pecan pAb: S, protein standard; 1, pecan extract; 2, rCar i 1 fusion protein; 3, rCar i 1; 4, nCar i 1; 5, rMBP. Protein load in each lane was 5 μg except lane 1 was10 μg.

RESULTS

Identification of Pecan 2S Albumin (Car i 1) Gene. The Car i 1 gene was amplified from the pecan nut cDNA library by PCR, and the resulting 432 bp amplicon found to code for a 143 amino acid protein (Figure 1). When the protein sequence was compared with the NCBI GenBank database, the sequence showed homology with 2S albumin seed storage proteins from other edible nut seeds (Table 1). We predicted the signal peptide of Car i 1 using the Signal P server, which indicated that the proprotein would be cleaving at amino acid position 19−20 in the process of post translational modification, resulting in mature protein with theoretical mass of 15 kDa.

Native Pecan 2S Albumin (nCar i 1) Characterization. Affinity chromatography was used to purify nCar i 1 from pecan extract. Recombinant 2S albumin fusion protein was bound to CNBr, and rabbit pAb specific for rCar i 1 was purified (Figure 2A, lane 4). This specific pAb was further used to purify the nCar i 1 (Figure 2A, lane 5). Though multiple bands were observed under silver staining of nCar i 1, one band had a molecular mass of ∼16 kDa, which is close to the recombinant counterpart (marked by an arrow in Figure 2B). The N-terminal amino acid sequence of 16 kDa band under reducing conditions was TTITTMEIDEDI, showing exact identity with the mRNA derived amino acid sequence from position 22−33. Both large and small subunits of nCar i 1 were subjected to MALDI-TOF and analyzed by MS/MS sequencing. The MS/MS sequencing analyses of the small subunit (Figure 2B) matched peptide "EQIQRQQYLNRCQDYLRQQCR" of rCar i 1 covering 17% of mature protein sequence. The large subunit of affinity purified nCar i 1 did not give significant MALDI-TOF data. This may be due to the presence of minor contaminating polypeptides at ∼11 kDa, since all the polypeptides in that region did not bind the small subunit to yield the mature 16 kDa protein under nonreducing condition (Figure 2B). However, one of the spots from the 2-dimensional gel of pecan whole protein extract (data not shown) matching the molecular mass of Car i 1 large subunit exhibited a peptide match with rCar i 1 at amino acid position 80−98 (QCCQQLSQMEEQCQCEGLR).

Reactivity of the Recombinant Protein (rCar i 1) with Human IgE. The Car i 1 cDNA was subcloned into the maltose binding protein (MBP) fusion protein expression vector

pMAL-c4x. The rCar i 1-MBP fusion protein was ~57 kDa, and factor Xa cleaved the fusion protein into two polypeptides: MBP, 40 kDa, and rCar i 1, 17 kDa (see Figure 3A, lanes 3 and 5). Serum from 28 patients with self-reported allergic reactions to pecan nut were used to determine the IgE immunoreactivity with the recombinant protein. Table 2 summarizes the patient characteristics along with age, sex, clinical symptoms, other food allergies and pecan ImmunoCAP score. Out of the 28 patients tested, IgE from 22 sera (79%) bound to the recombinant protein (Figure 4). Though individual serum dilutions were the same for the patients tested, the ImmunoCAP score did not correlate with the dot-blot intensity or the reported clinical severity. For example, patient 20 exhibited high reactivity with rCar i 1 in dot-blot, but had relatively low pecan-specific IgE value (16 kU_A/L) compared to patient 22 demonstrating comparatively low reactivity in dot-blot, but high pecan-specific IgE value (38.6 kU_A/L). Also, some patients had high pecan-specific IgE values (patients 26 and 27 had pecan-specific IgE 100 and 65 kU_A/L, respectively), but did not react with rCar i 1.

Comparison of rCar i 1 and nCar i 1 Immunoreactivity. The immunoreactivity of recombinant and native 2S albumin was compared using pAbs raised against whole pecan nut as well as pecan allergic human serum IgE. The rabbit anti-pecan IgG recognized the nCar i 1 as well as rCar i 1 (both fusion and cleaved). However, the intensity of nCar i 1 was markedly different from the factor Xa cleaved rCar i 1. The rCar i 1 exhibited reduced immunoreactivity upon cleavage with factor Xa (Figure 3B). Inhibition experiments were performed to determine the concordance of rCar i 1 with nCar i 1. Adsorption of rabbit anti-pecan IgG with nCar i 1 completely abolished the reactivity of rCar i 1 in Western blotting (Figure 5). This suggests the presence of immunologically similar protein in the pecan capable of binding to pAbs. However, the nCar i 1 was not inhibited by the rCar i 1. Similar results were found when Western blotting was done using human sera pool (data not shown). Treatment with different denaturants exhibited

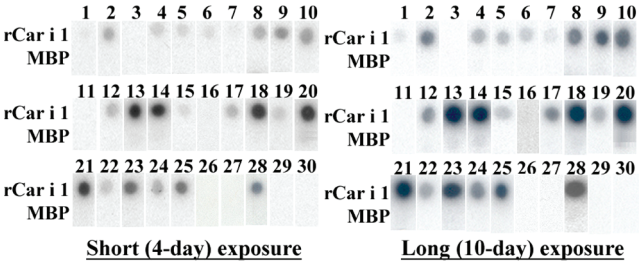

Figure 4. Dot-blot of rCar i 1 fusion protein with pecan allergic human serum (3× dilution). 1−28 are pecan allergic sera; 29 is atopic control; 30 is nonatopic control. Protein dotted on the membrane was 1 μg.

Table 2. Clinical Characteristics of Pecan Allergic Subjects[a]

no.	age	sex	history	other food reactions	ImmunoCAP to pecan (kU/L)
1	25	M	A, U	PN, nuts/beans/seeds, soybean, cottonseed	24
2	41	M	AD, A, U, anaph	milk or milk products, eggs, wheat, nuts/beans/seeds, fish, fruits	32.5
3	33	F	U, anaph	PN, nuts/beans/seeds, shellfish, fruits, cereal grains, parsley, vegetables	7.49
4	23	F	A, anaph	nuts/beans/seeds	30.8
5	33	F	A, U, anaph	PN, nuts/bean/seeds, legumes, chocolate, vegetables	13
6	30	F	AD	nuts/bean/seeds, chocolate, peanut butter	4.4
7	32	F	AD, A	milk or milk products, nuts/beans/seeds, chocolate	2.13
8	3	F	A, AR, FA	PN, TN, milk	18.1
9	6	M	AR, FA	PN, al-hives	21.8
10	7	F	FA, A, AD	wal-anaph	100
11	7	F	A, FA	PN, TN	52.3
12	15	M	FA, AD	TN-anaph, seed	57.7
13	7	F	A, FA, AD	milk, PN, TN, seed	54.9
14	10	M	FA, A	PN, TN, seed, milk	26.4
15	13	M	FA, A, AD, AR	PN, TN	15.2
16		F			16.2
17	18	F	A, FA	TN, shellfish	68.1
18	2	M	A, FA, AD	wal-ae, vom	63.1
19	24	M	FA, A	PN-anaph; TN-anaph	15
20		M			16
21	12	M		TN	>100
22	11	F	A, FA	wal-anaph; pecan: A, vom, ae	38.6
23	6	M	A, FA, AD	PN-hives, milk	54.6
24	8	F	A, FA, AD	PN-hives, vom; wal-hives	34.6
25	2	F	FA	pi, cash-hives, vom, ae	33
26	10	M	A, FA, AD, AR	PN, TN-anaph	100
27	8	M	FA, AD, A	TN	65
28	15	F	A, FA, AD, AR	TN never eaten; mustard-anaph	50

[a] A, asthma; AR, allergic rhinitis; FA, food allergy; AD, atopic dermatitis; U, urticaria; PN, peanut; TN, tree nuts; al, almond; wal, walnut; anaph, anaphylaxis; ae, angioedema; vom, vomiting; pi, pistachio; cash, cashew; M, male; F, female.

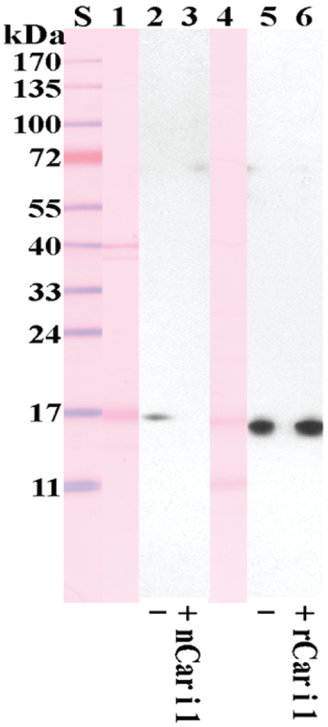

Figure 5. Inhibition blot of pecan 2S albumin rCar i 1 (lanes 2, 3) or nCar i 1 (lanes 5, 6) incubated with rabbit anti-pecan pAb. No inhibitor was added in lanes 2 and 5; anti-serum was preincubated with nCar i 1 (10 μg) and rCar i 1 (10 μg) in lanes 3 and 6, respectively. Protein load in each lane was 5 μg. Ponceau stained rCar i 1 and nCar i 1 are shown in lanes 1 and 4, respectively.

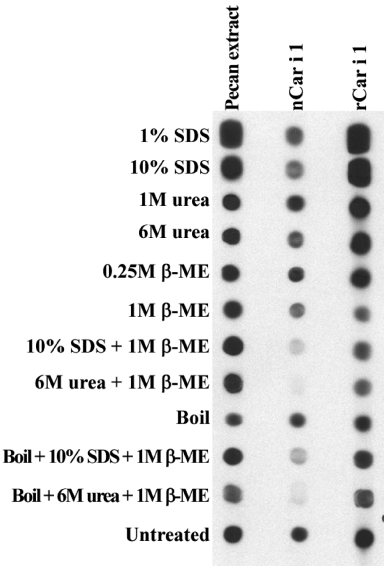

Figure 6. Effect of different denaturants on immunoreactivity of pecan 2S albumin. Protein dotted on the membrane was 0.2 μg of pecan extract, 0.3 μg of nCar i 1, and 0.9 μg of rCar i 1. The greater amount of rCar i 4 was loaded to account for the mass of MBP, which is roughly twice the mass of Car i 1.

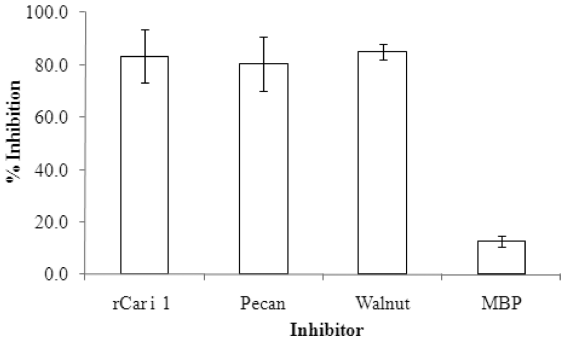

Figure 7. Inhibition ELISA with pecan allergic human sera pool (patients 9, 14, 19, and 23) showing the inhibition of rCar i 1 by pecan and walnut extract (n = 3).

significant loss of n Car i 1 immunoreactivity when treated with β-mercaptoethanol, which further lost most of its reactivity to rabbit pAb when treated in combination with other denaturants such as SDS or urea (Figure 6).

Car i 1 Cross-Reactivity. Since pecan and walnut belong to same family and their 2S albumins show high sequence homology, it was of interest to determine whether 2S albumins are cross-reactive between pecan and walnut. Inhibition ELISA of the fusion rCar i 1 protein with pecan and walnut extract when probed with a serum pool (patients 9, 14, 19, and 23) is shown in Figure 7. Both pecan and walnut extracts equally inhibited the IgE binding to rCar i 1. Adsorption of sera with MBP (negative control) did not significantly reduce IgE binding to rCar i 1. The data suggest that Car i 1 is IgE reactive and cross-reacts with the homologous walnut allergen, Jug r 1. The two patients' sera were analyzed for specific IgE against walnut proteins, and they indicated high walnut-specific IgE (walnut-specific IgE for patients 9 and 23 were, respectively, 19 and 85 kU_A/L).

Car i 1 Sequential Epitopes. The entire amino acid sequence of rCar i 1 was screened for IgE-binding sequential epitopes by probing 45 overlapping solid-phase synthetic peptides with sera from 23 of the 28 pecan allergic patients. The sera were divided into 3 pools depending on their reactivity to rCar i 1 in dot-blot assay so that each pool included sera from 2 patients that reacted strongly to Car i 1, and thus the variation in the SPOT intensity between the pools could be minimized. Two pools contained 8 and one pool contained 7 patients' sera. Epitope mapping using these pooled sera identified various IgE-binding epitopic regions within amino acid sequence of Car i 1. As presented in Table 3, the 3 pools collectively reacted weakly with 12 peptides, moderately with 7 peptides, and strongly with 5 peptides, many of them overlapped with one another. It should be noted that strong reactivity with any epitope may not necessarily relate to severe clinical symptoms of the patient. Eleven out of 24 reactive peptides were recognized by all 3 patients' serum pools. Out of these 11 peptides, 5 peptides were strongly reactive, which were spaced in 3 different regions of the Car i 1 at amino acid positions 43−57, 67−78, and 106−120. Each IgE-binding region may be composed of one or more epitopes. On the other hand, those reactive peptides that partially overlap with the adjacent reactive peptide may collectively represent a single epitope. Epitope region 43−57 was strongly recognized by pools 1 and 3 while moderately recognized by pool 2, whereas region 106−120 was strongly reactive to pool 2 and moderately reactive to pools 1 and 3. Epitope region 67−78 reacted strongly, moderately, and weakly to pools 1, 2, and 3, respectively.

Since 2S albumins share conserved features among tree nuts, we aligned the sequences of rCar i 1 with Jug r 1 and Ana o 3 using

ClustalW alignment program, and highlighted their IgE-binding peptides for comparison (Figure 8). Though Robotham et al.[31] found only 1 strongly reactive IgE-binding epitope, recently Sordet et al.[32] reported 4 epitopic regions that bind allergic patient sera IgE in Jug r 1. We show all 5 epitopes for Jug r 1 and compare this with our finding for Car i 1 and previously reported for Ana o 3.[22] Many of the epitopes in these allergens shared both sequence similarity and positional homology. Those epitopic regions that reacted strongly with patient sera are boxed in blue. Many amino acids within these strongly reactive epitopic regions were solvent accessible (colored red in Figure 8). It should be noted that the blue boxed regions in Figure 8, referred to as epitopic regions, may be composed of more than one epitope each. Two of the strongly reacting Car i 1 epitopes (67−78 and 106−120) showed significant sequential overlap with known epitopes of Jug r 1 and Ana o 3, while one strongly reacting epitope (43−57) of Car i 1 showed partial sequential overlap with Ana o 3 epitope.

Table 3. Sequential Car i 1 Epitopes and Relative IgE-Binding Signal Intensity

			IgE-binding intensity		
epitope	AA position	AA sequence	pool 1[a]	pool 2	pool 3
1[b]	4−15	VAALLVALLFVA	+	+	+
2	7−18	LLVALLFVANAA	+	+	+
3	25−36	TTMEIDEDIDNP	+		
4	28−39	EIDEDIDNPRRR	+		
5	31−42	EDIDNPRRRGES	+		
6	34−45	DNPRRRGESCRE	++	++	++
7	37−48	RRRGESCREQIQ		+	
8[c]	**43−54**	**CREQIQRQQYLN**	++	+	+++
9	**46−57**	**QIQRQQYLNRCQ**	+++	++	+++
10	49−60	RQQYLNRCQDYL	++	++	++
11	52−63	YLNRCQDYLRQQ		+	+
12	55−66	RCQDYLRQQCRS	++	+	++
13	58−69	DYLRQQCRSGGY	+	+	++
14	**67−78**	**GGYDEDNQRQHF**	+++	++	+
15	70−81	DEDNQRQHFRQC	+		+
16	73−84	NQRQHFRQCCQQ	+		+
17	76−87	QHFRQCCQQLSQ	+	+	
18	97−108	LRQAVRQQQQEE	+		
19	100−111	AVRQQQQEEGIR	++		
20	103−114	QQQQEEGIRGEE	++		+
21	**106−117**	**QEEGIRGEEMEE**	+	+++	++
22	**109−120**	**GIRGEEMEEMVQ**	++	+++	++
23	130−141	GISSRSCEIRRS		++	+
24	133−143	SRSCEIRRSWF		+	+

[a] Pool 1 included patients 2, 5, 8, 10, 12, 13, 20, and 24; pool 2 included patients 4, 7, 9, 14, 15, 17, 21, and 25; pool 3 included patients 6, 16, 18, 19, 22, 23, and 28. [b] Epitopes bound by sera from all 3 patient pools are underlined. [c] Bold print denotes strongly reacting epitopes.

Molecular Modeling and Surface Mapping. The three-dimensional homology model of Car i 1, Jug r 1, and Ana o 3 was built using NMR structure of rapeseed 2S albumin[33] as a template. Homology-based molecular modeling of Car i 1, Jug r 1, and Ana o 3 showed that they share common three-dimensional structural motifs composed primarily of α-helices connected by loops (Figure 9). Since walnut and pecan 2S albumins share high homology, it was not surprising to observe similar ribbon structures and molecular surface features for these proteins. Strongly reacting IgE-binding epitopes were labeled on the 2S albumins in the ribbon diagrams and surface model (Figure 9). Two IgE-binding regions were identified that were present on the surface of Car i 1, Jug r 1 and Ana o 3 (circled in Figure 9). A third epitopic region was found common in Car i 1 and Ana o 3 (rectangle in Figure 9). We could not label Jug r 1 epitope IDNPRR (29−34) and CGISSQRCEIRR (125−136) as the corresponding region was not present in the model. The ribbon model suggests these epitopes are present in the third epitopic region since the N- and C-terminal region of the protein lies there. The electrostatic potential of Car i 1 was compared with those of Jug r 1 and Ana o 3 (Figure 10). Car i 1 had high electronegatively charged regions (colored red) compared to Jug r 1 and Ana o 3. All epitopic regions contained charged areas on the molecular surface of the allergen, dominated by electronegative potential in Car i 1, while evenly distributed electropositive and electronegative potential in Jug r 1 and Ana o 3. However, no

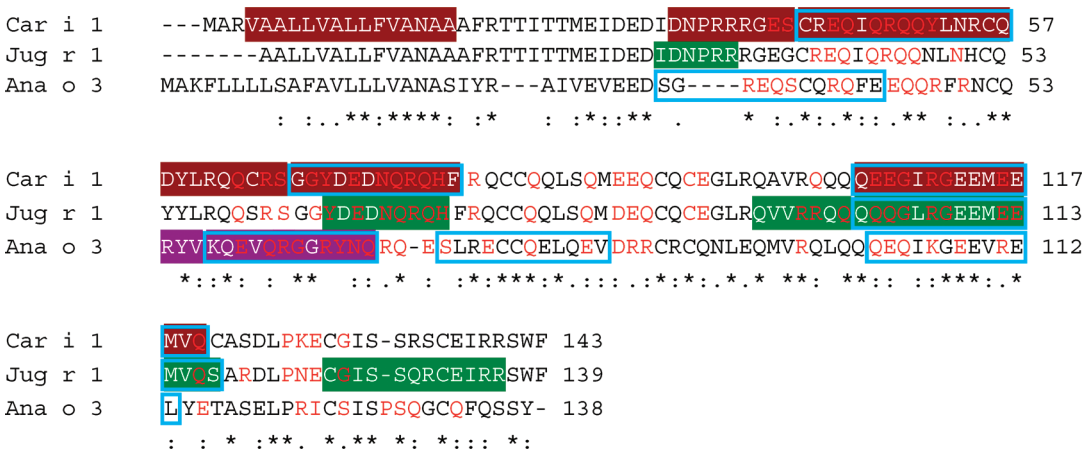

Figure 8. Amino acid sequence and sequential epitopic region comparison of pecan (Car i 1), walnut (Jug r 1),[31,32] and cashew (Ana o 3)[22] 2S albumin allergens. Epitope stretches deduced from IgE SPOTs assays identified by sera from all patient pools are highlighted in different colors (pecan, brown; walnut, green; and cashew, purple). Strongly reacting epitopes are boxed in blue. "*" indicates identical residues, ":" indicates conserved subsitution, and "." indicates semiconserved substitutions are observed. Solvent exposed amino acid residues are colored red.

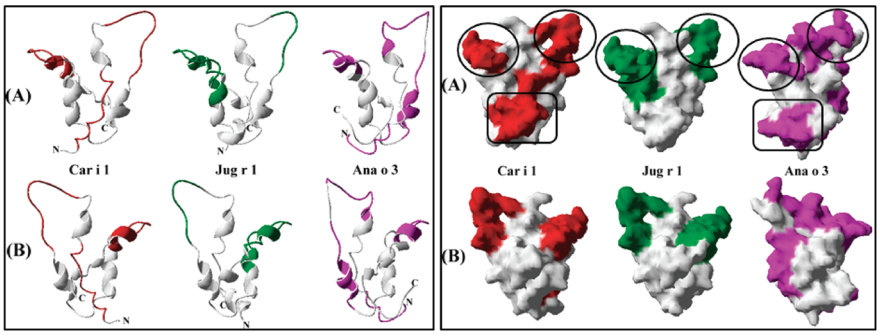

Figure 9. Ribbon diagram (left) and molecular surface (right) of front (A) and back (B) of the modeled Car i 1, Jug r 1, and Ana o 3. Strongly reacting Car i 1 epitopes (brown colored) are compared with Jug r 1 (green) and Ana o 3 (purple) epitopes. Amino acids residues used to build model: pecan, 41−130; walnut, 39−126; cashew, 35−133. Epitopes shown: pecan, 43−57, 67−78, 106−120; walnut, 65−73, 95−117; cashew, 35−44, 57−68, 72−83, 102−113.

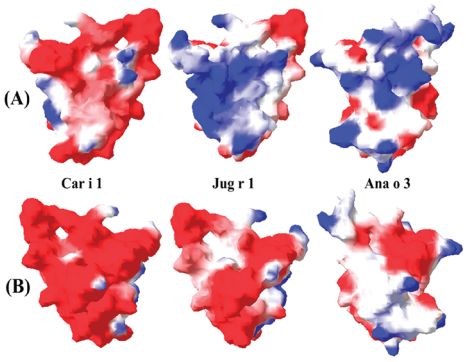

Figure 10. Mapping of electrostatic potentials on the molecular suface of Car i 1, Jug r 1, and Ana o 3. (A) and (B) indicate the front and back of the surface, respectively. Red = electronegatively charged regions, blue = electropositively charged regions, and white = neutral regions.

discrete presumption could be made about the IgE-binding epitope dependency on the electrostatic potential.

DISCUSSION

Among the seed storage proteins, 2S albumins, which are defined based on their sedimentation coefficient, have been identified as major allergens in several plants including tree nuts and peanut. The 2S albumins belong to the prolamin superfamily whose members are characterized by similar structural and/or functional features, and have a well-conserved skeleton of cysteine residues. The 2S albumin is synthesized as a 12−16 kDa polypeptide, which is cleaved post-translationally into a large (9−12 kDa) and a small (3−6 kDa) subunit held together by disulfide bonds. We amplified the 2S albumin gene from a pecan cDNA library. Since pecan, walnut, and hazelnut belong to the order Fagales, it was not surprising to see high sequence identity between their 2S albumins. The significant identity between walnut and pecan 2S albumin may be attributed to both tree nuts being in the Juglandaceae family. All of the homologous 2S albumins listed in Table 1 have been identified as allergens. The high homology in botanically related plant species may contribute to strong IgE-binding cross-reactivities observed between pecan, walnut and hazelnut.[34]

In our study, 28 patients with convincing histories of allergic reactions to pecan nut were used to determine the IgE-binding ability of pecan 2S albumin. Twenty-five patients also reported allergy to other tree nuts, while history of reactions to other foods were lacking for two patients (16 and 20). The majority of these patients also exhibited severe reactions following the ingestion of walnut. Most of the patients were allergic to multiple tree nuts with varying symptoms, which is not uncommon and may occur as a result of cross-reactivity or coexisting allergy. Twenty-two out of these 28 sera bound to the recombinant pecan 2S albumin (Car i 1).

Patients allergic to walnut are also often found to be allergic to pecan. We demonstrated *in vitro* cross-reactivity between Car i 1 and the corresponding walnut allergen Jug r 1. We have also observed that pAbs raised against pecan protein extract are cross-reactive with walnut proteins (data not shown). Cross-reactivity between pecan and walnut has been reported not only with respect to IgE but also in IgG-based immunoassay developed to detect walnut in foods.[35] However, Sordet et al.[32] did not find rabbit anti-Jug r 1 pAb to recognize Car i 1, but did find that it recognized the 2S albumin from sesame seed. Inhibition immunoblots employing sera from patients with life-threatening allergic reactions to walnut demonstrated that pecan and walnut extract equally inhibited walnut proteins.[14] The *in vitro* inhibition also correlated with the clinical reports of cross-reactivity in those patients. It is likely that the close phylogenetic relationship between pecan and walnut, which results in a high degree of amino acid identity (88%) and presumably tertiary structure between Car i 1 and Jug r 1, leads to a situation in which patients sensitized by one allergen produce a significant amount of IgE capable of binding to both allergen (i.e., cross-reactivity) resulting in allergic symptoms upon ingestion of the either nut.

We sought to identify and characterize Car i 1 in the native extract by affinity purification and proteomic tools. The majority of nCar i 1 was dissociated into a large and small subunit under reducing conditions. However, a 16 kDa polypeptide was also observed under reducing conditions, indicating (1) that the mature protein may be present in precursor form with covalently bound large and small subunit, (2) that the disulfide bond between these subunits may be only partially accessible, or (3) reassociation of the subunits after reduction. Similar findings have also been observed in walnut 2S albumin, where the intact heterodimer at 14 kDa was observed in reducing gels along with the 10 kDa large subunit and 4 kDa small subunit.[24] The rCar i 1 was expressed as a single polypeptide containing both large and small subunits. Though rCar i 1 and nCar i 1 polypeptides were similar in molecular mass (∼16−18 kDa), the rCar i 1 band

appeared to migrate slightly more slowly than nCar i 1 on SDS−PAGE. The difference may be attributed to post-translational modification(s), which can also result in several isoforms. Similar differences have been observed for cashew 2S albumin, Ana o3, where the native 2S albumin (12.6 kDa) was ∼3 kDa smaller than the predicted mass of Ana o 3 (16.3 kDa).[22]

The reduced immunoreactivity of cleaved rCar i 1 may be due to the change in structure of the polypeptide upon removal of MBP from the fusion protein indicative of the presence of conformational epitopes. Since the nCar i 1 had reactivity in the 16 kDa nonreduced region, and not in the 11 kDa large subunit region, suggests that either the disulfide bond or the close proximity of large and small subunits is important in maintaining the immunoreactivity of the protein. Denaturation studies with reducing agent and denaturants further confirmed the role of disulfide bonds in maintaining the immunoreactivity when probed with rabbit anti-pecan pAb. The study of conformational epitopes poses a challenge since protein folding may result in a loss/gain of epitopes. The high resistance of 2S albumins to thermal and gastrointestinal pH conditions is well-known.[33,36−38] This property enhances the possibility that 2S albumins may remain substantially intact following ingestion and interaction with the gut immune system, thereby preserving the linear and possibly some of the conformational epitopes.

Tree nut 2S albumins, including Jug r 1 (walnut) and Ana o 3 (cashew), have already been analyzed for their sequential epitopes. Since allergic IgE responses are polyclonal in nature often involving multiple epitopes, it is not surprising that 24 different epitopic regions were identified throughout the Car i 1 sequence or that different patients recognized different sets of epitopes. The shared homology between Car i 1, Jug r 1, and Ana o 3 may partially explain the observed cross-reactivity between some of the tree nut allergens. Mutational analysis of the sole immunodominant sequential epitope of Jug r 1 reported by Robotham et al.[31] revealed that the core amino acids RGEE (positions 36−39) and an additional glutamic acid residue (position 42) were necessary for maximum IgE binding to this epitope. This core region of Jug r 1 shares 100% identity and positional homology with a Car i 1 immunodominant epitope. Further mutational analyses of Car i 1 epitopes would be beneficial in understanding immunological similarities between the 2S albumins from botanically related species, thereby facilitating the improved understanding of the cross-reactivity. Conformational analysis of the linear IgE-binding epitope of Ara h 2, a peanut allergen, has been reported to exhibit no structural similarity with 2S albumin allergens from walnut (Jug r 1) and pecan nut (Car i 1).[39]

The rapeseed 2S albumin shared 31−34% identity with Car i 1, Jug r 1, and Ana o 3, and covered the target amino acid sequence range 41−131, 39−127, and 35−134, respectively. Molecular modeling and sequential epitope surface mapping of Car i 1 show that the 3 strongly reactive epitopic regions are exposed on the surface of Car i 1, since most of the amino acids in these epitopes are solvent accessible. The solvent accessible and flexible regions of 2S albumins are believed to have high IgE-binding capabilities, thereby representing a determinant of allergenicity.[33] Knowledge of protein structures may be of vital importance to study conformational epitopes, their mode of binding to IgE, and their role in IgE-antigen interactions.

In summary, pecan 2S albumin, Car i 1, is a major IgE binding allergen in 22 of 28 patients (79%) with documented IgE sensitivity and a convincing history of pecan allergy that exhibits *in vitro* cross-reactivity with walnut major allergen Jug r 1. The presence of disulfide bonds is important in maintaining the immunoreactivity of nCar i 1, at least with respect to rabbit anti-pecan IgG, suggesting the important role of conformational epitopes in pecan 2S albumin immunoreactivity. The sequential epitopes of Car i 1 share sequential and positional homology with known sequential epitopes of walnut (Jug r 1) and cashew (Ana o 3) 2S albumin allergens, which may be responsible for some of the observed cross-reactivity among these tree nuts.

AUTHOR INFORMATION

Corresponding Author

*402 Sandels Bldg., 120 Convocation Way, NFES, CHS, The Florida State University, Tallahassee, FL 32306-1493. Tel: +1-850-644-5837. Fax: +1-850-645-5000. E-mail: ssathe@fsu.edu.

Present Addresses

‡Immunobiology Branch, Center for Food Safety and Applied Nutrition, Food and Drug Administration, Laurel, MD 20708.

Funding Sources

This work was supported by USDA grants (No. 9901530, No. 2004-35503-14117, and No. 2006-35503-17229, S.K.S. and K.H. R.) and the College of Human Science (Dissertation Award Program, G.M.S.)

ACKNOWLEDGMENT

We thank Bodhana Dhole for her help with the development of IgE immunoblots.

REFERENCES

(1) Sicherer, S. H.; Sampson, H. A. Food allergy: recent advances in pathophysiology and treatment. *Ann. Rev. Med.* **2009**, *60*, 261–77.

(2) Sampson, H. A. Update on food allergy. *J. Allergy Clin. Immunol.* **2004**, *113* (5), 805–816.

(3) Sicherer, S. H.; Muñoz-Furlong, A.; Sampson, H. A. Prevalence of peanut and tree nut allergy in the United States determined by means of a random digit dial telephone survey: A 5-year follow-up study. *J. Allergy Clin. Immunol.* **2003**, *112* (6), 1203–1207.

(4) Bock, S. A.; Munoz-Furlong, A.; Sampson, H. A. Fatalities due to anaphylactic reactions to foods. *J. Allergy Clin. Immunol.* **2001**, *107* (1), 191–193.

(5) Fleischer, D. M.; Conover-Walker, M. K.; Matsui, E. C.; Wood, R. A. The natural history of tree nut allergy. *J. Allergy Clin. Immunol.* **2005**, *116* (5), 1087–1093.

(6) Sicherer, S. H.; Furlong, T. J.; Muñoz-Furlong, A.; Burks, A. W; Sampson, H. A. A voluntary registry for peanut and tree nut allergy: characteristics of the first 5149 registrants. *J. Allergy Clin. Immunol.* **2001**, *108* (1), 128–132.

(7) Sathe, S. K.; Monaghan, E. K.; Kshirsagar, H. H.; Venkatachalam, M. Chemical composition of edible nut seeds and its implication in human health. In *Tree nuts: composition, phytochemicals, and health effects*; Alasalvar, C., Shahidi, F., Eds.; CRC Press: Boca Raton, FL, 2009; pp 65−84.

(8) Morgan, W. A.; Clayshulte, B. J. Pecans lower low-density lipoprotein cholesterol in people with normal lipid levels. *J. Am. Diet. Assoc.* **2000**, *100*, 312–318.

(9) Rajaram, S.; Burke, K.; Connell, B.; Myint, T.; Sabate, J. A monounsaturated fatty acid−rich pecan-enriched diet favorably alters the serum lipid profile of healthy men and women. *J. Nutr.* **2001**, *131*, 2275–2279.

(10) Venkatachalam, M.; Sathe, S. K. Chemical composition of selected edible nut seeds. *J. Agric. Food Chem.* **2006**, *54*, 4705–4714.

(11) USDA. National Nutrient Database for Standard Reference, Agricultural Research Service, U.S. Department of Agriculture, 2006.

(12) Jeyaretnam, B.; Levi, A.; Phatak, S. C.; Wetzstein, H. Y. Changes in growth, water content and protein reflect embryo development in pecan (*Carya illinoinensis*). *J. Hort. Sci. Biotechnol.* **1999**, *74* (3), 315–320.

(13) Venkatachalam, M.; Roux, K. H.; Sathe, S. K. Biochemical characterization of soluble proteins in pecan [*Carya illinoinensis* (Wangenh.) K. Koch]. *J. Agric. Food Chem.* **2008**, *56* (17), 8103–8110.

(14) Teuber, S. S.; Sathe, S. K.; Roux, K. H.; Peterson, W. R. Cross-reactivity of walnut and other tree nuts by IgE immunoblot inhibition. *J. Allergy Clin. Immunol.* **2000**, *105* (1), S140.

(15) Venkatachalam, M.; Teuber, S. S.; Peterson, W. R.; Roux, K. H.; Sathe, S. K. Antigenic stability of pecan [*Carya illinoinensis* (Wangenh.) K. Koch] proteins: effects of thermal treatments and in vitro digestion. *J. Agric. Food Chem.* **2006**, *54*, 1449–1458.

(16) Malanin, K.; Lundberg, M.; Johansson, S. G. Anaphylactic reaction caused by neoallergens in heated pecan nut. *Allergy* **1995**, *50* (12), 988–991.

(17) Berrens, L. Neoallergens in heated pecan nut: products of Maillard-type degradation. *Allergy* **1996**, *51* (4), 277–278.

(18) Moreno, F. J.; Clemente, A. 2S albumin storage proteins: what makes them food allergens?. *Open Biochem. J.* **2008**, *2*, 16–28.

(19) Burks, A. W.; Williams, L. W.; Connaughton, C.; Cockrell, G.; O'Brien, T. J.; Helm, R. M. Identification and characterization of a second major peanut allergen, Ara h II, with use of the sera of patients with atopic dermatitis and positive peanut challenge. *J. Allergy Clin. Immunol.* **1992**, *90*, 962–969.

(20) Poltronieri, P.; Cappello, M. S.; Dohmae, N.; Conti, A.; Fortunato, D.; Pastorello, E. A.; Ortolani, C.; Zacheo, G. Identification and characterization of the IgE-binding proteins 2S albumin and conglutin γ in almond (*Prunus dulcis*) seeds. *Int. Arch. Allergy Immunol.* **2002**, *128*, 97–104.

(21) Pastorello, E. A.; Farioli, L.; Pravettoni, V.; Ispano, M.; Conti, A.; Ansaloni, R.; Rotondo, F.; Incorvaia, C.; Bengtsson, A.; Rivolta, F.; Trambaioli, C.; Previdi, M.; Ortolani, C. Sensitization to the major allergen of Brazil nut is correlated with the clinical expression of allergy. *J. Allergy Clin. Immunol.* **1998**, *102* (6), 1021–1027.

(22) Robotham, J. M.; Wang, F.; Seamon, V.; Teuber, S. S.; Sathe, S. K.; Sampson, H. A.; Beyer, K.; Seavy, M.; Roux, K. H. Ana o 3, an important cashew nut (*Anacardium occidentale* L.) allergen of the 2S albumin family. *J. Allergy Clin. Immunol.* **2005**, *115* (6), 1284–1290.

(23) Ahn, K.; Bardina, L.; Grishina, G.; Beyer, K.; Sampson, H. A. Identification of two pistachio allergens, Pis v 1 and Pis v 2, belonging to the 2S albumin and 11S globulin family. *Clin. Exp. Allergy* **2009**, *39*, 926–934.

(24) Teuber, S. S.; Dandekar, A. M.; Peterson, W. R.; Sellers, C. L. Cloning and sequencing of a gene encoding a 2S albumin seed storage protein precursor from English walnut (*Juglans regia*), a major food allergen. *J. Allergy Clin. Immunol.* **1998**, *101* (6), 807–814.

(25) Bradford, M. M. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **1976**, *72*, 248–254.

(26) Acosta, M. R.; Roux, K. H.; Teuber, S. S.; Sathe, S. K. Production and characterization of rabbit polyclonal antibodies to almond (*Prunus dulcis* L.) major storage protein. *J. Agric. Food Chem.* **1999**, *47*, 4053–4059.

(27) Levi, A.; Galau, G. A.; Wetzstein, H. Y. A rapid procedure for the isolation of RNA from high phenolic containing tissues of pecan. *Hort. Sci.* **1992**, *27* (12), 1316–1318.

(28) Fling, S. P.; Gregerson, D. S. Peptide and protein molecular weight determination by electrophoresis using a high-molarity tris buffer system without urea. *Anal. Biochem.* **1986**, *155*, 83–88.

(29) Towbin, H.; Staehlin, T.; Gordon, J. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 4350–4354.

(30) Arnold, K.; Bordoli, L.; Kopp, J.; Schwede, T. The SWISS-MODEL Workspace: A web-based environment for protein structure homology modelling. *Bioinformatics* **2006**, *22*, 195–201.

(31) Robotham, J. M.; Teuber, S. S.; Sathe, S. K.; Roux, K. H. Linear IgE epitope mapping of the English walnut (*Juglans regia*) major food allergen, Jug r 1. *J. Allergy Clin. Immunol.* **2002**, *109* (1), 143–149.

(32) Sordet, C.; Culerrier, R.; Granier, C.; Rance, F.; Didier, A.; Barre, A.; Rouge, P. Expression of Jug r 1, the 2S albumin allergen from walnut (*Juglans regia*), as a correctly folded and functional recombinant protein. *Peptides* **2009**, *30*, 1213–1221.

(33) Pantoja-Uceda, D.; Palomares, O.; Bruix, M.; Villalba, M.; Rodríguez, R.; Rico, M.; Santoro, J. Solution structure and stability against digestion of rproBnIb, a recombinant 2S albumin from rapeseed: relationship to its allergenic properties. *Biochemistry* **2004**, *43* (51), 16036–16045.

(34) Goetz, D. W.; Whisman, B. A.; Goetz, A. D. Cross-reactivity among edible nuts: double immunodiffusion, crossed immunoelectrophoresis, and human specific IgE serologic surveys. *Ann. Allergy, Asthma, Immunol.* **2005**, *95* (1), 45–52.

(35) Niemann, L.; Taylor, S. L.; Hefle, S. L. Detection of walnut residues in foods using an enzyme-linked immunosorbent assay. *J. Food Sci.* **2009**, *74* (6), T51–T57.

(36) Murtagh, G. J.; Archer, D. B.; Dumoulin, M.; Ridout, S.; Matthews, S.; Arshad, S. H.; Alcocer, M. J. C. In vitro stability and immunoreactivity of the native and recombinant plant food 2S albumins Ber e 1 and SFA-8. *Clin. Exp. Allergy* **2003**, *33*, 1147–1152.

(37) Lin, J.; Fido, R.; Shewry, P.; Archer, D. B.; Alcocer, M. J. C. The expression and processing of two recombinant 2S albumins from soybean (*Glycine max*) in the yeast *Pichia pastoris*. *Biochim. Biophys. Acta* **2004**, *1698*, 203–212.

(38) Moreno, F. J.; Maldonado, B. M.; Wellner, N.; Mills, E. N. C. Thermostability and in vitro digestibility of a purified major allergen 2S albumin (Ses i 1) from white sesame seeds (*Sesamum indicum* L.). *Biochim. Biophys. Acta* **2005**, *1752*, 142–153.

(39) Barre, A.; Borges, J.-P.; Culerrier, R.; Rouge, P. Homology modeling of the major peanut allergen Ara h 2 and surface mapping of IgE-binding epitopes. *Immunol. Lett.* **2005**, *100*, 153–158.

(40) Ling, M.; Ye, J.; Beyer, K.; Cockrell, G.; Bannon, G. A.; Stanley, J. S.; Helm, R. M.; Sampson, H. A.; Burks, A. W. Cloning, identification, and epitope-mapping two black walnut (*Juglans niger*) allergens. *J. Allergy Clin. Immunol.* **2003**, *111*, S248.

(41) Garino, C.; Zuidmeer, L.; Marsh, J.; Morati, M.; Versteeg, S.; Brettlova, V.; Schilte, P.; Shewry, P.; Arlorio, M.; van Ree, R. Isolation, cloning, and characterization of 2S albumin, a new hazelnut allergen (unpublished). Direct submission to NCBI. 2008.

(42) Bashir, M.; Hubatsch, I.; Leinenbach, H. P.; Zeppezauer, M.; Panzani, R. C.; Hussein, I. H. Ric c 1 and Ric c 3, the allergenic 2S albumin storage proteins of *Ricinus communis*: complete primary structures and phylogenetic relationships. *Int. Arch. Allergy Immunol.* **1998**, *115* (1), 73–82.