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Cloning and Characterization of an 11S Legumin, Car i 4, a Major Allergen in Pecan

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ABSTRACT: Among tree nut allergens, pecan allergens remain to be identified and characterized. The objective was to demonstrate the IgE-binding ability of pecan 11S legumin and characterize its sequential IgE-binding epitopes. The 11S legumin gene was amplified from a pecan cDNA library and expressed as a fusion protein in *Escherichia coli*. The native 11S legumin in pecan extract was identified by mass spectrometry/mass spectrometry (MS/MS). Sequential epitopes were determined by probing the overlapping peptides with three serum pools prepared from different patients' sera. A three-dimensional model was generated using almond legumin as a template and compared with known sequential epitopes on other allergenic tree nut homologues. Of 28 patients tested by dot blot, 16 (57%) bound to 11S legumin, designated Car i 4. MS/MS sequencing of native 11S legumin identified 33 kDa acidic and 20−22 kDa basic subunits. Both pecan and walnut seed protein extracts inhibited IgE binding to recombinant Car i 4, suggesting cross-reactivity with Jug r 4. Sequential epitope mapping results of Car i 4 revealed weak, moderate, and strong reactivity of serum pools against 10, 5, and 4 peptides, respectively. Seven peptides were recognized by all three serum pools, of which two were strongly reactive. The strongly reactive peptides were located in three discrete regions of the Car i 4 acidic subunit sequence (residues 118−132, 208−219, and 238−249). Homology modeling of Car i 4 revealed significant overlapping regions shared in common with other tree nut legumins.

KEYWORDS: food allergy, allergen, pecan, seed storage protein, 11S legumin, Car i 4, sequential epitope, homology model

INTRODUCTION

Per capita consumption of tree nuts in the United States has increased steadily over the past decade and is currently at ∼3.5 lb (http://www.ers.usda.gov/data/foodconsumption/FoodAvailSpreadsheets.htm#nuts). The consumption of tree nut depends on the age group and is highest in adults aged 40−59 years.[1] Pecan belongs to the Juglandaceae family, a family that includes hickory nuts and walnuts. Pecans (*Carya illinoinensis*) are one of the popular tree nut seeds that are eaten as a snack or are used as an ingredient in other food products, including bakery (e.g., pecan pie), confectionery (e.g., ice cream, candy), and snack foods (e.g., trail mix). It has been suggested that pecans offer health benefits, especially due to their potential to reduce the risk of cardiovascular disease.[2,3] Although safely enjoyed by most, pecans pose the threat of eliciting adverse reactions, for example, allergy/anaphylaxis, in sensitive individuals.

With the apparent rise in food allergies in Western countries, the safety of sensitive consumers is of concern. In the United States, the prevalence of reported food allergy among children <18 years of age has increased by 18% from 1997 through 2007.[4] Recent studies indicate up to 20% of the U.S. population has concerns about food allergy, whereas 4−8% children and 1−3% adults suffer from confirmed food allergies.[5] Severe food-induced allergic reactions, including fatal anaphylactic reactions, have been often associated with the consumption of peanuts and tree nuts.[6,7] Although initially believed to be a life-long allergy, studies have shown that about 9% of the patient population outgrow tree nut allergies.[8] Estimated peanut allergies in U.S. children and adults are 1 and 0.6%, respectively, of the population, with corresponding figures for tree nut allergies being 0.5 and 0.6%. Fatalities are primarily reported from allergic reactions to peanuts and tree nuts and therefore are of particular concern.[9] Among tree nuts, allergy to pecan nut ranks fourth after walnut, cashew nut, and almond.[10]

Allergic reaction to pecan nut seed proteins has been reported.[7] Venkatachalam et al.[11] identified multiple IgE-reactive polypeptide bands (range 7−66 kDa) when protein extracts, prepared from unprocessed and variously processed defatted pecan seeds, were probed with a pool of sera from pecan allergic patients. Recently, Sharma et al.[12] demonstrated Car i 1, a pecan 2S albumin, reactivity with pecan-allergic patient serum IgE. In vitro immunogenic cross-reactivity between pecan and walnut is well documented.[13]

Pecan seed storage proteins are primarily composed of 2S albumin, 7S vicilin, and 11S legumin. Legumin occurs as a hexamer composed of ∼50−60 kDa monomeric subunits. The hydrophobic signal peptide is removed post-translationally, and the resulting molecule is cleaved into an acidic and a basic polypeptide. An acidic subunit (∼30−40 kDa) is linked to a

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basic subunit (∼20−30 kDa) by intermolecular disulfide bond(s) to form the ∼50−60 kDa monomer. Six such monomers constitute the native hexameric legumin. Several legumin proteins in tree nut seeds, including almond [amandin, Pru du 6],[14] Brazil nut [Ber e 2],[15] cashew [Ana o 2], [16] hazelnut [Cor a 9],[17] pistachio [Pis v 2],[18] and walnut [Jug r 4][19], and in other seeds including sesame [Ses i 6],[20,21] soy [Gly m 6],[22] peanut [Ara h 3],[23] and mustard [Sin a 2][24] are reported as allergens. As tree nut and peanut legumins share considerable amino acid sequence identity as well as structural homology, IgE-binding cross-reactivity between peanut and tree nuts can be expected.[25−27] Earlier, we reported fractionation and biochemical characterization of soluble proteins in pecan seeds.[28] Here, the sequences of two cDNAs encoding subunits of the 11S legumin in pecan are reported. Furthermore, one clone was expressed (designated Car i 4) and the ability of the recombinant protein to bind serum IgE from patients with allergy to pecan nut was demonstrated. The sequential epitopes of Car i 4 were determined and mapped on the molecular surface of modeled Car i 4.

MATERIALS AND METHODS

Pecan Protein Extract. Defatted pecan flour was prepared as described by Sharma et al.[29] Pecan protein extract was prepared by vortexing defatted 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 (∼25 °C). The soluble protein in the supernatant was estimated,[30] and the supernatant was stored at −20 °C until further use.

Production of Rabbit Polyclonal Antibodies (pAb). Antibodies against soluble pecan proteins were produced by immunizing two New Zealand white female rabbits each with BSB extracted pecan proteins (500 μg) in 0.5 mL of RIBI adjuvant as described earlier.[31] Three booster doses were administered in RIBI adjuvant at 4 week intervals. Each rabbit was subsequently bled, and the serum was collected and stored at −20 °C until further use.

Human Sera. Blood samples from patients with a convincing history of pecan allergy and/or detectable pecan-specific IgE were either collected at the Mount Sinai School of Medicine (New York, NY) following approval by the institutional review boards or purchased from PlasmaLab International (Everett, WA). The allergic and control (patients with no history of pecan hypersensitivity) sera were stored at −70 °C until further use. The clinical characteristics of these pecan-allergic patients have been reported previously by Sharma et al.[12]

Isolation of Total RNA and Pecan cDNA Library Construction. Total RNA was isolated from maturing pecan nuts stored at −80 °C as described by Levi et al.[32] The 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 (PolyATtract kit, Promega Corp., Madison, WI) and a cDNA library was synthesized (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 λ Uni-ZAP XR expression vector, packaged in vitro, and transfected in *Escherichia coli* XL1-Blue MRF' strain to amplify the cDNA library.

Identification of 11S Legumin cDNA. The 11S legumin sequence was identified from the cDNA library using a set of primers in all possible combinations [forward, CGTACCATTGAGCCCAATGGCCTTC (F1), GCCTTCTCTTGCCTCAATACAGCAATG (F2), and T3 promoter; reverse, T7 promoter, GATGTTCTCCCTCAACCTCAAGGTG (R1), and AGGACTGGGAGGGTGTGGCTGTTG (R2)]. The primers were based on the conserved nucleotide sequence of the legumin gene in walnut, which exhibits sequence homology with other tree nuts. On the basis of the deduced nucleotide sequence of the amplicon, a new forward primer (CATGGCCAAGCCCATCTTGCTATCC) was designed and the complete sequence was amplified using T7 promoter as the reverse primer. The amplicon was cloned into pCR 2.1-TOPO (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions and plasmid purified using QIAprep spin plasmid miniprep kit (Qiagen Inc., Valencia, CA) for sequencing purposes.

Nucleotide Sequencing and Analysis of Legumin cDNA. The cDNAs were sequenced from both directions with 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 amino acid sequences were performed using the BLAST program (www.ncbi.nlm.nih.gov/BLAST/). SignalP 3.0 server (www.cbs.dtu.dk/services/SignalP/) was used to predict the signal peptide cleavage site. The alignment of the nucleotide and amino acid sequences was performed using the ClustalW program (www.ebi.ac.uk/clustalw/).

Subcloning, Expression, and Purification of Recombinant Proteins. The cDNA insert produced by PCR amplification with *Bam*HI and *Hin*dIII restriction sites at the 5′ end of the forward and reverse primers, respectively, was subcloned into the maltose binding protein (MBP) expression vector pMAL-c4x (New England BioLabs Inc., Beverly, MA). The expression and purification of the recombinant (r) Car i 4 fusion protein were carried out as described earlier.[12] The recombinant protein was stored at −20 °C until further use.

Sodium Dodecyl Sulfate−Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Two-Dimensional (2D) Gels. SDS-PAGE was done by using the method of Fling and Gregerson.[33] Suitable aliquots of seed protein extract or recombinant protein samples boiled in sample buffer [50 mM Tris-HCl, pH 6.8, 1% (w/v) SDS, 0.01% (w/v) bromophenol blue, 30% (v/v) glycerol, and 2% (v/v) β-mercaptoethanol] for 10 min were loaded on the gel and run at a constant current of 12 mA/gel until the tracking dye passed through the gel. The gels were stained with 0.25% Coomassie Brilliant Blue R (CBBR) in 50% methanol and 10% acetic acid (unless used for protein transfer) and destained with 50% methanol containing 10% acetic acid. For 2D gel separation, isoelectric focusing (IEF) of the protein sample was run in the first dimension using an 11 cm Immobiline DryStrip pH 3−11 (GE Healthcare Bio-Sciences Corp., Piscataway, NJ) for 33000 V-h on the Protean IEF system (Bio-Rad Laboratories, Inc., Hercules, CA) as per the manufacturer's instruction. The strip was laid horizontally on 8−25% acrylamide gradient SDS-PAGE gel, sealed with 1% (w/v) agarose containing 0.01% (w/v) bromophenol blue, and run in the second dimension at a constant current of 12 mM/gel overnight until the tracking dye passed the gel edge. The gels were stained and destained as described above.

Immunoblotting and Inhibition Immunoblotting. SDS-PAGE was carried out as described above, and proteins were transferred to a 0.22 μm nitrocellulose (NC) membrane as described by Towbin et al.[34] For dot blotting, 1 μg of rCar i 4 was dotted on the NC membrane and allowed to air-dry for 5 min. NC membranes were blocked for 1 h at room temperature (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), followed by incubation with diluted human sera (1:3 v/v dilution) or rabbit anti-pecan pAb (1:10000 v/v dilution) overnight at 4 °C. The membranes were then washed three 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 v/v dilution) overnight at 4 °C or goat anti-rabbit IgG (1:40000 v/v dilution) for 1 h at room temperature. The membranes were washed three times, 15 min each (30 min for human sera blots), in TBS-T, and exposed to X-ray film. The exposure time was generally 30 s for the blot

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(A)
Pec11S-1,2   CATGGCCAAGCCCATCTTGCTATCCATTTATCTGTGCCTTATAATTGTGGCTCTCTTCAA 60

Pec11S-1     TGGTTGCCTTGCTCAAAGCGGCGGACGGCAACAACACAAATTTGGCCAGTGCCAGCTCAA 120
Pec11S-2     ******************T******************************************* 120

Pec11S-1,2   TAGGCTCGATGCCCTTGAACCTACTAACCGCATTGAGGCCGAAGCCGGTGTTATTGAGTC 180
Pec11S-1,2   CTGGGACCCGAACCACCAGCAGCTCCAGTGCGCTGGGGTTGCCGTTGTTCGTCGTACCAT 240
Pec11S-1,2   TGAGCCCAATGGCCTTCTCTTGCCTCATTACAGCAATGCTCCTCAGCTCGTCTACATTGC 300
Pec11S-1,2   CAGAGGTAGGGGTATCACCGGGGTTCTATTTCCCGGCTGTCCAGAAACATTCGAAGAATC 360
Pec11S-1,2   TCAACGACAATCTCAACAAGGACAGAGACGCGAGTTCCAACAAGACAGGCACCAGAAGAT 420
Pec11S-1,2   TCGACACTTCCGAGAGGGCGACATAATCGCATTCCCGGCAGGAGTAGCCCATTGGTGCTA 480
Pec11S-1,2   CAACGACGGCAGTAGCCCAGTAGTTGCAATCTTTCTCCTTGACACCCACAACAATGCCAA 540
Pec11S-1,2   TCAGCTTGACCAGAACCCCAGAAATTTCTACCTTGCTGGGAACCCCGACGATGAATTTCG 600
Pec11S-1,2   GCCACAAGGTCAGCAGGAATACGAGCAGCACCGCCGACAGCAACAACATCAACAACGTCG 660
Pec11S-1,2   TGGCGAGCACGGCGAGCAACAGAGGGACTTAGGCAACAATGTGTTCAGTGGCTTCGATGC 720
Pec11S-1,2   TGAGTTTTTGGCGGACGCTTTCAACGTGGATACTGAAACGGCCAGAAGACTTCAGAGCGA 780
Pec11S-1,2   GAATGATCACAGGGGGAGCATAGTGAGAGTGGAAGGCCGTCAGCTTCAAGTGATCAGGCC 840
Pec11S-1,2   ACGATGGTCACGTGAGGAACAGGAGCATGAGGAGAGGAAAGAGAGAGAGCGAGAGCGAGA 900

Pec11S-1     GTCGGAAAGCGAGCGCCGCCAGAGCCGACGTGGCGGACGTGATGATAATGGGCTGGAGGA 960
Pec11S-2     *****G*************************************************** 960

Pec11S-1,2   GACTATCTGCACCTTGAGTTTGAGGGAGAACATCGGCGACCCTTCACGCGCCGACATTTA 1020
Pec11S-1,2   CACTGAAGAAGCCGGTCGCATCAGCACCGTCAACAGCCACAACCTCCCAATCCTGCGCTG 1080
Pec11S-1,2   GCTGCAACTCAGCGCCGAGAGGGGAGCTCTCTATAGTGATGCTCTGTATGTTCCACACTG 1140
Pec11S-1,2   GAACCTGAACGCCCACAGTGTGGTGTATGCTTTAAGGGGTCGTGCCGAGGTTCAGGTGGT 1200
Pec11S-1,2   GGACAACTTTGGTCAGACAGTGTTCGATGATGAGCTTAGAGAGGGTCAGCTCCTGACCAT 1260

Pec11S-1     TCCACAGAACTTTGCGGTTGTGAAAAGGGCTCGCGATGAGGGTTTCGAGTGGGTTTCATT 1320
Pec11S-2     *********************************A************************ 1320

Pec11S-1,2   CAAGACAAATGAGAATGCCATGGTTAGTCCACTTGCTGGCCGAACCTCGGCTATCAGGGC 1380
Pec11S-1,2   ATTGCCGGAGGAAGTGCTTGTCAATGCGTTCCAAATTCCGAGAGAGGATGCTAGGAGGCT 1440
Pec11S-1,2   TAAATTTAACAGGCAGGAGTCGACCTTGGTTCGTTCAAGGTCAAGGTCTTCAAGGTCTGA 1500
Pec11S-1,2   GAGGAGGGCTGAAGTTTAATGTTGTGAACTGCGCGCAATGTAGAGTGCCATATATATATG 1560

Pec11S-1     AGGCTCGCTCGATCGATCGTAGTTTGCTTTTTACATATGTTAATGCTCGATCCGCACTTG 1620
Pec11S-2     *********T********************************************** 1620

Pec11S-1     GTCGATCATCATCTAGACAATAAAAAAGTTGCTAGCCTTTTACTGGCATTTATAATCAAA 1680
Pec11S-2     ******************************************************AA*** 1680

Pec11S-1     AAAAAAAAAAAAAAAAAA 1697
Pec11S-2     **************---- 1693

(B)
Pec11S-1,2   MAKPILLSIYLCLIIVALFNGCLAQSGGRQQHKFGQCQLNRLDALEPTNRIEAEAGVIES 60
Pec11S-1,2   WDPNHQQLQCAGVAVVRRTIEPNGLLLPHYSNAPQLVYIARGRGITGVLFPGCPETFEES 120
Pec11S-1,2   QRQSQQGQRREFQQDRHQKIRHFREGDIIAFPAGVAHWCYNDGSSPVVAIFLLDTHNNAN 180
Pec11S-1,2   QLDQNPRNFYLAGNPDDEFRPQGQQEYEQHRRQQQHQQRRGEHGEQQRDLGNNVFSGFDA 240
Pec11S-1,2   EFLADAFNVDTETARRLQSENDHRGSIVRVEGRQLQVIRPRWSREEQEHEERKERERERE 300
                                        ↓
Pec11S-1     SESERRQSRRGGRDDNGLEETICTLSLRENIGDPSRADIYTEEAGRISTVNSHNLPILRW 360
Pec11S-2     *G***************************************************** 360

Pec11S-1,2   LQLSAERGALYSDALYVPHWNLNAHSVVYALRGRAEVQVVDNFGQTVFDDELREGQLLTI 420

Pec11S-1     PQNFAVVKRARDEGFEWVSFKTNENAMVSPLAGRTSAIRALPEEVLVNAFQIPREDARRL 480
Pec11S-2     ***********N*************************************************** 480

Pec11S-1,2   KFNRQESTLVRSRSRSSRSERRAEV 505
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Figure 1. Nucleotide (A) and derived amino acid sequence (B) of the 11S legumin cDNA clones *Pec11S-1* and *Pec11S-2*. The presumed isoform allelic difference between the two genes is indicated in bold type. The presumed start and stop codons are indicated in bold type and underlined. An asterisk (*) denotes identity. The presumed signal peptide is underlined. The presumed cleavage site between the acidic and basic subunit is indicated by an arrow. GenBank accession numbers for Pec11S-1 and Pec11S-2 are EU113051 and EU113052, respectively.

using rabbit antisera as primary antibody and 4−10 days at −80 °C for the blot using human sera.

For inhibition dot-blot, 50 μg of native protein extract or rCar i 4 was preincubated overnight at 4 °C with human serum (1:3 v/v dilution). The preincubated serum was incubated overnight at 4 °C with NC membrane previously dotted with 1 μg of rCar i 4 and blocked with 5% (w/v) NFDM. The strips were then treated as described above for Western blotting.

Table 1. Sequence Comparison of Car i 4 with Other 11S Legumins

source	accession no.	% identity	% similarity	allergen designation[a] [reference]
Juglans regia (English walnut)	AY692446	95	97	Jug r 4 [19]
Corylus avellana (hazelnut)	AF449424	72	82	Cor a 9 [17]
Ficus pumila (creeping fig)	EF091697	63	79	NI
Quercus robur (English oak)	X99539	66	79	NI
Anacardium occidentale (cashew nut)	AF453947	57	72	Ana o 2 [16]
Ricinus communis (castor bean)	EQ973941	60	75	ND [39]
Pistacia vera (pistachio)	EU410073	58	73	Pis v 2 [18]
Chenopodium quinoa (quinoa)	AY562549	53	70	ND [40]
Amaranthus hypochondriacus (amaranth)	X82121	52	69	NI
Actinidia chinensis (kiwifruit)	DQ251185	55	70	NI
Sesamum indicum (sesame)	AF240004	51	65	Ses i 6, Ses i 7 [20]
Prunus dulcis (almond)	GU059261	54	70	Pru du 6.02 [14]

[a] NI, not identified; ND, not designated allergen by International Union of Immunological Societies, Allergen Nomenclature Sub-committee, http://www.allergen.org/Allergen.aspx.

N-terminal Amino Acid Sequencing. The polypeptides from SDS-PAGE were transferred to 0.2 μm polyvinylidene fluoride (PVDF) membrane (Whatman Inc., Piscataway, NJ) presoaked in 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). Briefly, protein bands excised from 2D gels were washed with 50 mM NH_4HCO_3/acetonitrile (1:1) and dehydrated 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 room temperature. The gel pieces were washed several times in 50 mM NH_4HCO_3 and dehydrated with acetonitrile. The proteins were digested with trypsin (Promega, modified trypsin) overnight at 37 °C, and the resulting peptide mixtures were analyzed by MALDI-TOF peptide mass fingerprint; 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 one missed cleavage permitted, and (3) peptide tolerance set at 60 ppm.

Solid-Phase Peptide Synthesis. Synthetic overlapping 12 amino acid peptides, offset by 3 amino acids, were synthesized by Sigma Genosys (JPT Peptide Technologies, Acton, MA), based on the deduced amino acid sequence of Car i 4 and used for immunoblotting, as per the manufacturer's instructions described previously.[12]

Molecular Modeling. Homology modeling of Car i 4 was carried out under the automated mode of protein structure homology-modeling server SWISS-MODEL[35−37] by submitting the amino acid sequence of Car i 4 11S legumin and comparing it with the almond 11S legumin crystal structure (RCSB protein data bank code 3ehk chain F). The ribbon diagram and molecular surface 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.

RESULTS

Gene Identification and Characterization. A ∼800 bp amplicon was obtained upon PCR of the pecan cDNA library with internal reverse primer R1 and T3 promoter from the phagemid as the forward primer. The full-length coding sequence (∼1.7 kb) was amplified from the cDNA library using the newly designed forward primer (based on the nucleotide sequence of 800 bp amplicon) and T7 promoter reverse primer and cloned in the TA vector. Upon sequencing two of the plasmids and comparison with other genes in the GenBank, both plasmids were found to be homologous to the legumin family of seed storage proteins. The nucleotide sequences of two legumin cDNAs (accession numbers EU113051 and EU113052 in the GenBank database) from pecan and their corresponding deduced amino acid sequences are shown in Figure 1. The open reading frame of both clones was 1518 nucleotides and 505 amino acids in length, corresponding to a predicted protein molecular mass of 55.4 kDa and an isoelectric point at pH 6.2. The first 24 amino acid residues were predicted to be signal peptide, which is cleaved during transport into the endoplasmic reticulum. The open reading frames of the two clones are different at three nucleotide positions (positions 79, 906, and 1295) and two amino acid positions (positions 302 and 432), suggesting the existence of legumin isoforms.

Table 1 lists the proteins of the legumin group having high identity (51−95%) and similarity (65−97%) with Car i 4. Highest identity (95%) and similarity (97%) were observed with the walnut legumin allergen, Jug r 4, followed by legumins from hazelnut, creeping fig, English oak, cashew nut, castor bean, pistachio, quinoa, amaranth, kiwifruit, sesame, and almond (Table 1). The predicted cleavage site separating the acidic and basic subunits of pecan legumin is between residues 316 and 317 (indicated by an arrow in Figure 1B). The N-terminal amino acid sequences of two basic polypeptides in pecan extract were GLEETCTLR and GLEETFCTMR, further confirming the cleavage site and also suggesting the presence of different isoforms of legumin proteins. The cleavage site between the acidic and basic subunits is conserved among tree nuts (Figure 2) and peanut.[25,27] Also, the four cysteine residues (positions 37, 70, 113, and 323) involved in intra- and interpolypeptide disulfide bonding between acidic and basic subunits were found to be conserved in tree nut legumin allergens, except the Pru du 6.02 isoform of almond legumin, which conserved cysteine at only two positions (indicated by bold type in Figure 2).

Reactivity of Recombinant Protein (rCar i 4) with Rabbit IgG and Human IgE. The cDNA encoding Pec11S-1

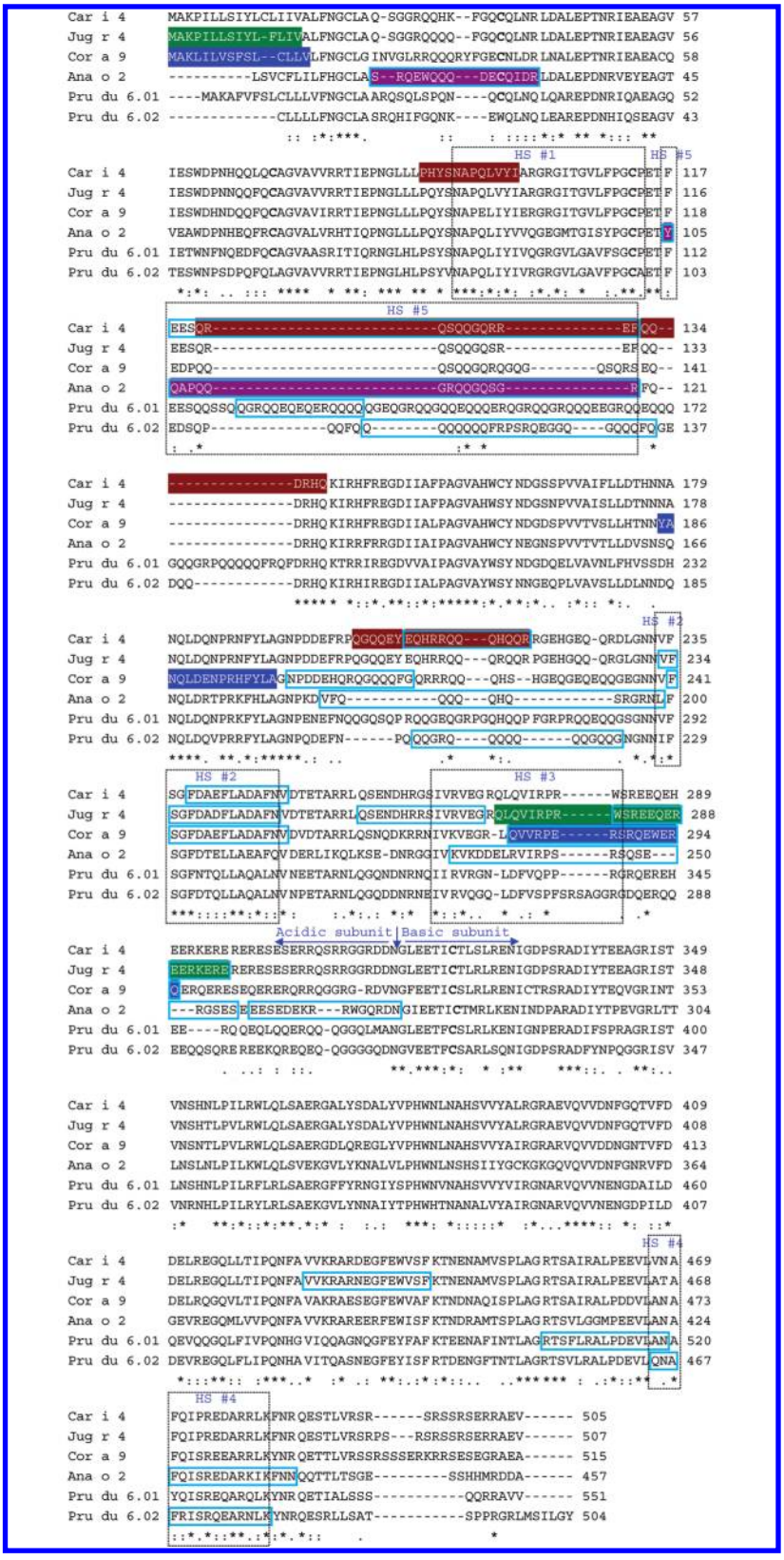

Figure 2. Amino acid sequence and sequential epitopic region comparison of pecan (Car i 4), walnut (Jug r 4),[27] hazelnut (Cor a 9),[27] cashew (Ana o 2),[16] and almond (Pru du 6.01 and 6.02)[14] 11S legumin allergens. Conserved cystine residues are indicated with bold type. Epitope stretches deduced from IgE SPOTs assays identified by sera from all patient pools are highlighted in different colors (pecan, brown; walnut, green; hazelnut, blue; and cashew, purple). Strongly reacting epitopes are boxed in blue. Positions where a space was added to enhance alignment are indicated with a dash (-). Identical residues are indicated with an asterisk (*), conserved subsitution with a colon (:), and semiconserved substitutions with a period (.). The five hot spots (HS 1−5) determined by Robotham et al.[27] and Willison et al.[14] are indicated by dotted black boxes.

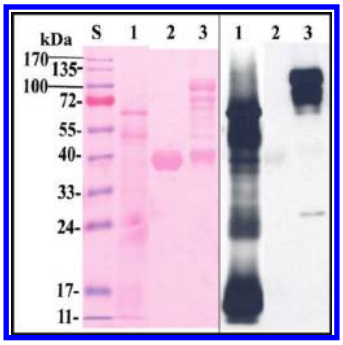

Figure 3. Ponceau S stain (left) and Western blot (right) of rCar i 4 with rabbit anti-pecan pAb. Lanes: S, protein standard; 1, pecan extract; 2, MBP; 3, rCar i 4 fusion protein; protein load in each lane was 5 μg except lane 1 (10 μg).

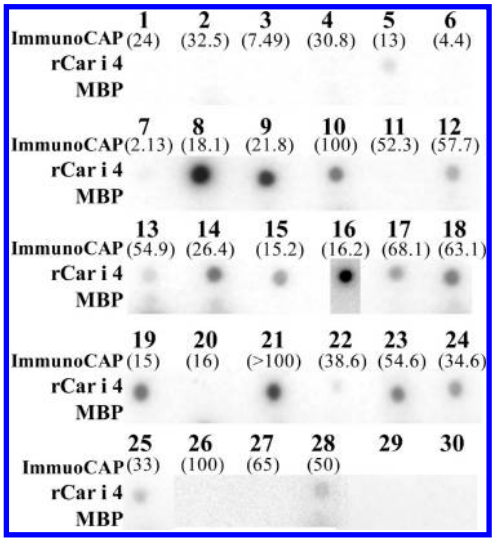

Figure 4. Dot blot of rCar i 4 fusion protein with pecan-allergic human serum (3× dilution). 1−28 are pecan allergic sera; 29 is atopic control; 30 is non-atopic control. MBP was used as a negative protein control. Sixteen of 28 pecan allergic sera are positive to rCar i 4.

(designated Car i 4), excluding the signal peptide, was cloned into the pMAL-c4x expression vector and transformed in *E. coli* BL21 cells. The resulting rCar i 4 fusion protein was ∼98 kDa, composed of 55 kDa rCar i 4 and 43 kDa MBP. Immunoblotting of the purified fusion protein revealed reactivity with rabbit anti-pecan pAb, whereas the MBP itself was not reactive (Figure 3). The IgE binding capacity of rCar i 4 fusion protein was studied by dot-blot using serum from 28 different allergic patients' containing pecan-specific IgE. IgE from 16 of 28 pecan allergic sera (57%) bound the recombinant protein (Figure 4).

Identification of Native (n) Car i 4. Proteins in pecan extract were separated on a 2D gel, and 35 polypeptide spots were subjected to in-gel trypsin digestion and MALDI-TOF analysis (Figure 5). Of these, five spots demonstrated significant identity with the legumin amino acid sequences. The molecular masses of the five spots and sequence coverage of legumin are shown in Table 2. Peptides further selected for MS/MS sequencing are summarized in Table 3. Spot 3 was ∼55 kDa and covered 40% of the legumin sequence, including the acidic and basic subunits. This spot could be the precursor form of the legumin protein, or the disulfide bond may not be accessible to reduction for effective

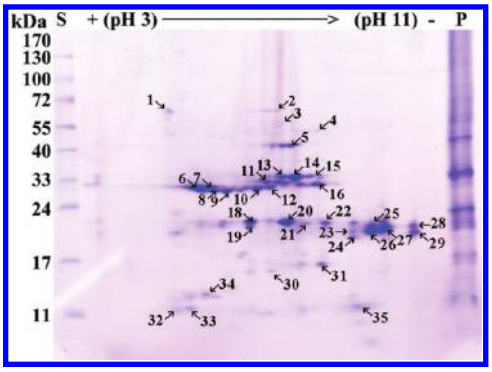

Figure 5. Two-dimensional electrophoresis (IEF + SDS-PAGE) of pecan extract. 1−35 indicate the spots selected for MALDI-TOF. Lanes: S, protein standard; P, pecan extract used for IEF ran on SDS-PAGE.

separation of the acidic and basic subunits. One acidic (spot 11) and three basic subunits (spots 20, 21, and 22) were confirmed by MS/MS sequencing (Table 3). On the basis of the 2D gel and MS/MS sequence, the presence of at least three isoforms of legumin proteins in pecan is confirmed. The presumed molecular masses of the acidic and basic subunits are 34 and 21.5 kDa, respectively. The observed molecular mass of spot 11 was ∼33 kDa and those of spots 20−22 were ∼20−22 kDa, which were close to the predicted mass of acidic (34 kDa) and basic (21.5 kDa) subunits, respectively.

Inhibition immunoblots with rabbit pAbs were performed to confirm the identity of nCar i 4 and determine the relative immunoreactivity of nCar i 4 subunits. When pecan protein extract was separated on a 2D gel and used for inhibition immunoblotting, two regions were found to be inhibited by rCar i 4 (Figure 6). One of these, ∼55 kDa (shown by the dotted rectangles in Figure 6) was similar to spot 3 from MALDI-TOF analysis, whereas the other region, ∼33 kDa (shown by bold rectangles in Figure 6), was similar to spot 11 of the MALDI-TOF analysis.

Car i 4 Cross-Reactivity. Rabbit anti-pecan IgG and human serum IgE from three pecan-allergic patients (patients 8, 9, and 21) were used in inhibition immunoblot to determine the possible cross-reactivity with legumin proteins in walnut extract (Figure 7). Both pecan and walnut extracts completely inhibited the rabbit IgG binding to rCar i 4, whereas adsorption of IgG to MBP resulted in no change in binding intensity. This result suggested that native pecan and walnut extract proteins contain epitopes similar to those found on the rCar i 4. Similar results were obtained when each human serum sample was preincubated with MBP, pecan, or walnut extract. All tested patients completely inhibited IgE binding to rCar i 4 upon preincubation with pecan extract. Serum from patients 8 and 21 showed complete inhibition to rCar i 4 upon preincubation with walnut extract, whereas serum from patient 9 showed partial inhibition.

Car i 4 Sequential Epitopes. Sequential epitopes of Car i 4 were determined by probing 165 overlapping solid-phase synthetic peptides (12 amino acid long, offset by 3 amino acids) with sera from 17 pecan allergic patients assigned to 3 pools. Two pools consisted of six patients' sera, whereas one had sera from five patients. The pools consisted of individual sera representing an equal distribution of strong and weak anti-rCar i 4 reactivities. Sequential epitopes identified by serum pools are listed in Table 4. Collectively, the three pools reacted weakly with 10, moderately with 5, and strongly with 4 sequential epitopes. Because there is no fixed number of amino acids required to

Table 2. Identified 11S Legumin Isoforms of Pecan after In-Gel Trypsin Digestion under Reducing Conditions and MALDI-TOF Analysis

polypeptide spot[a]	polypeptide mass[a] (kDa)	identified isoform	Mascot score	no. of matched peptides	sequence coverage (%)
3	55	Pec11S-2	254	19	40
11	33	Pec11S-1	425	14	31
20	22	Pec11S-1	141	4	12
21	21	Pec11S-2	85	11	24
22	21	Pec11S-2	415	16	34

[a] Polypeptide spot and molecular mass corresponding to Figure 4.

Table 3. Summary of In-Gel Tryptic Digested Peptides Analyzed by MS/MS Sequencing

			polypeptide spots[a]				
monoisotopic mass	sequence	amino acid position	3	11	20	21	22
1543.75	QQHKFGQ[b]CQLNR	30−41		X			
2579.39	TIEPNGLLLPHYSNAPQLVYIAR	79−101		X			
2337.12	GRGITGVLFPG[b]CPETFEESQR	102−122		X			
2124.00	GITGVLFPG[b]CPETFEESQR	104−122		X			
3094.46	NFYLAGNPDDEFRPQGQQEYEQHRR	188−212		X			
1510.76	LQSENDHRGSIVR	257−269		X			
1009.64	QLQVIRPR	274−281	X	X			
1463.85	ISTVNSHNLPILR	347−359	X			X	X
1002.55	WLQLSAER	360−367	X			X	X
2829.55	GALYSDALYVPHWNLNAHSVVYALR	368−392			X		
1813.00	EGQLLTIPQNFAVVKR	414−429					X
1696.01	ALPEEVLVNAFQIPR	460−474			X		X
1249.68	FNRQESTLVR	482−491	X				X

[a] Polypeptide spots correspond to Figure 4. [b] Carbamidomethylation of Cys.

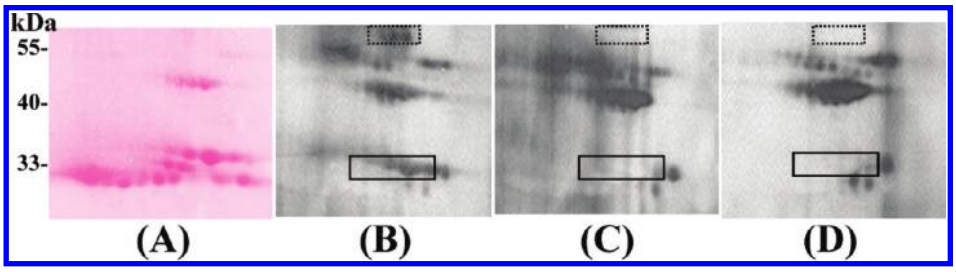

Figure 6. (A) Ponceau S stain of pecan extract transferred from 2D gel onto NC membrane. (B) Immunoblot of pecan extract with rabbit pAb. (C) and (D) are immunoblots of pecan extract with rabbit pAb preincubated with 100 and 500 μg of rCar i 4, respectively. The inhibited spots are enclosed by rectangles (solid and dotted) within the blots.

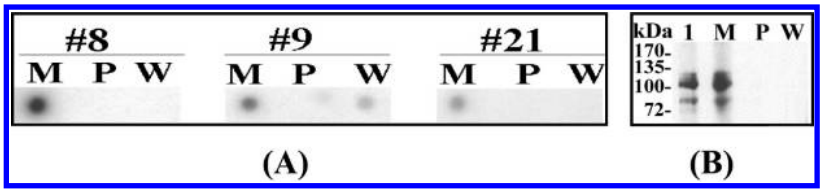

Figure 7. (A) Inhibition dot blot with pecan-allergic human sera (patients 8, 9, and 21) and (B) inhibition Western blot with rabbit anti-pecan IgG showing the inhibition of rCar i 4 by pecan and walnut extract. Lanes: 1, no inhibitor; M, P, and W denote MBP, pecan extract, and walnut extract were used as inhibitors, respectively.

make up an epitope, it should be noted that each of the 12 amino acid peptides may have more than one epitope, and two or more successive peptides may represent a single epitope. Seven epitopes were bound by patients' sera from all three pools, of which two were strongly reactive (epitopes 4 and 11, Table 4). The strongly reactive epitopes were located in the acidic subunit

(amino acid residues 1−316) at positions 118−132, 208−219, and 238−249.

Car i 4, Jug r 4, Cor a 9, Ana o 2, and Pru du 6 were aligned by their amino acid sequence using the ClustalW program. The epitopes of Car i 4 identified by all tested patient serum pools (color highlighted) and the strongly reactive epitopes (boxed) were compared with those of other tree nut legumins (Figure 2). The majority of the reactive epitopes were located in the C-terminal region of the acidic subunit. Residues 118−132 and 208−219 in Car i 4, which were strongly reactive and identified by all serum IgE pools, exhibited overlap with Ana o 2 epitopic regions 105−119 and 185−199. However, the third strongly reactive region of Car i 4 (residues 238−249) did not overlap with Ana o 2 and, instead, showed positional and sequential overlap with the strongly reacting epitopic regions of Jug r 4 (residues 233−247) and Cor a 9 (241−255).

Table 4. Sequential Car i 4 Epitopes and Relative IgE-Binding Signal Intensity

			IgE-binding intensity[a]		
reactive peptide[b]	AA position	AA sequence	pool 1	pool 2	pool 3
1	28−39	GRQQHKFGQCQL	+		+
2	88−99	PHYSNAPQLVYI	++	+	+
3	**118−129**	**EESQRQSQQGQR**		+++	+
4	**121−132**	**QRQSQQGQRREF**	++	+	+++
5	124−135	SQQGQRREFQQD	+	+	+
6	127−138	GQRREFQQDRHQ	+	+	+
7	130−141	REFQQDRHQKIR			++
8	133−144	QQDRHQKIRHFR			+
9	202−213	QGQQEYEQHRRQ	++	++	+
10	205−216	QEYEQHRRQQQH	+	+	+
11	**208−219**	**EQHRRQQQHQQR**	+++	+	++
12	232−243	NNVFSGFDAEFL			+
13	235−246	FSGFDAEFLADA			+
14	**238−249**	**FDAEFLADAFNV**		+++	+
15	304−315	ERRQSRRGGRDD			++
16	382−393	LNAHSVVYALRG	++		
17	385−396	HSVVYALRGRAE	+		
18	421−432	PQNFAVVKRARD	+		
19	424−435	FAVVKRARDEGF	+		

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

Molecular Modeling and Surface Mapping. We built the homology based three-dimensional monomeric model of Car i 4 and select tree nut legumins from the X-ray crystal structure of almond legumin, prunin (PDB code 3EHK). Prunin shared 61.2, 61.9, 60.3, and 51.1% amino acid sequence identity with modeled Car i 4, Jug r 4, Cor a 9, and Ana o 2 amino acid sequences, respectively. The ribbon model of all legumins, Car i 4, Jug r 4, Cor a 9, and Ana o 2, showed the presence of the cupin motif made from β-sheets, whereas most of the helices were located toward the periphery of the monomer model (Figure 8).

The strongly reacting IgE-binding sequential epitopes were mapped on the ribbon model as well as the molecular surface model of legumins. Three strongly reactive epitopic regions (Figure 9; 1−3 in Car i 4) of Car i 4 varied in their secondary structure. Epitopic region 1 (residues 118−132) consisted of both β-sheet (residues 118−119) and α-helix (residues 122−129), whereas epitopic regions 2 (residues 208−219) and 3 (residues 238−249) contained β-sheet (residues 211−213) and α-helix (241−247), respectively. Jug r 4, Cor a 9, and Ana o 2 consist of four, three, and six strong sequential epitopic regions (boxed blue in Figure 2) and are composed of different secondary structures, making it difficult to predict the property of epitopes on the basis of their secondary structure (Figure 9A,B). The epitopes were mapped on the surface model to assess their location in three dimensions as well as to determine if the epitope is accessible to solvent or buried in the monomer (Figure 9C,D). A structurally similar epitopic region (circled in Figure 9C) was found to be present in Car i 4, Jug r 4, and Cor a 9.

DISCUSSION

Legumins belonging to tree nuts,[18,38] sesame,[20] castor bean,[39] and quinoa[40] have been implicated with allergic reactions in sensitive individuals. Pecan legumin also demonstrated binding to allergic patient serum IgE. Although all patients' sera were diluted in equal proportion (3× diluted in blocking buffer) for dot-blotting, there was significant variation in the intensity that did not correlate with the ImmunoCAP values. For example, patients 8 and 16 exhibited strong reaction with rCar i 4, but had ImmunoCAP values of 18.1 and 16.2, respectively, whereas patients 13 and 22 exhibited weak reactivity, but their ImmunoCAP values were 54.9 and 38.6, respectively. Patients 12 and 26 exhibited severe anaphylactic reactions upon ingestion of pecan or tree nuts, but their IgE either did not bind or bound weakly with rCar i 4. Hence, the in vitro immunoblot reactivity with rCar i 4 could not be correlated with the ImmunoCAP values or the severity of clinical symptoms of the sensitive individuals. It should be noted that the ImmunoCAP scores were against pecan extract and the discrepancy between immunoblot and

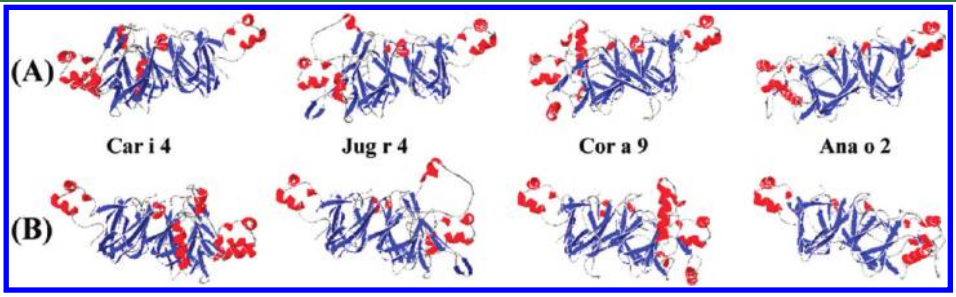

Figure 8. Ribbon diagram of front (A) and back (B) of the modeled Car i 4, Jug r 4, Cor a 9, and Ana o 2. The helices and sheets are colored red and blue, respectively.

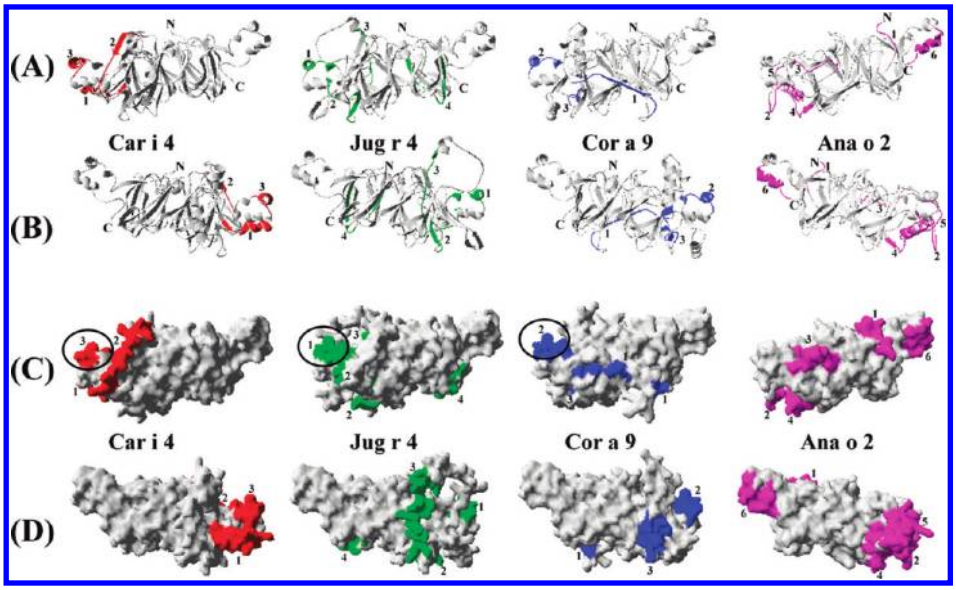

Figure 9. Ribbon diagram (A, B) and molecular surface (C, D) of front (A, C) and back (B, D) of the modeled Car i 4, Jug r 4, Cor a 9, and Ana o 2. Strongly reacting Car i 4 epitopes (red; 1, 118−132; 2, 208−219; 3, 238−249) are compared with Jug r 4 (green; 1, 233−247; 2, 253−271; 3, 281−295; 4, 425−439), Cor a 9 (blue; 1, 201−215; 2, 241−255; 3, 281−295), and Ana o 2 (purple; 1, 15−29; 2, 105−119; 3, 185−199; 4, 233−255; 5, 257−271; 6, 425−439) epitopes.

ImmunoCAP may be due to allergens other than Car i 4 affecting the ImmunoCAP score.

Legumin proteins have conserved structural motifs and amino acid sequence regions that could be potential epitopes for cross-reactivity among this group of proteins in different plant species. Inhibition immunoblots suggest Car i 4 is similar to walnut allergen Jug r 4. However, there may be some epitopes specific to pecan legumins that are absent in walnut legumin as indicated by incomplete inhibition when probed with IgE from patient 9. Teuber et al.[13] demonstrated in vitro cross-reactivity of pecan and walnut proteins by IgE immunoblot inhibition studies. Walnut legumin IgE cross-reactivity with cashew, hazelnut, and peanut extract was studied by Wallowitz et al.[19] using three different walnut allergic patients' sera. Cashew extract eliminated IgE reactivity to rJug r 4 fusion protein from two patients and reduced reactivity from one patient, whereas hazelnut and peanut extract eliminated IgE reactivity to rJug r 4 fusion protein from two and one patient, respectively.

The basic subunit of Car i 4 did not exhibit significant reactivity with pAb, suggesting the acidic subunit is more immunoreactive than the basic subunit. Stronger immunoreactivity of the acidic subunit than of the basic subunit has been observed in soy glycinin.[41] Fifteen of 19 patient IgE epitopes, including the strongly reactive ones, were located on the acidic subunit of Car i 4. Also, epitopes recognized by all three serum pools were on the acidic subunit. Together these findings suggest the acidic subunit is more immunoreactive than the basic subunit. Similarly, several investigators reported high human IgE-immunoreactivity located in the legumin acidic subunit as compared to the basic subunit of peanut,[23] cashew,[16] hazelnut,[27] walnut,[27] and soybean.[42]

Robotham et al.[27] identified linear IgE-binding epitopes of walnut and hazelnut legumin, and comparison with other known legumin proteins revealed four hot spot regions. An additional hot spot region was further identified by Willison et al.[14] upon evaluation of almond legumin IgE-binding linear epitopes (HS 1−5, boxed in black dots, Figure 2). HS 1 partially overlapped an epitopic region (residues 92−99) of Car i 4 that was identified by all serum IgE pools, whereas the HS 3 and HS 4 regions were not bound by IgE in Car i 4. The HS 5 region included a strongly reactive epitopic region of Car i 4, which was identified by all serum pools. This region also included strongly reactive epitopic regions of Ana o 2, Pru du 6.01, and Pru du 6.02. The HS 2 region had a strongly reactive epitopic region of Car i 4 (residues 238−249), which was also found in Jug r 4 and Cor a 9. Most of this epitopic region (HS 2) is solvent accessible in the legumin monomer and partly accessible in the trimeric and hexameric forms.[27] Walnut, pecan, and hazelnut form a group of strongly cross-reactive tree nuts as demonstrated by human IgE inhibition ELISA.[43] HS 2 may consist of a significant epitope responsible for IgE cross-reactivity between Car i 4, Jug r 4, and Cor a 9.

All three epitopic regions of Car i 4 are located on the surface with the majority of amino acids being solvent accessible. The epitopic regions 2 and 4 in Jug r 4 had few solvent-accessible amino acids, making the epitopes partly buried in the monomer. Similarly, epitopic region 1 in Cor a 9 and epitopic region 4 in Ana o 2 are partly buried in the monomer. Epitopic region 3 in Car i 4 is structurally similar to epitopic region 1 of Jug r 4 and epitopic region 2 of Cor a 9 (circled in Figure 9). This region is identified as HS 2 and is also present in the peanut legumin allergen, Ara h 3.[27] The shared structural homology in this region suggests the possible basis for the observed cross-reactivity among tree nuts and between tree nut and peanut.

In summary, this is the first evidence for the identification of Car i 4 as a major pecan allergen. The IgE-reactive sequential epitopes of Car i 4 are mapped, and the cross-reactive epitopes are indicated. We also show evidence for the occurrence of several isoforms of Car i 4 in the native pecan. Future investigations on clinically relevant isoforms lacking IgE binding may be useful in developing hypoallergenic seeds.

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