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Jug r 4, a Legumin Group Food Allergen from Walnut (*Juglans regia* Cv. Chandler)

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Allergy to walnut is the most frequently reported tree nut allergy in the United States. Walnut 2S albumin, a vicilin-like protein, and a lipid transfer protein allergen have previously been described. Our objective was to clone and express a cDNA encoding a legumin group protein, assess IgE-binding with sera from walnut allergic patients, and investigate cross-reactivity with selected nuts. Primers were used to obtain the cDNA by 5′ and 3′ rapid amplification of cDNA ends from walnut mRNA. The cDNA was subcloned into the pMAL-c2X vector and the recombinant fusion protein, named rJug r 4, was expressed in *Escherichia coli*. The obtained cDNA encoded a precursor protein with a predicted molecular weight of 58.1 kD, which showed significant sequence homology to hazelnut and cashew legumin allergens. Serum IgE from 21 of 37 (57%) patients bound the rJug r 4 fusion protein. In vitro cross-reactivity was demonstrated with hazelnut, cashew, and peanut protein extracts.

KEYWORDS: Food allergy; tree nut allergy; walnut; legumin group protein; Jug r 4; peanut allergy; cDNA

INTRODUCTION

Juglans regia (*J. regia*), the English walnut or Persian walnut, is an important tree nut associated with allergic reactions to food. About 0.5% of the United States population is estimated to be allergic to tree nuts on the basis of recent random digit dial surveys conducted by the Food Allergy and Anaphylaxis Network (FAAN) (*1*, *2*). In these surveys and in the FAAN voluntary peanut and tree nut allergy registry, walnut allergy is the most frequently reported tree nut allergy in the United States (*1*−*3*). Data from the FAAN registry shows 34% of self-reported tree nut allergic subjects report reactions to walnut, followed by cashew (20%), and almond (15%) (*3*). Allergic reactions to walnut can be severe, and fatal reactions have been reported (*4*, *5*). Thus, full characterization of the major allergens in walnut is important for several reasons, including improvement and standardization of specific IgE assays, refinement of walnut detection kits for the food industry, and development of immunomodulation therapies using recombinant proteins.

We have previously cloned cDNAs encoding the major walnut allergens Jug r 1, a 2S albumin, and Jug r 2, a vicilin-like protein (*6*, *7*). Pastorello et al. recently published data that Jug r 3, a nonspecific lipid transfer protein, is the major allergen in an Italian population with walnut food allergy (IgE binding in 80% of patients) (*8*). This result parallels other reports from the Mediterranean region that the lipid transfer protein family of polypeptides is important in fruit and nut allergy in that specific population (*9*). Legumin group proteins, or 11S globulins, are potential major allergens in tree nuts and seeds, and cDNAs encoding these have now been cloned and expressed from cashew (Ana o 2) (*10*), hazelnut (Cor a 9) (*11*), sesame (Ses i 6) (*12*), soy (Gly A3b4) (*13*), and peanut (Ara h 3) (*14*). We previously identified legumin group proteins in walnut as likely allergens in two patients who had severe systemic reactions initially to walnut but went on to develop sensitivity to coconut. Presumably, the newly developed sensitivity was due to cross-reactive IgE that bound coconut legumin group proteins (determined by electrophoresis pattern) (*15*). We have also shown extensive cross-reactivity of IgE-binding proteins among walnut cultivars and species (*16*).

Legumin group proteins are seed storage proteins that function as nitrogen donors for seedling growth during germination. Usually, legumins are encoded by many genes whose translation products are proteolytically processed by removal of the hydrophobic signal peptide and are further cleaved, resulting in two polypeptides called “acidic” and “basic” subunits (*17*, *18*). The acidic subunits are generally of a larger molecular weight on sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS-PAGE), around 35−40 kDa, while the basic subunits migrate through a gel at around 20−30 kDa. Synthesis of the precursor proteins occurs on the rough endoplasmic reticulum, and the signal peptides are removed during translocation into the lumen. After assembly into trimers (with help from molecular chaperones (*19*)), the protein precursor travels through the Golgi apparatus to the protein bodies (*20*−*22*). It

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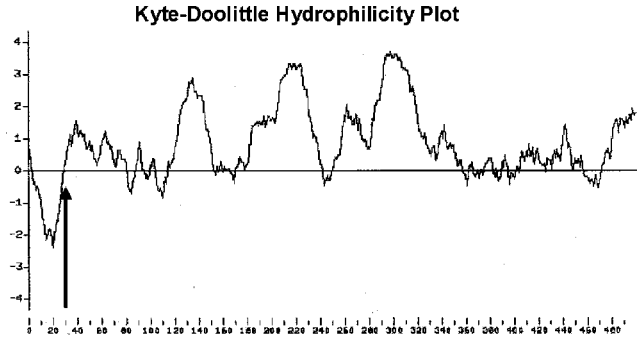

Figure 1. Kyte–Doolittle hydrophilicity plot of the deduced Jug r 4 polypeptide, residues 1–500. Calculated hydrophilicity is on the *x*-axis and amino acid position is on the *y*-axis. The black arrow indicates the point where the hydrophobic sequence ends.

Table 1. Sequence Comparisons Using BLAST 2 Sequence Engine (*36*)

source	accession #	identity (%)	similarity (%)
Cor a 9 (hazelnut)	AAL73404	63	70
Ana o 2 (cashew)	AAN76862	53	67
Ses i 6 (sesame)	AAK15087	48	61
Ara h 3 (peanut)	AAT39430	40	55
Gly A3b4 (soybean)	1OD5A	40	52

is within the protein bodies that each subunit of the trimer is further processed and cleaved. Herein, the first cloning and characterization of a cDNA encoding a single walnut legumin group precursor protein is described, and inhibition immunoblotting with extracts from cashew, hazelnut, and peanut is performed.

MATERIALS AND METHODS

Cloning of a Full-Length Legumin Group cDNA from *J. regia* Cultivar (Cv.) Chandler. mRNA was isolated from frozen Chandler embryos using the manufacturer's instructions (RNeasy Plant Mini Kit, Qiagen, Valencia, CA). This mRNA was used as a template for cDNA synthesis (Marathon cDNA Amplification Kit, BD Bioscience, Palo Alto, CA). 3′ and 5′ rapid amplification of cDNA ends (RACE) was performed using primers (3′ RACE primer: GGTTAGTCCGCTTGCTGGCCGAACCTC, 5′ RACE primer: GACCTTGACGGCCTTGAACGAACCAAGG) synthesized by Invitrogen, (Carlsbad, CA) on the basis of pWE241 (which contains a partial coding sequence for a walnut legumin group protein) from a *J. regia*, cv. Chandler, embryo expressed sequence tag database (http://cgf.ucdavis.edu). Polymerase chain reaction (PCR) products were cloned into pCR4-TOPO according to the manufacturer's instructions (Invitrogen), and the corresponding inserts were sequenced by Davis Sequencing (Davis, CA). On the basis of this deduced sequence, new 5′ and 3′ primers (5′ primer: CAATCATCAGATATCACCATGGCCAAGCC, 3′ primer: TTAATTTATAAGGCTTTATTGACTAACTGATCTCG) were synthesized and used in a reverse transcriptase (RT)-PCR reaction to clone the full-length legumin group cDNA. Products from the RT-PCR reaction were cloned and sequenced as above.

Expression of Recombinant Walnut Legumin Group Precursor Protein. The insert was subcloned into the maltose binding protein (MBP) expression vector pMAL-c2X (New England BioLabs Inc., Beverly, MA) and was transformed into *E. coli* BL21-codon Plus-RIL (Stratagene, La Jolla, CA). Positive clones were identified by PCR screening. Single colonies were grown in Luria−Bertani broth with ampicillin (50 μg/mL) to an optical density of 0.9 at 600 nm and then were induced with 1 mM isopropyl thiogalactoside for 4 h at 37 °C. The cells were harvested, lysed with mild sonication, and centrifuged at 12000*g*. The lysate supernatant was incubated with amylose affinity resin (NEB, Beverly, MA), and the fusion protein was eluted with 10 mM maltose. Poor expression was initially obtained. Since the N-terminal sequence of the polypeptide precursor contained an extremely hydrophobic signal sequence that was predicted to be cleaved off in normal post-translational processing, deletion of nucleotides coding this region might aid in the expression of the fusion protein. The hydrophobic region is shown in the Kyte−Doolittle hydophilicity plot in **Figure 1** (*23*). The initial 96 bases from the 5′ end encoding 32 amino acids at the N-terminus were deleted by selection of a new forward primer (5′ TTT GGC CAG TGC CAG CTC AAT 3′) to select cDNA encoding from residue 33 onward. The PCR product was subcloned as above and was expressed well.

Human Subjects. The study was approved by the institutional review board of the University of California at Davis. Blood samples were drawn after informed consent from 37 patients with histories of potentially life-threatening systemic reactions to walnut involving bronchospasm, hypotension, or laryngoedema/throat swelling. Food challenges were not performed in these individuals because of the severity of reactions. The sera were frozen at −70 °C until use. The presence of walnut-reactive IgE was confirmed by Phadia ImmunoCAP (Phadia, Inc., Uppsala, Sweden) in our hospital pathology laboratory with two exceptions. Two patients were previously positive for walnut-specific IgE by modified radioallergosorbent testing in our hospital laboratory but were negative on repeated assays by ImmunoCAP performed on the same blood samples. Control sera from patients with a history of pollinosis but not food allergy were used in each experiment.

Protein Extraction for Inhibition Immunoblotting. All protein extracts were prepared as described previously (*16*). Raw peanuts (Spanish type), raw cashews, and raw hazelnuts were purchased from a local produce market. Walnuts were obtained from the Department of Plant Sciences at the University of California, Davis. Briefly, two grams of nuts were chopped with a razor blade, and then 10 mL Buffer D (50 mM Tris-HCl, pH 8.0, 22% v/v glycerol, 1% w/v polyethylene glycol 8000, 7 mM citric acid, 6 mM L-cysteine, 6 mM L-ascorbic acid, 2 mM ethylenediaminetetraacetic acid, and 4 g polyvinylpolypyrrilidone) was added. The nuts were homogenized on ice and were spun at 13000*g* for 30 min, and the lipid plug was removed. The supernatant was passed through a 5.0 μm Millex-SV syringe filter (Millipore) and was frozen at −70 °C. Protein concentration determinations were made with the Coomassie Plus Protein Assay kit (Pierce, Rockford, IL).

SDS-PAGE. Samples were boiled for 5 min in sample buffer [60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, and 0.01% bromophenol blue (100 mM dithiothreitol was included for walnut extract)], and electrophoresis was carried out on a SE 250 Mini-Vetical Electrophoresis Unit (Amersham Biosciences Corp, Piscataway, NJ) at a constant current of 20 mA. 10% and 15% gels were used. Proteins were transferred to 0.22 μm nitrocellulose (Schleicher & Schuell, Keen, NH) membrane (BioRad, Hercules, CA) overnight at 30 V by using a TE 22 Mini Tank Transfer Unit (Amersham Biosciences Corp).

IgE Immunoblotting and Inhibition Immunoblotting. Nitrocellulose strips were incubated for 30 min in blocking buffer [phosphate-buffered saline (PBS) and 3% nonfat dried milk with 0.1% Triton X-100]. Strips were then incubated overnight at 4 °C with sera diluted 1:20−1:100 in blocking buffer. All patient sera were blotted against MBP alone to check for IgE binding to the carrier protein. For inhibitions of total walnut extract with rJug r 4, individual sera were preincubated overnight with 10 μg rJug r 4 or control protein per 50 μL patient serum. Strips were subsequently washed in PBS/0.1% Triton X-100 and then were incubated overnight with ^{125}I-antihuman IgE (Hycor) diluted 1:10. Strips were washed, dried, and exposed to film for 72 h (Kodak BioMax MS). For inhibition of rJug r 4 with cashew, English walnut, hazelnut, or peanut extracts, 200 μg/mL of whole extract was added to blocking buffer with individual serum samples diluted 1:20. Strips were then incubated with preabsorbed primary antibodies overnight, were washed with PBS/0.05% Tween-20, and were incubated 1 h with horseradish-peroxidase-labeled mouse antihuman IgE (Zymed, San Francisco, CA). Strips were washed first in PBS/0.05% Tween-20 and then in PBS before incubation with SuperSignal West Femto chemiluminescent substrate (Pierce) according to the manufacturer's instructions. Strips were exposed for 1 min on Kodak BioMax Light X-ray film.

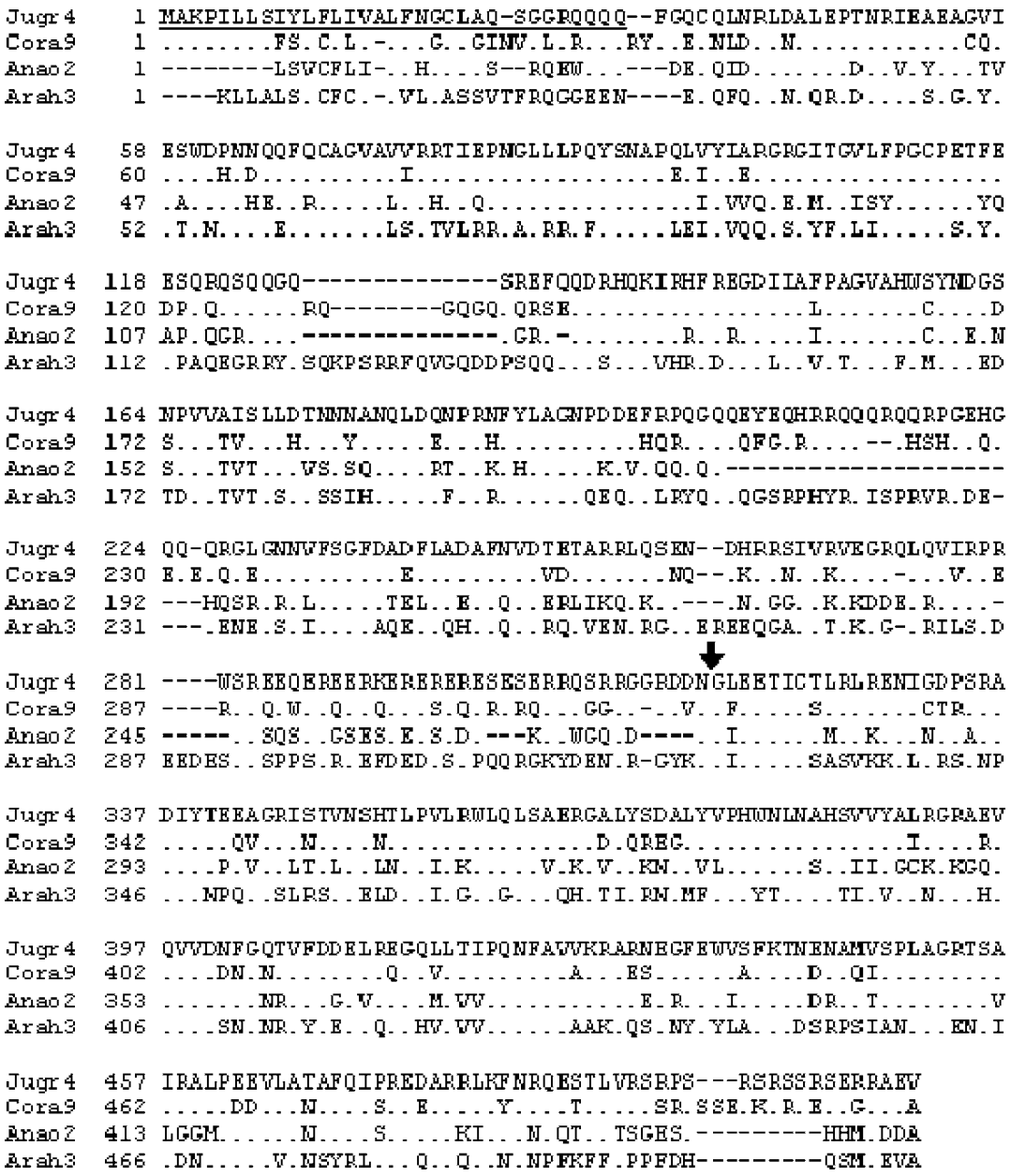

Figure 2. Sequence alignment of the legumin group proteins from walnut, hazelnut, and cashew. The amino acids of the predicted signal sequence in the walnut legumin group protein are underlined. A predicted cleavage site, found between residues 315 and 316, is identified with a dark arrow.

RESULTS

Identification of cDNA Encoding a Legumin Precursor Protein. A full-length 1.7 kb clone was obtained that, when translated, included a hydrophobic sequence (**Figure 1**), with the first 23 amino acid residues predicted to be a signal peptide (with lower levels of probability extending out to the 31st residue) (SignalP V1.1 World Wide Web Prediction Server www.cbs.dtu.dk/services/SignalP/www.cbs.dtu.dk/services/SignalP/) (**Figure 1**). The open reading frame was 507 amino acids in length, corresponding to a predicted molecular weight of 58.1 kDa and predicted isoelectric point at pH 6.8. The encoded polypeptide was designated as Jug r 4 and was submitted to the International Union of Immunological Societies' Allergen Nomenclature Subcommittee. The cDNA sequence was submitted to GenBank and is accessible as AY692446.

Sequence comparisons (**Table 1**) of Jug r 4, Cor a 9 (hazelnut legumin), and Ana o 2 (cashew legumin) revealed 63% identity between Jug r 4 and Cor a 9 and 53% identity between Jug r 4 and Ana o 2. Positives (similar, nonidentical residues) were 70% for Cor a 9 and 67% for Ana o 2 when compared to Jug r 4. The results from multiple sequence alignments (**Figure 2**), which also includes peanut Ara h 3, suggest the majority of the differences between the legumin proteins lie in one of three sections: the first 34 amino acids (which include the signal peptide), amino acids 210−231, and the last residues, 500−518. The predicted major cleavage site separating the protein into larger (acidic) and smaller (basic) legumin group subunits is between residue 315 and 316 and is indicated in **Figure 2** (*17*, *18*).

rJug r 4 IgE Immunoblotting. The rJug r 4 fusion protein (minus the initial 32 amino acid hydrophobic region) was detected in the stained gel as a single band at approximately 97 kD (55 kDa legumin group precursor protein fused to 42 kDa MBP) (**Figure 3**). However, in **Figure 4**, IgE from some sera appear to bind additional truncated products not visible in Coomassie-stained gels. Regardless, the fusion protein bound IgE from 21 of 37 patients (57%) but none of the controls (**Figure 4**). An additional patient serum appeared to show faint

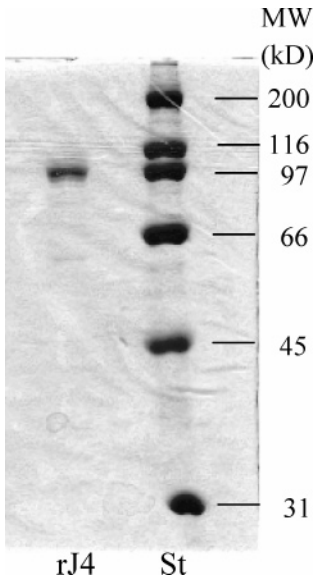

Figure 3. Representative 10% acrylamide gel of the Jug r 4 fusion protein (rJ4) and molecular weight standards (St) stained with Coomassie Brilliant Blue.

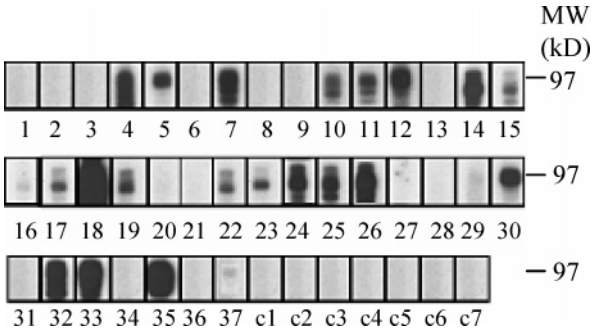

Figure 4. IgE immunoblot of rJug r 4 blotted with 37 walnut allergic sera (#1–37) and 7 controls with pollinosis, peanut allergy, or brazil nut allergy without allergy to walnuts (#c1–c7).

IgE binding (serum #16), but this patient also showed IgE faintly recognizing the MBP negative control (data not shown) indicating that IgE binding was to MBP, not rJug r 4. Information on patient characteristics, reactions, and ImmunoCAP values is given in **Table 2**.

rJug r 4 IgE Inhibition Immunoblotting. Legumin precursors typically undergo proteolytic post-translational processing into polypeptides of varying size. To determine the molecular weight of the native peptides derived from the Jug r 4 precursor protein, immunoblotting with patient sera was performed before and after incubation of the sera with rJug r 4. **Figure 5** shows the results of inhibition immunoblots after preincubation with rJug r 4. The proteins from whole walnut extract were reduced to enhance separation of the acidic and basic subunits. Four bands of approximately 42, 26, 23, and 19 kDa were absorbed out by rJug r 4, and, interestingly, this varied by patient. N-terminal sequencing of the absorbed proteins was attempted but no useful sequence information was obtained.

The IgE binding profiles of the sera in **Figure 5** show the diversity of the IgE response that exists in this walnut allergic population. Cleaved and processed legumin group polypeptide subunits from seeds are classically characterized as larger acidic subunits (~36 kDa) or smaller basic subunits (~22 kDa) (*17*, *18*). rJug r 4 inhibited serum #7 IgE binding to a legumin subunit that corresponded to the acidic portion of the protein. Reactivity to the lower molecular weight fractions, the area consistent with the predicted basic subunits of the walnut legumin, was eliminated by preincubation of sera #5, #7, and #17 with rJug r 4, including polypeptides that did not show up well with Coomassie staining.

Cross-Reactivity. In **Figure 6**, sera from three walnut allergic patients are individually blotted for IgE reactivity against rJug r 4, which under these conditions (optimized for detecting cross-reactivity) was detected on immunoblotting as a single band at 97 kDa. Each serum was preincubated with a series of extracts to determine possible IgE cross-reactivity with other tree nuts. Patient #17 has a history of reactions to walnut, cashew, and hazelnut (**Table 2**). With this patient's serum, IgE reactivity to rJug r 4 was nearly or completely eliminated by preincubation with walnut, cashew, or hazelnut extracts. The small dot present in the cashew column is an artifact. Similar results were observed with serum from patient #24 (severe reactions to walnut and cashew, mild reactions to hazelnut) though inhibition was not complete after incubation with cashew extract. With serum from patient #26 (severe walnut, cashew, and hazelnut reactions), only cashew extract was able to eliminate reactivity to rJug r 4, although walnut extract greatly decreased IgE binding. There was little or no inhibition of IgE reactivity to rJug r 4 after incubation of this patient's serum with hazelnut extract. These results indicate a high level of cross-reactivity among the legumin group proteins in the tree nuts tested.

Inhibition immunoblots using peanut extract were also performed (last columns in **Figure 6**). Serum #17 is from a peanut-tolerant donor who experiences no clinical reactions after peanut consumption but has quantifiable IgE titers to peanut as measured by in-vitro assays (1.06 kIU/L by ImmunoCAP). The peanut extract virtually eliminated IgE reactivity to rJug r 4 in this patient's serum, suggesting that clinically irrelevant cross-reactivity between walnut legumin and peanut legumin group proteins likely exists in this case. In contrast, serum from a severely peanut allergic individual, patient #24, showed no decrease in IgE binding to walnut legumin group protein after preincubation with peanut extract, suggesting no cross-reactivity. The serum from patient #26 showed no inhibition, and this patient has no detectable peanut IgE (<0.35 kIU/L by ImmunoCAP). Together, these results indicate that the cross-reactivity of the legumin proteins may vary substantially among sensitized populations.

DISCUSSION

We wish to emphasize that we have cloned only one cDNA encoding one possible acidic/basic subunit pair. This is important to point out because in soy, for example, four different acidic subunits have been identified (*24*, *25*) suggesting that the legumin hexamer may be a dynamic combination of multiple acidic and basic subunits. The interesting results in **Figure 5**, which showed varying patterns of inhibition between patient sera, are evidence that most likely several genes encoding other related subunits are involved or that multiple different post-translational processing changes are in effect that variably affect patient serum IgE binding. The primary cleavage site into acidic/basic subunits is well conserved among a wide variety of plant species and contains an Asn-Gly peptide bond (*26*). This NG site is part of the NGLEET deduced amino acid sequence of Jug r 4 (*27*), NGFEET of Cor a 9 (*28*), and NGIEET of both Ana o 2 (*10*) as well as Ara h 3 (*14*). After cleavage, on the basis of studies with soy glycinin legumin group proteins, the peptides are reassembled, resulting in acidic and basic polypeptides held together by a single disulfide bond (*29*). These

Table 2. Patient Characteristics

no.	age	type of reaction to walnut	other nut allergies (CAP to selected)	CAP[g] (kIU/L)	IgE to rJug r 4
1	63	A[a], throat swelling, SOB[b], U[c]	peanut, pecan	5.68	–
2	43	wheezing, SOB, A, stridor	almond	0.92	–
3	63	wheezing, A, throat swelling, U	peanut, sesame	1.8	–
4	26	wheezing, SOB, A, throat swelling, V[d], U	peanut, pecan, almond	1.31	+
5	24	throat swelling, wheezing	peanut	0.86	+
6	32	stridor, A, U	peanut, pecan, almond	2.21	–
7	14	A, throat swelling	almond	0.64	+
8	8	wheezing, SOB, stridor, A, U, V		0.72	–
9	57	throat swelling, V, D[e], U	peanut, almond	1.73	–
10	38	wheezing, SOB, throat swelling, A, V, U	peanut, almond, pistachio, brazil nut	8.98	+
11	5	wheezing, A		1.92	+
12	73	wheezing, SOB, throat swelling, A, U	peanut, multiple tree nuts	13.7	+
13	57	throat swelling	macadamia	5.36	–
14	59	wheezing, throat swelling, A, V, D	peanut, coconut, almond	22.01	+
15	23	LOC[f], hypotension, SOB	less severe reactions to almond, cashew, pistachio, and peanut	27.0	+
16	27	A, throat swelling, U	macadamia	8.21	–[h]
17	53	wheezing, SOB, A, throat swelling	pecan, pistachio (7.24), cashew (1.62), hazelnut (6.82)	<0.35	+
18	41	wheezing, SOB, throat swelling, V	peanut	18.2	+
19	22	wheezing, SOB, throat swelling, V, U, light-headed	pecan	3.94	+
20	68	wheezing, SOB, throat swelling, V	pecan	6.61	–
21	52	throat swelling, A	Brazil nut, less severe to pecan, almond, hazelnut	1.92	–
22	50	wheezing, SOB, A, V	pecan, multiple tree nuts	10.2	+
23	28	throat swelling, V	pecan, hazelnut, brazil nut	30.8	+
24	30	hypotension, wheezing, SOB, A, throat swelling, V, U, light-headed	cashew (4.04), less severe to hazelnut (2.42), peanut (17.9)	0.47	+
25	26	wheezing, SOB, hypotension, V,D, A, throat swelling	Brazil nut, hazelnut, cashew	<0.35	+
26	33	wheezing, stridor, SOB,V, U	cashew (36.1), pecan, almond, hazelnut (3.83)	1.17	+
27	55	throat swelling, wheezing, A, U, V, D	pecan, hazelnut, cashew, pistachio	10.2	–
28	40	wheezing, A, throat swelling, stridor	less severe to peanut, pecan	1.04	–
29	48	LOC, wheezing, stridor, A,V, U	almond, cashew, less severe to pecan and hazelnut	4.27	–
30	38	wheezing, SOB, throat swelling, U, V	pecan, almond, cashew, pistachio, hazelnut	3.94	+
31	39	throat swelling, A, U		2.90	–
32	50	LOC, wheezing, SOB, A	peanut, pine nut, almond	5.28	+
33	38	LOC, hypotension, bronchospasm, throat swelling, U, A, V, D	hazelnut, cashew, pistachio	0.43	+
34	48	throat swelling, A, SOB	pecan	1.42	–
35	13	throat swelling	peanut, pecan	1.43	+
36	51	throat swelling, SOB, wheezing, stridor, U, V, D	less severe to pecan	2.74	–
37	62	wheezing, SOB, throat swelling, U, V	peanut, less severe to almond	0.45	+

[a] A: angioedema. [b] SOB: shortness of breath. [c] U: urticaria. [d] V: vomiting. [e] D: diarrhea. [f] LOC: loss of consciousness. [g] CAP: Phadia ImmunoCAP specific IgE assay. [h] This patient showed trace IgE binding to rJug r 4 fusion protein but was also faintly positive to maltose binding protein used alone as a control and is thus negative to the walnut allergen.

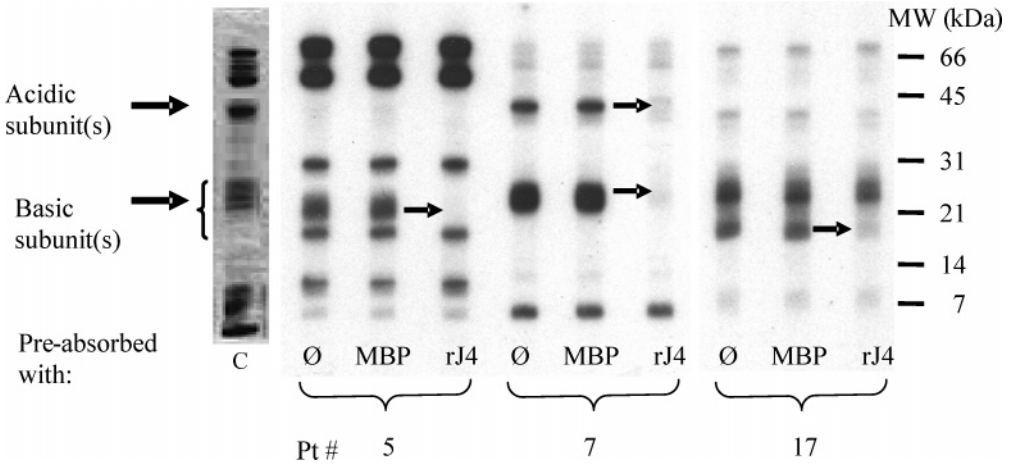

Figure 5. Representative 15% reducing gel of English walnut extract stained with Coomassie Brilliant Blue dye (C) and inhibition immunoblots of three representative serum samples preincubated, as follows: ∅, blocking solution alone; MBP, maltose binding protein (negative control); rJ4, rJug r 4 fusion protein.

polypeptides subsequently aggregate to form hexamers which are known as 11S storage globulins on the basis of the sedimentation rate (*30*). As an example of the number of genes that may be involved, the legumin of soybean has been well characterized and is encoded by five glycinin genes (*Gy*1−*Gy*5) (*29, 31, 32*) at four genetic loci. *Gy1* and *Gy2* are alleles at one locus, while the other three occur as single genes at different loci (*33*). However, the full post-translational processing of legumin proteins is unknown and certainly is not described as yet in walnut.

Using the deduced translated amino acid sequence from our cloned cDNA, we determined the homology to legumin proteins from other seeds and nuts. Comparisons of sequence alignment profiles showed nearly 70% amino acid similarity between the

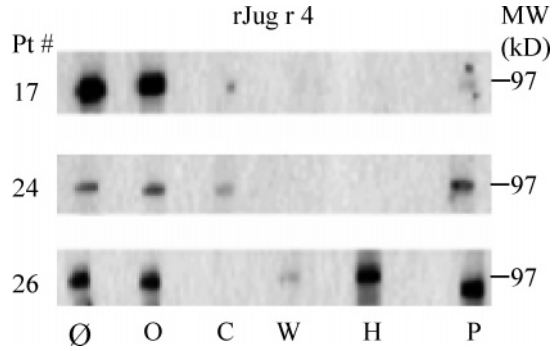

Figure 6. Cross-reactivity of rJug r 4. Inhibition immunoblot of rJug r 4 preincubated with three individual serum samples, as follows: Ø, blocking solution alone; O, ovalbumin as a control; C, cashew; W, walnut; H, hazelnut; P, peanut.

legumin proteins of walnut, hazelnut, and cashew (**Table 1**). Sequence homology was slightly higher between the walnut and hazelnut legumins compared to cashew although the significance of this was not explored in this study.

We examined potential cross-reactivity by inhibition immunoblotting. Whole extracts from other nut species eliminated or reduced the reactivity of IgE toward the rJug r 4 fusion protein (**Figure 6**). These results suggest that the native tree nut proteins contain similar epitopes as those found on the recombinant protein. In addition, these results suggest that at the IgE level, reactivity toward conserved epitopes exists among the tree nut legumin group proteins (**Figure 6**). The observation that walnut extract did not completely eliminate IgE reactivity to rJug r 4, using serum from patient 26 in **Figure 6**, has several possible explanations. The IgE titer to this walnut legumin may have been extremely high and unable to be competed out, or certain patient-specific epitopes may be hidden in the native protein extracts under the inhibition conditions used but exposed on the rJug r 4 bound to nitrocellulose. Additionally, the rJug r 4 may contain additional IgE binding epitopes not represented in the walnut extract used.

We also examined potential cross-reactivity with peanut. We found that peanut extract could inhibit IgE binding to rJug r 4 in one out of three sera tested (**Figure 6**). Therefore, the proteins present in the peanut extract contain similar epitopes to those found on rJug r 4 for this particular patient. Interestingly, it was a serum sample from a patient who tolerates peanut (#17) which demonstrated cross-reactivity, while a serum sample from a patient with anaphylaxis to both peanut and walnut showed no in-vitro cross-reactivity under these conditions (**Figure 6**). If, as is likely, the proteins inhibiting IgE binding to rJug r 4 are peanut legumin proteins, then Ara h 3, a described peanut legumin group allergen, may not always be responsible for clinical reactivity to peanut and could be implicated in some cases of clinically irrelevant IgE cross-reactivity. This might also suggest that IgE against only legumin group proteins in peanut is not enough to result in clinical reactivity. However, the results of Restani and colleagues (*34*) suggested that Ara h 3 was an important allergen in their population of patients, with some exhibiting IgE only toward this protein, particularly the basic subunit. Their patients who showed this reactivity tended to have coexistent hazelnut allergy. It is also possible that the cross-reactive antibodies detected in serum #17 against peanut are not of adequate affinity or are otherwise unable to activate mast cells and basophils. Further investigation of peanut and tree nut cross-reactivity and further efforts to obtain sequence data on IgE binding proteins that are the likely native legumin subunits are currently underway.

Characterization of the major allergens in walnut is important so that producers of commercial walnut extracts used for prick skin testing and of extracts used for construction of in-vitro specific IgE assays can ensure inclusion of all major allergens. Failure to do so could result in misleading skin test or specific IgE assays. We present indirect evidence to suggest that the legumin group allergen of walnut may not be adequately represented in some commercial preparations. Two of the patient sera used in this study were from patients with negative walnut ImmunoCAP assays despite a history of clinical reactions to walnut. These two sera, #17 and #27, which are positive for IgE binding to rJug r 4, are negative for specific IgE to black walnut pollen, English walnut pollen, perennial ryegrass pollen, and birch pollen. It is interesting that these sera were Class IV and Class III, respectively, in a previous modified RAST to walnut in our hospital laboratory (with positive results considered at Class II and above), had been successfully used for immunoblotting against walnut multiple times in our laboratory, and yet returned negative upon Phadia ImmunoCAP (repeated twice for each sera, <0.35 kIU/L). This suggests that at least some of the walnut legumin proteins are difficult to extract in the buffer system used by particular manufacturers. Indeed, Sathe demonstrated that a highly alkaline extraction system showed the greatest efficiency in protein extraction of walnut (*35*).

In summary, a cDNA encoding a precursor protein for a major allergen from walnut, the most common tree nut implicated in food allergy in the United States, has been cloned and expressed. The clear potential for cross-reactivity of the recombinant walnut legumin protein with other nut extracts has been demonstrated on the basis of inhibition immunoblotting. The availability of another major allergen sequence could aid future design of immunotherapeutics for severe walnut allergy, while on a practical level for both the food and healthcare industry today, the characterization of the major allergens from tree nuts should improve standardization of immunodetection kits and patient diagnostic tests.

ABBREVIATIONS USED

FAAN, Food Allergy and Anaphylaxis Network; MBP, maltose binding protein; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; RT, reverse transcriptase; SDS-PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis.

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