

Purification and Characterization of a Black Walnut (*Juglans nigra*) Allergen, Jug n 4

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ABSTRACT: Tree nuts as a group cause a significant number of fatal anaphylactic reactions to foods. Walnuts (*Juglans* spp.) are one of the leading causes of allergic reactions to tree nuts in the U.S. and Japan. The purpose of this study was to purify and characterize potential food allergens from black walnut. Here, we report the isolation of the black walnuts allergen Jug n 4 (an 11S globulin) by ammonium sulfate precipitation, hydrophobic interaction, and size exclusion chromatography. Reducing SDS-PAGE analysis indicated that purified Jug n 4 consists of three major bands. N-Terminal sequencing data of these bands indicated that they were the results of a post-transcriptional protease cleavage of the mature protein at a site that consists of a known conserved protease recognition motif, NGXEET. Western blot experiments revealed that 32% of the sera from 25 patients with double-blind, placebo-controlled clinical walnut allergy contained IgE antibodies that recognized Jug n 4, indicating that it is a walnut allergen. Identifying this and additional allergens may facilitate the understanding of the allergenicity of seed storage proteins in tree nuts and their cross-reactivity.

KEYWORDS: Jug n 4, dbpcfc, food allergen, post-transcriptional modification, storage protein

INTRODUCTION

Food allergy is a major global health concern.^{1,2} To date, there is no cure for food allergies, although in some cases of egg, milk, and peanut allergy, temporary desensitization by immunotherapy using very small, but increasing amounts of food or recombinant allergens may be considered promising treatment for some individuals.^{3–7} Food allergies impact not just the quality of life of allergic patients and their families but also the utilization of agricultural products and the operations of the food industry. In recent years, the presence of undeclared sources of food allergens has become one of the leading reasons for expensive food recalls.^{8,9} Most immediate type food allergies are triggered when the offending food allergens are recognized by immunoglobulin E (IgE).¹⁰ Among thousands of protein families, all known food allergens belong to just a few such families. Peanut (*Arachis hypogaea* L.) and tree nuts are the sources of food allergens that cause the majority of near-fatal and fatal food allergy cases in the United States.^{11,12} In addition to understanding the conditions of the immune systems that underlie the pathological mechanisms of food allergy, identifying new food allergens and studying the allergenicity of food proteins are also important aspects of understanding food allergies. The present study is focused on walnut (*Juglans* spp.) allergens.

Black walnut (*Juglans nigra* L.) is also known as American walnut or eastern black walnut.¹³ It is a deciduous hardwood

tree species of the walnut (*juglandaceae*) family native to eastern north America.^{14,15} Black walnut is a high-value crop best known for its use as lumber and veneer.^{14,15} The nutshell, green husk, and kernel oil are used in various industries^{14,16} and in traditional medicine.¹⁵ As a food product, the kernels of black walnut have been consumed by wildlife and humans for millennia. In recent years, black walnut is becoming more popular with consumers due to its ample oil, high protein content, rich aromatic flavor, and perceived health benefits.^{17,18}

As in other plant seeds, most of the protein content in walnut kernels are seed storage proteins that accumulate during seed development, with no known functions other than storing carbon, nitrogen, sulfur, and amino acids for the germination and early seedling growth of the future plant.¹⁹ On the basis of their ultracentrifugation sedimentation coefficient (S) and solubility, common seed storage proteins are divided into 11S, 7S, and 2S storage proteins.²⁰ These seed storage proteins provide an indispensable protein source for human nutrition. Unfortunately, they are also the culprits for causing millions of people worldwide to suffer from food allergies.^{21,22} The orthologues of the 11S globulin have been characterized as

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food allergens in many tree nuts and legumes.^{23–26} The 11S globulin is a hexameric protein. Each chain is translated as a single peptide but is processed by a protease. The protease recognition site is known to contain six amino acids. The sequence of the protease site is NGLEET in English walnut allergen²⁷ Jug r 4 and almond allergen²⁸ Pru du 6, NGFEET in hazelnut allergen²⁹ Cor a 9, and NGIEET in cashew allergen³⁰ Ana o 2 and peanut allergen³¹ Ara h 3. The cleavage occurs between the Asn and Gly, and it is well conserved among a wide variety of plant species.³² Tree nut allergies as a group are as prevalent as peanut allergy,³³ and walnut is one of the leading causes of allergic reactions to tree nuts in the U.S. and Japan.^{34–36} However, only five allergens have been identified in English (or Persian) walnut (*Juglans regia* L.)^{27,37,38} and two in black walnut (Jug n 1, a 2S albumin, and Jug n 2, a 7S vicilin³⁹) while there are eight food allergens defined in hazelnut (*Corylus avellana* L.). The 11S protein in English walnut is a known food allergen, Jug r 4.²⁷ Whether its orthologue in black walnut is a food allergen remains an open question. Here, we report the isolation of black walnut 11S globulin and its identification as a food allergen.

MATERIALS AND METHODS

Preparation of Protein Extract. Whole unripe nuts of cultivar 'Sparrow' were collected 21 June and 7 Sept 2016 from a 22-year-old grafted black walnut tree at the research orchard at University of Missouri's Southwest Research Center in southwest Missouri and shipped overnight with dry ice to the laboratory at USDA. Thirty grams of immature walnut kernels were ground in 300 mL of extraction buffer (10 mM Gly, pH 10, 200 mM NaCl) in a KitchenAid blender. After filtering with cheese cloth, the extract was defatted by adding 1/10 volume of hexane and vortexed at room temperature for 10 min. The sample was centrifuged at 13000 rpm (using an F18-12 × 50 rotor from Thermo Scientific) for 30 min. The aqueous layer was collected, and the lipid-rich layer at the top was discarded. The collected sample was defatted with 1/10 volume of hexane again and subjected to centrifugation. The aqueous partition was labeled as walnut extract and used immediately for protein isolation.

Isolation of Black Walnut 11S Globulin. The extract was mixed with 1.5 volumes of saturated ammonium sulfate and spun at 4000 rpm for 10 min at room temperature. The pellet was collected and redissolved in the extraction buffer. The sample was subjected to centrifugation at 13000 for 30 min as described above. Black walnut 11S globulin (hereafter also referred to as Jug n 4) was purified from the supernatant by repeated consecutive hydrophobic interaction chromatography (HIC) and size exclusion chromatography (SEC) using a Pharmacia FPLC system at room temperature. For HIC, 36 mL of the sample was mixed with 18 mL of saturated ammonium sulfate. A 5 mL HiTrap Phenyl HP column (GE Healthcare, Piscataway, NJ) was equilibrated with the extraction buffer plus 33% saturated ammonium sulfate. The sample was passed through a 0.22 μ m syringe filter before it was loaded onto the HIC column. The column was washed with extraction buffer plus 35% saturated ammonium sulfate. A 75 mL linear gradient of 35%–0% saturated ammonium sulfate was applied to elute the bound proteins. Fractions containing mostly the protein later determined to be Jug n 4 were pooled and purified with the size exclusion column. SEC was carried out using a 300 mL Superdex 200 column (XK 26/70, GE Healthcare) equilibrated and eluted with the extraction buffer.

SDS-PAGE Analysis. SDS-PAGE was carried out using 4–20% poly acrylamide gels and a Tris-HEPES-SDS running buffer (100 mM Tris, 100 mM HEPES, 3 mM SDS, pH 8.0). Prestained protein molecular weight standards of 10, 15, 20, 25, 37, 50, 75, 150, and 250 kDa (Precision Plus All Blue, Bio-Rad, Hercules, CA) were used as references. Samples were boiled in an SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) containing 100 mM β -mercaptoethanol for 5 min before loading. Gels

were stained as previously described⁴⁰ and documented with an ImageQuant LAS 400 Imager (GE Healthcare).

N-Terminal Protein Sequencing. Purified Jug n 4 was subjected to SDS-PAGE as described above and electrophoretically transferred to a PVDF membrane (GE Healthcare) in a transfer buffer (48 mM Tris, 39 mM glycine, pH 9.2). The blot was stained with Coomassie Brilliant Blue and the three major peptide bands from P1 and P2 were excised and sent to the analytical core facility at Tufts Medical School for N-terminal amino acid sequencing.

Circular Dichroism (CD). CD spectroscopy was used to study the property of purified Jug n 4. Far-UV spectra were collected in the 195–270 nm region using a J-815 CD spectrometer (Jasco, Tokyo) and a quartz cuvette with a light path length of 1.0 mm. Wavelength scans were performed with a speed of 50 nm/min at 20 °C. The spectra were averaged for 10 scans.

Guanidinium Hydrochloride (GuHCl) Denaturation. Two stock solutions of the purified protein were prepared. Both solutions contained 3.4 μ M of the protein in the extraction buffer. One of the stock solutions also contained 6.0 M of GuHCl. Thirty samples were prepared with various ratios of the two stock solutions to obtain samples at 0.2 M intervals of GuHCl concentration from 0.2 to 6.0 M. The samples were equilibrated at room temperature for 24 h. The CD signal of these samples at 220 nm were measured at 20 °C for 30 s with data collected every second. The free energy of denaturation of the protein was calculated by fitting the data to a two-state transition using Origin Pro9 software (OriginLab Corp., Northampton, MA).

Preparation of Genomic DNA. To isolate genomic DNA, 100 mg of developing endosperm from the center portion of unripe black walnut seeds were used. Genomic DNA was purified using the RNeasy Plant Mini Kit and QIAprep Spin Miniprep Kit (QIAGEN, Valencia, CA) with a procedure combining the manufacturer's protocols for the two kits. Briefly, the sample was placed in a mortar containing liquid nitrogen and ground with a pestle. Liquid nitrogen was added to keep the sample submerged at all times. After grinding, the sample along with some liquid nitrogen was transferred to a liquid-nitrogen-cooled RNase-free, DNase-free 2 mL microcentrifuge tube. Buffer RLT (450 μ L) was added to the sample as soon as the liquid nitrogen was evaporated. The sample was then vigorously vortexed and transferred to a QIAshredder spin column and centrifuged for 2 min at 13000 rpm in a microcentrifuge. The supernatant of the flow-through (the lysate) was transferred to a QIAprep spin column placed and spun at 13000 rpm for 2 min in a 2 mL collection tube. Column washing and DNA elution were carried out following the miniprep kit protocol. Then 60 μ L of Milli-Q water was used to elute the genomic DNA.

PCR and Cloning. Different primers were designed based on the coding sequence for legumin of English walnut. A PCR product covering the 3' region of the target gene was first obtained and named fragment I. On the basis of the sequencing results of fragment I, an additional primer was designed. A second fragment (fragment II) was obtained in a PCR experiment using the new primer and a primer matching the start of the coding sequence of Jug r 4. These two overlapping fragments covered the whole region of the Jug n 4 gene that encompasses the coding sequence for the mature protein. Introns were identified using the GENSCAN⁴¹ server at MIT⁴² and manually based on the sequence alignment with the legumin sequences of pecan [*Carya illinoensis* (Wangenh.) K. Koch] and English walnut.

Patient Sera. Twenty-five sera (nos. 1–3, 5, and 7–27 corresponding to deidentified patient codes 5720, B5312, 5858, 5647, B5333, 2429, 5478, 6856, 5374, 5422, 6051, 5863, 6202, 5823, 2252, 2251, 5648, 2341, 6415, 5532, 2154, 6204, 6380, 5524, and 5426, respectively) were collected from patients with a positive oral food allergy challenge to walnut. The patients were enrolled in the food allergy study at Stanford University under institutional review board approval with informed consent (IRB approval certificate no. 8629). In addition, two allergic patients' sera (nos. 4 and 6) were acquired from PlasmaLab International (Everett, WA).

IgE Recognition of Jug n 4. In addition to the protein extracting procedures described above, raw immature black walnut kernels were extracted with 10 M urea. Walnut protein extract and the purified Jug n 4 were heated to 96 °C for 10 min in 1× SDS sample buffer

containing 100 mM β -mercaptoethanol and separated by electrophoresis with 4–20% gels. Multiple SDS-PAGEs were performed for protein detection by Coomassie Brilliant Blue (CBB) staining and for IgE binding analysis by Western blot. For Western blot, protein bands in the SDS-gels were transferred to PVDF membranes with a Trans-Blot SD semidry transfer cell (Bio-Rad, Hercules, CA). The membranes were blocked for 30 min at room temperature in TBST (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl, 0.1% Tween 20) containing 5% nonfat dry milk. In the meantime, individual serum (120 μ L) was incubated with 12 μ L of protein A immobilized with agarose (Pierce, Rockford, IL, USA) for 1 h at 4 °C in 3 mL of TBST with 1% nonfat milk. After incubation, protein A was removed by centrifugation at 1000 rpm for 2 min. The membranes were then incubated with individual serum overnight at 4 °C. The membranes were washed three times with TBST for a total of 15 min before being incubated 1 h at room temperature with an antihuman IgE secondary antibody conjugated to peroxidase (Sigma). The antibody was diluted 5000 times with TBST containing 1% nonfat milk before use. The membranes were then washed 5 $\times$ 5 min with TBST, and the bound IgE was detected using an ECL Western Blotting substrate (Pierce, Rockford, IL, USA) and an ImageQuant LAS4000 imaging system.

RESULTS AND DISCUSSION

The prevalence of food allergies has increased in recent years.^{4,43} A very limited number of protein families contain most of the known food allergens. From plant sources, these include the pathogen resistance proteins-10, the nonspecific lipid transfer proteins, profilins, and the 2S, 7S, and 11S seed storage proteins. Peanut, soybean, milk, egg, fish, crustacean shellfish, wheat, and tree nuts are the eight most common food allergen sources as defined by the Food and Agriculture Organization of the United Nations.⁴⁴ The U.S. Food and Drug Administration (FDA) recognizes that these eight food sources account for more than 90% of all food allergies in the United States.^{45,46} The FDA listed 19 nuts in the tree nut group of allergen sources⁴⁶ for the purpose of food labeling (although not all of them are nuts in the botanical sense). Among those tree nuts, the 11S orthologous proteins in seven of them (i.e., almond, Brazil nut, cashews, English walnut, hazelnuts, pecan, and pistachios) have been designated as food allergens by the World Health Organization and International Union of Immunological Societies (WHO/IUIS) Allergen Nomenclature Subcommittee. Black walnut 11S globulin is very likely a food allergen; therefore, an objective of this study was to purify this 11S seed storage protein and study its biochemical and immunological properties related to food allergy.

Purification of Mature Jug n 4. As in peanuts, almond, and other tree nuts, the 7S vicilin and the 11S legumin make up most of the protein content of the nut kernel. The molecular weights of the 7S and the 11S proteins translated from their coding sequences are similar (~50–70 kDa). The mature proteins, however, are post-translationally processed and they are separated to smaller peptide bands in SDS gels at reduced conditions. In their native states, the 7S protein is a trimer and the 11S protein is a hexamer. Their size difference, however, did not always facilitate their separation as both of these proteins from some species also form higher-order structures or oligomers. Ion exchange chromatography was not always effective for separating them either. To purify the 11S protein from black walnut, one has to overcome these difficulties. Characterized 11S seed storage proteins from other species are known to be processed by protease cleavage after the first amino acid of a consensus peptidase recognition motif (NGXEET),^{32,47} yielding a mature protein with a N-terminal acidic subunit and a C-terminal basic subunit covalently linked

by a disulfide bond. In this study, 60% ammonium was used to first precipitate proteins from the extract. The precipitate was further purified by HIC and fractions containing a strong pattern of bands consistent with those for the 11S protein were further purified by SEC. The SEC elution profile showed two major peaks (Figure 1a). SDS-PAGE analysis indicated that

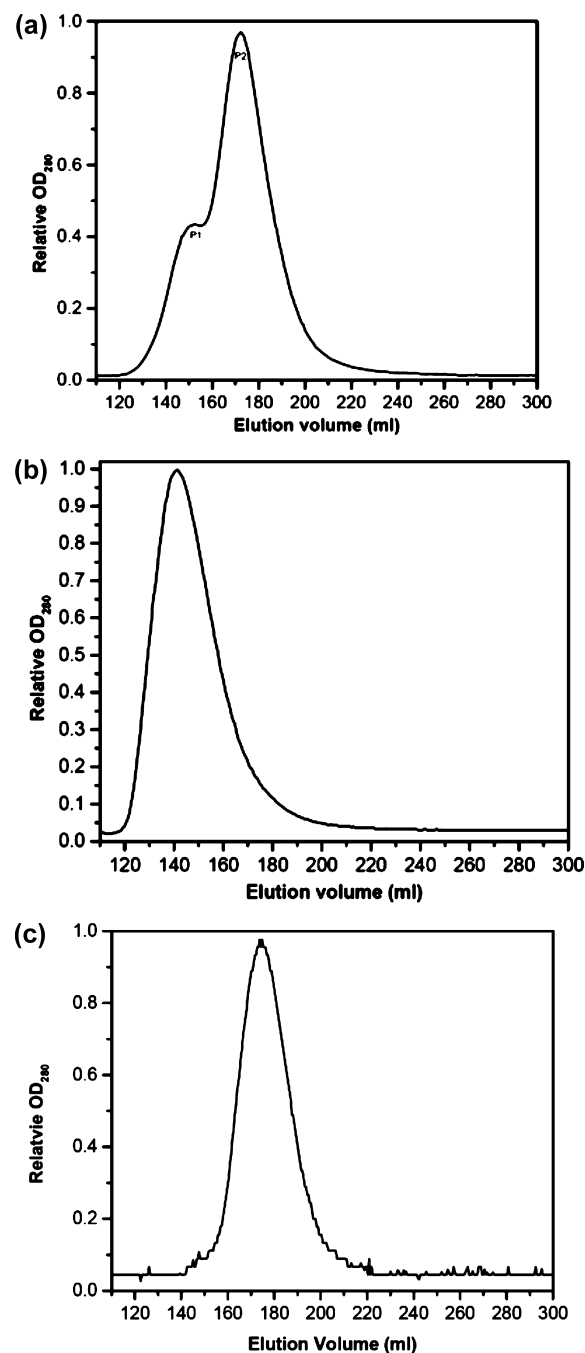


Figure 1. Purification of Jug n 4. Following hydrophobic interaction chromatography, the protein was further purified with a Superdex 200 column (a). The two major peaks were further purified by repeated HIC and SEC. The major component of peak 1 had a front edge at 122 mL in the SEC elution profile (b). The molecular mass of this component (P1) was estimated, based on the column calibration, to be 754 kDa. The front edge of the elution peak of the second component (P2) was 152 mL, and its molecular mass was estimated to be 287 kDa (c).

both peaks contained peptide fragments with molecular masses compatible with those of the 11S protein. We further separated these two components by repeated HIC and SEC (Figure 1b,c) and named them P1 and P2, respectively. Their band patterns as revealed by SDS-PAGE are shown in Figure 2. To determine

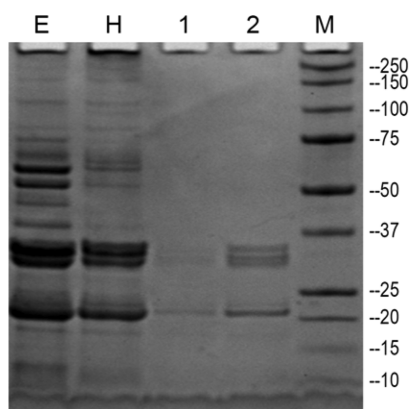


Figure 2. SDS-PAGE analysis of Jug n 4. Samples of 60% ammonium precipitated walnut protein extract (lane E), the sample after HIC chromatography (lane H), the component further purified from peak 1 of Figure 1a (lane 1), and the component further purified from peak 2 of Figure 1a (lane 2) were analyzed with SDS-PAGE using a homemade 4–20% gel. The molecular masses of the reference bands in the protein marker (lane M) are shown to the right of the gel image.

the identity of P1 and P2, the three major bands in each of the samples were sent for N-terminal peptide sequencing. The results were the same for both samples. The first amino acid of bands 1 and 2 (at ~34 kDa) was a serine, indicating that the proteins had a signal peptide removed (see below). This suggested that these two bands might have originated from the N-terminal acidic domain similar to those found in the almond allergen Pru du 6.⁴⁸ The first five residues of band 3 (at ~22 kDa) were GLEET, consistent with the N-terminal sequences of the basic domain of the 11S protein resulting from protease digestion. On the basis of the calibration of the Superdex 200 column, the molecular mass of the P1 and P2 were 754 and 286.2 kDa, respectively. While the latter is consistent with a hexameric 11S protein, P1 seems to be in an unknown configuration of a multimeric state possibly containing 12 molecules of the 11S protein.

Chemical Stability of Jug n 4. Circular dichroism spectrometry was used to study the chemical denaturation of Jug n 4. Far-UV CD spectra indicated that the hexameric Jug n 4 contained a mixture of α -helix and β -sheet (Figure 3), consistent with secondary structure composition of other 11S proteins with known structures.^{48–50} The GuHCl-induced folding-unfolding equilibrium of Jug n 4 was studied by measuring the CD signal at 220 nm (Figure 4). The data points appeared to make a sigmoidal shape, suggesting that Jug n 4 was either in its folded or unfolded state, and no stable intermediate was measurably populated. Thus, the measured CD signal was considered to be the sum of those of the folded and unfolded proteins, both of which are generally a linear function of the concentration of the denaturant. The changes in the total CD signal represented changes in the concentrations of the protein in the folded [F] and unfolded [U] states. Nonlinear curve fitting can be used to estimate the Gibbs free energy of the equilibrium transition,^{40,51} $\Delta G = \ln([U]/[F])$,

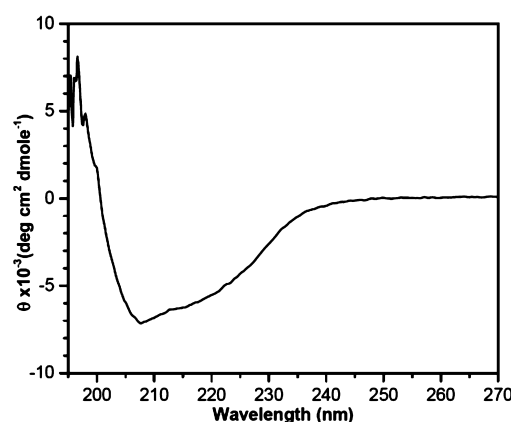


Figure 3. CD spectra of Jug n 4. The spectrum of the hexameric black walnut legumin (P2) in the extraction buffer (see text) was recorded from 270 to 195 nm at 20 °C.

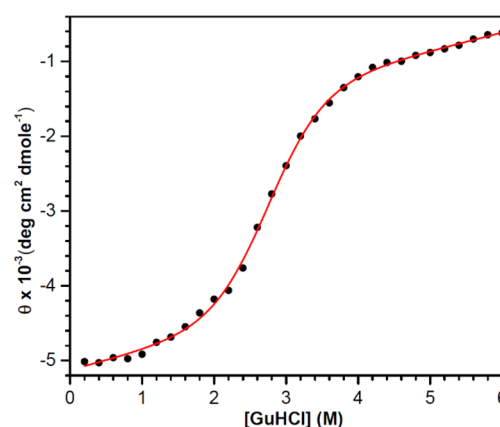


Figure 4. Urea induced unfolding of Jug n 4. The CD signal of Jug n 4 as a function of GuHCl was recorded at 220 nm at 20 °C. The solid red line shows the result of fitting the data to a two-state model (see text).

which is a linear function of the denaturant concentration, C , to the first-order approximation:

$$\Delta G = \Delta G_0 - mC$$

where ΔG_0 is the free energy of the transition when there is no denaturant. When the unfolded and folded states are equally populated ($[U] = [F]$) at the midpoint of the transition, ($C = C_m$), $\Delta G = 0$, and $m = \Delta G_0/C_m$. The results of the nonlinear fitting are shown in Figure 4 and Table 1. In addition, no

Table 1. Equilibrium Parameters^a for GuHCl-Induced Unfolding of Jug n 4^a

ΔG_0 (kcal mol ⁻¹)	C_m (M)	m (kcal mol ⁻¹ M ⁻¹)	R^2
4.1 ± 0.3	2.75 ± 0.03	1.5 ± 0.3	0.999 ± 0.002

^aDerived from CD data at 220 nm in the extraction buffer (see text).

significant change in the CD signal of the protein at 220 nm was observed when it was monitored while the temperature of the sample was raised to 96 °C (data not shown). These data indicated that Jug n 4 is a stable protein with a heat resistant structure.

Isolation of the Black Walnut Legumin Gene and Deduction of the Sequence of the Mature Protein. A series of oligos matching various positions of the coding

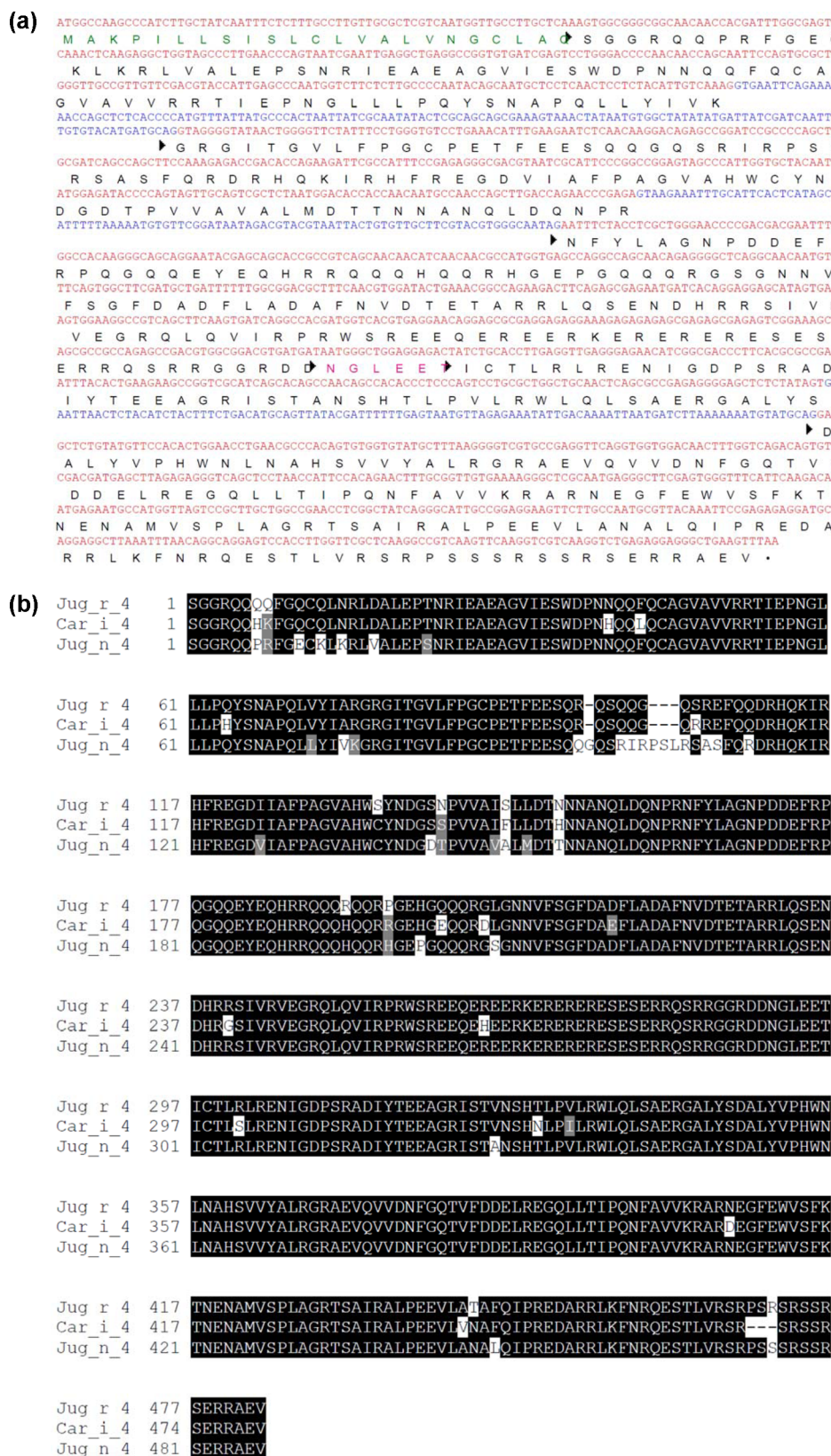


Figure 5. Isolation of black walnut legumin gene, deducing its amino acid sequence and N-terminal amino acid sequencing. (a) The DNA sequence of the black walnut legumin gene is presented with the coding sequence shown in red and the introns shown in blue. The protein sequence predicted with GENSCAN and based on sequence alignment (see text) is shown using one-letter amino acid code. The signal peptide was determined based on the N-terminal sequencing results of the purified mature protein. The protease recognition motif between the acidic and basic domain is shown in magenta. N-Terminal sequencing results indicated that protease cleavage was between the first and second residues of this motif. (b) Sequence alignment of English walnut (top), pecan (middle), and black walnut (bottom) legumins using clustalW.

sequence of Jug r 4 (GenBank: AY692446.1) were designed. PCR experiments were carried out with different primer pair combinations using genomic DNA isolated from young black walnut kernels as a template. Unfortunately, only one of the reactions yielded any DNA with the expected size (data not shown). The first successful PCR product was obtained with primers 302 (forward, ggctcagcaggaatatgagca, which matched a sequence 900 base pairs upstream from the end of the coding sequence of Jug r 4) and 301 (reverse, ttaacttcagcctctctc, which complemented the 3' end of the aforementioned coding sequence). The PCR product was named fragment I and inserted in an in-house cloning vector (pB) to produce pB-jugn3 and sent for DNA sequencing. The result revealed a 1058 base pair sequence homologous to the 3' half of the coding sequence of Jug r 4, with a short (103 base pair) intron (see below). New primers were designed based on the sequencing results. A subsequent PCR experiment was performed with primers 333 (atggccaagcccatcttgctatc, forward), which matched the beginning of the coding sequence for English walnut legumin and 325 (ctgaacacattgtgcct, reverse), which complemented a stretch of sequence close to the 5' end of fragment I. The PCR product (named fragment II) was cloned into the pB vector to make pB-jugn5. DNA sequencing results revealed a 941 base pair sequence homologous to the 5' half of the coding sequence of the Jug r 4, with two short introns (134 and 94 base pairs, respectively, see below). Fragments I and II overlapped 101 base pairs. Thus, the whole gene sequencing encompassing the coding sequence of Jug n 4 was determined to be 1884 base pairs. Introns were determined manually by sequence alignment of black and English walnut legumins (Figure 5a). The same exon–intron boundaries were identified when the GENSCAN⁴¹ server at MIT⁴² was used to predict exon–intron boundaries for the Jug n 4 gene. The sequence of the legumin DNA has been deposited to the GenBank with accession number KX891230. N-Terminal sequencing results indicated that the first 23 amino acids were removed in the mature protein, most likely as a signal peptide. N-terminal sequencing results also indicated that the protease separation of the acidic subunit and the basic subunit was between N318 and G319. The protein sequences of Jug n 4 and those of pecan and Jug r 4 are aligned in Figure 5b. Pairwise alignment showed that the sequence identity between the two walnut allergens is 91%, and the identity between the legumins of black walnut and pecan is 89%.

Identification of Black Walnut Legumin As an Allergen. In this study, a total of 27 sera were used to test whether they contained IgE molecules that recognize Jug n 4. Two sera with confirmed IgE to walnuts were obtained from PlasmaLab International. All patients enrolled in the food allergy study at Stanford University whose sera were used in this study were diagnosed by double-blind placebo-controlled food challenge (DBPCFC), considered the gold standard method for food allergy diagnosis) to be allergic to walnut. Their age range was 5–21 (average 11.36). All or some of them also had an allergy to one or more of the following foods: pecan, hazelnut, peanut, almond, cashew, milk, egg, pistachio, and sesame. The clinical information on the patients is shown in Table 2.

These patients' sera were tested by Western blot to determine if black walnut legumin is a food allergen. Ten molar urea extract of raw black walnut and purified Jug n 4 were separated by SDS gels and analyzed. More than half of the sera either recognized some other proteins but not the purified Jug

Table 2. Clinical Findings of Walnut-Allergic Patients

patient no.	age	symptoms ^a	IgEb (kU/L) ^b	diagnosis	known allergy to other foods
1	8	A, GI		dbpcfc	peanut, pecan
2	13	A		dbpcfc	pecan
3	10	R		dbpcfc	pecan
4	24		43.6	immunoCAP	cashew, hazelnut, pistachio, pecan
5	9	R		dbpcfc	hazelnut, pecan
6	56		60	immunoCAP	almond, hazelnut, pecan
7	14	A, R		dbpcfc	pecan
8	11	A, GI		dbpcfc	pecan
9	21	GI		dbpcfc	cashew pecan
10	16	U		dbpcfc	pecan
11	6	U		dbpcfc	pecan
12	8	R		dbpcfc	peanut, sesame, pecan
13	5	U		dbpcfc	pecan
14	7	U		dbpcfc	pecan
15	11	U		dbpcfc	almond, peanut, pecan
16	12	R		dbpcfc	pecan
17	8	U		dbpcfc	pecan
18	20	U		dbpcfc	pecan
19	13	R		dbpcfc	pecan
20	15	R		dbpcfc	egg, milk, pecan
21	7	GI		dbpcfc	pecan
22	6	A, U		dbpcfc	pecan
23	14	R		dbpcfc	milk, pecan
24	9	A		dbpcfc	pecan
25	11	GI		dbpcfc	pecan
26	17	GI		dbpcfc	pecan
27	13	U		dbpcfc	pecan

^aAbbreviations for symptoms: A, asthma; GI, gastrointestinal symptoms; R, rhinitis; U, urticaria. dbpcfc = double-blind, placebo-controlled food challenge. ^bImmunoCAP specific IgE.

n 4 (serum 18 is shown in Figure 6a as an example) or did not recognize any of the proteins in the extract (serum 19 is shown in Figure 6b as an example). Two of the sera (nos. 14 and 16) recognized both the basic and the acidic subunits of purified Jug n 4 as well as bands in the walnut urea extract at the same positions (Figure 6a), indicating that these sera contained at least two isoforms of IgE antibodies that recognized linear epitopes in its acidic and basic subunits, respectively. Three of the sera (nos. 1, 3, and 4) recognized the acidic subunit but not the basic subunit (Figure 6b), while four of the sera (nos. 2, 20, 22, and 26) recognized the basic subunit but not the acidic subunit (Figure 6c). As the sera recognized different patterns of peptide bands in the walnut urea extract, no sera from normal subjects were included for negative controls because the result for one serum could be used as controls for other sera.

In summary, food allergy is not only a health problem for millions of food allergy patients. It is also a food safety problem that impacts the safe use of agricultural products. The 11S legumin belongs to one of a few protein families that account for most of the food allergens. It makes up a large proportion of the protein content of tree nut kernels and seeds of other plants. However, the levels of difficulty in developing protocols for purifying the 11S globulin from different plant seeds varies. This might be one of the reasons why the 11S globulin from black walnut has not been characterized. This study reports, for the first time, the purification of black walnut legumin from the

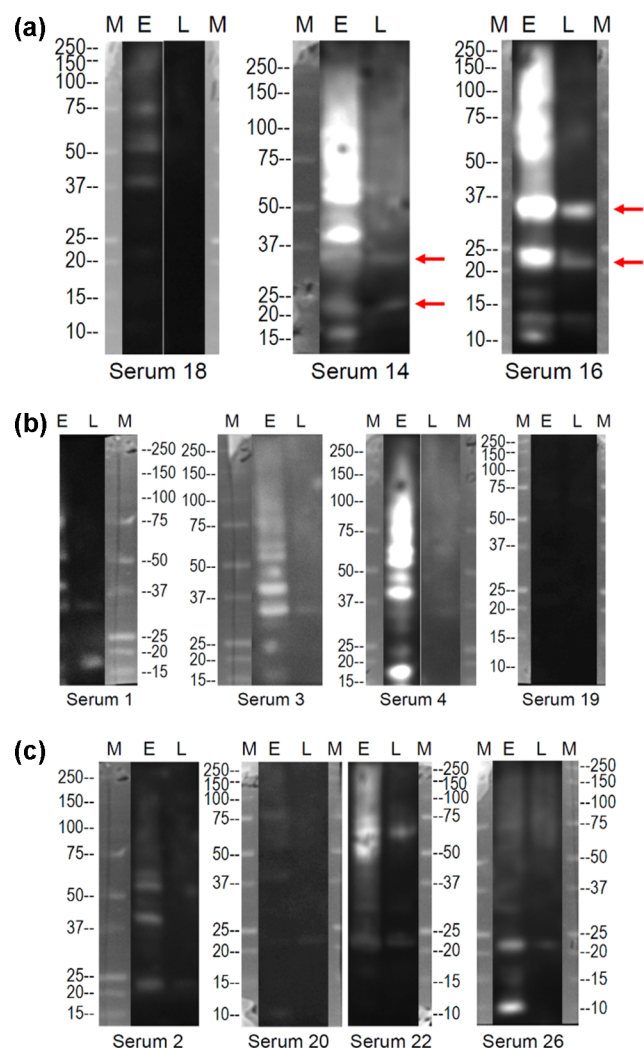


Figure 6. Western blot showing patient sera recognizing Jug n 4. Purified Jug n 4 (lane L) and proteins in black walnut urea extract (lane E) were denatured and separated with 4–20% SDS gels and transferred to PVDF membranes. An imaging system was used to document the Western blot results in the chemiluminescence mode. White light images of the membranes were also taken without moving the membranes. The positions of the bands of the prestained standards in the second image where overlaid with the chemiluminescence image. The sizes of the standards are shown to the side of the marker lane (M). The recognition of both the acidic and the basic subunits of Jug n 4 by two sera (as indicated by the red arrows) is shown in (a) along with the image of a blot showing serum 18 not recognizing Jug n 4. An image of the membrane strips stained by BBC was also taken. Overlaying this image with the Western blot images showed that the latter could not resolve the doublet bands of the acidic subunit. The recognition of acidic subunits of Jug n 4 by three sera is shown in (b) along with the image of a blot showing serum 19, which had no detectable recognition of any black walnut protein. The recognition of the basic subunits of Jug n 4 by four sera is shown in (c).

nut kernels and its characterization, the isolation of the legumin gene, the determination of the peptide sequence of the protein, and the identification of this proteins as a food allergen. There are two aspects of a protein being a food allergen. One is its ability to sensitize people who are at risk. One is its ability to trigger adverse reactions in sensitized patients. The latter sometimes can be a result of allergen cross-reaction. Why a few protein families contain most of the allergens is poorly

understood and information about walnut allergens is limited. Additional studies are needed to define the important factors that contribute to the allergenicity of food proteins. Information obtained in this study will facilitate better understanding of the allergenicity of walnut allergens and enhance the marketability of walnuts and other tree nuts in the future.

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ABBREVIATIONS USED

HIC, hydrophobic interaction chromatography; SEC, size exclusion chromatography; CBB, Coomassie brilliant blue

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