

Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions

Kirsten Beyer, MD, Galina Grishina, MS, Ludmilla Bardina, MS,
Alexander Grishin, PhD, and Hugh A. Sampson, MD *New York, NY*

Background: Hazelnuts are a common cause of food allergy. Allergic reactions to hazelnuts range from oral allergy syndrome caused by cross-reactivity between tree pollen and hazelnut proteins to severe anaphylactic reactions. Little information is available regarding the identification of pollen-independent hazelnut allergens.

Objective: The aim of the study was to identify these pollen-independent allergens in patients with hazelnut allergy with systemic reactions.

Methods: Extracted hazelnut proteins were separated by means of 2-dimensional PAGE, and immunolabeling was performed with individual sera from 14 patients with hazelnut-induced systemic reactions. Edman sequencing was performed on a 40-kd protein identified as an allergen. In parallel, RNA isolated from hazelnuts was used to construct a cDNA library. By using the peptide sequence data, oligonucleotide primers were synthesized and used to screen the library. Full-length cDNA clones were isolated, sequenced, expressed, and screened with patient sera.

Results: By using 2-dimensional proteomics, a protein fraction at 40 kd was recognized by serum IgE from 86% (12/14) of the patients with hazelnut allergy with systemic reactions. Two internal amino acid sequences were determined by means of Edman sequencing. Screening of the prepared hazelnut cDNA library with oligonucleotides based on these internal peptide sequences resulted in isolation of a novel protein cDNA. The new protein, named Cor a 9, belongs to the 11S globulin seed storage protein family. This family comprises known food allergens in peanut (Ara h 3) and soybean (glycine max). The pairwise homology among these 3 proteins ranges from 45% to 50%. Interestingly, one known IgE-binding epitope of Ara h 3 shares 67% of homologous amino acid residues with the corresponding area of Cor a 9. The amino acids that differ were previously shown not to be critical for IgE binding in Ara h 3. **Conclusion:** Cor a 9 is the first tree pollen-unrelated hazelnut allergen isolated, sequenced, and cloned. The identification of food allergens is the first step toward generating recombinant allergens for use in future immunotherapeutic approaches. In addition, the detection of conserved IgE epitopes in common

food allergens, such as seed storage proteins, might be a useful tool for predicting cross-reactivity to certain foods. (*J Allergy Clin Immunol* 2002;110:517-23.)

Key words: Cor a 9, hazelnut, food allergy, tree nut, seed storage protein, peanut

The leading causes of severe food-induced allergic reactions are allergies to peanuts and tree nuts. The prevalence rate for peanut allergy, tree nut allergy, or both is 1.1% in the US population; allergy to tree nuts alone is found in 0.5%.¹ Hazelnuts (*Corylus avellana*) are among the common tree nuts that lead to allergic reactions. In Europe the estimated prevalence of hazelnut allergy is 0.1% to 0.5%.^{2,3} Allergy to hazelnuts is often found in patients with tree pollen allergy. These patients usually present with oral allergy syndrome caused by cross-reactivity between tree pollen allergens and hazelnut proteins. It has been shown that proteins similar to Cor a 1, the major hazel pollen allergen, and hazel profilin are also expressed in hazelnut and explain the sensitivity to hazelnut in patients allergic to tree pollen.⁴ More recently, a Bet v 1-related major allergen from hazelnut, Cor a 1.0401, has been cloned, expressed, characterized, and compared with the homologue in hazel pollen, Cor a 1.01.⁵ On the other hand, severe allergic reactions to hazelnuts are reported in patients without any association to tree pollen allergy.^{6,7} These patients usually have urticaria, angioedema, asthma, vomiting, pharyngeal edema, diarrhea, and/or anaphylaxis. The hazelnut allergens responsible for these reactions have not yet been identified.

The world production of hazelnut has almost doubled in the last 2 decades, reaching greater than 600,000 tons of in-shell nuts.^{8,9} In the United States 99% of hazelnuts are grown in Oregon, and the main cultivars are Barcelona, occupying 80% of the plantations.^{8,9} Only 10% of the hazelnuts are eaten as in-shell nuts; the majority of the crop is cracked, and the kernels are sold to bakers, candy makers, and other processors.⁸ Hazelnuts are commonly found in chocolate, cakes, cookies, breakfast cereals, ice cream, pastries, breads, and confectionary products.

Despite the fact that hazelnuts are used increasingly in the United States and Europe and that allergic reactions are not limited to oral allergy syndrome through cross-reactivity with tree pollen allergens, the identification of hazelnut allergens is still incomplete. By using 2D proteomics and Edman sequencing, followed by subsequent cloning and sequencing, this article reports a new major food allergen that seems to be involved in the majority of non-tree pollen-associated hazelnut allergy.

From the Division of Pediatric Allergy and Immunology and the Jaffe Institute for Food Allergy, The Mount Sinai School of Medicine, New York. Supported in part by a grant from the Food Allergy Initiative and in part by a grant from the National Center of Research Resources-NIH (MO1 RR-00071) awarded to the Mt Sinai School of Medicine.

Received for publication March 8, 2002; revised June 11, 2002; accepted for publication June 12, 2002.

Reprint requests: Kirsten Beyer, MD, The Mount Sinai School of Medicine, Division of Pediatric Allergy and Immunology, Box No. 1198, One Gustave L. Levy Place, New York, NY 10029-6574.

© 2002 Mosby, Inc. All rights reserved.

0091-6749/2002 \$35.00 + 0 1/87/127434

doi:10.1067/mai.2002.127434

TABLE I. Clinical reactions to hazelnut and to hazelnut- and hazel pollen-specific IgE for the 14 patients with hazelnut allergy

Patient ID	Clinical reaction	Hazelnut IgE (kU/L)	Hazel pollen IgE (kU/L)	Pollen-related rhinoconjunctivitis	Recognition of 11S globulin
A	GI, urticaria	>100.00	<0.35	No	Yes
B	GI, resp	15.8	4.78	Yes	Yes
C	GI, urticaria	6.33	13.5	Unsure	Yes
D	OAS, angioedema, urticaria	11.1	19.1	No	Yes
E	Urticaria, resp	8.44	37.7	Yes	Yes
F	Urticaria, resp	0.81	<0.35	No	Yes
G	Urticaria	0.76	0.71	No	Yes
H	Angioedema, resp	0.93	<0.35	No	Yes
I	Resp	1.1	8.25	Yes	Yes
J	GI	0.75	<0.35	No	No
K	Urticaria	8.64	<0.35	No	Yes
L	GI	2.51	<0.35	No	Yes
M	OAS, urticaria, resp	3.78	0.57	Yes	Yes
N	Urticaria, GI, resp	12.7	4.8	Unsure	No

GI, Gastrointestinal symptoms; resp, respiratory symptoms; OAS, oral allergy syndrome.

METHODS

Preparation of hazelnut extract

Hazelnuts were ground until a smooth paste was achieved by using a coffee grinder (Krupps, Peoria, Ill) followed by a mortar and pestle. The paste was defatted by means of washing with at least 20 volumes (wt/vol) of cold acetone and dried overnight at 4°C. Dried acetone powder was stored at -80°C. Protein was extracted from the defatted dried powdered pulp by agitating with PBS and a protease inhibitor cocktail without EDTA (Roche, Indianapolis, Ind) overnight at 4°C. After centrifugation at 2500g for 15 minutes at 4°C, the supernatant was collected, filtered, and centrifuged at 12,000g for 3 minutes. Protein concentrations were determined by using the Coomassie Plus Protein Assay (Pierce, Rockford, Ill). All extracts were stored at -80°C.

2D Proteomics mapping

Crude hazelnut extract was suspended in Rehydration Buffer (9.8 mol/L urea, 4% Chaps, 2 mmol/L tributylphosphine, 0.2% Ampholytes, and 0.001% Bromophenol blue; Biorad, Hercules, Calif) and applied to 7-cm immobilized pH 5-8 gradient strips (Biorad). After focusing for 17 hours with a linear voltage slope of up to 20,000 volt-hours, immobilized pH 5-8 gradient strips were equilibrated with urea, SDS, and glycerol containing Tris-HCl buffer in the presence of 5 mmol/L tributylphosphine for 10 minutes and then 135 mmol/L iodoacetamide for 10 minutes, followed by 2D gel electrophoresis with NuPAGE 4%-12% Bis-Tris ZOOM Gels (Invitrogen, Carlsbad, Calif). The resolved proteins were subsequently transferred to Immobilon-P membrane (Millipore, Bedford, Mass) and then stained with 2.2% Coomassie Blue for total protein analysis. For molecular weight determination, MultiMark Multi-Colored Standard (Invitrogen) was used.

Probing immunoblots with hazelnut-sensitized patient sera

For detection of IgE binding to the separated hazelnut proteins, immunolabeling was performed with individual patient sera from 14 individuals with hazelnut allergy. The diagnosis of hazelnut allergy was based on clinical history and IgE measurement. Double-blind, placebo-controlled food challenges were not performed because of the severities of the systemic reactions. Clinical

history and hazelnut-specific IgE levels detected with the Pharmacia FEIA-Cap System are shown in Table I. In addition, reactions to other tree nuts, as well as peanuts, including the specific IgE levels against these foods, are shown in Table II. Normal human serum (Pharmacia, Peapack, NJ) was used as a negative control. Patient and control sera were diluted from 1:4 to 1:10 (depending on the level of hazelnut-specific IgE) in PBS-Tween plus 1% BSA and 10% normal goat serum and incubated with immunoblots for 1 hour by means of gentle agitation at room temperature. After brief rinsing with PBS, phosphatase-labeled goat anti-human IgE (KPL, Gaithersburg, Md) at a concentration of 1 µg/mL goat IgG/mL in PBS-Tween plus 1% BSA and 10% normal goat serum was applied. After agitation for 45 minutes at room temperature and washing with PBS, followed by 50 mmol/L TRIS at pH 9.6, immunoblots were developed with phosphatase substrate BCIP/NBT (KPL). Protein images were analyzed with GelDoc 2000 (Biorad) by using Quantity One Quantitation Software.

MALDI MS analysis and Edman sequencing

According to the procedure described in the "2D Proteomics mapping" section, the hazelnut proteins were separated with 2D PAGE, and the gels were stained with Coomassie Blue. The protein at 40 kd was analyzed at the Wistar Institute Protein Microchemistry/Mass spectrometry Facility. Briefly, the spot was excised and digested with trypsin. The tryptic digests were separated by means of microbore HPLC, followed by MALDI MS analysis of selected peaks and subsequent Edman sequencing of 2 peaks by using standard methods.

Construction of a cDNA library

A cDNA library was constructed from green Barcelona hazelnuts, which were a kind gift from Shawn Mehlenbacher from the Oregon State University. Total RNA was extracted according to the method of Chang et al,¹⁰ and mRNA was purified on an Oligotex mRNA Spin column from Quiagen (Valencia, Calif). Synthesis and cloning of cDNA was performed by using a cDNA synthesis kit from Stratagene (La Jolla, Calif). The double-stranded cDNA was ligated into the lambda vector Uni-ZAP, packaged in vitro (Gigapak Gold, Statagene), and then transfected into *Escherichia coli* XL1-Blue cells.

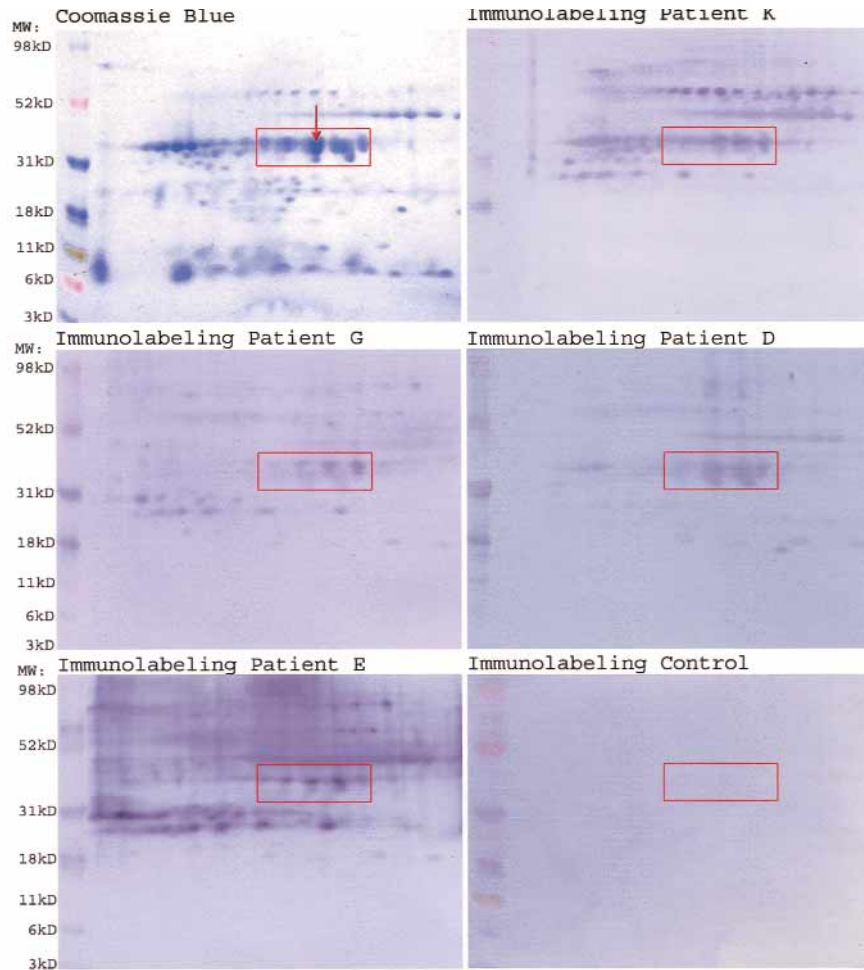


FIG 1. 2D Proteomics map for the water-soluble hazelnut proteins. Shown is the labeling with Coomassie blue for the total protein analysis and the immunolabeling of sera from 4 representative patients and the control serum. Molecular weight standards (*MW*) are shown on the left. The *square* marks the area recognized by serum IgE from 86% (12/14) of the patients with hazelnut allergy with systemic reactions. The *arrow* marks the 11S globulin-like protein that was identified as Cor a 9.

Construction of 11S globulin- and Cor a 1.04-specific primers, screening of the cDNA library, and sequencing of identified clones

The 11S globulin-specific primers were designed by using the 2 short amino acid sequences identified through Edman sequencing: CAG/A GGI CAG/A CAG/A CAG/A TTC/T GGI CAG/A and CAC TTC TAC CTI GCT/C GGI AAC CCA/T. By using these primers, the cDNA library was screened, and the isolated clones were sequenced at the Mount Sinai Core Facility. In addition, the library was screened for Cor a 1.04. Two primers were designed by using the published sequence of this protein: ATG AGG CCA CCT CCG TTA TC and GGA GGA GGA TCC ATC TTG AA. The sequenced clone was 100% identical to Cor a 1.0403 (GI:11762103).

Probing identified clones with patient sera

One of the isolated 11S globulin clones was incubated with *E coli* (strain Y 1090) for 15 minutes at 37°C and plated in a layer of Luria-Bertani soft agar at a density of 100 plaques per plate. The plates were incubated for 5 hours at 42°C. After soaking in 10 nmol/L isopropyl thiogalactose, a Hybond C Extra membrane filter (Amersham Pharmacia Biotech, Piscataway, NJ) was dried, placed

on the agar plate, and further incubated overnight at 37°C. Then filters were preblocked in PBS-Tween plus 1% BSA and 10% normal goat serum for 1 hour. Individual sera from 2 patients who recognized the 11S globulin on the 2D gel were diluted 1:10 in PBS-Tween plus 1% BSA and 10% normal goat serum and incubated overnight at 4°C with the filter. Iodine 125-labeled anti-human IgE (Diamed, Windham, Me) diluted 1:5 was added after washing and incubated for 5 hours at room temperature. Filter was exposed to a X-OMAT film (Kodak, Rochester, NY) with an intensifying screen at -20°C for 24 to 48 hours and developed. Similar experiments were performed for Cor a 1.0403 for all 14 patients.

RESULTS

Proteins were separated by means of 2D gel electrophoresis and either stained with Coomassie Blue for total protein analysis or transferred to membranes and immunolabeled with sera from 14 individuals with hazelnut allergy to identify the major allergenic proteins of hazelnut. All individuals had systemic allergic reactions after consumption of hazelnut (Table I). Several of these

	M A K L I L V S F S L C																				
1	AAA	CAA	CAT	CAG	ATA	TAT	ATT	ACC	ATG	GCC	AAA	CTT	ATC	TTG	GTC	TCT	TTT	TCT	CTG	TGC	60
	L	L	V	L	F	N	G	C	L	G	I	N	V	G	L	R	R	Q	Q	Q	
61	CTT	CTT	GTG	CTC	TTC	AAT	GGC	TGC	CTG	GGT	ATC	AAT	GTA	GGA	CTA	AGG	CGG	CAG	CAA	CAG	120
	R	Y	F	G	E	N	L	D	R	L	N	A	L	E	P	T	N	R	I		
121	CGA	TAT	TTT	GGC	GAG	TGC	AAC	CTC	GAC	AGG	CTC	AAT	GCC	CTT	GAA	CCC	ACA	AAC	CGC	ATC	180
	E	A	E	A	C	Q	I	E	S	W	D	H	N	D	Q	Q	F	Q	C	A	
171	GAG	GCT	GAA	GCT	TGC	CAG	ATC	GAG	TCT	TGG	GAC	CAC	AAC	GAC	CAG	CAA	TTC	CAG	TGC	GCT	240
	G	V	A	V	I	R	R	T	I	E	P	N	G	L	L	L	P	Q	Y	S	
241	GGG	GTT	GCC	GTC	ATC	CGG	CGA	ACC	ATT	GAG	CCC	AAT	GGC	CTT	CTC	TTG	CCC	CAA	TAC	AGC	300
	N	A	P	E	L	I	Y	I	E	R	G	R	G	I	T	G	V	L	F	P	
301	AAT	GCT	CCA	GAA	CTC	ATA	TAC	ATT	GAG	AGA	GGT	AGG	GGA	ATC	ACC	GGG	GTC	CTG	TTT	CCT	360
	G	C	P	E	T	F	E	D	P	Q	Q	Q	S	Q	Q	G	Q	R	Q	G	
361	GGG	TGT	CCC	GAA	ACA	TTT	GAA	GAT	CCT	CAA	CAA	CAA	TCT	CAA	CAG	GGA	CAG	AGA	CAG	GGA	420
	Q	G	Q	S	Q	R	S	E	Q	D	R	H	Q	K	I	R	H	F	R	E	
421	CAG	GGA	CAG	AGC	CAG	AGG	TCT	GAA	CAA	GAC	CGG	CAT	CAG	AAG	ATT	CGA	CAC	TTC	CGA	GAG	480
	G	D	I	I	A	L	P	A	G	V	A	H	W	C	Y	N	D	G	D	S	
481	GGA	GAC	ATC	ATC	GCA	TTG	CCA	GCT	GGA	GTA	GCC	CAT	TGG	TGC	TAT	AAC	GAC	GGT	GAC	TCC	540
	P	V	V	T	V	S	L	L	H	T	N	N	Y	A	N	Q	L	D	E	N	
541	CCA	GTT	GTC	ACA	GTC	TCT	CTC	CTT	CAC	ACC	AAC	AAC	TAT	GCT	AAC	CAG	CTT	GAC	GAG	AAC	600
	P	R	H	F	Y	L	A	G	N	P	D	D	E	H	Q	R	Q	G	Q	Q	
601	CCT	AGA	CAC	TTC	TAC	CTT	GCC	GGG	AAC	CCA	GAC	GAC	GAG	CAT	CAG	CGA	CAG	GGT	CAG	CAG	660
	Q	F	G	Q	R	R	R	Q	Q	Q	H	S	H	G	E	Q	G	E	Q	E	
661	CAA	TTC	GGG	CAG	CGT	CGC	CGC	CAG	CAA	CAG	CAT	AGC	CAT	GGT	GAG	CAA	GGC	GAG	CAA	GAG	720
	Q	Q	G	E	G	N	N	V	F	S	G	F	D	A	E	F	L	A	D	A	
721	CAG	CAG	GGC	GAA	GGA	AAC	AAC	GTA	TTC	AGT	GGC	TTC	GAT	GCT	GAG	TTT	TTG	GCG	GAC	GCG	780
	F	N	V	D	V	D	T	A	R	R	L	Q	S	N	Q	D	K	R	R	N	
781	TTC	AAC	GTG	GAT	GTT	GAC	ACG	GCC	AGA	AGG	CTT	CAG	AGC	AAC	CAA	GAC	AAA	AGG	AGG	AAC	840
	I	V	K	V	E	G	R	L	Q	V	V	R	P	E	R	S	R	Q	E	W	
841	ATC	GTC	AAA	GTG	GAA	GGC	AGG	CTT	CAG	GTG	GTG	AGG	CCG	GAA	AGG	TCG	CGT	CAG	GAA	TGG	900
	E	R	Q	E	R	Q	E	R	E	S	E	Q	E	R	E	R	Q	R	R	Q	
901	GAG	CGA	CAG	GAG	AGA	CAA	GAG	AGG	GAG	AGT	GAG	CAA	GAG	CGG	GAA	CGC	CAG	CGT	CGC	CAG	960
	← Acidic subunit →										← Basic subunit →										
	G	G	R	G	R	D	V	N	G	F	E	E	T	I	C	S	L	R	L	R	
961	GGA	GGA	CGT	GGA	CGT	GAT	GTC	AAT	GGC	TTT	GAG	GAG	ACA	ATA	TGC	AGC	TTG	AGG	CTC	AGG	1020
	E	N	I	C	T	R	S	R	A	D	I	Y	T	E	Q	V	G	R	I	N	
1021	GAA	AAC	ATC	TGC	ACC	CGC	TCA	CGC	GCT	GAC	ATT	TAC	ACC	GAA	CAA	GTC	GGT	CGC	ATC	AAC	1080
	T	V	N	S	N	T	L	P	V	L	R	W	L	Q	L	S	A	E	R	G	
1081	ACC	GTC	AAC	AGC	AAC	ACC	CTC	CCA	GTC	CTC	CGC	TGG	CTC	CAG	CTC	AGC	GCT	GAG	AGA	GGA	1140
	D	L	Q	R	E	G	L	Y	V	P	H	W	N	L	N	A	H	S	V	V	
1141	GAT	CTT	CAA	AGG	GAG	GGT	CTA	TAT	GTA	CCG	CAC	TGG	AAC	CTC	AAT	GCC	CAC	AGT	GTG	GTG	1200
	Y	A	I	R	G	R	A	R	V	Q	V	V	D	D	N	G	N	T	V	F	
1201	TAT	GCC	ATA	CGG	GGT	CGC	GCC	CGA	GTT	CAG	GTG	GTG	GAC	GAC	AAC	GGT	AAC	ACC	GTG	TTC	1260
	D	D	E	L	R	Q	G	Q	V	L	T	I	P	Q	N	F	A	V	A	K	
1261	GAC	GAT	GAA	CTT	AGA	CAG	GGT	CAG	GTG	TTG	ACC	ATC	CCC	CAG	AAC	TTC	GCG	GTG	GCG	AAA	1320
	R	A	E	S	E	G	F	E	W	V	A	F	K	T	N	D	N	A	Q	I	
1321	CGG	GCA	GAG	AGC	GAG	GGT	TTC	GAG	TGG	GTG	GCC	TTC	AAG	ACC	AAC	GAC	AAC	GCC	CAA	ATT	1380
	S	P	L	A	G	R	T	S	A	I	R	A	L	P	D	D	V	L	A	N	
1381	AGT	CCT	CTT	GCC	GGA	CGA	ACC	TCA	GCC	ATC	AGG	GCC	TTG	CCG	GAC	GAT	GTC	CTT	GCC	AAC	1440
	A	F	Q	I	S	R	E	E	A	R	R	L	K	Y	N	R	Q	E	T	T	
1441	GCG	TTT	CAG	ATT	TCA	AGG	GAG	GAA	GCC	AGG	AGG	CTG	AAA	TAC	AAC	AGG	CAG	GAG	ACC	ACC	1500
	L	V	R	S	S	R	S	S	S	E	R	K	R	R	S	E	S	E	G	R	
1501	TTG	GTT	CGC	TCA	AGC	AGG	TCT	TCA	TCT	GAG	AGG	AAG	AGG	AGG	TCT	GAG	TCT	GAG	GGC	AGA	1560
	A	E	A	*																	
1561	GCT	GAA	GCT	TAA	TTG	AAG	ATC	AAA	AAA	TGC	TAA	TGT	AAT	GCG	GCA	CGT	AGT	TTT	ATT	TTG	1620
1621	TGG	TTG	CTA	AGC	TTA	GGG	TTT	AGG	GCC	ATC	ACT	ATG	CAA	CAG	TCC	TTC	CTA	ACT	AGT	ATA	1680
1681	ATC	TGA	GTG	TAA	AAA	TAC	GCA	CAA	GGG	CAA	CCT	TTA	CTA	CAA	AGA	ATA	AAA	GTG	ACC	GCT	1740
1741	TTT	TCT	AAA	AAA	AAA	AAA	AAA	AAA	AAA	AAA	1767										

FIG 2. Nucleotide sequence of the 11S globulin cDNA clone and the deduced amino acid sequence. Marked in blue is the assumed cleavage site for the acidic and basic subunit, and marked in green are the 4 conserved cysteine residues. The 2 peptides identified by means of Edman sequencing are highlighted in yellow.

patients were also sensitized against other tree nuts, peanuts, or both (Table II).

Fig 1 shows the water-soluble hazelnut proteins separated by 2D PAGE. In addition to the Coomassie

blue-stained membrane that shows all proteins, immunolabeling with sera from 4 representative patients and the negative control subject is presented. The IgE-binding pattern was quite different in all patients. However, sever-

al spots at 40 kd were recognized by sera from 12 of 14 patients with hazelnut allergy (Fig 1 and Table I). To further analyze the spot at 40 kd, in-gel digestion and Edman sequencing was performed, and 2 internal sequences were obtained: QGQQQFGQR and HFYLAGNPDDEHQR. Through database screening, these sequences were shown to possess a significant homology to the legumin precursor of English oak and the pruning 2 precursor of almond, both of which are 11S globulin seed storage proteins. In parallel, RNA isolated from the Barcelona variety of hazelnuts was used to construct a cDNA library. The prepared cDNA library was screened with 2 phosphate 32-labeled oligonucleotides designed from the determined internal amino acid sequences to identify clones encoding the 11S globulin of hazelnut. The isolated clones were full-length sequenced. Analysis of the DNA sequence revealed that the obtained cDNA comprises an open reading frame of 1767 bp and encodes a 59-kd protein, apparently a precursor of the identified 40-kd polypeptide. The nucleotide sequence of the 11S globulin cDNA clone and the deduced amino acid sequence are shown in Fig 2. The nucleotide sequence was submitted to the GenBank Database under accession number AF449424, and the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Sub-Committee has designated the 11S globulin-like protein of hazelnut (*C avellana*) as Cor a 9.

Furthermore, one of the identified clones was screened with individual sera from 2 of the patients with hazelnut allergy. Both patients showed IgE binding, and the results for one of them are shown in Fig 3.

The 11S globulins described undergo posttranslational modification, with cleavage into 2 chains linked by a disulfide bond. The asparaginyl cleavage site that split the Cor a 9 into the acidic and basic subunits, as well as the conserved cysteines involved in the disulfide bond between the subunits, were identified by alignment with other known 11S globulins from peanut (Ara h 3), soybean (glycinin), sesame, almond, and oak (data not shown) and highlighted in Fig 2. Our identified protein on the 2D gel had a molecular weight of 40 kd and appears to be the acidic subunit of the protein cleaved under the reducing conditions of the gel electrophoresis (Figs 1 and 2).

Cor a 1.04 was previously described as a major allergen in hazelnut. A clone encoding Cor a 1.04 was also isolated from the cDNA library to clarify the role of Cor a 1.04 and Cor a 9 in the patients from the present study. The isolated clone was full-length sequenced and showed 100% identity with Cor a 1.0403. Screening with individual sera from all 14 patients with hazelnut allergy showed IgE binding in 7 (50%) of 14 patients (data not shown).

DISCUSSION

With the present study we identified an 11S globulin as a major food allergen for hazelnuts. 11S Globulins belong to the family of seed storage proteins. Seed storage proteins have been known to be allergenic for many decades. They are divided into albumins and globulins. Albumins

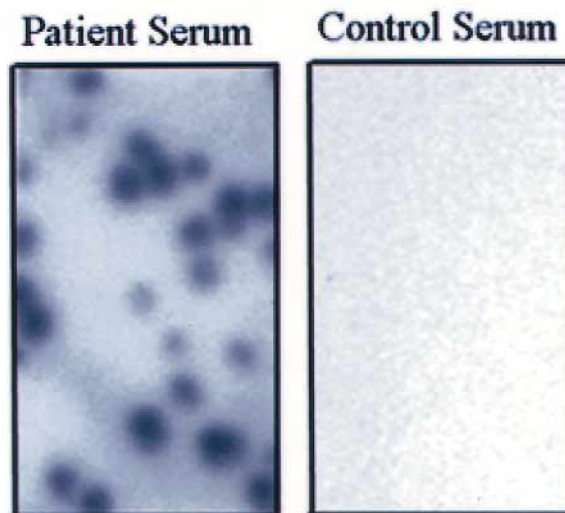


FIG 3. A Cor a 9 clone is labeled with serum from a patient with hazelnut allergy.

are water soluble at low salt concentrations, and globulins are water soluble at high salt concentrations.

The newly identified 11S globulin protein from hazelnut has been classified as a new major food allergen, Cor a 9. Thus far, 2 hazelnut allergens have been identified, Cor a 1¹¹ and Cor a 8, a lipid transfer protein (accession no. AF329829). All allergens from the order Fagales (alder, birch, hornbeam, chestnut, hazel, and white oak) are considered to belong to one group of homologous proteins. Therefore, the number 9 has been assigned to the new allergen, regardless of whether it is a food allergen, a pollen allergen, or both.

11S Globulin seed storage protein from peanut (Ara h 3) and soybean (glycinin) have previously been identified as allergens. Looking at the entire sequence, the pairwise homology among these 3 proteins ranges from 45% to 50% (36%-41% identity), with the newly identified Cor a 9 having 49% homology with Ara h 3 and 50% with glycine max.

The 11S globulins are nonglycosylated proteins that form hexameric structures. This family represents the precursor protein, which is cleaved into 2 chains. Both chains are linked together by disulphide bonds.¹² The protein we identified on the 2D gel had a molecular weight of 40 kd and appears to be the acidic subunit of the protein, dissociated from the smaller subunit under the reducing conditions of the gel electrophoresis. Similar to Cor a 9, binding of IgE from individuals with soy allergy has been found to soybean glycinin acidic chains.¹³ In parallel, the majority of the IgE-binding sites of the glycinin G2 correspond to the acidic chain of the protein.¹⁴ Similar results were found with regard to the peanut Ara h 3. All 4 identified B-cell epitopes¹⁵ are located within the acidic chain.

In the present study 86% of patients with systemic reactions after the ingestion of hazelnuts recognized the hazel pollen-unrelated Cor a 9. Of these patients, 42% had no specific IgE against hazel pollen and did not present with pollen-related rhinoconjunctivitis, whereas the

Ara h 3: 237 GN**F****S****G****F****T****P****E****F****L****E****Q****A** 251

Cor a 9: 250 NN**V****F****S****G****F****D****A****E****F****L****A****D****A** 264

FIG 4. The homology of amino acid sequences for peanut Ara h 3 and hazelnut Cor a 9 is shown in the area of an IgE-binding epitope of Ara h 3. Identical amino acids are depicted in **bold** and underlined, and homologous amino acids are underlined only. Amino acids that are highlighted in *gray* are known to lead to a loss of IgE binding when substituted with another amino acid.¹⁵

TABLE II. Clinical reactions and specific IgE to other tree nuts and peanuts, as well as ages for the 14 patients with hazelnut allergy

Patient ID	Clinical reaction (specific IgE [kU/L]) to:					Age (y)
	Peanut	Walnut	Brazil nut	Cashew nut	Almond	
A	0 (3.45)	NE (6.97)	NE (20.4)	NE (10.0)	NE (7.66)	8
B	0 (53.5)	G, R, T (14.8)	NE (5.94)	G, R, T (19.1)	0 (8.7)	34
C	G, U (>100)	G (6.93)	NE (4.48)	NE (88.4)	0 (2.47)	8
D	A, T (>100)	NE (38.9)	NE (10.4)	NE (0.5)	NE (0.58)	4
E	NE (27.8)	NE (9.11)	NE (4.64)	U, R (4.45)	NE (6.51)	6
F	0 (<0.35)	NE (<0.35)	NE (<0.35)	NE (37.0)	NE (<0.35)	12
G	0 (1.24)	0 (0.54)	NE (0.76)	U, T (7.53)	0 (1.38)	9
H	0 (<0.35)	G, A, R (0.79)	NE (1.09)	NE (1.04)	NE (0.87)	2
I	T (3.72)	NE (4.52)	NE (<0.35)	NE (<0.35)	0 (<0.35)	20
J	0 (<0.35)	U, A (7.45)	NE (<0.35)	0 (<0.35)	0 (<0.35)	10
K	NE (8.64)	NE (8.70)	NE (6.16)	NE (14.6)	NE (4.69)	1
L	0 (0.65)	NE (<0.35)	NE (0.39)	NE (1.16)	NE (<0.35)	2
M	U, R, T (24.2)	G, R (15.8)	NE (1.13)	NE (28.0)	NE (0.6)	11
N	G (6.82)	G, U, R (31.2)	G, U, R (6.52)	G, U, R (24.5)	G, U, R (2.6)	8

0, No reactions; NE, no known exposure; G, Gastrointestinal symptoms; R, respiratory symptoms; T, throat tightness; U, urticaria; A, angioedema.

other might also recognize cross-reacting allergens. In addition, only 50% of the patient population of the present study recognize Cor a 1.04, another hazelnut allergen, suggesting that Cor a 9 might be the major hazelnut allergen for several individuals.

Very recently, Pastorello et al¹⁶ identified 4 major allergens at 18, 32, 35, and 47 kd in hazelnut-sensitized patients with positive double-blind, placebo-controlled food challenge results.¹⁶ All of these patients presented with oral allergy syndrome, but 8 had gastrointestinal or mild systemic symptoms also. By using the partial N-terminal sequence, the 35-kd protein was further identified as a protein belonging to the legumin family, with 67% similarity to *Calocedrus decurrens*, a species of conifer.¹⁶ Comparing this N-terminal sequence with our identified Cor a 9 showed a 99% homology.

It is still not understood why some individuals have clinical reactions to only one nut or seed whereas others react to several. Peanuts and tree nuts, as well as peanuts and seeds or tree nuts and seeds, are not closely related botanically. It is of interest that one of the 4 known IgE-binding epitopes of Ara h 3 has 67% identity with the corresponding area of Cor a 9 (Fig 4). This epitope on Ara h 3 was recognized in previous studies by 38% of the study

population.¹⁵ The different amino acids were previously shown not to be critical for IgE binding in Ara h 3 (Fig 4).¹⁵ One might speculate that recognition of this IgE-binding site in a patient could result in clinical reactivity to both peanut and hazelnut. However, future studies to identify B-cell epitopes on Cor a 9 and subsequent screening of similar IgE-binding sites with sera from patients allergic to peanut, hazelnut, or both will be necessary.

The hazelnuts used in the present study were Barcelona hazelnuts from Oregon in the United States. By using SDS-PAGE and silver staining, it was shown previously that the protein patterns of different hazelnut varieties are quite similar.¹⁷

With the present study, we were able to identify and full-length sequence the first major allergen in hazelnuts that is unrelated to tree pollen. Interestingly, we could show sequence homology between an IgE-binding site of Ara h 3 and the newly identified Cor a 9 that might explain cross-reactivity in certain patients.

We thank Shawn Mehlenbacher from the Oregon State University for providing us with the green hazelnuts, as well as Kaye Speicher and David Reim from the Wistar Institute Protein Microchemistry/Mass spectrometry Facility for their great work and helpful discussions.

REFERENCES

1. Sicherer S, Munoz-Furlong A, Burks AW, Sampson HA. Prevalence of peanut and tree nut allergy in the US determined by a random digit dial telephone survey. *J Allergy Clin Immunol* 1999;103:559-62.
2. Groot H, Jong NW, Vuijk MH, Gerth van Wijk R. Birch pollinosis and atopy caused by apple, peach, and hazelnut; comparison of three extraction procedures with two apple strains. *Allergy* 1996;51:712-8.
3. Tariq SM, Stevens M, Matthes S, Rideout S, Twiselton R, Hide DW. Cohort study of peanut and tree nut sensitization by age of 4 years. *BMJ* 1996;313:514-7.
4. Hirschwehr R, Valenta R, Ebner C, Ferreira F, Sperr WR, Valent P, et al. Identification of common allergenic structures in hazel pollen and hazelnuts: a possible explanation for sensitivity to hazelnuts in patients allergic to tree pollen. *J Allergy Clin Immunol* 1992;90:927-36.
5. Luettkopf D, Mueller U, Skov PS, Ballmer-Weber BK, Wuethrich B, Skamstrup Hansen K, et al. Comparison of four variants of a major allergen in hazelnut (*Corylus avellana*) Cor a 1.04 with the major hazel pollen allergen Cor a 1.01. *Mol Immunol* 2001;38:515-25.
6. Ortolani C, Ballmer-Weber BK, Skamstrup Hansen K, Ispano M, Wuethrich B, Bindslev-Jensen C, et al. Hazelnut allergy: a double-blind, placebo-controlled food challenge multicenter study. *J Allergy Clin Immunol* 2000;105:577-81.
7. Schocker F, Luettkopf D, Muller U, Thomas P, Vieths S, Becker WM. IgE binding of unique hazelnut allergens: identification of non pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions. *Eur J Nutr* 2000;39:172-80.
8. Tous Marti J. World hazelnut production 2001. Available at: <http://www.aoi.com.au/acotanc/papers/tous-1/author-n-test.htm>. Accessed November 13, 2001.
9. United State Department of Agriculture. Hazelnut situation and outlook 2000. Available at: <http://www.fas.usda.gov/htp2/ircular/1999/99-11/hazsit.htm>. Accessed November 13, 2001.
10. Chang S, Puryear J, Cairney J. A simple and efficient method for isolating RNA from pine trees. *Plant Mol Biol Reporter* 1993;11:113-6.
11. Breiteneder H, Ferreira F, Hoffmann-Sommergruber K, Ebner C, Breitenbach M, Rumpold H, et al. Four recombinant isoforms of Cor a 1, the major allergen of hazel pollen, show different IgE-binding properties. *Eur J Biochem* 1993;212:355-62.
12. Protein families database of alignments and HMMs of the Sanger Institute. Available at: <http://www.sanger.ac.uk>. Accessed February 14, 2002.
13. Pedersen HS, Djurtoft R. Antigenic and allergenic properties of acidic and basic peptide chains from glycinin. *Food Agric Immunol* 1989;1:101-9.
14. Helm RM, Cockrell G, Connaughton C, Sampson HA, Bannon GA, Beilinson V, et al. A soybean G2 glycinin allergen. 1. Epitope mapping and three-dimensional modeling. *Int Arch Allergy Immunol* 2000;123:205-12.
15. Rabjohn P, Helm EM, Stanley JS, West CM, Sampson HA, Burks AW, et al. Molecular cloning and epitope analysis of the peanut allergen Ara h 3. *J Clin Invest* 1999;103:535-42.
16. Pastorello EA, Vieths S, Pravettoni V, Farioli L, Trambaioli C, Fortunato D, et al. Identification of hazelnut major allergens in sensitive patients with positive double-blind, placebo-controlled food challenge results. *J Allergy Clin Immunol* 2002;109:563-70.
17. Mueller U, Luettkopf D, Hoffmann A, Petersen A, Becker WM, Schocker F, et al. Allergens in raw and roasted hazelnuts (*Corylus avellana*) and cross-reactivity to pollen. *Eur Food Res Technol* 2000;212:2-12.