

Recombinant lipid transfer protein Cor a 8 from hazelnut: A new tool for in vitro diagnosis of potentially severe hazelnut allergy

Frauke Schocker, PhD,^a Dirk Lüttkopf, PhD,^b Stephan Scheurer, PhD,^b
Arnd Petersen, PhD,^a Anna Cisteró-Bahima, MD,^c Ernesto Enrique, MD,^c
Mar San Miguel-Moncin, MD,^c Jaap Akkerdaas, MSc,^d Ronald van Ree, PhD,^d
Stefan Vieths, PhD,^b and Wolf-Meinhard Becker, PhD^a *Borstel and Langen, Germany,
Barcelona, Spain, and Amsterdam, The Netherlands*

Background: Cor a 1.04 has been identified as the major hazelnut allergen in 65 European patients with positive double-blind, placebo-controlled food challenge results to hazelnut. Recently, the 11S globulin Cor a 9 was shown to be a pollen-independent hazelnut allergen in the United States, whereas preliminary data suggest the lipid transfer protein (LTP) as an important birch pollen-unrelated hazelnut allergen in Europe.

Objective: We sought to recruit a group of European patients allergic to hazelnut without birch pollen allergy and to identify and clone the major food allergen(s) in this study population.

Methods: We recruited 26 such Spanish patients, including 10 patients with anaphylaxis. IgE immunoblotting was performed with hazelnut extract. Hazelnut LTP Cor a 8 was cloned by using a PCR strategy, purified, and subjected to IgE immunoblotting. Recombinant Cor a 8, rCor a 1.0401, and rCor a 2 (profilin) were further investigated by means of enzyme allergosorbent test. Immunoblot inhibition experiments were used to compare the immunologic properties of natural and recombinant LTP.

Results: A 9-kd major allergen was identified in hazelnut extract. Cloning, sequencing, heterologous expression, and inhibition experiments identified it as an LTP. The prevalence of specific IgE antibody reactivity to LTP was 62% in hazelnut extract and 77% when recombinant LTP was tested by means of immunoblotting. IgE immunoblot inhibition with hazelnut extract showed that natural Cor a 8 and rCor a 8 shared identical epitopes. Only one patient had positive reactivity to Cor a 1.04, and no patients had positive reactivity to Cor a 2. Two sera bound to high-molecular-weight allergens. The LTP was denominated as Cor a 8 and submitted to the allergen database

of the World Health Organization/International Union of Immunological Societies Allergen Nomenclature Subcommittee. **Conclusions:** Cor a 8 is a relevant allergen for a majority of Spanish patients with hazelnut allergy that can cause severe allergic reactions. (*J Allergy Clin Immunol* 2004;113:141-7.)

Key words: Hazelnut allergens, lipid transfer protein, Cor a 8, in vitro diagnosis, enzyme allergosorbent test, immunoblot

Hazelnut is a common cause of food-induced allergic reactions. In Europe the prevalence of hazelnut allergy is estimated to be between 0.1% and 0.5%.^{1,2} Recently, a multicenter study confirmed allergy to hazelnut by means of double-blind, placebo-controlled food challenge.³ Although food allergy to hazelnut was the only inclusion criterion, all patients recruited in that study had a birch pollen-related food allergy and developed almost exclusively oral allergy syndrome (OAS) during the challenge. Analysis of the allergen profile of the patients revealed that the Bet v 1-related major allergen Cor a 1.0401⁴ was detected by IgE from 97% of the patients.⁵ By contrast, Beyer et al⁶ very recently identified the first non pollen-related major allergen in hazelnut as an 11S globulin. This allergen had an IgE prevalence of 86% in 14 subjects with severe hazelnut allergy recruited in the United States. By contrast, Pastorello et al⁵ presented preliminary data indicating that a 9-kd lipid transfer protein (LTP) might be a major allergen in 7 Mediterranean patients with anaphylaxis to hazelnut.

LTPs are small proteins comprising 91 to 95 amino acids, with 8 conserved cysteines forming disulfide bridges, basic isoelectric points, and a common α -helical structure.⁷ Because of their stable structure, they have been supposed to be responsible for more severe allergic reactions to hazelnuts.⁸⁻¹⁰ The first fully characterized allergenic LTP was Pru p 3, the major allergen of peach.¹¹ LTPs have also been identified as major allergens in apricot,¹² maize,¹³ asparagus,¹⁴ and lettuce.¹⁵ It is remarkable that (1) almost all study populations in which LTPs were described as relevant allergens were from Mediterranean countries and that (2) LTP sensitization was found to be associated with severe food allergies

From ^aForschungszentrum Borstel, Division of Biochemical and Molecular Allergology, Borstel; ^bPaul-Ehrlich-Institut, Department of Allergology, Langen; ^cInstitut Universitari Dexeus, Allergy Department, UAB, Barcelona; and ^dSanquin Research at CLB, Department of Immunopathology, Amsterdam.

Received for publication May 2, 2003; revised August 22, 2003; accepted for publication September 2, 2003.

Reprint requests: Frauke Schocker, PhD, Forschungszentrum Borstel, Division of Biochemical and Molecular Allergology, Parkallee 22, D-23845 Borstel, Germany.

0091-6749/\$30.00

© 2004 American Association of Allergy, Asthma and Immunology
doi:10.1016/j.jaci.2003.09.013

Abbreviations used

EAST: Enzyme allergosorbent test
LTP: Lipid transfer protein
OAS: Oral allergy syndrome
SPT: Skin prick test

in the majority of cases. Using a panel of recombinant cherry allergens for component-resolved in vitro and in vivo diagnosis, Scheurer et al¹⁶ and Ballmer-Weber et al¹⁷ compared study populations from Italy and Spain with those from Germany and Switzerland and confirmed the apparent geographic differences in sensitization patterns: the Bet v 1 homologue was the dominating allergen in Swiss and German patients, whereas sensitization to the LTP Pru av 3 was almost exclusively detected in Mediterranean patients.

In the light of the study by Pastorello et al,⁵ we suspected an LTP as the major hazelnut allergen in South European patients. In the present investigation we have recruited the largest population of subjects allergic to hazelnuts without birch pollen allergy that has been studied thus far (n = 26). The aim of our study was to identify the major hazelnut allergen in this group of patients and, if possible, to determine its primary structure and to express a recombinant protein as a new tool for component-resolved diagnosis of hazelnut allergy.

METHODS**Patient sera**

Serum samples were collected from 26 Spanish patients with a clear history of adverse reactions after hazelnut ingestion in the past year with hazelnut as the only food implicated in the episode. Specific IgE to hazelnut was determined by means of in vivo and in vitro tests. Double-blind, placebo-controlled food challenges to hazelnut were performed in patients reporting OAS. Challenge tests to hazelnut were not performed in patients reporting systemic reactions except for in one patient in whom hazelnut-dependent exercise-induced anaphylaxis was diagnosed.

Allergy to pollens was assessed from a clinical history of respiratory symptoms during the season of the different pollens.

Skin prick tests (SPTs) were performed according to the standard procedure¹⁸ with a panel of inhalant allergens containing the main allergens prevalent in our environment: dust mites, molds, cockroach, cat and dog dander, latex, and pollens, such as grass, *Parietaria judaica*, *Artemisia vulgaris*, *Plantago lanceolata*, *Chenopodium album*, *Mercurialis annua*, *Olea europea*, *Cupressus arizonica*, *Corylus avellana*, *Platanus acerifolia* (Bial-Aristegui, Bilbao, Spain), and *Betula verrucosa* (ALK-Abello, Madrid, Spain).

SPTs with a panel of food allergens with commercially available extracts (Leti, Barcelona, Spain) were also performed, including milk, egg, mixed flours, peach, apple, banana, kiwi, cherry, hazelnut, peanut, chestnut, walnut, almond, maize, rice, mustard, celery, green bean, tomato, lettuce, mixed meat, mixed fish, shrimp, and *Anisakis simplex*. We also performed SPTs by using the prick-to-prick technique¹⁹ with fresh hazelnuts and implicated foods.

Histamine dihydrochloride, 10 mg/mL, and PBS solution served as positive and negative controls, respectively. An SPT response was considered positive if the wheal area was 3 mm greater than that elicited by the negative control 15 minutes after testing.

Written informed consent was obtained from all subjects included in this study.

Allergen extracts

Allergen extract from hazelnuts was prepared as previously described.²⁰

RNA purification and first-strand cDNA synthesis

Total RNA was isolated from 100 mg of hazelnut powder by using RNeasy Plant Mini kit (Qiagen, Hilden, Germany). Approximately 1 µg of total RNA was reversely transcribed with the First Strand cDNA Synthesis kit (Pharmacia, Freiburg, Germany). The cDNA synthesis was primed with a degenerated oligonucleotide primer (5'-T₁₆NN-3' [dTdeg]) at 37°C for 60 minutes, followed by heat denaturation at 99°C for 5 minutes.

PCR and 5'RACE (Rapid Amplification of cDNA Ends)

A degenerated primer selected on the basis of known homologous sequences of LTPs (LTP2: 5'-TGT GGW GTY AAY ATT CCT TAC-3') and the dTdeg primer were used for the PCR strategy. The first-strand cDNA amplification was performed as previously described¹⁶ at optimized PCR conditions. Rapid amplification of cDNA ends (5'RACE) was performed with the 5'RACE system (GIBCO BRL, Eggenstein, Germany), according to the manufacturer's instructions. Single-stranded cDNA was synthesized with a gene-specific antisense primer (GSP1: 5'-CAC TTC ACG TTG TTG CAG TTG G-3'). A homopolymeric dC-tail was ligated to the 3' end of the purified cDNA by means of a terminal desoxynucleotidyl transferase reaction. The dC-tailed cDNA was amplified by using an abridged anchor primer (provided with the 5'RACE system) and a nested second gene-specific antisense primer (GSP2: 5'-GCT GAT CTT GTA AGG AA-3').

Amplification of full-length coding DNA

The identified sequences of the 2 overlapping partial cDNAs were used to derive terminal primers: *Nde*-LTP(+) 5'-GG GAA TTC CAT ATG TCC CTG ACA TGC CCA CAG-3' and *Xho*I-LTP(-) 5'-TAA CCG CTC GAG TCA CTT CAC GTT GTT GC-3' (restriction sites are underlined, and start and stop codons are shown in italics).

Cloning and sequencing of hazelnut LTP cDNA

Amplified cDNA was purified from agarose gels by using the QIAEX kit (Qiagen), ligated into pGEM-T vector (pGEM-T Vector System I; Promega, Madison, Wis), and transformed into *Escherichia coli* XL1-Blue (Stratagene, Heidelberg, Germany) by means of electroporation. Plasmids from positive clones were subjected to sequence analysis, as previously described.¹⁶

Expression and purification of recombinant hazelnut LTP

The LTP cDNA was cloned into the vector pET-16b (Novagen, Schwalbach, Germany) through *Nde*I and *Xho*I restriction sites. For target gene expression, the plasmid was transformed into *E coli* BL21(DE3) Codon Plus RIL cells (Stratagene, Amsterdam, The Netherlands). The cells were grown at 37°C in LB medium containing 50 µg/mL carbenicillin and 35 µg/mL chloramphenicol to a cell density of 0.6 at a wavelength of 600 nm. Expression was induced with 1 mmol/L isopropyl-D-thiogalactopyranoside, and incubation was continued at 30°C for 2 hours. The cell pellet was dissolved in binding buffer (50 mmol/L NaH₂PO₄, 300 mmol/L NaCl, 10 mmol/L imidazole, and 175 U of benzonase, pH 8.0) and disrupted with a French pressure cell disruption apparatus (SLM Aminco; Spectronic Unicam Instruments Inc, Cambridge, United

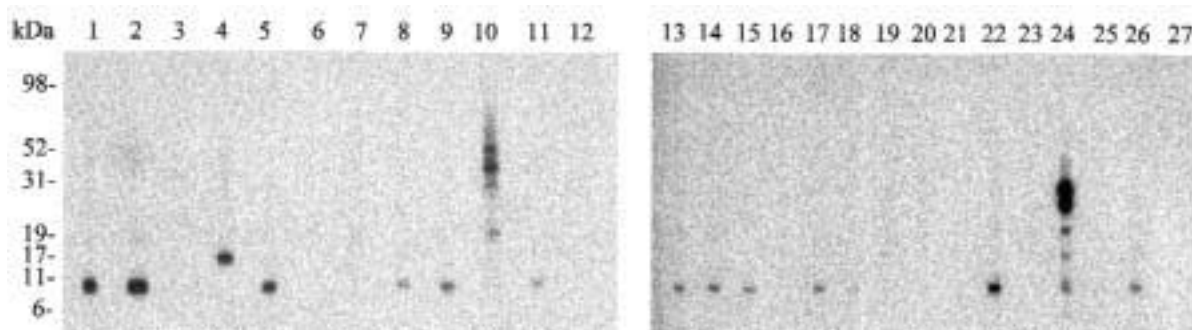


FIG 1. IgE immunoblotting of hazelnut extract: lanes 1 to 26, patient sera 1 to 26; lane 27, nonallergic control.

Kingdom). After centrifugation, the target protein was purified by means of Ni-chelate affinity chromatography. Protein concentrations of the eluates were measured by using the Coomassie Plus Protein Assay (Pierce, Rockford, Ill).

SDS-PAGE, immunoblot, and immunoblot inhibition

Protein samples were separated by means of SDS-PAGE (15%) under reducing conditions according to the method of Laemmli²¹ and were Coomassie stained. For immunoblot and immunoblot inhibition experiments, hazelnut extract (20 µg/cm total protein) and recombinant LTP (0.5 µg/cm) were separated by using NuPAGE-Bis-Tris gels (10%; Novagen, Groningen, The Netherlands) under nonreducing conditions and transferred onto nitrocellulose membranes (Schleicher and Schuell, Dassel, Germany).

For immunoblot analysis, the serum samples were diluted 1:20 in PBS containing 0.05% Tween 20. Immunoblot inhibition experiments were performed by preincubating a serum pool (Table I, no. 9 and 11) with 15 µg of hazelnut rLTP or 700 µg of crude hazelnut protein at room temperature for 2 hours. Then the samples were diluted to 1:20. Serum from a nonatopic subject was chosen as a negative control. For detection of bound IgE antibodies to hazelnut extract, the strips were incubated with iodine 125–radiolabeled sheep anti-human IgE (SH25-1-p7; Sanquin, Amsterdam, The Netherlands).

Ion exchange chromatography and N-terminal sequencing

DEAE Sepharose Fast Flow Resin (Amersham Pharmacia Biotech AB, Uppsala, Sweden) was loaded on a column (Pharmacia) for FPLC (Fast Protein Liquid Chromatography) purification. After equilibration with binding buffer (20 mmol/L Tris, pH 8.0), 5 mg of crude hazelnut protein was bound to the resin. The column was washed with binding buffer and finally eluted by means of stepwise increases of the salt gradient with elution buffer (20 mmol/L Tris and 0.5 mol/L NaCl, pH 8.0). Fractions containing the enriched low-molecular-weight proteins were electroblotted onto polyvinylidene difluoride membranes (Westran; Schleicher and Schuell) and stained with Coomassie stain. Microsequencing of a 9-kd protein was performed by means of Edman degradation with a 476-A pulsed liquid protein sequencer with an online phenylthiohydantoin amino acid analyzer (Applied Biosystems, Weiterstadt, Germany).

Determination of specific IgE by means of enzyme allergosorbent test and CAP

Recombinant hazelnut allergen LTP (0.3 µg per disk), Cor a 1.0401 (Genbank accession no. AF 136945; 0.25 µg per disk), and profilin Cor a 2 (Genbank accession no. AF 327622; 2 µg per disk) were coupled to cyanogen bromide–activated filter paper disks (Hyor, Kassel, Germany), as described elsewhere.²² The enzyme allergosorbent test (EAST) was performed in duplicates by using the “Allergopharma

Spez IgE ELISA” (Allergopharma), according to the manufacturer’s instructions. Specific IgE to hazelnut extract was determined by using the CAP assay (Pharmacia & Upjohn, Uppsala, Sweden).

RESULTS

Patients with hazelnut allergy

We recruited 26 patients (16 female and 10 male patients; age range, 7–58 years) with a history of adverse reactions to hazelnuts. All subjects included had positive responses to hazelnut both in SPTs with commercial extract and with the fresh food. Of 26 cases, history revealed 10 (38%) cases of anaphylaxis, 8 (31%) cases of OAS, 6 (23%) cases of angioedema, and 2 (8%) cases of urticaria (Table I).

Most of the patients were sensitized to other plant-derived foods, mainly peach and peanut (Table I), and several of the foods causing a positive SPT response were not tolerated by the patients. All had plane pollen allergy, and some of them (6/26 [22%]) were additionally sensitized to grass pollen. None of the patients were allergic to birch pollen.

Identification of a 9-kd major allergen in hazelnut

Sixteen of 26 patients’ sera displayed IgE reactivity to a 9-kd protein in hazelnut extract (nos. 1, 2, 5, 7–11, 13–15, 17, 18, 22, 24, and 26; Fig 1 and Table I). In 2 serum samples (nos. 10 and 24) allergenic hazelnut structures above 20 kd were detected in addition to the 9-kd protein. One of 26 sera contained IgE antibodies to an 18-kd allergen (no. 4). Incubation with an mAb against Bet v 1 identified this protein as Cor a 1.04, the Bet v 1–analogous hazelnut allergen (data not shown). The rest of the sera were monosensitized against the 9-kd allergen.

Identification of the 9-kd hazelnut allergen as an LTP

Enrichment of natural LTP and amino acid sequencing. The natural LTP from hazelnut was enriched by means of ion exchange chromatography. Because we focused not only on the isolation of LTP but also on further low-molecular-weight proteins, different resin materials with varying buffer systems were run and accessed in Coomassie-stained Laemmli gels (15%). The most effective enrichment of proteins below 14 kd was achieved by running a DEAE Sepharose Fast Flow Resin with an elution buffer

```

bp 1 atg ggt agc ctt aag ttg gta tgc gcg gtc ctc ttg tgc atg atg gtg gcc gca ccc gtt 60
aa  M  G  S  L  K  L  V  C  A  V  L  L  C  M  M  V  A  A  P  V

61 gcc cgg gcg tcc ctg aca tgc cca cag ata aaa ggc aac ctc acg cca tgc gtg ctc tac 120
   A  R  A  S  L  T  C  P  Q  I  K  G  N  L  T  P  C  V  L  Y

121 ctg aag aac ggc ggc gtt ctt cct ccc tct tgc tgc aag ggc gtc agg gct gta aac gac 180
   L  K  N  G  G  V  L  P  P  S  C  C  K  G  V  R  A  V  N  D

181 gcc tcc agg acc acg tcc gac cgc cag tcc gct tgc aac tgc ttg aaa gat aca gcc aaa 240
   A  S  R  T  T  S  D  R  Q  S  A  C  N  C  L  K  D  T  A  K

241 ggc atc gct ggc ctc aac cct aat ctt gct gct ggc ctc ccc ggc aag tgt ggt gtc aac 300
   G  I  A  G  L  N  P  N  L  A  A  G  L  P  G  K  C  G  V  N

301 att cct tac aag atc agc ccc tcc acc aac tgc aac aac gtg aag tga
   I  P  Y  K  I  S  P  S  T  N  C  N  N  V  K  *

```

FIG 2. Nucleotide sequence and deduced amino acid sequence of Cor a 8. The N-terminus determined in nCor a 8 is shown in bold, and the identified signal peptide is underlined. The nucleotide sequence has been submitted to GenBank under accession no. AF 329829.

of 20 mmol/L Tris and 0.5 mol/L NaCl, pH 8.0. The fractions with the low-molecular-weight proteins were eluted at highest purity at the beginning of the elution step (data not shown). The fractions containing a 9-kD protein were electroblotted onto a Western membrane and sequenced by means of Edman degradation. The amino acid sequence of the first 23 amino acids of the natural 9-kD protein confirmed the N-terminus of recombinant LTP (Fig 2).

Sequence analysis of hazelnut LTP cDNA. The hazelnut LTP cDNA was identified as a 345-bp open reading frame coding for 92 amino acids of the mature protein and a signal peptide of 23 amino acids (Fig 2). Computer-assisted sequence alignments on the amino acid level showed similarities to the group of nonspecific transfer proteins. The highest degrees of amino acid sequence identity were revealed with LTP from almond (62%), apple (61%), cherry (61%), and peach (59%). The sequence was submitted to the NCBI GenBank database under accession no. AF 329829. The deduced protein had a theoretic molecular mass of 9.4 kD and an isoelectric point of 10.7. It was denominated as Cor a 8 and submitted to the official World Health Organization/International Union of Immunological Societies Allergen Database.

Expression and purification of rCor a 8 from hazelnut. Complementary DNA encoding the mature protein Cor a 8 was expressed in *E coli* BL21(DE3) RIL cells at 30°C for 2 hours. Purification was performed under native conditions by means of metal affinity chromatography. Fractions containing rCor a 8 were pooled (Fig 3). Approximately 1.5 mg of recombinant protein was purified from 1 L of bacterial culture.

Allergen profile of the patients determined on the basis of component-resolved diagnosis

The 26 sera from Spanish patients with hazelnut allergy included in this study were investigated for IgE-binding capacity to rCor a 8 by means of immunoblotting and EAST.

As shown in Fig 4 and Table I, all the sera with IgE binding to nCor a 8 also recognized the recombinant protein in the immunoblot. Serum samples 6, 19, 20, and 25 bound to rCor a 8 but did not recognize natural LTP in hazelnut extract.

By means of EAST with rCor a 8, we determined 1 class 3, 6 class 2, and 7 class 1 results in this group of patients (Table I). After testing the sera with rCor a 1.0401, the Bet v 1-related major hazelnut allergen, and recombinant hazelnut profilin Cor a 2.01, the Bet v 2-analogous hazelnut allergen, only serum sample 4 showed IgE against Cor a 1.0401, and none showed IgE against profilin. Two sera of nonatopic subjects and 2 sera of patients only sensitized to Cor a 1.04 and Cor a 2, respectively, were chosen as controls (not shown in Table I). The nonatopic sera did not show reactivity to any of the recombinant allergens, whereas the positive controls confirmed the specificity of the EAST.

Twenty-one of the patients presented IgE to hazelnut extract in the CAP test.

nCor a 8 and rCor a 8 share identical epitopes

For IgE immunoblot inhibition experiments, a serum pool (nos. 9 and 11, Table I) from 2 patients with anaphylactic reactions to hazelnuts was selected. The serum pool was preincubated with 15 µg of rCor a 8 or 700 µg of protein of natural hazelnut extract before being tested on blot strips with hazelnut extract. IgE binding was completely inhibited with the recombinant hazelnut LTP and with the crude extract (Fig 5).

DISCUSSION

To identify birch pollen-independent allergens in hazelnut, we have studied 26 subjects with hazelnut allergy from Spain, the largest group of such patients studied thus far. Because 18 (69%) patients reported systemic symptoms after hazelnut ingestion, challenge tests were not performed in these subjects but were used to confirm OAS in

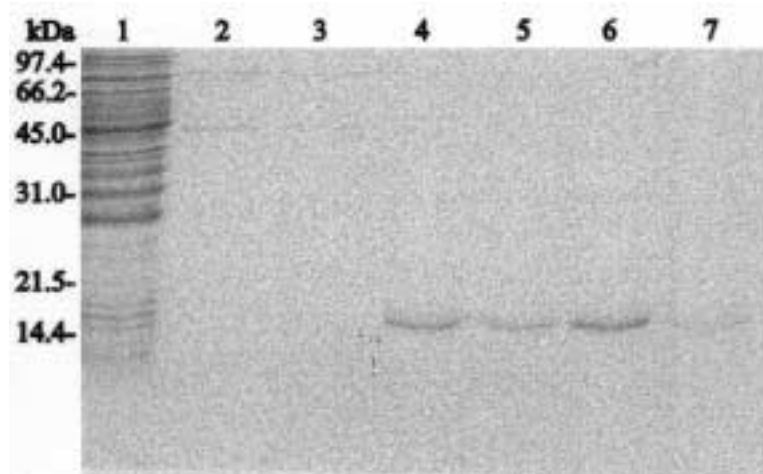


FIG 3. FPLC purification of recombinant (His₁₀)-tagged Cor a 8 with nickel-nitrilotriacetic acid superflow resin: *lane 1*, flow-through after binding to column; *lane 2*, eluates after the 10 mmol/L imidazole binding step; *lane 3*, eluates after the 50 mmol/L imidazole washing step; *lane 4*, eluates after the 100 mmol/L imidazole washing step; *lanes 5 to 7*, elution with 250 mmol/L imidazole buffer.

TABLE I. Summary of data for patients with hazelnut allergy

Patient no.	Age (y)	Sex	Food symptoms	Other sensitizations*		EAST, rCor a 8	EAST, rCor a 1.0401	EAST, rCor a 2	CAP, hazelnut	Immunoblot, nCor a 8	Immunoblot, nCor a 8
				Aero-allergens†	Foods‡						
1	30	F	Urticaria	P	pe, l, pn, m	2	0	0	3	+	+
2	29	F	Angioedema	P	pe, ch, pn, t	2	0	0	3	+	+
3	30	F	OAS	P+G	gb	0	0	0	0	—	—
4	41	F	OAS	P+G	pe, a	0	3	0	2	—	—
5	30	F	OAS	P+G	t, pn	1	0	0	2	+	+
6	33	M	Angioedema	P+C	pe	0	0	0	0	—	(+)
7	38	F	Anaphylaxis	P+D	pe, pn, c	0	0	0	1	(+)	+
8	27	F	Anaphylaxis	P+D	w, ch, al, m, pn, r	0	0	0	2	+	+
9	30	M	Anaphylaxis	P	w, che	1	0	0	2	+	+
10	36	M	OAS	P+G	pn, r, m, l	0	0	0	2	(+)	(+)
11	23	F	Anaphylaxis	P+G	pe, t, r, che, k, ch	0	0	0	2	+	+
12	58	F	Angioedema	P+L+D	pe, pn	0	0	0	0	—	—
13	33	M	Angioedema	P	pn, pe, ch	1	0	0	2	+	+
14	29	F	OAS	P	pn, pe, m, l	3	0	0	3	+	+
15	30	F	Angioedema	P	pn, pe, m, w	2	0	0	2	+	+
16	26	F	FDEIA	P	a, l, m, pn, ch	0	0	0	0	—	—
17	10	M	OAS	P+D		1	0	0	2	+	+
18	29	F	Anaphylaxis	P	pe, l, pn	1	0	0	2	(+)	+
19	33	M	OAS	P+G	w	0	0	0	0	—	(+)
20	35	M	Angioedema	P	b, av	0	0	0	1	—	+
21	29	F	Anaphylaxis	P	pe	0	0	0	2	—	—
22	25	F	OAS	P+D+C	pe, pn, k, m, l	2	0	0	3	+	+
23	35	M	Urticaria	P	pn	0	0	0	2	—	—
24	7	M	Anaphylaxis	P+D	pe, w, ch, pn, al, m	2	0	0	5	+	+
25	26	F	Anaphylaxis	P	w, pn, b, k, che	2	0	0	2	—	+
26	38	M	Anaphylaxis	P	pe, pn, ch, w, che	1	0	0	2	+	+

FDEIA, Food-dependent exercise-induced anaphylaxis.

*Determined by means of SPT.

†P, Plane; G, grass; C, cat dander; D, dust mite; L, latex.

‡pe, Peach; l, lettuce; pn, peanut; m, maize; ch, chestnut; t, tomato; gb, green bean; a, apple; c, celery; w, walnut; al, almond; r, rice; che, cherry; k, kiwi; b, banana; av, avocado.

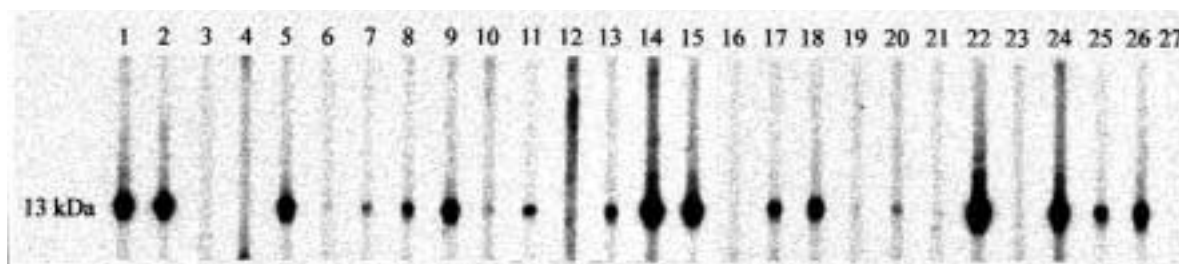


FIG 4. IgE immunoblotting of rCor a 8: lanes 1 to 26, patient sera 1 to 26; lane 27, nonallergic control.

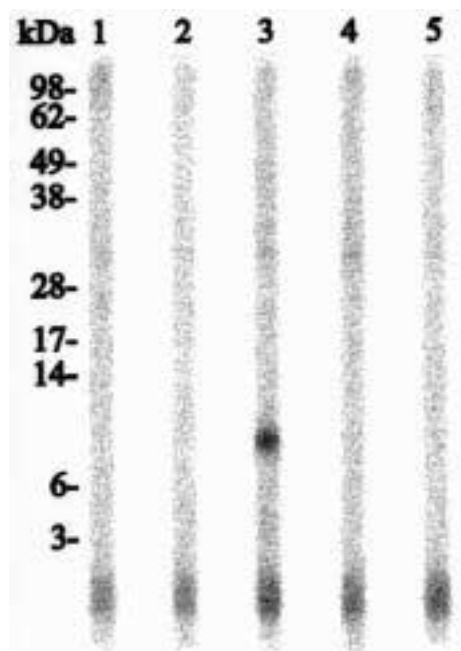


FIG 5. Immunoblot inhibition of immobilized nCor a 8 (20 µg of hazelnut extract per centimeter) with serum pool: lane 1, buffer control; lane 2, nonallergic serum; lane 3, no inhibitor; lane 4, 15 µg of rCor a 8; lane 5, 700 µg of hazelnut extract.

those reporting mild symptoms. A 9-kd major allergen in hazelnut extract was recognized by IgE from 16 (62%) of 26 of the study subjects. Both partial purification followed by N-terminal sequencing and PCR-based cloning identified this major allergen as hazelnut LTP, Cor a 8. Using rCor a 8 for IgE immunoblotting revealed superior diagnostic sensitivity of rCor a 8 compared with the extract because 4 additional subjects had positive IgE to the recombinant allergen, resulting in an IgE prevalence of 20 (77%) of 26. Moreover, several sera showed a stronger band intensity to rCor a 8 compared with the binding at 9 kd to hazelnut extract (Figs 1 and 4). Seven patients with anaphylaxis were monosensitized to Cor a 8 (nos. 7, 8, 9, 11, 18, 25, and 26). Interestingly, several patients with OAS were also monosensitized to LTP, indicating that LTP can trigger symptoms similar to pollen-related allergens in a subgroup of patients. Only one patient was positive to Cor a 1.04, and none to profilin Cor a 2. Two sera bound to high-molecular-weight allergens.

It is remarkable that the hazelnut CAP and EAST results were negative in 5 patients with a history of anaphylaxis, angioedema, or OAS to hazelnut, and a positive SPT and prick-to-prick test response to fresh nuts (nos. 3, 6, 12, 16, and 19), and in 2 cases positive challenge test results (nos. 3 and 16) and that sera from all these patients had negative results in the hazelnut extract immunoblot. These observations suggest a sensitivity problem of the in vitro diagnosis of hazelnut allergy, which is probably caused by important allergens that are weakly expressed in nuts. Because the use of rCor a 1.0401 by Lüttkopf et al⁴ and Cor a 8 in this study led to an increased sensitivity of in vitro diagnosis compared with hazelnut extracts, in the future, this problem might be overcome by additional recombinant hazelnut allergens, such as the recently described 11S globulin Cor a 9 or 2S albumin. The complete inhibition of IgE binding to natural hazelnut LTP after preincubation of the serum pool with recombinant hazelnut LTP confirmed that natural and recombinant hazelnut LTP share identical epitopes.

In this study we determined the complete primary structure of hazelnut LTP by means of cDNA cloning and sequencing. We found a typical LTP with a molecular weight of 9 kd (mature protein). The immature protein consisted of 115 amino acids with a typical signal peptide and cleavage site,²³ resulting in a mature LTP of 92 amino acids. The deduced N-terminus of hazelnut LTP could be verified by means of N-terminal sequencing of natural hazelnut LTP enriched in hazelnut extract by using ion exchange chromatography.

Because high amino acid sequence identities were found with important allergenic LTPs, such as those from almond (62%), apple (Mal d 3, 61%),²⁴ cherry (Pru av 3, 61%),¹⁶ and peach (Pru p 3, 59%),¹¹ IgE cross-reactivity between Cor a 8 and these allergens in Mediterranean patients is to be expected.

Our data also confirm regional differences in sensitization patterns reported for other birch pollen-related foods.^{16,17} As shown in this study, hazelnut LTP is relevant for a majority of patients recruited in Spain. By contrast, in our previous study carried out in a group of 53 patients allergic to hazelnuts with birch pollen allergy from Germany, we were able to show that the majority were sensitized to the pollen-related hazelnut allergen Cor a 1.04 and that only one serum sample from a patient without pollinosis reacted to non pollen-associated hazelnut allergens.²⁰ These results are well in accordance

with the findings of Pastorello et al.⁵ demonstrating that all 7 patients with hazelnut allergy but without birch pollen allergy from Italy had specific IgE antibodies to a 9-kd hazelnut allergen, presumably LTP, in contrast to not one single LTP-positive serum sample out of 65 patients allergic to hazelnuts with birch pollen allergy from Milan, Copenhagen, and Zurich.

Within the investigated patient group, all patients were sensitized to plane pollens (*Platanus acerifolia*). Enrique et al.²⁵ have recently described this clinical association between *Platanus acerifolia* pollinosis and plant-derived food allergy, with a high percentage of patients who are allergic to hazelnuts. Therefore it is also important to bear the existence of LTPs in these pollens as cross-reactive allergenic structures in mind, which has been shown for ragweed pollen and Par j 1 from *Parietaria judaica*.²⁶ This could account for a primary sensitization through the inhalational route and a subsequent cross-reaction with Cor a 8 and might explain the differences of IgE reactivity patterns between patients with hazelnut allergy in Northern and Southern Europe.

In conclusion, our results demonstrated that LTP from hazelnut is a major allergen for Spanish patients allergic to hazelnuts without birch pollen allergy. The hazelnut LTP was denominated as Cor a 8 and has been added to the official World Health Organization/International Union of Immunological Societies Allergen Database. Similar to other recombinant allergens, rCor a 8 was found to be a suitable tool to improve diagnostic sensitivity and to obtain a refined component-resolved diagnosis of a potentially severe food allergy to hazelnut.

We thank Mrs Daniela Warneke and Mr Jürgen Sarau (Forschungszentrum Borstel, Borstel, Germany) for their excellent technical assistance.

REFERENCES

- 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.
- 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.
- Ortolani C, Ballmer-Weber BK, Skamstrup Hansen K, Ispano M, Wüthrich 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.
- Lüttkopf D, Müller U, Skov PS, Ballmer-Weber BK, Wüthrich 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 hazelnut pollen allergen Cor a 1.01. *Mol Immunol* 2001;38:515-25.
- 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.
- Beyer K, Grishina G, Bardina L, Grishin A, Sampson HA. Identification of an 11S globulin as a major hazelnut food allergen in hazelnut-induced systemic reactions. *J Allergy Clin Immunol* 2002;110:517-23.
- Kader JC. Lipid-transfer proteins on plants. *Annu Rev Plant Physiol Plant Mol Biol* 1996;47:627-54.
- Asero R, Mistrello G, Roncarolo D, de Vries SG, Gautier MF, Ciurana CL, et al. Lipid transfer protein: a pan-allergen in plant-derived foods that is highly resistant to pepsin digestion. *Int Arch Allergy Immunol* 2000;122:20-32.
- Fernández-Rivas M, van Ree R, Cuevas M. Allergy to Rosacea fruits without related pollinosis. *J Allergy Clin Immunol* 1997;100:728-33.
- Pastorello EA, Pompei C, Pravettoni V, Brenna O, Farioli L, Trambaioli C, et al. Lipid transfer proteins and 2S albumins as allergens. *Allergy* 2001;56:45-7.
- Pastorello EA, Farioli L, Pravettoni V, Ortolani C, Ispano M, Monza M, et al. The major allergen of peach (*Prunus persica*) is a lipid transfer protein. *J Allergy Clin Immunol* 1999;103:520-6.
- Pastorello EA, Purrello Dambrosio F, Pravettoni V, Farioli L, Giuffrida G, Monza M, et al. Evidence for a lipid transfer protein as the major allergen of apricot. *J Allergy Clin Immunol* 2000;105:371-7.
- Pastorello EA, Farioli L, Pravettoni V, Ispano M, Scibola E, Trambaioli C, et al. The maize major allergen, which is responsible for food-induced allergic reactions, is a lipid transfer protein. *J Allergy Clin Immunol* 2000;106:744-51.
- Díaz-Perales A, Tabar AI, Sanchez-Monge R, Gracia BE, Gomez B, Barber D, et al. Characterization of asparagus allergens: a relevant role of lipid transfer proteins. *J Allergy Clin Immunol* 2002;110:790-6.
- San Miguel-Moncin M, Krahl M, Scheurer S, Enrique E, Alonso R, Conti A, et al. Lettuce anaphylaxis: identification of a lipid transfer protein as the major allergen. *Allergy* 2003;58:511-7.
- Scheurer S, Pastorello EA, Wangorsch A, Kästner M, Hausteiner D, Vieths S. Recombinant allergens Pru av 1 and Pru av 4 and a newly identified lipid transfer protein in the *in vitro* diagnosis of cherry allergy. *J Allergy Clin Immunol* 2001;107:724-31.
- Ballmer-Weber BK, Scheurer S, Fritsche P, Enrique E, Cistero-Bahima A, Haase T, et al. Component-resolved diagnosis with recombinant allergens in patients with cherry allergy. *J Allergy Clin Immunol* 2002;110:167-73.
- European Academy of Allergy and Clinical Immunology Subcommittee of Skin Tests and Allergen Standardization. Skin tests used in type I allergy testing. *Allergy* 1989;44(suppl 10):13-60.
- Dreborg S, Foucard T. Allergy to apple, carrot and potato in children with birch pollen allergy. *Allergy* 1983;38:167-72.
- Schocker F, Lüttkopf D, Müller U, Thomas P, Vieths S, Becker W-M. IgE binding to unique hazelnut allergens: identification of non pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions. *Eur J Nutr* 2000;39:172-80.
- Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970;227:680-5.
- Müller U, Lüttkopf D, Hoffmann A, Petersen A, Becker WM, Schocker F, et al. Allergens in native and roasted hazelnuts (*Corylus avellana*) and their cross-reactivity to pollen. *Eur Food Res Technol* 2000;212:2-12.
- Nielsen H, Engelbrecht J, Brunak S, von Heijne G. Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng* 1997;10:1-6.
- Pastorello EA, Pravettoni V, Farioli L, Ispano M, Fortunato D, Monza M, et al. Clinical role of a lipid transfer protein that acts as a new apple-specific allergen. *J Allergy Clin Immunol* 1999;104:1099-106.
- Enrique E, Cistero-Bahima A, Bartolome B, Alonso R, San Miguel-Moncin MM, Bartra J, et al. *Platanus acerifolia* pollinosis and food allergy. *Allergy* 2002;57:351-6.
- Duro G, Colombo P, Assunta CM, Izzo V, Porcasi R, Di Fiore R, et al. Isolation and characterization of two cDNA clones coding for isoforms of the *Parietaria judaica* major allergen Par j 1.101. *Int Arch Allergy Immunol* 1997;112:348-55.