

Evidence of Cross-Reactivity between Different Seed Storage Proteins from Hazelnut (*Corylus avellana*) and Walnut (*Juglans regia*) Using Recombinant Allergen Proteins

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Food allergy · Seed storage proteins · 2S albumins · Legumins · Walnut · Hazelnut · Cross-reactivity

Abstract

Seed storage proteins are extremely stable allergens in nuts, seeds, and legumes and are responsible for the most severe allergic reactions to these foods. The cross-reactivity between seed storage proteins from different sources has not been studied at a molecular level so far. This study aimed to ascertain the cross-reactivity between walnut and hazelnut seed storage proteins using recombinant allergens. Sera from 13 consecutive patients with severe primary walnut and/or hazelnut allergy and hypersensitive to both nuts were studied. IgE specific for rCor a 9, rCor a 14, and rJug r 1 was measured, and inhibition experiments were carried out by measuring IgE reactivity after absorption of patients' sera with freshly prepared walnut extract. All 13 sera showed strong IgE reactivity against walnut 2S albumin, Jug r 1, 12 reacted to hazelnut 2S albumin, Cor a 14, and 8 to the hazelnut legumin, Cor a 9. In inhibition experiments, absorption

of sera with whole walnut extract led to the complete disappearance of IgE reactivity to Jug r 1 in 12/13 cases, as expected, but also to the complete disappearance of specific IgE to Cor a 14 in 9/12 sera, and of IgE reactivity to Cor a 9 in 7/8. In the remaining cases a dramatic drop in IgE reactivity was observed. The study shows that patients primarily allergic to either walnut or hazelnut showing a skin or serological reactivity to the other nut also are potentially at risk of severe allergic reactions caused by cross-reactivity between 2S albumins and legumins.

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Hazelnut and walnut are by far the most popular tree nuts worldwide. Allergy to these two nuts is frequent in pollen-allergic patients primarily sensitized to birch pollen or strongly sensitized to the plant pan-allergen profilin due to the co-recognition of homologous allergens in walnut and hazelnut by pollen-specific IgE [1, 2]. In Med-

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iterranean countries, walnut and hazelnut allergy occurs quite frequently also in subjects primarily sensitized to the peach nonspecific lipid transfer protein (nsLTP) due to the largely cross-reacting nature of this allergen [3]. In contrast, primary (genuine) allergy to walnut and/or hazelnut is much less common, at least in Italy. In fact, in a multicenter study carried out in 33 Italian allergy centers only 36 allergic patients were recruited over 1 entire year [4]. In patients with a true walnut or hazelnut allergy the clinical consequences of the ingestion of these foods may be extremely severe or even life threatening due to the high stability of the relevant allergen proteins. Several allergens have been detected in walnut and hazelnut so far, including PR-10, profilin, lipid transfer protein, different seed storage proteins (2S albumin, vicilin, legumin) and thaumatin-like protein, although this list seems far from being complete [4]. In the Italian study [4], one third of those primarily sensitized to one of these two tree nuts showed a striking co-sensitization to the other one, suggesting possible co-recognition of homologous allergens. Thus, we carried out the present study with the aim to ascertain the cross-reactivity between walnut and hazelnut seed storage proteins using recombinant allergens.

Sera from 13 consecutive patients (males/females 8/5; mean age 18.7 years, range 5–50 years) diagnosed as having primary walnut or hazelnut hypersensitivity at the three participating allergy centers during the last year were studied. Walnut and/or hazelnut allergy was diagnosed on the basis of a clear-cut clinical history of allergic reaction within 2 h of ingestion of the offending food, confirmed by strong skin reactivity on SPT (ALK-Abellò, Madrid, Spain; 1:20 w/v), and further confirmed by *in vitro* testing. Due to the severity of reported allergic reactions confirmative oral challenges were not considered in patients with a history of systemic reactions. Those with local reactions had a history of multiple reactions induced by the same food, had spontaneously stopped eating it, and refused confirmative oral challenges. In all cases the mean diameter of the wheal produced on SPT exceeded 8 mm. Exclusion criteria included the reactivity to at least one out of PR-10, profilin, and/or nsLTP panallergen superfamilies, and/or cross-reactive carbohydrate determinant recognition. The clinical features of the study population are shown in Table 1. All patients had a history of severe reactions following the ingestion of either walnut ($n = 11$) or hazelnut ($n = 6$). Four patients had a history of allergic reactions to both nuts; in 2 cases allergy started with walnut and hazelnut, respectively, in 1 case allergy to the two tree nuts started at the same time, while in 1 case this information was not available because clinical allergy started

in infancy. In the remaining 9 cases the tolerance to the tree nut other than the offending one was unknown due to the fact that the patients had stopped eating it after experiencing the initial allergic reaction. Specific IgE to the allergenic components Cor a 9 (hazelnut 11S globulin, also called legumin), Cor a 14 (hazelnut 2S albumin), and Jug r 1 (walnut 2S albumin) (all produced by Thermo-Fisher Scientific) were measured by ImmunoCAP (Thermo-Fisher Scientific). The study allergens are the only recombinant seed storage proteins from hazelnut and walnut currently available on ImmunoCAP. Specific IgE levels >0.35 kU/L were considered positive. ImmunoCAP inhibition experiments were carried out by measuring IgE reactivity before and after absorption of patients' sera with freshly prepared walnut. In these experiments the immunoadsorbent was prepared coupling walnut extract on CNBr-activated Sepharose according to the manufacturer's instructions [4]. Briefly, 26 mg of walnut extract was added to 6 mL of the CNBr-activated Sepharose 4B resin in 0.1 M sodium bicarbonate NaHCO_3 , pH 8.3, containing 0.5 M NaCl (coupling buffer) under mild overnight stirring at 4°C. The excess ligand was washed away with 5 vol of coupling buffer. For blocking any remaining active groups, the medium was transferred into the 0.1 M Tris-HCl buffer, pH 8.0 for 2 h. The resulting suspension was washed with 3 cycles of 0.1 M sodium acetate, pH 4.0, with 0.5 M NaCl, followed by 0.1 M Tris-HCl, pH 8.0, with 0.5 M NaCl. The coupled walnut-Sepharose was then transferred to a column in phosphate-buffered saline. Undiluted patients' sera were added to the immunoadsorbent and stirred overnight at 4°C to eliminate walnut-specific IgE. The residual sera were then retrieved and newly retested by ImmunoCAP assay to verify their IgE-binding capacity against hazelnut allergens. All the patients (or their parents) gave an informed written consent to the use of their sera in an anonymous form for this investigation. The study protocol was approved by the Internal Review Board of participating clinical centers.

Results are summarized in Table 1. All 13 patients showed strong IgE reactivity against Jug r 1 with specific IgE levels ranging between 1.39 and 33.1 kU/L. As expected, serum absorption with whole walnut extract led to complete disappearance of IgE reactivity in all 13 cases. IgE to hazelnut 2S albumin, Cor a 14, was detected in 12/13 sera with levels ranging between 0.41 and 43.7 kU/L. The absorption of sera with whole walnut extract led to a complete disappearance of specific IgE to Cor a 14 in 9/12 sera, while the 3 remaining sera, 2 of which exhibited the highest baseline-specific IgE levels, showed a dramatic drop of IgE reactivity (Table 1).

Table 1. Clinical features of the study patients and specific IgE measurements

No.	Sex/age	Offending food	Symptoms	Cor a 9		Cor a 14		Jug r 1	
				pre	post	pre	post	pre	post
1	M/50	W	U-A	10.30	0.02	0.41	0	9.22	0.02
2	M/5	WH*	U	0.04	0.01	1.04	0	5.65	0.07
3	F/20	H	U-A	84.50	10.40	43.70	1.09	1.39	0.01
4	F/10	H	U-A	3.51	0.13	12.60	0.12	5.27	0.04
5	M/11	WH	U	3.81	0.06	2.49	0.25	2.85	0.08
6	M/22	W	U	1.22	0.03	3.78	0.02	4.15	0.08
7	M/36	W	Anaph	0.27	0.09	2.50	0.82	2.96	0.08
8	M/24	W	U-A	0.03	0.03	0.53	0.08	15.60	0.21
9	F/8	W	A: mouth	15.60	0.00	6.76	0	33.10	0.22
10	F/7	W*H	U-A	2.66	0.28	0.92	0	7.72	0.01
11	M/9	W	Anaph	0.25	0.01	0.24	0.03	5.12	0.05
12	M/9	W	U	17.00	0.22	31.32	1.99	30.60	0.18
13	F/24	WH	A: mouth/pharynx	0.35	0.00	1.87	0	1.72	0.02

Food: W, walnut; H, hazelnut. In patients with a clinical history of allergy to both foods an asterisk shows the foods that started the allergy (if known). *Symptoms:* U-A, generalized urticaria-angioedema; U, generalized urticaria; A, angioedema; Anaph, anaphylaxis. *Specific IgE levels:* pre, before serum absorption with whole walnut extract; post, after serum absorption with whole walnut extract. IgE levels are expressed as kU/L.

Eight sera also showed elevated IgE reactivity to Cor a 9, the hazelnut legumin, with levels ranging between 1.22 and 84.5 kU/L. Again, the absorption of sera with walnut extract led to a complete disappearance of IgE reactivity in 7/8 sera and to a drop from 84.5 to 10.4 kU/L in the remaining case (Table 1).

Although cross-reactivity seems more common between nuts from the same taxonomic family [5–7], possible co-recognition of taxonomically different nuts is found in about one third of allergic patients [8]. Specifically, the cross-reactivity between stable allergens in walnut (*Juglans regia*) and hazelnut (*Corylus avellana*), two botanically correlated tree nuts as they both belong to *Fagales* order, has been suspected for a long time, but hypersensitivity to walnut, hazelnut, and Brazil nut (*Ericales* order) in the same patient has been reported as well [9]. In this study, the use of recombinant allergens allowed us to demonstrate that the cross-reactivity between hazelnut and walnut seed storage proteins exists and that it occurs at the level of both 2S albumins and 11S globulins (legumins). Since not all patients primarily allergic to walnut or hazelnut show a co-sensitization to the other tree nut [4], it is probable that not all allergenic epitopes of seed storage proteins are equally conserved from a phylogenetic point of view. In effect, the alignment of amino acid sequences of 2S albumins and legumins from walnut and hazelnut shows an identity ranging between 61.8 and 71.7%, respec-

tively (Uniprot data, see online suppl. Fig. 1; see www.karger.com/doi/10.1159/000492399 for all online suppl. material). Seed storage proteins (including 2S albumins, vicilins, and legumins) are extremely stable plant proteins responsible for the most severe food-induced allergic reactions in adults worldwide. Some studies concluded that cross-reactivity between homologous seed storage proteins from botanically unrelated allergen sources is rather uncommon and variable [10]. However, as early as in 2001 Sicherer [6] reported that the probability of nut-allergic subjects to cross-react clinically to botanically unrelated nuts exceeds 35%. Our study shows that patients primarily allergic to either walnut or hazelnut showing a skin or serological reactivity to the other nut are also potentially at risk of severe allergic reactions caused by cross-reactivity between several seed storage proteins.

Disclosure Statement

The authors declare the absence of any conflict of interest and of any financing of the present study.

Author Contributions

D.V., E.S., and R.A. conceived the study and selected the study patients. D.V., E.S., S.A., and G.M. performed the in vitro work (IgE measurements and absorption of sera for inhibition experiments). R.A. wrote the manuscript.

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[illegible]

Cor a 14 (D0PWG2) vs Jug r 1 (P93198); Identity: 61,8% (Identical position: 92; Similar positions: 30)

Legumin (11s Globulin)



Suppl. Fig. 1. Comparison between 2S-albumins (A) and 11S-globulins (B) from walnut and hazelnut (UniProt data).