

IgE crossreactivity between mugwort pollen (*Artemisia vulgaris*) and hazelnut (*Abellana nux*) in sera from patients with sensitivity to both extracts

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Summary

Background An association between sensitization to *Compositae* pollens and hypersensitivity to hazelnut has been previously described. There is no previous *in vitro* study about crossreactivity between mugwort pollen and hazelnut.

Objectives To study mugwort pollen and hazelnut allergens and to assess if there is IgE crossreactivity between mugwort pollen and hazelnut.

Methods A serum pool formed by 28 individual sera with specific IgE to mugwort pollen and hazelnut was used to investigate IgE crossreactivity. RAST-inhibition, SDS-PAGE/IEF immunoblotting inhibition assays were performed by preincubation of the sera with mugwort pollen and hazelnut.

Results RAST to hazelnut was inhibited up to 63% by mugwort pollen, but the mugwort pollen RAST was only inhibited up to 36% by hazelnut. In SDS-PAGE immunoblotting mugwort pollen showed nine allergens ranging from <16 to 65 kDa and hazelnut had four main allergens: 42 kDa, 17 kDa and <16 kDa (two bands). In the SDS-PAGE immunoblotting inhibition hazelnut partially inhibited all the mugwort pollen bands, except that with 19 kDa, whereas mugwort pollen produced a nearly total inhibition of all the hazelnut allergens. In isoelectrofocusing immunoblotting mugwort pollen had two groups of allergens: pI 7.5–8.5 and pI 3.5–5.2 and hazelnut one group of allergens: pI 5.2–5.8. In the isoelectrofocusing immunoblotting inhibition hazelnut produced a partial inhibition of all the bands of mugwort pollen and mugwort pollen partially inhibited all the allergenic bands of hazelnut.

Conclusions The RAST and SDS-PAGE/IEF immunoblotting inhibition results provide evidence of IgE cross reactivity between mugwort pollen and hazelnut allergens. The inhibition of hazelnut by mugwort pollen is higher than the inhibition of mugwort pollen by hazelnut in both RAST inhibition and SDS-PAGE immunoblotting inhibition. These results suggest that mugwort pollen allergens would behave as primary immunogens in the association between sensitivity to mugwort pollen and hazelnut.

Keywords: *Artemisia vulgaris* pollen, hazelnut, cross reactivity, allergens, IgE

Clinical and Experimental Allergy, Vol. 27, pp. 1203–1211. Submitted 1 August 1996; revised 28 January 1997; accepted 11 March 1997.

Introduction

Different associations between pollinosis and sensitization to edible vegetables have been published during the last three decades. Therefore, there have been reports about the

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association between ragweed pollinosis and hypersensitivity to melon and banana [1,2], birch pollinosis and sensitization to hazelnut, apple, carrot, potato and other vegetables and kiwi [3–8] and mugwort pollinosis and sensitivity to celery, carrot, certain spices and legumes [9–12]. Other authors have published details about an association between grass pollinosis and sensitization to tomato, green-pea, peanut,

apple, potato, watermelon, melon and orange [13–16] and, between plantain sensitization and melon hypersensitivity [16]. In a previous study we described an association between sensitivity to mugwort pollen and sensitivity to some foods of vegetable origin, such as sunflower seed, chestnut, hazelnut, mustard and others [17]. To the best of our knowledge, there are no other references about IgE cross reactivity between hazelnut and mugwort pollen. In this study we performed RAST, SDS-PAGE immunoblotting and IEF immunoblotting inhibition assays to assess if there is crossreactivity between mugwort pollen and hazelnut.

Materials and methods

Selection of sera reacting with mugwort pollen and hazelnut

Serum samples were obtained from the Allergy Laboratory of Hospital 'La Paz'. Selection of sera with IgE antibodies reacting with both mugwort pollen and hazelnut was carried out by testing all the sera with high serum specific IgE to mugwort pollen for their positivity to hazelnut and vice-versa. Sera with specific IgE higher or equal to class 3 (3.5 U/L), measured by means of CAP-System FEIA (Pharmacia-Upjohn, Sweden), to both allergens were selected. No attempt was made to make a selection on a clinical basis. All sera were kept frozen at -20°C until use.

We used four serum pools (I–IV):

Serum pool I: We selected 28 sera from patients with IgE antibodies to both mugwort pollen and hazelnut (mugwort pollen $\geq$ CAP 3; hazelnut $\geq$ CAP 3) and mixed them. The total IgE of serum pool I was 1021 KU/L (IMx, Abbot Diagnostics, Chicago, Illinois, USA) and the specific IgE was 15.5 U/L to mugwort pollen and 12.4 U/L to hazelnut (CAP-System FEIA).

Serum pool II: It was formed by 11 sera with specific IgE to mugwort pollen (mugwort pollen $\geq$ CAP 3) and without specific IgE to hazelnut (hazelnut = CAP 0). The total IgE was 469 U/L and the specific IgE was 5.0 U/L to mugwort pollen and negative to hazelnut.

Serum pool III: It was constituted by seven sera with specific IgE to hazelnut (hazelnut $\geq$ CAP 3) and without specific IgE to mugwort pollen (mugwort pollen = CAP 0). The total IgE was 841 U/L and the specific IgE was negative to mugwort pollen and 15.1 U/L to hazelnut.

Serum pool IV: We also used a negative control serum pool, formed by 10 sera from atopic patients without specific IgE to pollens or vegetables (mugwort pollen = CAP 0; hazelnut = CAP 0). The total IgE of the resultant serum pool was 456 KU/L.

Allergen extracts

Raw hazelnuts were purchased from a local food store. Hazelnuts (200 g) were peeled, chopped into small pieces

and then ground. Extraction was performed by incubation in pH 8.0 0.05 M ammonium carbonate at 20% (w/v) during 4 h at 4°C with constant magnetic stirring. The mixture was filtered through a sterile gauze and subsequently defatted with ether. After centrifugation (three times for 20 min at 5800 g at 4°C) the supernatant was collected and dialyzed for 24 h at 4°C against 10 mM glycine. The dialyzed extract was frozen, lyophilized and stored at -20°C until use. The protein content of the hazelnut extract, assessed according to the method of Lowry *et al.* [18], was 62 mg per 100 mg of dry weight material. The mugwort pollen extract was kindly provided by Alergia e Inmunología Abelló (Madrid, Spain) and it was prepared as previously described [19].

RAST inhibition

Hazelnut and mugwort pollen extracts were coupled to cyanogen-bromide activated paper disks according to procedure of Ceska *et al.* [20].

RAST inhibition was performed according to Gleich *et al.* [21] in serum pools I, II and III. Briefly, 50 μL of the serum pool were incubated under continuous shaking overnight with 50 μL of different concentrations of mugwort pollen, hazelnut or phosphate buffered saline. By pilot titration experiments, these concentrations were found to be ideal for our assays. They were centrifuged at 4800 g at 4°C for 20 min and subsequently, RAST to hazelnut and mugwort disks was performed in the supernatants by the Phadebas RAST method (Pharmacia, Sweden). In each experiment the dose–response curves and regression lines were calculated. Parallel-line assay was used to analyse the linearity and the parallelism of the RAST inhibition curves. The antigen concentration necessary to obtain a 50% inhibition was also calculated.

One-dimensional electrophoresis (SDS-PAGE)

Protein separation was accomplished by polyacrilamide slab-gel electrophoresis in the presence of sodium dodecyl sulphate (SDS-PAGE) according to Laemmli's method [22]. A Protean II slab cell apparatus (BioRad, Chicago, USA) and a pH 8.3 Tris-glycine buffer were used. Polyacrilamide gel concentration was 5% for the stacking gel and 15% for the running gel. Electrophoretic migration was performed at 2.5°C and at 250 V constant voltage (stacking gel) and 80 V constant voltage (running gel) overnight until completion. Proteins bands were visualized by Coomassie brilliant blue R250 staining. Molecular weight markers were purchased from Bio-Rad (110, 84, 47, 33, 24, 16 kDa) and were included in each electrophoretic separation.

Analytical isoelectric focusing

Hazelnut and mugwort pollen extracts were subjected to

electrofocusing in 5% polyacrilamide gels in the presence of Ampholine at a pH range between 3.5 and 9.5 (Ampholine PAGplate; LKB Pharmacia, Uppsala, Sweden) in the model 2117 Multiphor cell (LKB Pharmacia) following the manufacturer's instructions. Electrofocusing was performed at 2.5°C and at 12 W constant power for 90 min. This procedure separated the proteins of the extracts according to their individual isoelectric points. The proteins were visualized by Coomassie brilliant blue R250 staining.

Immunoblotting

Immunoblotting was performed according to Towbin *et al.* [23] with serum pools I and IV. Mugwort pollen and hazelnut were subjected to SDS-PAGE or IEF and then electroblotted onto Trans-Blot nitrocellulose membranes (BioRad) in a Trans-blot cell system (BioRad) with a pH 8.3, 25 mM Tris, 20% glycine, 20% methanol buffer for SDS-PAGE and 0.7% acetic acid buffer for IEF. Transfer was performed at 550 mA for 2 h at 2.5°C. The quality of the transfer was checked by nitrocellulose staining with 0.15%

Amido Black. The nitrocellulose sheet was blocked (5% non-fatty milk, in phosphate buffered saline) at 37°C. After washing, the strips were incubated overnight with the serum pools diluted 1:3 in pH 7.2 phosphate buffered saline (PBS). The blots were washed extensively with 0.5% Tween 20 in PBS and subsequently incubated overnight with ^{125} I-labelled antihuman IgE (Pharmacia). A control strip which had only been incubated with ^{125} I-labelled antihuman IgE was routinely included. After extensive washing with 0.5% Tween 20 in PBS, the strips were dried, mounted on a cardboard support and loaded into a Curix cassette (Afga-Gevaert, Mortsel, Belgium) with photographic film (Kodak, Rochester, NY, USA) and exposed at -70°C for 14 days.

Immunoblotting inhibition

Aliquots of serum pool I were incubated overnight with equal volumes of hazelnut (1000 µg/mL) and mugwort

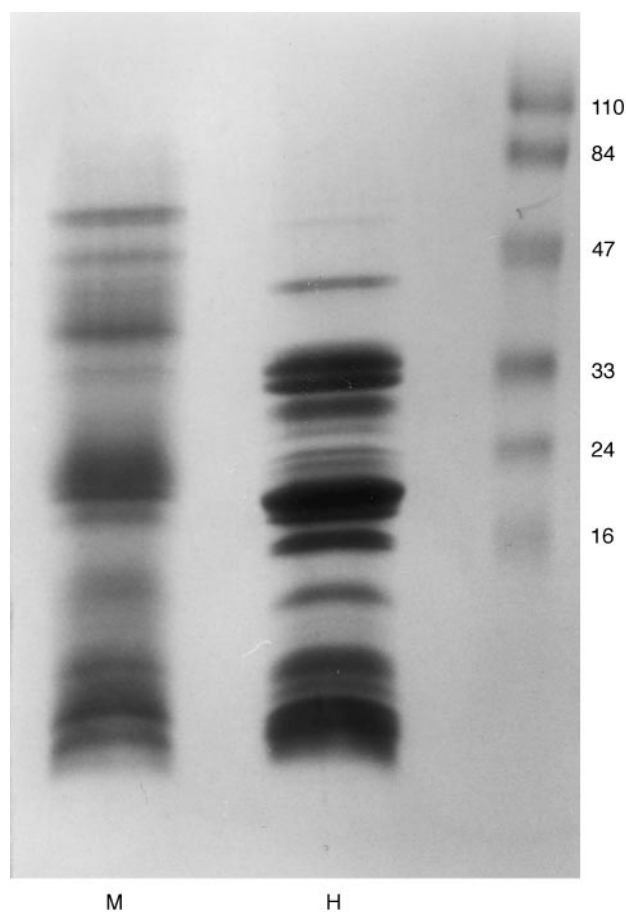


Fig. 1. SDS-PAGE of mugwort pollen (M) and hazelnut (H).

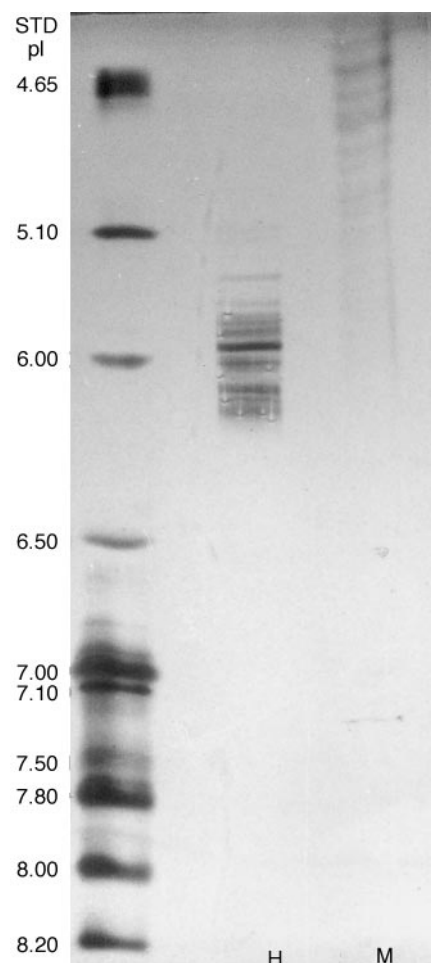


Fig. 2. IEF of mugwort pollen (M) and hazelnut (H).

pollen (500 µg/mL). Mixtures were then centrifuged and their supernatants incubated separately with both hazelnut and mugwort pollen blotted nitrocellulose strips, followed by immunodetection as described above.

Results

SDS-PAGE (Fig. 1)

Mugwort pollen: 14 antigens are detected with molecular weights between 63 and <16 kDa. Hazelnut: 15 antigens are observed with molecular weights between 56 and less than 16 kDa.

IEF (Fig. 2)

Mugwort pollen: eight antigens with pI between 5.06 and <4.65 can be seen. Hazelnut: 10 antigens are observed with pI between 5.45 and 6.16.

SDS-PAGE immunoblotting (Fig. 3)

Serum pool I Hazelnut: Four allergens were detected with molecular weights: 42, 17 and two bands with less than 16 kDa. Mugwort pollen: nine allergens were observed with molecular weights: 65, 50, 37, 33, 19 and four bands with less than 16 kDa.

IEF immunoblotting (Fig. 4)

Serum pool I Hazelnut: there was only a group of allergens with isoelectric point 5.2–5.8. Mugwort pollen: there were two groups of allergens with isoelectric points 3.5–5.2 and 7.5–8.5, respectively.

RAST inhibition

Serum pool I: the mugwort pollen RAST was maximally inhibited 86% by mugwort pollen (500 µg/mL) and 36% by hazelnut (1 mg/mL); the maximum inhibition of hazelnut RAST was 78% by hazelnut (1 mg/mL) and 63% by

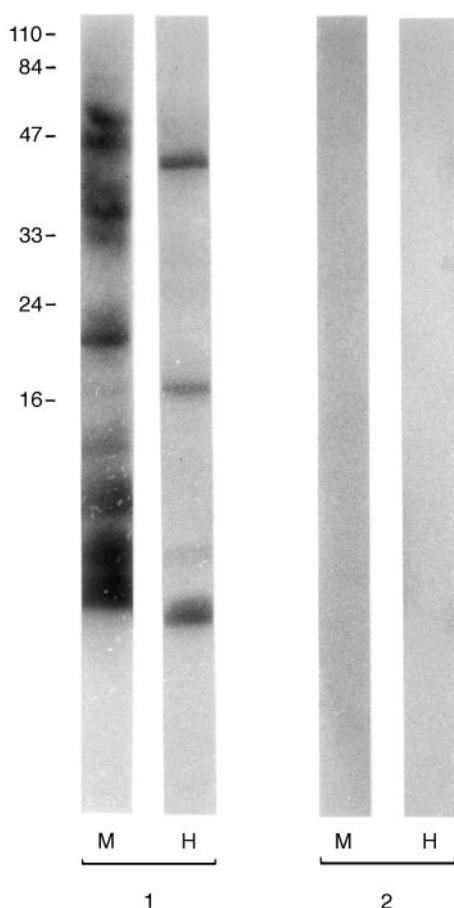


Fig. 3. SDS-PAGE of mugwort pollen (M) and hazelnut (H) followed by immunoblotting with serum pools I (1) and IV (2).

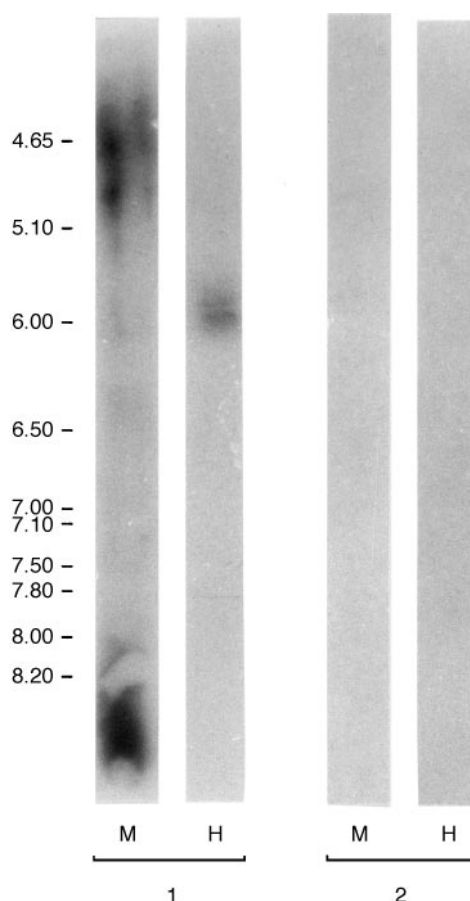


Fig. 4. IEF of mugwort pollen (M) and hazelnut (H) followed by immunoblotting with serum pools I (1) and IV (2).

mugwort pollen (500 $\mu\text{g/mL}$). Concentrations of antigen necessary to obtain a 50% inhibition of the RAST were: 17 $\mu\text{g/mL}$ of mugwort pollen for mugwort pollen RAST, 51 $\mu\text{g/mL}$ of hazelnut for hazelnut RAST and 235 $\mu\text{g/mL}$ of mugwort for hazelnut RAST (Fig. 5). The inhibition of mugwort RAST by hazelnut did not reach the 50% inhibition. The study of parallelism showed no significant difference between the slopes of the regression lines (Student's *t*-test).

Serum pool II: the IgE binding to mugwort pollen was autoinhibited a 95.3% by mugwort pollen. Hazelnut extract (1000 $\mu\text{g/mL}$) did not inhibit the mugwort pollen RAST (Fig. 6).

Serum pool III: the hazelnut RAST was inhibited a 74% by the own hazelnut, but could not be inhibited by pre-incubation of the serum pool with mugwort pollen (1000 $\mu\text{g/mL}$) (Fig. 7).

SDS-PAGE immunoblotting inhibition

Serum pool I (Fig. 8) The IgE binding to mugwort pollen extract was highly inhibited by hazelnut extract. Hazelnut completely inhibited the bands between 33 and 65 kDa and partially the bands with <16 kDa in mugwort pollen, but

only weakly the 19 kDa band. Mugwort pollen produced a nearly complete inhibition of IgE binding of all the hazelnut allergenic bands.

IEF immunoblotting inhibition (Fig. 9)

Serum pool I The IgE binding to mugwort pollen extract was highly inhibited by hazelnut extract, disappearing nearly totally the two groups of allergens (acid and basic). The IgE-binding to hazelnut extract was inhibited by preincubation of the serum pool with mugwort pollen, disappearing almost totally the allergen with pI 5.2–5.8.

Discussion

The interest in the association between pollinosis and vegetable hypersensitivity comes from 1942, when Juhlin–Dannfeldt described for the first time the association between birch pollinosis and fruit or nuts hypersensitivity [24]. Since then several studies have studied in depth the possible explanations for this association. First, Bet v 1, a pathogenesis related protein, was shown responsible for cross reactivity between birch pollen and vegetable foods [25]. Secondly, profilin, a protein that controls actin polymerization in eucariotic cells, was involved in cross-reactivity between different pollens (birch, timothy grass,

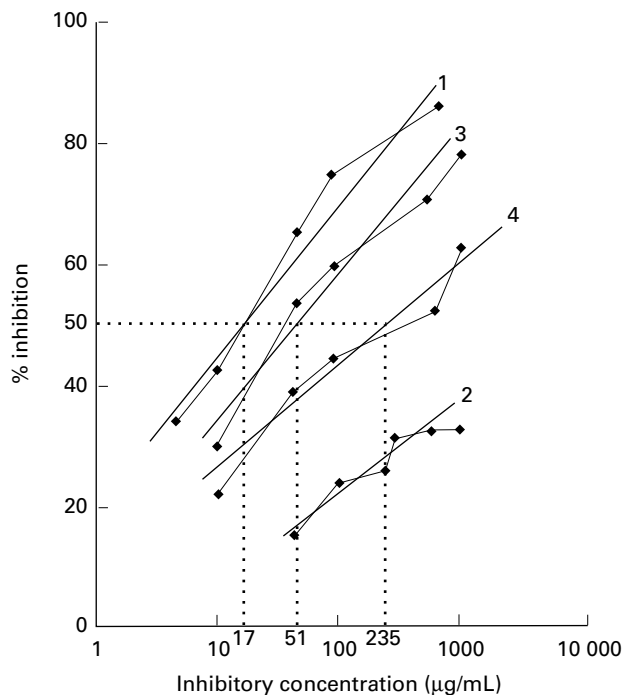


Fig. 5. Mugwort pollen and hazelnut RAST inhibition in serum pool I. 1, Mugwort pollen RAST inhibited by mugwort pollen. 2, Mugwort pollen RAST inhibited by hazelnut. 3, Hazelnut RAST inhibited by hazelnut. 4, Hazelnut RAST inhibited by mugwort pollen. 50% inhibitory concentrations are shown with spotty lines.

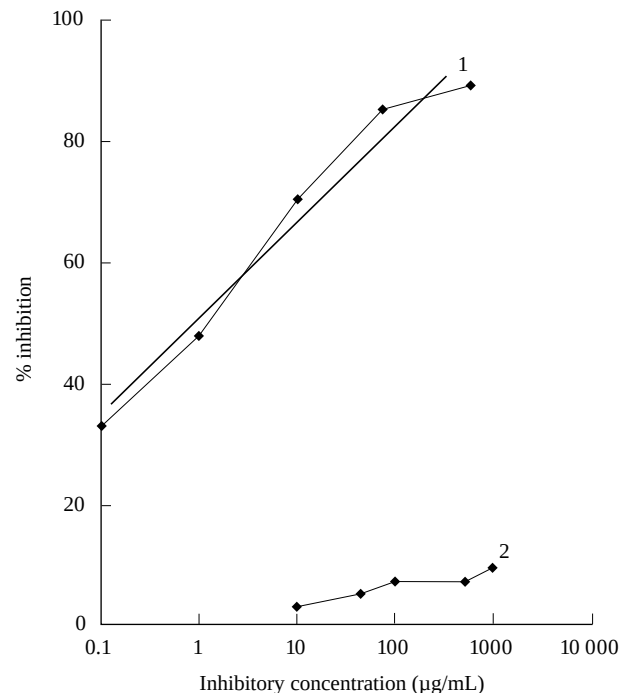


Fig. 6. Mugwort pollen RAST inhibition in serum pool II. 1, Mugwort pollen RAST inhibited by mugwort pollen. 2, Mugwort pollen RAST inhibited by hazelnut.

mugwort, ...) and vegetable foods [26]. Finally, carbohydrate structures on glycoproteins (cross reactive carbohydrate determinants: CCD) were also involved in cross reactivity between pollens and edible vegetables [27].

The observation of a high frequency of associated sensitizations between mugwort pollen and hazelnut in our everyday practice was confirmed in a previous study about association between pollinosis and vegetable sensitization [17]. So we designed this study to confirm the presence of common allergens between mugwort pollen and hazelnut.

Some authors had previously reported the association between birch pollinosis and hazelnut hypersensitivity [3,5]. However, only a few *in vitro* studies confirmed the existence of common allergens between hazelnut and birch pollen [28–31] or hazelnut pollen [31]. On the other hand, several papers described the existence of cross reactivity between mugwort pollen and some vegetables, such as celery, carrot, spices and legumes [9–12]. As far as we know, there are no previous *in vitro* studies about IgE cross reactivity between mugwort pollen and hazelnut.

Allergens

We detected nine allergens in *Artemisia vulgaris* pollen extract by means of SDS-PAGE immunoblotting with

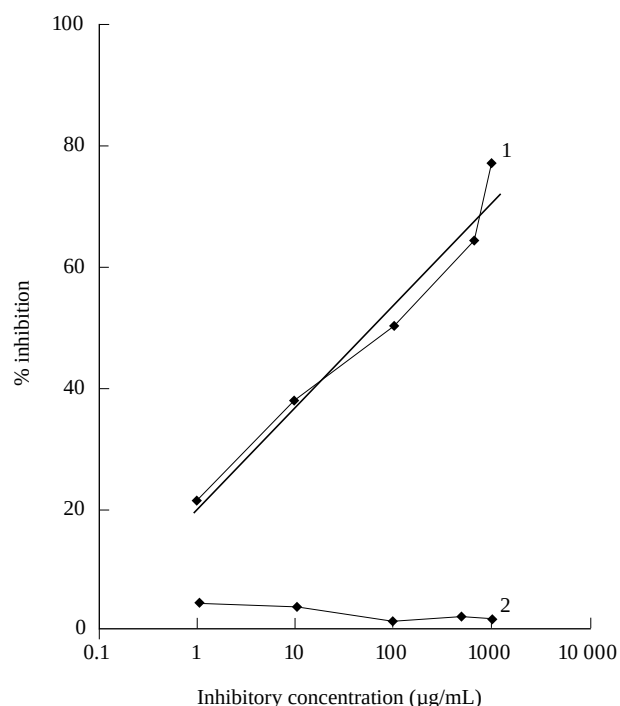


Fig. 7. Hazelnut RAST inhibition in serum pool III. 1, Hazelnut RAST inhibited by hazelnut. 2, Hazelnut RAST inhibited by mugwort pollen.

serum pool I. In previous studies 10 allergens [19,32] or 15 allergens [33] could be demonstrated in the soluble fraction of *Artemisia vulgaris* pollen. Dreborg *et al.* found 10 allergens with molecular weights between 10 and 90 kDa [32]. On the other hand, Nilsen *et al.* detected at least 15 allergenic bands with molecular weights between 12 and 80 kDa [33]. Lastly, de la Hoz *et al.* detected 10 allergens with molecular weights between <14 kDa and 100 kDa [19]. The range of molecular weights of *Artemisia vulgaris* pollen allergens is similar in all the studies published. In this study we did not find allergens with molecular weights over 65 kDa. This discrepancy could be due to differences in the pollen extracts (variations in the extraction process), in the concentrations of the gels or in the sera used. The 50 kDa allergen could correspond to Art v 1, with a reported MW 47–60 kDa [19]; the 19 kDa allergen could be Art v 2, with MW 20 kDa [34]. Lastly, the 33 kDa allergen could correspond to Art v 3, with MW 30–35 kDa [35]. In IEF

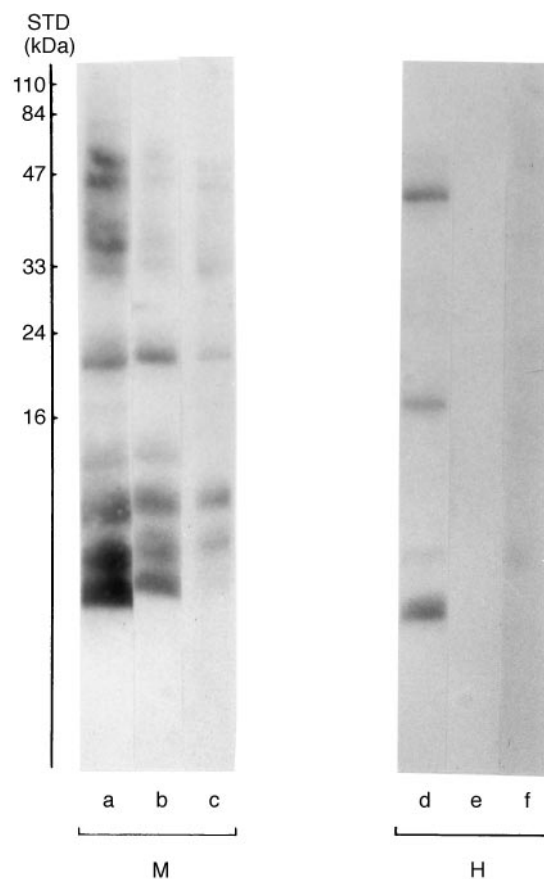


Fig. 8. SDS-PAGE immunoblotting inhibition of mugwort pollen (M) and hazelnut (H). a, mugwort pollen + PBS; b, mugwort pollen inhibited by hazelnut; c, mugwort pollen inhibited by mugwort pollen; d, hazelnut + PBS; e, hazelnut inhibited by hazelnut; f, hazelnut inhibited by mugwort pollen.

immunoblotting two groups of allergens were recognized in mugwort pollen by serum pool I, one basic (pI 7.5–8.5) and other acid (pI 3.5–5.2). These results are similar to those published by de la Hoz *et al.* [19].

Four allergens from hazelnut were detected in SDS-PAGE immunoblotting with serum pool I. Although hazelnut has been described as one of the main food allergens, there is only one published study about its allergens [31]. In this paper Hirschwehr *et al.* [31] found that the main hazelnut allergen had 18 kDa. Other minor allergens had 69, 46, 40, 39 and 14 kDa. The 17 kDa hazelnut allergen of our study could correspond to the 18 kDa allergen described by Hirschwehr *et al.* in hazelnut. These authors demonstrated that the 18 kDa allergen from hazelnut corresponded to a 17 kDa allergen from hazelnut pollen and that both were related to Bet v1, as the preincubation with rBet v1 inhibited the IgE binding to this allergen in the SDS-PAGE immunoblotting. In IEF immunoblotting one group of allergens with pI 5.2–5.8 is detected in hazelnut. To the best of our

knowledge there is no other study that analyze the pI of all the hazelnut allergens. Only Hirschwehr *et al.* [31] studied the pI of the 18 kDa hazelnut allergen, that had three isoforms with pI 5.7, 6.1 and 6.3, different from the pIs found in this study. These differences in the hazelnut allergens recognized in the two papers could be due to variations in the selection of sera.

Crossreactivity

The results from the RAST inhibition assays in serum pool I confirm that mugwort pollen and hazelnut share some allergenic determinants. RAST inhibition results with serum pool II and III discard an unspecific inhibition of IgE binding to mugwort pollen and hazelnut.

Though there is a reciprocal inhibition between both extracts, the mugwort pollen extract behaves as a more potent inhibitor than the hazelnut extract. The fact that the pollen act as a more potent inhibitor than the vegetable food had been previously reported [12,13,30,31,36–39]. Some authors suggested that this might be due to a loss of activity of the food extracts used [12]. Others proposed that all the allergens of the food extracts would be contained in the pollen extracts but not vice versa [37]. Most authors pointed out that the common allergens would behave as major allergens in the edible vegetables and minor allergens in the pollens [13,30,38,39]. However, it was shown subsequently that some of the common allergens behaved really as major allergens in the pollen extracts (Bet v 1, Cor a 1) [31]. Thus, Hirschwehr *et al.* pointed out that the allergens responsible for the cross reactivity in edible vegetables had less antigenic determinants than those corresponding to pollens (Cor a 1, Bet v 1). This was confirmed in later studies with purified allergens [40,41]. Only Enberg *et al.* showed some discrepant results with respect to the reciprocity of the inhibition between pollens and vegetable foods [2]. They described a reciprocal inhibition between ragweed pollen and watermelon. This suggests that in this model the allergens responsible for cross reactivity had the same relevance in both ragweed pollen and watermelon. According to our data mugwort pollen would be a more potent inhibitor than hazelnut. Then, the allergens responsible for cross reactivity between mugwort pollen and hazelnut would have less immunogenic determinants in hazelnut than in mugwort pollen and mugwort pollen allergens would behave as primary immunogens in the association between sensitivity to mugwort pollen and hazelnut.

The fact that in SDS-PAGE immunoblotting inhibition mugwort pollen extract inhibited the IgE binding to several allergens and vice versa, together to the finding that the molecular weights of hazelnut and mugwort pollen allergens are not coincident do not allow us to identify a specific

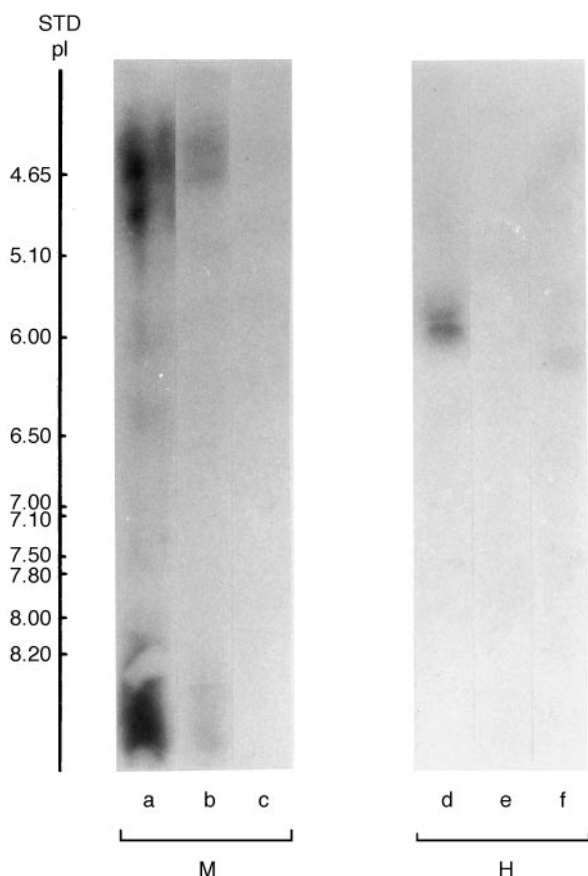


Fig. 9. IEF immunoblotting inhibition of mugwort pollen (M) and hazelnut (H). a, mugwort pollen + PBS; b, mugwort pollen inhibited by hazelnut; c, mugwort pollen inhibited by mugwort pollen; d, hazelnut + PBS; e, hazelnut inhibited by hazelnut; f, hazelnut inhibited by mugwort pollen.

allergen as the responsible for the crossreactivity between both extracts. Lombardero *et al.* [42] had suggested previously that the cross reactivity between different pollens (Chenopodiaceae and Amaranthaceae) could be due to the existence of common epitopes in different allergens with different molecular weights. Recently, it has been shown that different allergens from the same pollen (Lol p 1, Lol p 2/3) had common epitopes for B cells, that would be responsible for the IgE cross reactivity among those allergens from a single pollen [43]. Then, we hypothesize that in this model the existence of cross-reactivity could be due to the presence of common epitopes among the diverse allergens of hazelnut and mugwort pollen. More studies should be conducted in order to assess this hypothesis.

Nevertheless, the 19 kDa allergen of mugwort pollen and the 17 kDa allergen in hazelnut, that we found in this study, could correspond to the 18 kDa allergen reported in hazelnut by Hirschwehr *et al.* and related to Bet v1 [31]. However, the finding that the 19 kDa allergen of mugwort pollen is poorly inhibited by hazelnut plus the observation that the pIs of hazelnut allergens in this study differ from those of the 18 kDa hazelnut allergen in Hirschwehr's *et al.* study [31] are against this hypothesis. On the other hand, one of the allergens with MW <16 kDa could correspond to profilin, an allergen with 14–15 kDa reported as responsible for cross reactivity between pollens and vegetables [26].

In summary, the RAST and immunoblotting inhibition results confirm the existence of cross reactivity between mugwort pollen and hazelnut, due to the presence of several common epitopes that could be shared by different allergens.

Acknowledgments

The authors are indebted to Francisco de la Hoz and José Carreira, from Alergia e Inmunología Abelló (Madrid, Spain) for providing the mugwort pollen extract. This study was supported in part by Fondo de Investigaciones Sanitarias (Grant 93/0731).

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