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ORIGINAL CONTRIBUTION

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IgE binding to unique hazelnut allergens: Identification of non pollen-related and heat-stable hazelnut allergens eliciting severe allergic reactions

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Abstract *Background:* Usually hazelnut allergic patients suffer from the tree pollen associated oral allergy syndrome (OAS) caused by cross-reactive structures.

Anaphylactic reactions elicited by hazelnuts happen rarely but are of high clinical significance. Considering that hazelnuts are ingredients in processed foods, hazelnuts may play an important role as hidden allergens for these high risk patients. Therefore, we analyzed the IgE reactivity of a young woman with severe allergic reactions after ingestion of hazelnuts without any association to tree pollen allergy.

Aim of the study: The aim of this study was to identify and characterize these potent hazelnut-specific allergens. We compared these allergens to structures displayed by sera from patients with a completely or partially non pollen-related hazelnut allergy and with birch pollen-related hazelnut allergy. None of the sera had a clinical history of anaphylaxis. Special emphasis was placed on the heat stability and cross-reactivity of these allergens.

Methods/Results: Using Western blotting with extract from birch pollen and EAST inhibition techniques we were able to show that the allergens in the serum sample of the young woman were not cross-reactive with birch pollen. Immunoblot experiments with extracts from native and heated hazelnuts and EAST inhibition tests further characterized these allergens to be heat-stable. Unlike the IgE binding pattern of the sera from the patients with pollen-related hazelnut allergy, low molecular weight proteins below 10 kDa were identified by the sera from the patients without pollinosis.

Conclusions: Since the binding pattern of the serum sample of the young woman was different from that of the sera from patients without pollen allergy but less severe symptoms, we assume an association between single non pollen-dependent hazelnut allergens in the low molecular range and severe allergic reactions.

These results enable us to approach a subgroup of hazelnut allergens which we believe to be responsible for anaphylactic reactions in hazelnut allergic patients after ingestion of heat-stable hazelnut structures in processed food stuff, independent of pollinosis.

Key words Non pollen-related hazelnut allergens – birch pollen-related hazelnut allergens – low molecular weight allergens – Western blotting – EAST inhibition – anaphylaxis

Introduction

The prevalence of allergic reactions to foods is increasing. These reactions are mediated by IgE antibodies specific for allergens found in fruits and vegetables. Food allergy is often induced by hypersensitivity to pollen. According to previous publications, up to 70 % of patients with birch pollen allergy suffer from allergy to a variety of raw fruits, vegetables and nuts, most frequently to apples and hazelnuts [1–9]. The "birch pollen related food allergy" (BPRF) is due to cross-reactivity between food and pollen proteins [2, 4, 7, 8, 10]. Most of the parallel appearance of birch and food allergy can be explained by proteins sharing common epitopes (IgE binding regions) with the major birch pollen allergen Bet v 1 which shows a high sequence homology to a group of so-called "pathogenesis-related proteins" [2, 4, 11–13]. In addition, profilins, a group of cross-reacting allergens which are ubiquitously found in pollens and food [11, 14, 15], and carbohydrate determinants of glycoproteins (CCD) from pollen and food [7, 16] may be responsible for the phenomenon of birch pollen-related food allergy.

Due to this, hazelnut allergy is highly prevalent among the birch pollen-related food allergies [4, 5, 17–24]. Recently, two prominent IgE binding proteins, Bet v 1- and Bet v 2 (profilin)-analogous structures, were identified in hazelnuts which are responsible for the allergenic cross-reactions between birch and hazel pollen, and hazelnuts [2, 4]. Cross-reactive carbohydrate determinants, partially heat-resistant, were found as allergic components in hazelnut extract at high molecular mass by Müller et al. [25]. Clinically, the oral allergy syndrome (OAS) affects most hazelnut allergic patients suffering from pollinosis and is described as the most common food allergy in allergic adults [26]. In contrast, anaphylactic reactions provoked by hazelnuts are rare but not uncommon [27–29].

Native and roasted hazelnuts are ingredients of many processed foods which sometimes cannot be recognized by the allergic consumer [30]. Since life-threatening allergic reactions may be caused by stable allergens, hazelnut allergens in processed food stuff have to be taken into consideration as hidden allergens and are therefore a serious problem for highly sensitized patients. Up to now, there has been lack of molecular information about hazelnut allergens which can cause severe allergic reactions.

For this purpose, we analyzed the serum of a young woman who had severe anaphylactic reactions after ingestion of hazelnuts. According to her case history and CAP results (binding assay to quantify specific IgE against allergens) the patient's hazelnut allergy was not pollen-associated. Likewise, we included serum from one patient who was also not affected by birch pollen allergy, one serum with IgE reactivity to pollen- and non pollen-dependent hazelnut allergens and 53 sera from patients with hazelnut allergy and pollinosis. Except for the young woman, none of the patients studied suffered from anaphylactic reactions.

The aim of this study was to obtain access to hazelnut allergens which are not related to tree pollen and which provoke anaphylaxis. By means of Western blots and EAST inhibition experiments, we focused on non pollen-associated allergens and their heat stability.

On the basis of the individual IgE binding of non pollen-associated allergens, it should be possible to improve diagnostics by differentiating between high risk hazelnut allergic patients and patients with a birch pollen-related hazelnut allergy who suffer from milder allergic reactions like OAS.

Materials and Methods

Patients' sera and antisera

Fifty-three serum samples were collected from patients suffering from pollinosis to birch and hazel pollen and sensitization to hazelnut with milder forms of allergic reactions to hazelnuts like OAS (relying on case history, positive skin prick test or CAP classes). The sera had CAP classes to birch and hazel pollen > 4 and to hazelnut between 1 and 6 (CAP classes according to the classes of CAP FEIA [Pharmacia, Uppsala, Sweden]). Of these sera, 40 were obtained from Borstel Hospital, Borstel (Dr. U. Lepp), Germany, 12 samples were kindly supplied by Borkum Riff Hospital, Borkum, Germany (Dr. H. Aulepp), and one serum was provided by Mast Diagnostica, Reinfeld, Germany.

Serum MS (named using the initials of the serum donor) was collected from a young woman who reacted anaphylactically after ingestion of hazelnuts but did not suffer from pollinosis to birch pollen (LMU Hospital, Dermatologische Klinik und Poliklinik, München, Germany). Determination of specific IgE antibodies by the CAP test in serum MS showed CAP classes of birch pollen 0, hazelnut 6 and almond 5. Patient PEI 130 provided by the Paul-Ehrlich-Institute, Langen, Germany, with moderately severe allergic reactions (gastrointestinal symptoms, urticaria) revealed CAP classes of birch pollen 0, hazelnut 4, walnut 4, brazil nut 3 and cashew nut 1. Serum of patient PEI 65 (Paul-Ehrlich-Institute, Langen) with birch and hazel pollen allergy displayed CAP classes of birch pollen 5, hazel pollen 4 and hazelnut 4. According to the case report this patient reacted with asthma and mouth itching to hazelnuts. For these three sera EAST classes were determined as described by Müller et al. [25]. Using extract from native hazelnuts, patient MS revealed EAST classes of 4, PEI 130 of 3 and PEI 65 of 4. With extract from roasted hazelnuts serum sample MS displayed EAST classes of 4, PEI 130 of 3 and PEI 65 of 4.

Four sera (PEI 17, 48, 62, 63) from patients with pollen-related hazelnut allergy (Paul-Ehrlich-Institute, Langen) were pooled for the EAST inhibition assay.

As a negative control, serum of a nonatopic subject with

no history of any allergies to inhalants or foods was used. A rabbit anti-celery profilin antibody (kindly supplied by Dr. P. Deviller (Immuno-Virologie Moleculaire et cellulaire, Lyon, France)) and a monoclonal antibody (moab) against Bet v 1 were tested for IgE-binding to hazelnut extract.

Allergen extracts

Hazelnuts (unknown variety) were purchased in a local food store. After mechanical homogenization at –80°C the meal of native hazelnuts was incubated in a 0.1 M NH_4CO_3 buffer (pH 8.0) for 2 h at 4°C, followed by centrifugation at 20,000 *g* for 30 min. The supernatant was vacuum filtered and desalted by dialysis at 4°C against double-distilled water. The extract from native hazelnuts, variety "Piemonte", was prepared as previously described [25]. The extract from roasted hazelnuts heated at 140°C for 40 min prior to extraction was prepared in the same way. Birch pollen extract was obtained from Mast Diagnostica, Reinfeld, Germany.

All extracts were stored freeze-dried at –20°C until used. For SDS-PAGE, 20 µg of the extract from native and roasted hazelnut and 5 µg of birch pollen extract were applied per cm gel after reconstituting the freeze-dried extracts in double-distilled water.

SDS-PAGE and peptide electrophoresis

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of the hazelnut extracts and birch pollen extract were carried out according to Laemmli [31], with a polyacrylamide stacking gel (T = 4 %, C = 2.7 %) on top of a homogeneous separation gel (T = 15 %, C = 2.7 %). The sample buffer contained 20 mM Tris/HCl (pH 8.0), 2 mM EDTA, 2 % (w/v) SDS, 25 % (v/v) glycerol, 1 % (w/v) DTE, 3 % (v/v) 2-mercaptoethanol and 0.02 % (w/v) bromophenol blue as the tracking dye.

NuPAGE™-Bis-Tris gels (10 %) were commercially purchased from Novex (San Diego, USA). These gels are based upon a Bis-Tris-HCl buffered (pH 6.4) polyacrylamide gel. The electrophoresis was run with NuPAGE™ MES SDS running buffer containing 50 mM MES (2-(N-morpholino) ethane sulfonic acid), 50 mM Tris base, 3.5 mM SDS and 1 mM EDTA. The samples were reduced with NuPAGE™ sample buffer (0.293 M sucrose, 141 mM Tris base, 106 mM Tris HCl, 69.5 mM SDS, 0.51 mM EDTA, 0.22 mM serva blue G250, 0175 mM phenol red) and NuPAGE™ reducing agent according to the manufacturer's instructions. Additionally, NuPAGE™ antioxidant was added to the NuPAGE™ MES SDS running buffer to prevent reduced proteins from reoxidizing during electrophoresis.

Electrophoretic blotting

For immunoblotting, the separated proteins were transferred from the SDS-PAGE separation gel onto nitrocellulose (NC) membranes by semi-dry blotting [32] at 0.8 $mA \cdot cm^{-2}$ for 30 min. After transfer, unspecific binding was blocked by incubation with Tris buffered saline (TBS), pH 7.4, containing 0.5 % Tween 20 for 30 min. NuPAGE™-Bis-Tris gels were blotted onto NC membranes for 1.5 h at 25 V according to the manufacturer's instructions using NuPAGE™ transfer buffer which contains 25 mM Bicine, 25 mM Bis-Tris, 1.0 mM EDTA and 0.05 mM chlorobutanol. For prevention of unoccupied protein-binding sites, the membranes were also treated with 0.5 % Tween 20 in TBS. For total protein staining, the blocked NC membrane was incubated in India ink (Pelican, Hannover, Germany) [33].

Immunologic detection

The NC membrane was cut into strips and incubated on a shaker overnight at room temperature with patients' sera, a rabbit anti-celery profilin antibody and a moab against Bet v 1, respectively. The serum samples were diluted 1:20, the anti-celery profilin antibody and the moab against Bet v 1 1:100 in TBS, pH 7.4, containing 0.5 % Tween 20. For detection of bound IgE antibodies the strips were incubated with alkaline phosphatase-labeled anti-human IgE (Allergopharma, Reinbek, Germany; dilution 1:2000), anti-mouse IgG/M (Dianova, Hamburg, Germany; dilution 1:10000) and polyclonal anti-rabbit antibody (DAKO, Hamburg, Germany; dilution 1:1000).

EAST inhibition assay

EAST inhibition assays were performed as described by Vieths et al. [34] with native and roasted hazelnut extract, variety "Piemonte". The serum pool from patients with pollen-related hazelnut allergy (PEI 17, 48, 62, 63) was diluted 1:6, serum sample PEI 65 1:3 and serum sample MS 1:10 in 0.05 M Tris buffer, pH 7.4, containing 0.15 M NaCl, 0.3 % (w/v) bovine serum albumin (BSA), and 0.1 % Tween 20. A 10-fold dilution series of the inhibitor extracts was prepared in the same buffer. The serum pool was incubated with the inhibitor solutions on allergen disks coupled with native hazelnut extract, serum sample MS and PEI 65 on allergen disks coupled with native and roasted hazelnut extract overnight. Extracts from native hazelnut (positive control), heated hazelnut, birch pollen and milk (negative control) were used as the inhibitors. The EAST was performed according to the standard procedure (Allergopharma, Reinbek, Germany). The results were expressed as % inhibition. Extract potencies were quantitatively compared by estimating the protein concentration responsible

for a 50 % inhibition of the IgE binding to the solid phase (C_{50}) from the inhibition graphs.

Results

Immunoblot studies with extracts from hazelnut and birch pollen

We performed Western blotting with membrane strips from native hazelnut extract of a commercially distributed unknown variety to investigate the IgE reactivity of 53 patients' sera with hazelnut and tree pollen allergy. As indicated in Fig. 1 the IgE-binding proteins could be detected in the range from 12 to 100 kDa. 40/53 patients (75 %) showed IgE binding to a 18 kDa protein. Regarding this 18 kDa allergen, the IgE of 20 patients bound exclusively to it whereas the IgE of the other 20 patients also displayed binding to other structures. 10/53 sera (19 %) contained IgE antibodies to a 14 kDa protein. None of the sera tested reacted exclusively with this component. Incubation with a specific monoclonal antibody against Bet v 1 and a rabbit anti-celery profilin antibody (Fig. 1B, strips 55 and 54; marked by arrows) identified these two proteins as Bet v 1- and Bet v 2- (profilin)-analogous structures in hazelnut extract. Additionally, IgE-binding proteins could be detected at 20 kDa (17 %), 23 kDa (6 %), 31 kDa (13 %), 50 and 55 kDa (9 %), and 62 kDa (9 %). A broad spectrum of IgE specifities above 30 kDa was identified from 9/53 sera (17 %). However, the binding patterns of the 53 sera tested were very individual.

Analyzing the IgE reactivity of the hazelnut allergic patient MS without birch pollen allergy in a SDS-PAGE immunoblot with membrane strips from native hazelnut extract of the unknown hazelnut variety, 5 dominating bands with apparent molecular weights of 50, 42, 38, 9 and 7 kDa were found (Fig. 2). Serum sample PEI 130, positive to native and roasted hazelnut with EAST classes of 3, detected proteins in the low molecular mass region of 12–14 kDa and reacted with further structures in the high molecular

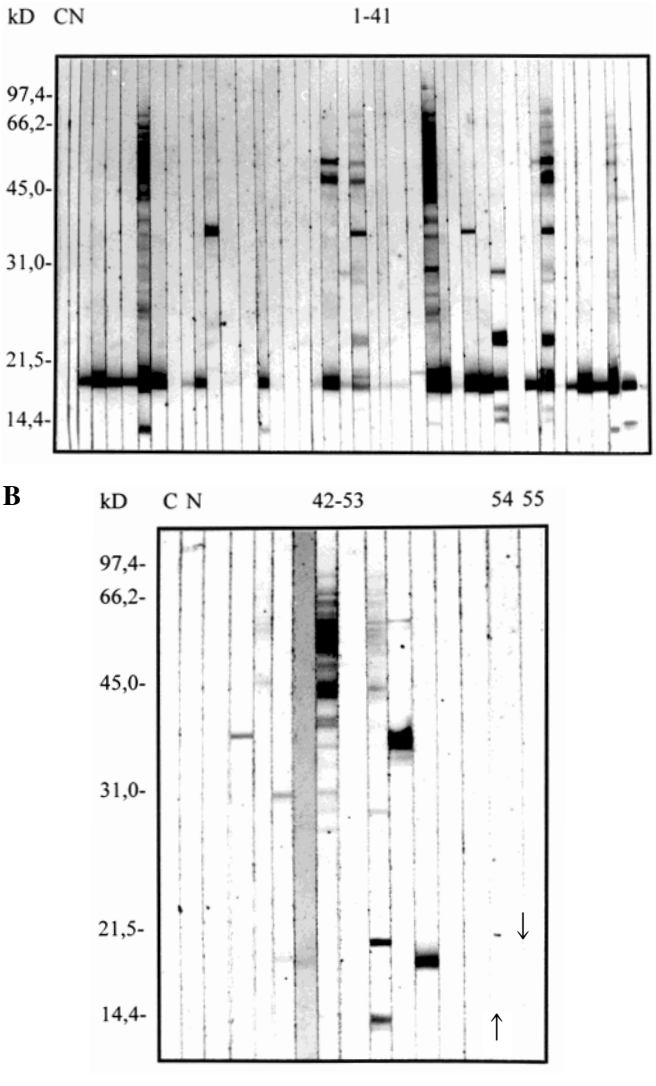

Fig. 1 A, B IgE-binding patterns of sera from patients with hazelnut and pollen allergy to native hazelnut extract, separated by SDS-PAGE and transferred to NC.
C buffer control, *N* negative control serum, *1–53* sera from patients with pollen-associated hazelnut allergy, *54* rabbit anti-celery profilin, *55* moab against Bet v 1.

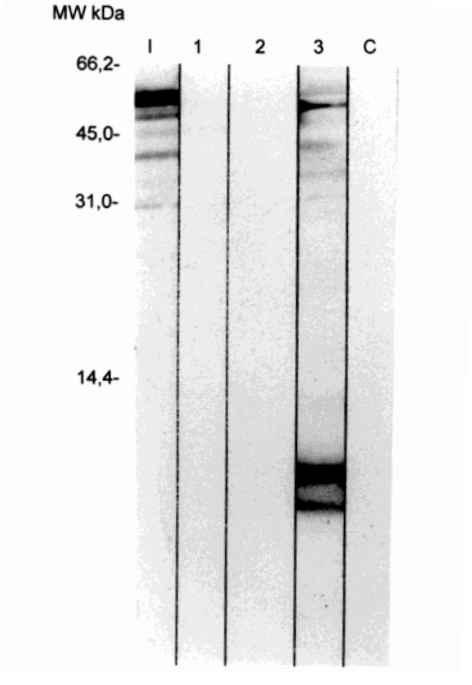

Fig. 2 IgE-binding pattern of serum sample MS with severe allergic reactions to hazelnut without pollinosis to native hazelnut extract, separated by SDS-PAGE and transferred to NC. *I* Protein staining by India ink, *1* negative control serum, *2* serum sample No. 20, *3* serum sample MS, *C* buffer control.

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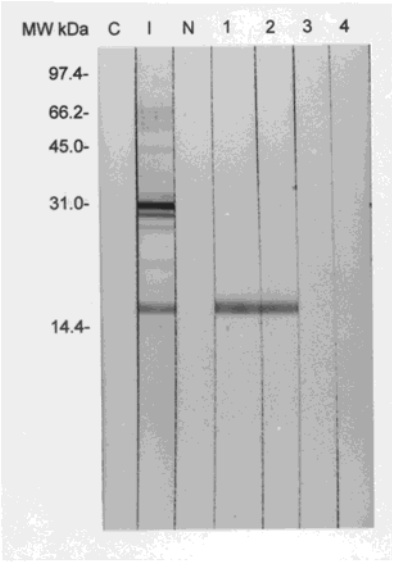


Fig. 3 Immunoblot of birch pollen extract with serum samples PEI 65, PEI 130 and MS, separated by SDS-PAGE and transferred to NC. *I* Protein staining by India ink, *C* buffer control, *N* negative control serum, *1* serum sample No. 3, *2* serum sample PEI 65, *3* serum sample PEI 130, *4* serum sample MS.

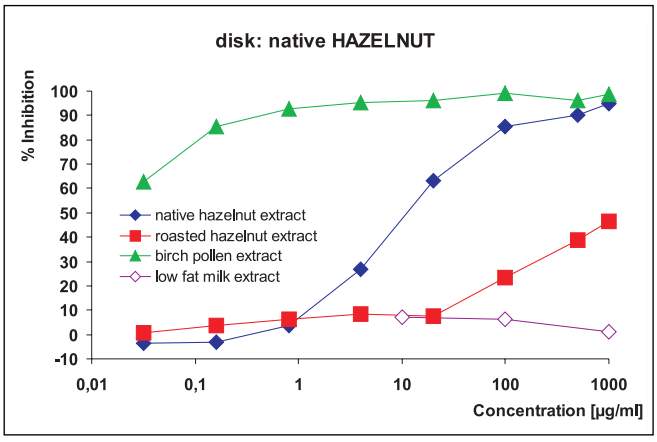


Fig. 4 EAST inhibition experiments with allergen disks coupled with native hazelnut protein and a serum pool (1:6) of patients with birch pollen-related hazelnut allergy, performed to study IgE cross-reactivity to birch pollen.

range (not shown). IgE from serum sample PEI 65 bound to allergens of the molecular range of 12–14 kDa and of 18 kDa (shown by [25]). In order to confirm the negative CAP results of birch pollen, Western blotting with birch pollen extract was carried out with serum samples MS, PEI 130 and PEI 65 (Fig. 3). One birch pollen-associated serum (No. 3) was chosen as a positive control identifying Bet v 1 (Fig. 3, lane 1). Actually, no IgE reactivity to any birch pollen allergen could be determined for sera MS and PEI 130. IgE of serum PEI 65 reacting to pollen and non pollen-dependent hazelnut components bound exclusively to Bet v 1 and not to any structures in the low molecular range in birch pollen extract.

EAST inhibition assay

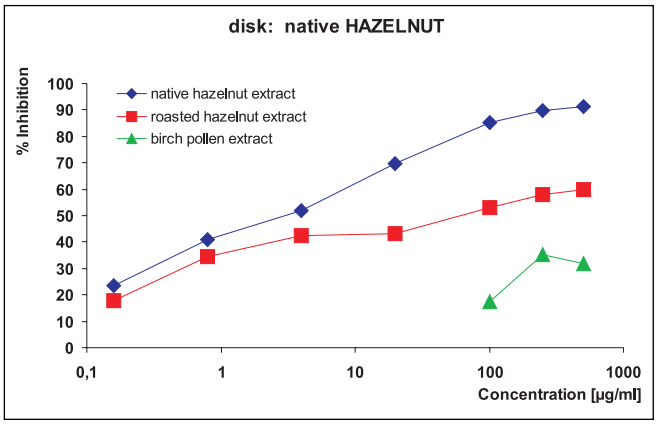
To determine any cross-reactivity in hazelnut allergic patients with or without birch pollen allergy EAST inhibition assays were performed. Allergen disks coupled with native hazelnut proteins were incubated with a serum pool from four patients (PEI 17, 48, 62, 63) with pollen-related hazelnut allergy and IgE binding to the 18 kDa hazelnut allergen in a Western blot (not shown). Extract from native and roasted hazelnut, birch pollen and milk were used as the inhibitors. The results are summarized in Fig. 4. In contrast to extract from native hazelnut used as the inhibitor which was able to inhibit the IgE binding up to 90 % (positive control), extract from roasted hazelnut was only able to inhibit the IgE binding up to 30 to 50 %. This clearly indicates that the IgE of the serum pool is to a great extent directed against heat-labile allergens.

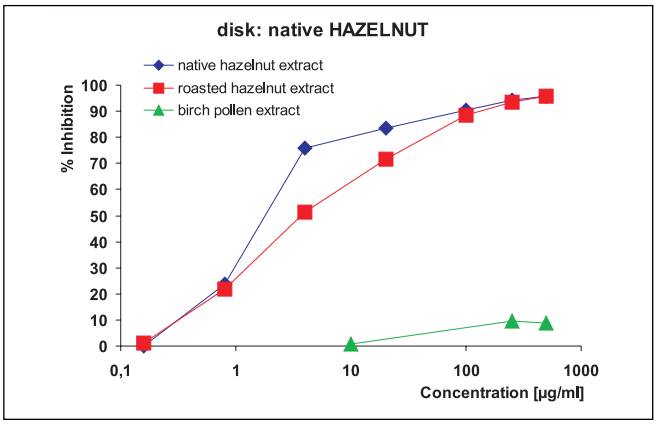
With birch pollen extract, a 50 % inhibition (C_{50}) was obtained at inhibitor concentrations of less than 0.03µg which is about a 1000-fold lower than C_{50} of extract from native hazelnut, revealing its high cross-reactivity in patients with birch pollen-associated hazelnut allergy. No significant inhibition was observed with milk extract, serving as a negative control.

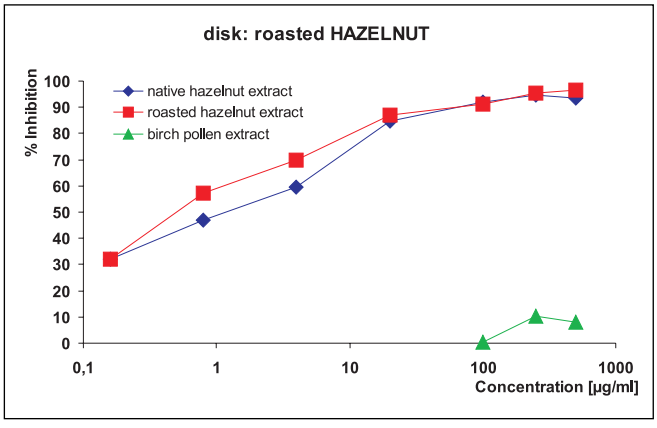
The results of the inhibition assay performed with serum PEI 65 with IgE reactivity to pollen and non pollen-dependent hazelnut components are shown in Fig. 5. Using disks with native hazelnut proteins and roasted hazelnut proteins as the inhibitor (Fig. 5A) about 60 % inhibition was obtained. Applying birch pollen extract as the inhibitor on allergen disks with native hazelnut proteins, a 35 % inhibition was achieved showing that this serum sample contains IgE against cross-reactive proteins. Running the same experiment on allergen disks with roasted hazelnut proteins an inhibition of less than 10 % reveals that the cross-reactive hazelnut proteins are heat-labile (Fig. 5B).

To investigate serum MS in regard to any cross-reactivity to birch pollen, IgE binding to allergen disks coupled with native and roasted hazelnut were inhibited by extract from roasted hazelnut, native hazelnut and birch pollen as indicated in Fig. 6. Using disks with native hazelnut proteins and roasted hazelnut as the inhibitor (Fig. 6A) more than 90 % inhibition could be achieved showing that more than 90 % IgE is directed against heat-stable allergens. Running the reverse experiment (disks with roasted hazelnut proteins and native hazelnut as the inhibitor; Fig. 6B) the results could be confirmed.

Applying birch pollen extract as the inhibitor on the allergen disk with native or roasted hazelnut proteins, no sig-







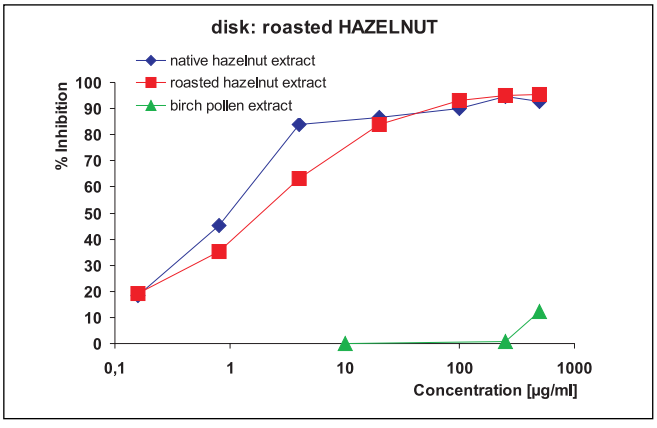


Fig. 5 EAST inhibition experiments with allergen disks coupled with native (**A**) and roasted (**B**) hazelnut proteins and serum sample PEI 65 (1:3), performed to study its IgE cross-reactivity to birch pollen.

Fig. 6 EAST inhibition experiments with allergen disks coupled with native (**A**) and roasted (**B**) hazelnut proteins and serum sample MS (1:20), performed to study its heat stability and IgE cross-reactivity to birch pollen.

nificant inhibition was obtained indicating that only allergens were involved which are independent of birch pollen.

These data demonstrated cross-reactivity of birch and hazelnut structures regarding hazelnut allergic patients with birch allergy, and the lack of cross-reactivity of birch pollen in a non pollen-related hazelnut allergic (serum MS) patient, respectively. Thus, the results of the inhibition assays agreed closely with the IgE binding observed by Western blotting (Fig. 3).

Immunoblot studies with extracts from native and roasted hazelnut

To obtain access to the low molecular weight proteins which were identified by serum samples MS, PEI 130 and PEI 65 in the SDS-PAGE immunoblot, we used NuPAGE™-Bis-Tris gels followed by Western blotting for better resolution of the small proteins.

Furthermore, to investigate any differences in heat stability between birch pollen- and non birch pollen-associated hazelnut allergens on Western blots we performed immunoblot experiments with extracts from native and roasted hazelnut, variety "Piemonte" respectively (Fig. 7). For this purpose we included a positive control serum from a patient with pollen-related hazelnut allergy (No. 3). Interestingly, the Bet v 1-analogous band was almost depleted in the roasted extract (Fig. 7B, lane 1), indicating its heat lability. Likewise, the 18 kDa protein detected by serum PEI 65 disappeared, whereas the low molecular weight structures remained (Fig. 7B, lane 2). Regarding the binding pattern of serum samples MS, PEI 130 and PEI 65 in the low molecular range in Fig. 7A, all sera bound to different proteins and/or with different intensities. Besides the Bet v 1-analogous structure at 18 kDa, PEI 65 identified a doublet band at 5–6 kDa and a band at 10 kDa. Serum sample PEI 130 bound to protein bands of approximately 8 kDa and further to bands higher. Serum MS revealed a doublet band of approximately 5–7 kDa and a protein of 9–10 kDa in addition to components in the higher molecular weight range. Figure 7B shows that the non pollen-dependent structures identified by these sera did not change their

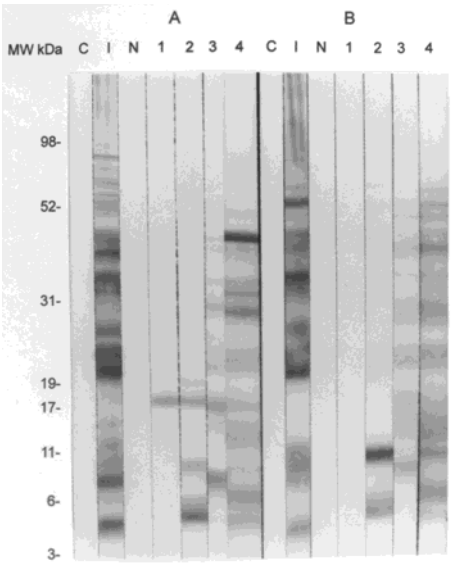


Fig. 7 A, B Comparison of the IgE binding patterns from serum samples PEI 65, PEI 130 and MS to blotted extracts from native and roasted hazelnut after separation in NuPAGE™-Bis-Tris gels.
A Extract from native hazelnut, *B* extract from roasted hazelnut. *I* Protein staining by India ink, *C* buffer control, *N* negative control serum, *1* serum sample No. 3, *2* serum sample PEI 65, *3* serum sample PEI 130, *4* serum sample MS.

binding pattern on Western blot with extract from roasted hazelnut, although the bound proteins appeared as less distinct. The results underline that these structures belong to heat-stable components which were not cross-reactive to birch pollen.

Discussion

Hazelnut allergy is a common disease of patients who suffer from tree pollen allergy. Hirschwehr et al. [4] found proteins of 14 kDa and 18 kDa in hazel pollen and hazelnuts and described these structures as cross-reactive allergens. The authors identified the major allergen of hazelnut (18 kDa) as an allergen similar to Cor a 1, the major allergen of hazel pollen, which also shares IgE epitopes with Bet v 1. They showed that hazelnuts contained fewer immunologic determinants than proteins from tree pollens that belong to the order *Fagales* (e. g. birch, alder, hazel, hornbeam). Additionally, the second prominent IgE binding protein of hazelnuts (14 kDa) could be identified as profilin. Recently, Müller et al. reported on cross-reactive carbohydrate determinants (CCD) in hazelnuts [25].

Normally, hazelnut allergics with pollinosis suffer from OAS [26]. Patient PEI 65 chosen for our investigations belongs to this group revealing OAS and asthma after ingestion of hazelnuts. However, the case histories of some patients show that hazelnuts can also cause food allergy, independent of pollinosis. We studied patients without pollinosis but with severe (patient MS) and moderately severe reactions (patient PEI 130) after ingestion of hazelnuts. Patient PEI 65 represents a mixed type hazelnut allergy which is pollen and non pollen-associated. This study was designed to identify hazelnut-specific, pollen-independent allergens eliciting severe allergic symptoms incorporating hazelnut allergens shared by tree pollen. The sera included in the study were collected from patients whose hazelnut allergy was proven by clinical history, skin prick test, CAP test and/or EAST test. For the identification of non pollen-related hazelnut allergens three sera were used with the following features. Serum PEI 65 revealed a partially pollen-related hazelnut allergy and serum samples MS and PEI 130 no association to pollen allergy. Serum sample MS showed a clear clinical history of a severe hazelnut allergy without association to tree pollen allergy. The characteristics of these three sera were compared with 53 sera from patients who suffer from pollen-associated hazelnut allergy.

Proteins with MWs ranging from 12 to 100 kDa were detected as allergenic structures by the 53 sera. Of the BPRF patients 75 % identified IgE reactivity to an 18 kDa component, which appears to be the major hazelnut allergen. By means of a specific monoclonal antibody against Bet v 1, this allergen could be recognized as the Bet v 1-analogous structure in hazelnuts. Of these patients 19 % showed IgE reactivity to a 14 kDa protein, which could be identified as profilin by a rabbit anti-celery profilin antibody. These results confirmed the data of Hirschwehr et al. [4] who published the first report on cross-reactive proteins in hazelnuts and hazel pollen. In our own Western blot experiments, further allergens were detected at 20, 23, 31, 50, 55 and 62 kDa. Moreover, a broad spectrum of IgE reactivities was identified above 30 kDa which Müller et al. [25] identified as glycoproteins with cross-reactive carbohydrate determinants (CCD). However, the binding pattern of the 53 sera differed individually which could also be shown in Hirschwehr's studies although not explicitly pointed out.

We analyzed the IgE reactivity of the putative non pollen-related sera. Our first aim was to make sure that the serum samples of MS and PEI 130 did not react with birch pollen extract. This was proven by CAP and Western blot to birch pollen extract and for serum MS also by EAST inhibition test. For serum sample PEI 65 IgE reactivity to birch pollen extract determined by CAP and EAST inhibition test was exclusively directed against Bet v 1 as demonstrated by Western blot. On immunoblots with hazelnut extract, serum sample MS displayed IgE-binding to allergens at 50, 42, 38, 9 and 7 kDa. Serum PEI 130 also bound to low molecular weight proteins at 12–14 kDa and detected further protein bands above 14 kDa. PEI 65 identified allergic components at 12–14 kDa and at 18 kDa. Since the serum samples MS, PEI 65 and PEI 130 showed IgE reactivity to allergens below 14 kDa which are only poorly separated by SDS-PAGE according to Laemmli [31], we ap-

plied peptide electrophoresis (NuPAGE™-Bis-Tris gels) to obtain a better resolution of the proteins in the low molecular range. By this technique, the allergens below 10 kDa could be identified more precisely for sera MS, PEI 130 and PEI 65 all of which showed an individual IgE binding pattern. The fact that the allergen pattern identified by serum sample MS was not detected by the other two sera (PEI 65 and 130) supports our opinion that these single structures are responsible for the anaphylactic reactions of patient MS. Our hypothesis is in line with findings of proteins below 10 kDa belonging to the family of lipid transfer proteins which can provoke severe allergic reactions in patients without pollinosis as described for peach and apple [35, 36]. Since patients often react to hidden allergens ingested by processed food stuff, the heat stability is an important criterion to calculate the allergenic potential. We analyzed this by EAST inhibition and Western blot. Using allergen disks with native and roasted hazelnut proteins, we could show that the IgE from serum MS was directed against heat-stable allergens. We confirmed these results by investigating the heat stability on Western blots with extract from native and roasted hazelnut. In contrast to the pollen-associated proteins to which IgE binding was destroyed (Bet v 1) on the blots with roasted hazelnut compared to blots with native hazelnut (sera No. 3 and PEI 65), the IgE-reactive components detected by patient MS and PEI 130, respectively, remained. The fact that the Bet v 1-analogous allergen of hazelnut was heat-labile corresponds to data obtained with Mal d 1 and Api g 1, the major apple and celery allergens, and underlines the heat lability of Bet v 1-related food allergens [37, 38]. However, the IgE-binding bands identified by patients MS and PEI 130 were displayed in both extracts from native and roasted hazelnut. The individuality of the patients' IgE binding may be the explanation for the different clinical reactions. Literature data underline the occurence of life-threatening allergic reactions after ingestion of food containing heated or processed hazelnuts [39]. One explanation could be the existance of single heat-stable hazelnut components influencing the clinical severity of hazelnut allergy found in hazelnut allergics without pollinosis.

Although hazelnut allergy in patients allergic to tree pollens is well documented, very little is known about hazelnut allergy which is provoked independently of pollinosis. According to our hypothesis there is a subgroup of high risk patients among the hazelnut allergics whose severe reactions are caused by individual non pollen-related allergens. Our study showed that the hazelnut allergens involved in patients allergic to birch pollen differ from the allergen pattern of the patient allergic to hazelnuts without pollinosis as no low molecular weight allergens could be identified. Among the non pollen-dependent hazelnut allergics, however, the IgE binding patterns were not identical which may be the answer for the grade of severity of allergic reactions.

Although this form of hazelnut allergy is rare, it is worthwhile to analyze because of the obvious severity of clinical reactions involved.

In conclusion, our findings enable us to access hazelnut allergens which are not pollen-associated and can cause anaphylactic reactions after ingestion of hazelnut containing food stuff. In the next step, a larger group of high risk hazelnut allergics without pollinosis has to be investigated to confirm our findings and to identify further hazelnut-specific allergens which provoke severe allergic reactions and are therefore of high clinical relevance.

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