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Journal of Chromatography B, 756 (2001) 239–248

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Determination of the allergenicity of various hazelnut products by immunoblotting and enzyme allergosorbent test inhibition

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Abstract

Although allergic reactions to hazelnuts are common especially in Europe, there are only a few investigations with regard to the influence of processing on the IgE-binding potency of hazelnut proteins. In this study the allergenicity of different hazelnut products, such as chocolate, nougat products, croquant or cookies, was examined by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE), immunoblotting and enzyme allergosorbent test (EAST) inhibition experiments using sera of 17 hazelnut-allergic individuals. In only a few cases did the immunoblotting experiments yield positive results as regards the allergenicity of the investigated products. By means of EAST inhibition a residual IgE-binding potency could be detected in almost all of the product extracts. Therefore hazelnuts are a potential hazard to allergic people even as an ingredient of processed foods.

Keywords: Food allergy; Hazelnuts; Immunoblotting; Enzyme allergosorbent test

1. Introduction

The immediate-type allergic reaction to foodstuffs is a common disease the prevalence of which has been estimated at 1–3% among the adult population and up to 5–8% among the pediatric population [1]. Especially in Europe hazelnuts are considered a widespread cause of food allergies among both children [2] and adults [3]. Hazelnut allergy is part of the phenomenon of pollen-associated food allergies, which are the most common food-induced allergic reactions in Europe [4,5]. Cross-reactivities often occur among the major allergens of birch pollen and the proteins of, for instance, hazelnut, apple, carrot, potato and kiwi [6–11]. Fifty to 93% of patients with birch pollinosis also suffer from hazelnut and/or apple allergy [12]. Cross-reactivities between hazelnut and further tree pollens, such as alder, hazel, hornbeam, oak and mugwort, are documented as well [7,12–16]. Various authors have demonstrated the existence of a partial immunological identity between birch pollen and hazelnut proteins [8,17]. In a further work [18] the existence of two common allergens in hazel pollen and hazelnut has been revealed: a 17-kDa allergen in hazel pollen and an 18-kDa allergen in hazelnut, respectively. An additional 14-kDa allergen has been shown in both hazel pollen and hazelnut, which is considered a profilin and is cross-reactive to the 14-kDa birch pollen profilin. The 18-kDa allergen in hazelnut is considered cross-reactive to the 17-kDa major allergen in birch pollen Bet v 1. Additional IgE-binding proteins in hazelnut have been detected at 37, 40, 46 and 69 kDa.

Although even near-fatal anaphylactic reactions to processed and heated hazelnuts have been described

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0378-4347/01/$ – see front matter
PII: S0378-4347(01)00084-6

[19,20], very little is known about the influence of processing on the IgE-binding potency of the allergens [21]. This is hardly comprehensible, because hazelnuts are used as ingredients of many foodstuffs and are marketed in many forms. Besides, in Germany hazelnuts belong to the most important so-called hidden allergens in foodstuffs [22].

Whilst in a previous study we dealt with the influence of different heat treatments and storage on the allergenicity of the IgE-binding proteins in hazelnuts [23], the aim of this study is the investigation of the allergenicity of the wide spectrum of foodstuffs which contain hazelnuts, such as chocolates, nougats and cookies, for example, by means of SDS–PAGE, immunoblotting and enzyme-allergosorbent test (EAST) inhibition.

2. Experimental

2.1. Patients sera

A total of 16 patients' sera were collected at the University Hospital Eppendorf, Department of Dermatology and Allergology, Hamburg, Germany. Serum Mast 127 was supplied by Mast Diagnostica (Reinfeld, Germany). The relevant clinical information is summarized in Table 1. The patients with known anamnesis of allergy to hazelnuts suffered from itching in the mouth or throat, swelling of the lips, heartburn, nausea or difficulties in breathing. None of the patients with known anamnesis of allergy shows anaphylaxis. Many patients showed further sensitivities to different fruits, nuts and tree pollen. All sera were EAST class ≥ 2 when tested for specific IgE to hazelnut extract by enzyme allergosorbent test (Allergopharma, Reinbek, Germany). A serum from a non-atopic individual with no history of food hypersensitivity (EAST class 0) was used as negative control. The pooled serum used for EAST inhibition experiments contained 13 of the listed sera (3–15).

2.2. Hazelnut products

The raw hazelnuts used as reference material were purchased at a local food store. The investigated commercial hazelnut products, such as five hazelnut chocolates, one milk chocolate as a negative control, two nougat chocolates, two nougat masses, two nougat cremes, one hazelnut cake, three hazelnut cookies, one muesli bar and two hazelnut croquant samples, were obtained at a local food store as well.

2.3. Preparation of allergen extracts

Native protein extracts were obtained by extracting 1 g of peeled, ground and defatted hazelnuts with 30 ml phosphate-buffered saline [PBS (0.01 *M* potassium phosphate buffer, pH 7.4, 0.13 *M* sodium chloride)] for 60 min at room temperature. The suspension was centrifuged (4°C, 10 500 *g*, 60 min) and the supernatant filtered. The solution was divided into aliquots of 2 ml, lyophilized and stored at −20°C until use. In the case of the hazelnut products containing whole hazelnuts or hazelnut pieces (hazelnut chocolates, croquant and muesli bar), the nuts were isolated, defatted and extracted as described above. Homogeneous products (nougat products, cookies and cake) in which an isolation of the nuts was not possible were completely homogenized, defatted and extracted with PBS.

2.4. Protein determination

The protein contents of the extracts were determined by the method of Bradford [24].

2.5. Sodium dodecyl sulfate–polyacrylamid gel electrophoresis (SDS–PAGE)

SDS–PAGE was performed in 10% acrylamide gels using NuPAGE® vertical electrophoresis system according to the manufacturer's recommendations (Novex, San Diego, CA, USA). Protein samples dissolved in Tris–HCl/SDS sample buffer (pH 6.8) containing 5% (w/v) β-mercaptoethanol were boiled for 3 min. Applying about 20 μg protein per well, electrophoresis was performed at 200 V constant voltage for 35 min on the NuPAGE® electrophoresis system (Novex) using a MultiDrive XL power supply (Pharmacia, Uppsala, Sweden). Gels were silver stained [25].

Table 1
Characterization of patients' sera with IgE binding to hazelnut proteins[a]

No.	Patient (sex, age)	Clinical symptoms	Specific IgE (U ml^{-1})	EAST class	Further sensitivities
1	KDH (male, 45)	IM	5.76	3	Carrot, potato, apple, mango, lychee, birch pollen, mugwort pollen
2	JGM (nk, nk)	nk	>17.5	4	nk
3	TR (male, 34)	IM, SL, DB, HB	1.39	2	Apple, cherry, plum, peanut
4	TS (male, 27)	IT	14.01	3	Hen's egg, wheat, milk, yeast, pineapple, apple, birch pollen, grass pollen
5	LS (male, 27)	IT	4.56	3	Apple, birch pollen
6	SK (female, 28)	IM	5.72	3	Peach, apple, pear, strawberry, birch pollen, hazel pollen
7	UW (male, 26)	IM, SL, DB	4.24	3	Apple, potato, walnut, birch pollen, hazel pollen, alder pollen
8	BS 127 (nk, nk)	nk	6.04	3	nk
9	IB (female, 29)	IM, IT	4.91	3	Celery, carrot, mango, mugwort pollen, birch pollen
10	HN (male, 25)	IM, SL	0.88	2	Walnut
11	IK (female, 28)	IM, SL	1.98	2	nk
12	SM (male, 29)	IM, IT	0.76	2	Almond, birch pollen
13	BvW (female, 40)	IM, IT	1.70	2	Apple, cherry, plum
14	HRF (female, 52)	IM, IT	3.50	3	Birch pollen, alder pollen, hazel pollen
15	JA (female, 26)	IM, SL	5.54	3	Almond, apple, tree pollen, grass pollen
16	MM (female, 20)	IM, SL, IT, DB, N	3.55	3	Apple, potato, birch pollen
17	TV (male, 22)	IM, N	1.22	2	Apple, birch pollen

[a] DB, difficulty in breathing; HB, heartburn; IM, itching in mouth; IT, itching in throat; SL, swelling of lips; N, nausea; nk, not known.

2.6. Immunoblotting

Proteins were electrotransferred from slab gels to nitrocellulose (NC membrane 0.2 μm, Schleicher & Schuell, Dassel, Germany) at 0.8 mA/cm^2 for 80 min using a NovaBlot semidry blotting apparatus (Pharmacia) according to Kyhse-Andersen [26] with discontinuous buffer system as described by Vieths et al. [27]. Afterwards the membrane was dried for 30 min and cut into strips of 2 mm. Immunostaining of IgE was performed as described previously [27,28]. To check the efficiency of protein transfer, from each gel one strip including separated proteins and a molecular mass (M_r) marker (Pharmacia) was colloidal gold stained (Bio-Rad, Munich, Germany) according to Moeremans et al. [29].

2.7. Enzyme allergosorbent test (EAST) inhibition

For inhibition experiments the pooled serum was diluted 1+1 in incubation buffer (PBS containing 0.3% (w/v) bovine serum albumin (BSA) and 0.1% (v/v) Tween 20). A 10-fold dilution series of the inhibitor extracts from native hazelnuts and hazelnut products was prepared in five to six steps using the same incubation buffer. Fifty μl of the diluted serum

were mixed with 50 μl of the inhibitor solution and a hazelnut allergen disk. The solutions were incubated overnight at room temperature in the dark. After washing three times with 1% Tween 20 in PBS (v/v), 50 μl of anti-human IgE alkaline phosphatase conjugate (Allergopharma), diluted 1+1 in incubation buffer, were added and incubated overnight. The disks were washed again and enzyme activity was stained with *p*-nitrophenylphosphate (PNPP) for 60 min at 37°C. Absorbance was measured at 405 nm [28].

3. Results

Fig. 1 illustrates the silver stained proteins in the extracts of the native hazelnut and the investigated hazelnut products separated by SDS–PAGE. The molecular mass marker protein bands ranged from 14 to 94 kDa (M_r). The electrophoretic patterns for the chocolate, nougat croquant and muesli bar extracts (2–6, 8–13, 16, 18–20) were except for slight differences similar to the native hazelnut extract (1). Extracts 10–13 and 16 showed additional bands in the lower molecular mass area, which are considered to be milk proteins. Extract 7, which represents the milk chocolate as a negative control, showed strong milk protein bands <30 kDa, which distinguish it from the remaining extracts. The extracts of the cookies and cakes (14, 15 and 17) revealed only a weak staining.

Fig. 2 shows the EAST inhibition experiments, applied to compare the IgE binding potency of the different nougat products. An additional homologous inhibition was carried out to check the allergenic activity of the native hazelnut extract. The resulting C_{50} concentrations of protein extracts responsible for a 50% inhibition of the IgE binding, which reflect the extracts' allergenic potencies, are listed in the legend of Fig. 2. It is clearly recognizable that the IgE binding potency of each extract was reduced. Whilst both nougat chocolate extracts reach similar C_{50} values (790 and 852 mg/l), the remaining extracts show maximum inhibitions of less than 40%.

The immunoblots of the nougat chocolate extract 2 and the native hazelnut extract as a positive reference are shown in Fig. 3. Incubation was carried out using

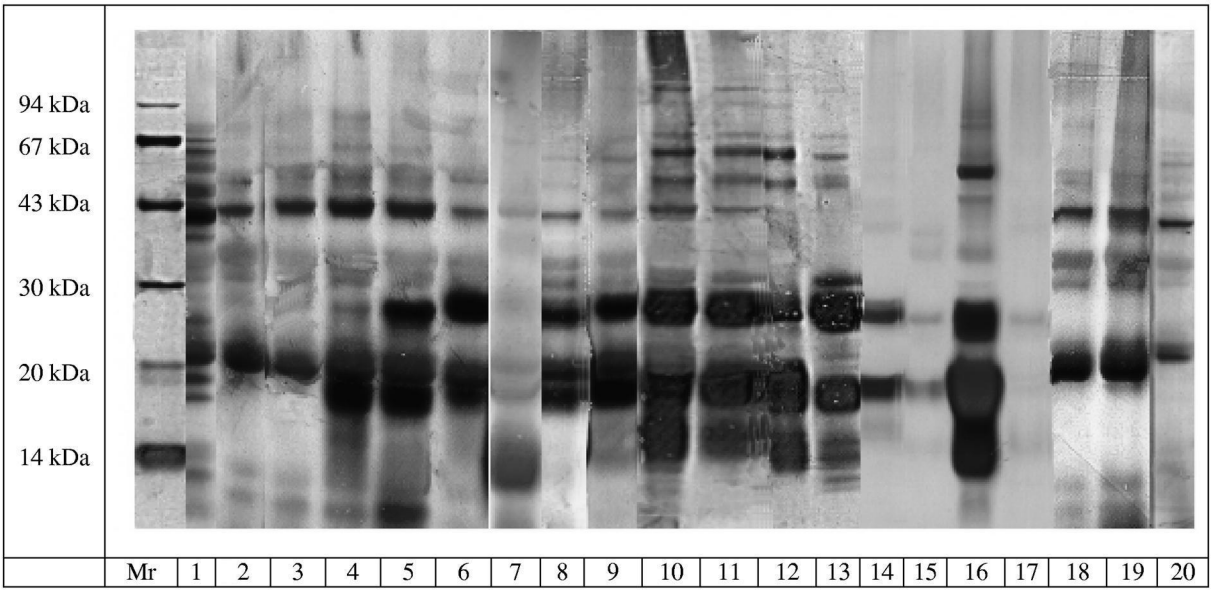

Fig. 1. SDS–PAGE/silver staining of protein extracts from native hazelnuts and hazelnut products. M_r, molecular mass marker proteins: (1) native hazelnut extract; (2) chocolate 5; (3) chocolate 1; (4) chocolate 4; (5) chocolate 2; (6) chocolate 3; (7) milk chocolate (control); (8) nougat 2; (9) nougat 1; (10) nougat chocolate 2; (11) nougat chocolate 1; (12) nougat creme 2; (13) nougat creme 1; (14) hazelnut cookie 2; (15) hazelnut cake; (16) hazelnut cookie 1; (17) hazelnut cookie 3; (18) croquant 1; (19) croquant 2; (20) muesli bar.

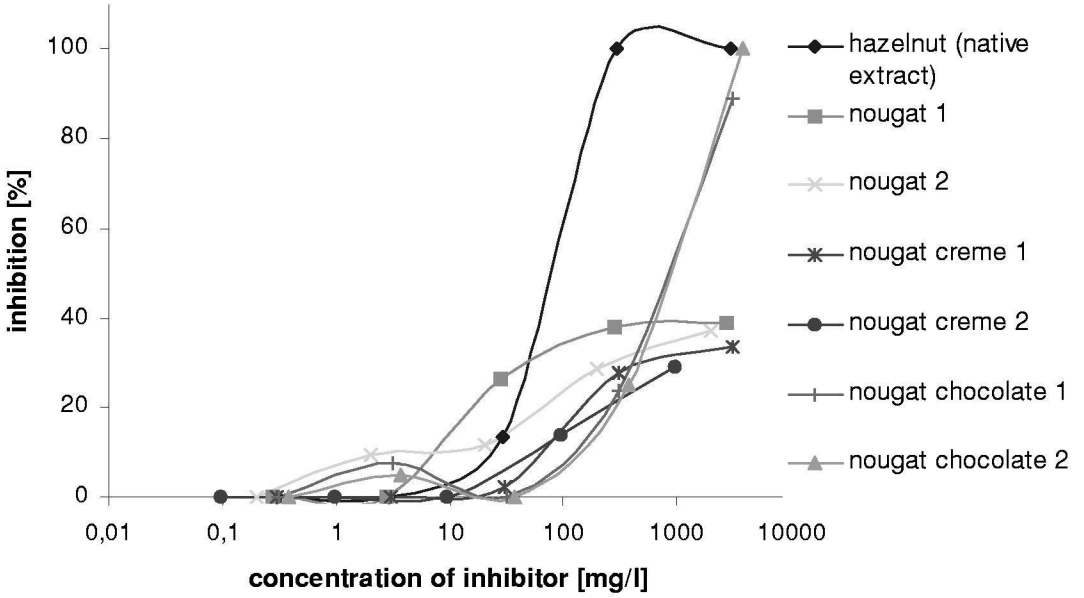

Fig. 2. EAST-inhibition of IgE-binding to hazelnut allergen disks by nougat extracts using a pooled serum of 13 allergic individuals. Native hazelnut extract was used as positive control. Results are expressed in percent inhibition. The following C_{50} concentrations were obtained: native extract, 85 mg/l; nougat chocolate 1, 790 mg/l; nougat chocolate 2, 852 mg/l. The remaining extracts do not reach a 50% inhibition.

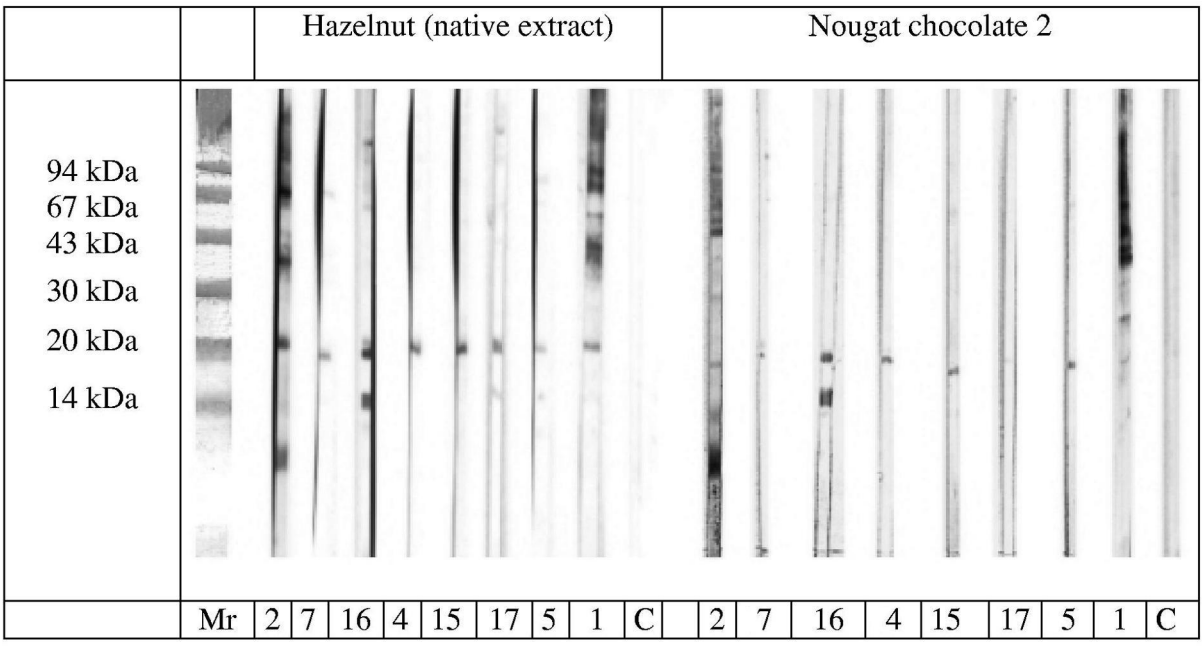

Fig. 3. Immunoblot of the extracts of native hazelnuts and nougat chocolate 2. Lanes marked represent incubation with sera from eight different hazelnut allergic patients (see Table 1). Negative control C was incubated using a serum of a non allergic individual. Molecular mass marker (M_r) was colloidal gold stained.

sera of eight allergic patients and a control serum of a non allergic individual. In the native extract the 18-kDa major allergen was detected by each of the sera. In addition sera 5, 16 and 17 reacted to the 14-kDa profilin. Serum 2 exhibited additional allergens of approximately 60, 40 and <14 kDa, and serum 1 showed additional bands between 30 and 70 kDa. In the extract of nougat chocolate 2 the serum from each patient exhibited similar IgE binding patterns with slight differences as regards the intensity of detection. Sera 2, 7, 4, 15, 5 and 1 detected the major 18-kDa allergen less distinctly and serum 17 did not detect it any longer. The allergen detection using serum 16 was the same as in the native extract. In the extract of the nougat chocolate extract 1 the detection of allergen bands was similar to the detection in the nougat chocolate extract 2. The extracts from the remaining nougat products revealed no positive results in immunoblotting experiments (data not shown).

The results of the EAST inhibition investigations using extracts of hazelnuts separated from different chocolates are illustrated in Fig. 4. The graphs of the homologous inhibition and the inhibition of a milk chocolate extract as a negative control are shown as well. The resulting C_{50} concentrations responsible for a 50% inhibition are listed in the legend. Although the IgE binding potency of each extract was reduced compared to the native extract (C_{50}=63 mg/l), each extract except for the negative control showed remaining allergenic activity. The C_{50} values vary between 366 mg/l (chocolate 1) and 1586 mg/l (chocolate 5).

By means of immunoblotting experiments IgE-binding activities were obtained by the extracts of chocolate 1 and 2. The immunoblotting results of the protein extract of chocolate 2 using seven different patients' sera are shown in Fig. 5. Each patient serum exhibited similar IgE binding patterns as in the native extract (see Fig. 3) with slight differences as regards the intensity of detection. Sera 16, 4, 15, 5 and 1 detected the major 18-kDa allergen less distinctly; serum 2 did not detect it any longer. The detection of the 14-kDa allergen by sera 16 and 5

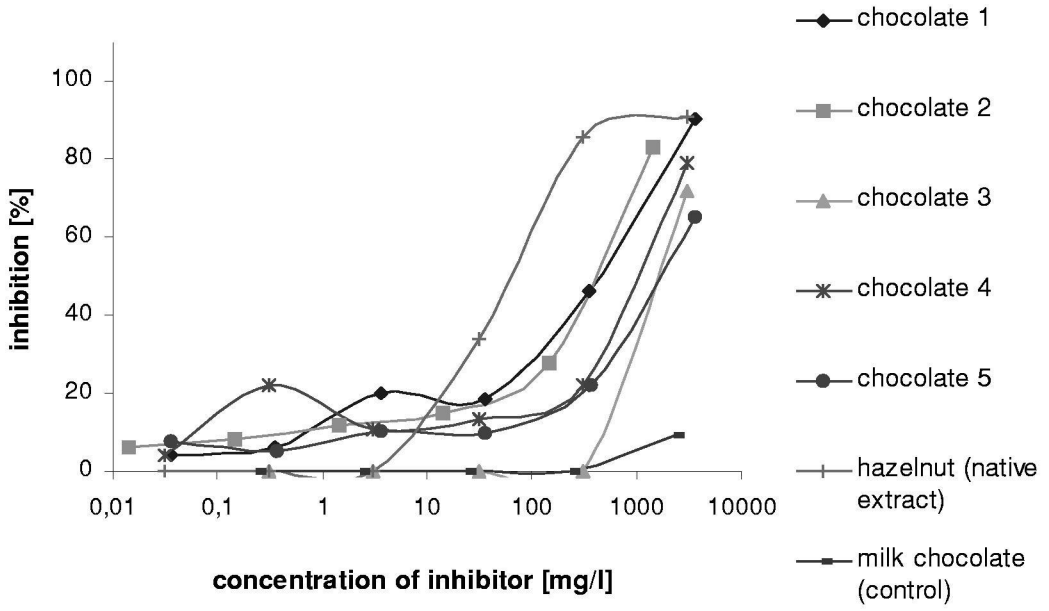

Fig. 4. EAST-inhibition of IgE-binding to hazelnut allergen disks by hazelnut chocolate extracts using a pooled serum of 13 allergic individuals. Native hazelnut extract was used as positive control and milk chocolate extract as negative control. Results are expressed in percent inhibition. The following C_{50} concentrations were obtained: native extract, 63 mg/l, chocolate 1, 366 mg/l; chocolate 2, 441 mg/l; chocolate 3, 1529 mg/l; chocolate 4, 950 mg/l; and chocolate 5, 1586 mg/l. The control extract of milk chocolate does not reach a 50% inhibition.

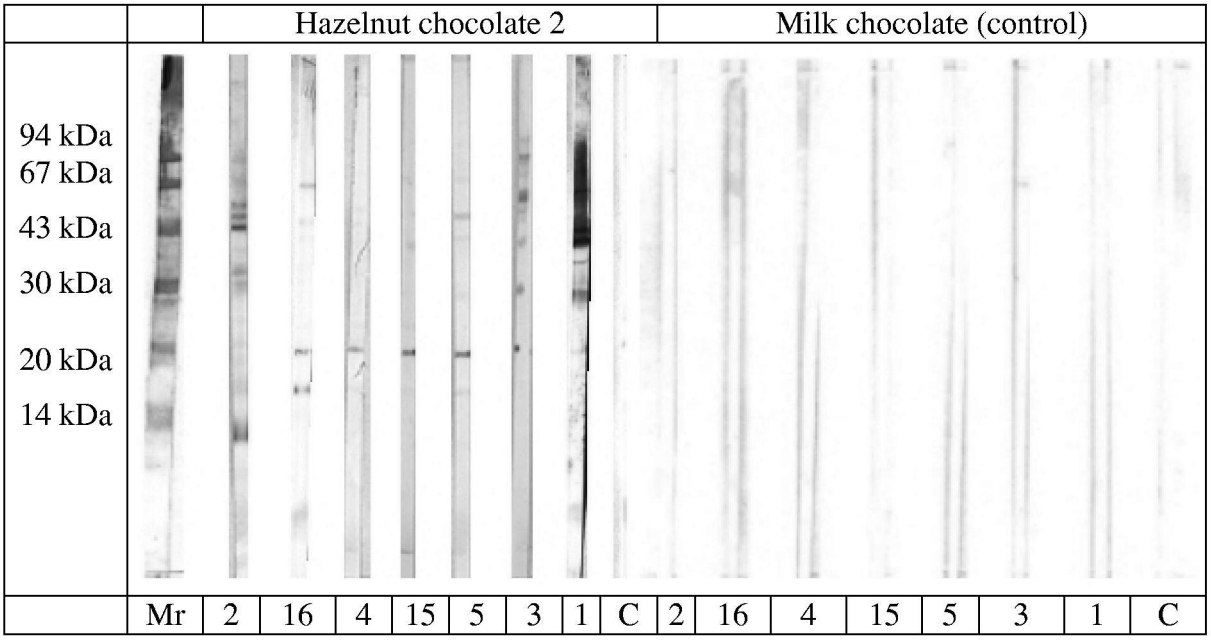

Fig. 5. Immunoblot of the hazelnut extract of hazelnut chocolate 2 and the milk chocolate as a negative control. Lanes marked represent incubation with sera from seven different hazelnut allergic patients (see Table 1). Negative control C was incubated using a serum of a non allergic individual. Molecular mass marker (M_r) was colloidal gold stained.

was also less strong than in the native extract. The negative control using a milk chocolate extract revealed no bands with any of the sera.

Fig. 6 reveals the EAST inhibition curves of two extracts from hazelnut croquant. An additional homologous inhibition was carried out to check the allergenic activity of the native hazelnut extract. The C_{50} values of croquant extract 1 and 2 (1374 and 1992 mg/l, respectively) expressed a reduction of IgE binding activities. By means of immunoblotting no allergen bands were detectable in the croquant extracts 1 and 2 (data not shown).

The EAST inhibition graphs of further products containing hazelnuts are shown in Fig. 7. Whilst the allergenic activity of the extract obtained from a muesli bar (C_{50}=63 mg/l) was similar to the native extract (C_{50}=45 mg/l), the IgE binding potency was distinctly reduced in the remaining extracts of hazelnut cake or cookies. The immunoblotting results of the muesli bar extract (see Fig. 8) did not differ from the results obtained by the native extract. Each of the sera detected IgE-binding proteins in the same way as in the native hazelnut extract (see Fig. 3). None of the remaining extracts from hazelnut cookies or cake showed allergen bands in immunoblotting experiments (data not shown).

4. Discussion

Although immediate-type reactions to hazelnuts are a widespread disease among children and adults in Europe [2,3], there are only a few studies with regard to the influence of processing on the allergenicity of hazelnuts [21]. Changes in IgE binding activity of food proteins and glycoproteins could be induced by various technological processes and treatments. Furthermore the modification of epitopes by conformational changes, thermal or hydrolytic fragmentation of proteins is possible. Except for a single study, in which the allergenicity of different tree nut oils including hazelnut oil was investigated [30], no literature is available concerning the allergenic potency of hazelnut products.

The aim of this study was to determine the IgE binding activity of different hazelnut products by

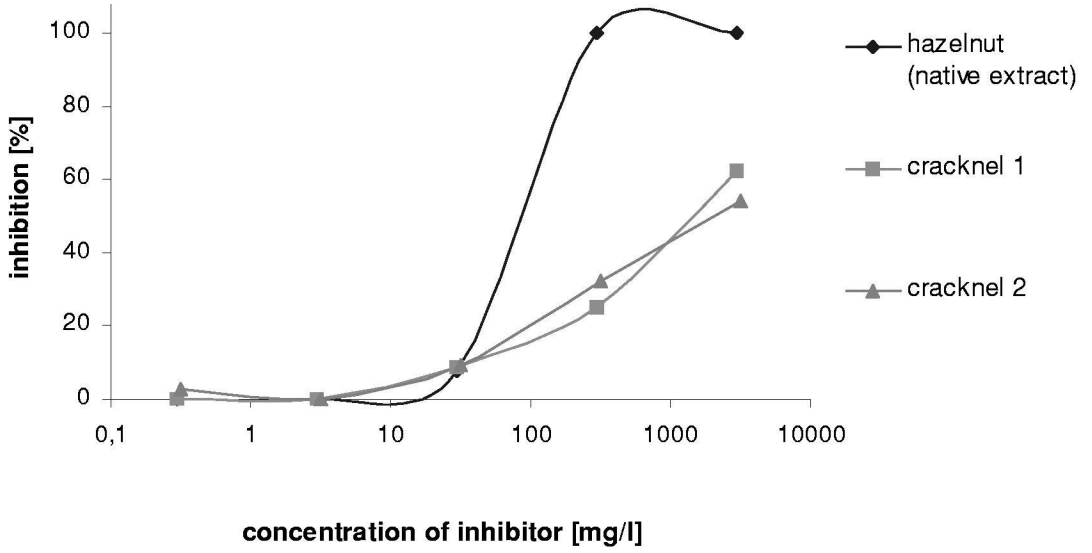

Fig. 6. EAST-inhibition of IgE-binding to hazelnut allergen disks by hazelnut croquant extracts using a pooled serum of 13 allergic individuals. Native hazelnut extract was used as positive control. Results are expressed in percent inhibition. The following C_{50} concentrations were obtained: native extract, 85 mg/l; croquant 1, 1374 mg/l; croquant 2, 1992 mg/l.

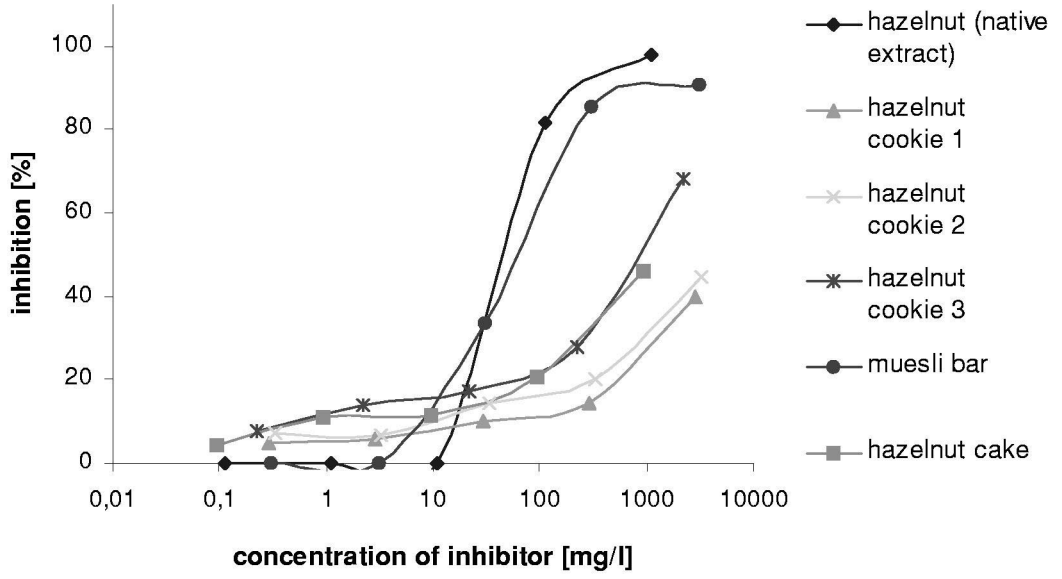

Fig. 7. EAST-inhibition of IgE-binding to hazelnut allergen disks by hazelnut cookie, cake and muesli bar extracts using a pooled serum of 13 allergic individuals. Native hazelnut extract was used as positive control. Results are expressed in percent inhibition. The following C_{50} concentrations were obtained: native extract, 45 mg/l; hazelnut cookies 3, 779 mg/l; muesli bar, 63 mg/l. The remaining extracts do not reach a 50% inhibition.

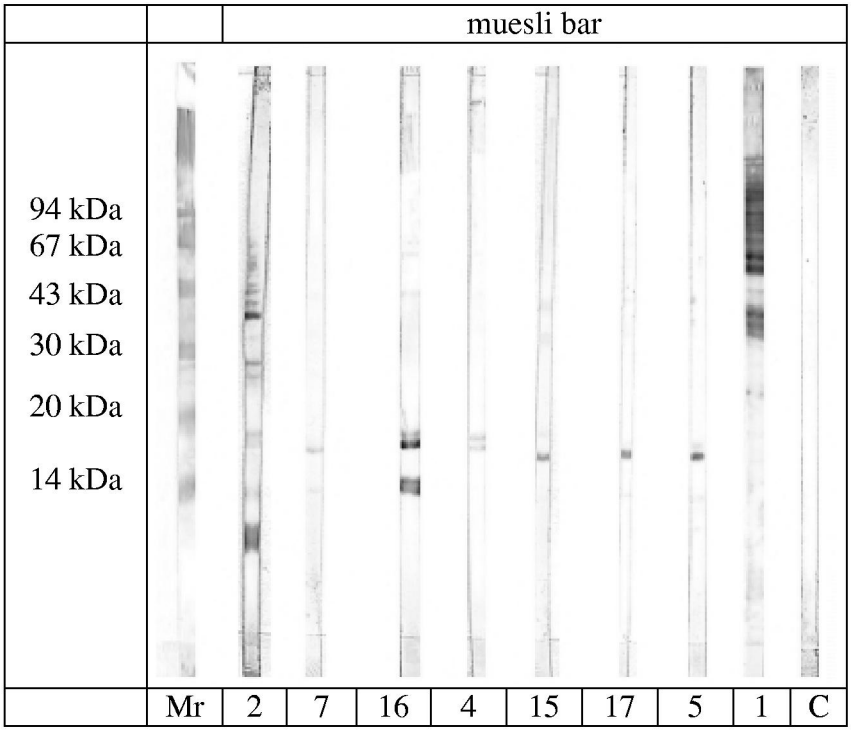

Fig. 8. Immunoblot of the hazelnuts of the muesli bar. Lanes marked represent incubation with sera from eight different hazelnut allergic patients (see Table 1). Negative control C was incubated using a serum of a non-allergic individual. Molecular mass marker (M_r) was colloidal gold stained.

means of immunoblot and EAST inhibition experiments. Whilst the protein extracts of only a few products (a muesli bar, two hazelnut chocolates and two nougat chocolates) showed allergen bands in immunoblot, almost all of the investigated extracts from different hazelnut products revealed a residual IgE-binding activity in EAST inhibition. A possible explanation for the higher sensitivity of EAST inhibition experiments could be the formation of low-molecular mass peptides during processing, which are no longer detectable by electrophoretic methods, such as immunoblotting.

Great differences were recognizable with regard to the allergenic potencies of the different products. The C_{50} values of the investigated hazelnut chocolates for example varied between 366 mg/l (chocolate 1) and 1586 mg/l (chocolate 5). In addition allergen bands were detectable by means of immunoblot in the extract of chocolate 1 and 2 but not in the remaining chocolate extracts. It is also remarkable that the extracts of the nougat chocolates 1 and 2 possessed the highest allergenic activity among the nougat products. A possible reason for the discrepancies between the IgE-binding activities of the hazelnut allergen extracts obtained from comparable products could be different applied roasting temperatures or roasting times during processing of the hazelnuts. In a previous study [23] we were able to show that hazelnut allergens are stable to 15 min heat treatment up to 155°C.

Unlike further birch pollen associated fruit and vegetable allergens which are considered heat labile, such as the major allergens of celery (Api g 1) [31] or apple (Mal d 1) [32,33], the hazelnut allergens are quite stable to heat and processing. This is not an untypical phenomenon of nuts. Bargman et al. [34] for example investigated the allergenicity of almonds and their products. They were able to show heat stable allergens as well. In a further study [35] the heat stability of peanut allergens was revealed.

Nordlee et al. [36] determined the allergenicity of 19 different peanut products. Those investigations did not result in a considerable reduction of the allergenic activity of peanut products compared with native extracts.

Although the IgE-binding activities of some of the investigated hazelnut products were reduced compared to the native extracts, no elimination of the allergenic potency was detectable. Therefore it is supposed that the investigated products are a potential hazard to hazelnut allergic individuals. This is significant especially with regard to the importance of hazelnuts as so-called hidden allergens in foods [22].

5. Conclusions

It has been demonstrated that the IgE binding activity of the proteins in different hazelnut products varies strongly. Although in some products a reduction of the allergenic potency is detectable, in none of the investigated products was the allergenicity eliminated. Therefore the diversity of processed foodstuffs containing hazelnuts is a potential hazard to hazelnut allergic individuals.

Acknowledgements

We would like to thank Dr. R. Weßbecher, University Hospital Eppendorf, Hamburg, Germany for kindly supplying sera of hazelnut-allergic patients. Special thanks to Mrs S. Schubert and Mr O. Bendokat for their technical support.

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