

Influence of Varieties, Storage and Heat Treatment on IgE-Binding Proteins in Hazelnuts (*Corylus avellana*)

M. WIGOTZKI, H. STEINHART AND A. PASCHKE

University of Hamburg, Institute of Biochemistry and Food Chemistry, Hamburg, Germany

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The allergenicity of four hazelnut varieties (Runde Römer, Levantiner, Neapler, Contorta) and different types of heat treated and stored hazelnuts was examined and compared by SDS-PAGE/immunoblotting and EAST-inhibition experiments using sera of 19 hazelnut allergic individuals. The immunoblot and EAST-inhibition investigations of the four varieties revealed only slight differences with regard to the allergenic activities. Heat treatment at 100°C for up to 90 min had no influence on the allergenicity of hazelnut proteins. The IgE binding activity of the main hazelnut allergens decreased after 15 min heat treatment at temperatures between 100 and 185°C and was no longer detectable at 170°C. A protein < 14 kDa appeared to be very stable to heat and could be detected even after treatment at 185°C. Microwave treatment and storage of ground hazelnuts up to 19 weeks at room temperature had no influence on the allergenicity of hazelnut proteins.

Keywords: Food allergy, hazelnut, varieties, heat treatment, microwave, storage, IgE-binding, SDS-PAGE, immunoblot, EAST, EAST-inhibition

INTRODUCTION

Immediate-type allergic reactions to foodstuffs are usually caused by water-soluble proteins, or glycoproteins with molecular weights between approximately 10 and 70 kDa which are natural components of food (Lehrer *et al.*, 1996). These reactions are mediated by human immunoglobulin E (IgE) antibodies specific to these allergens. The prevalence of immediate-type food allergy has been estimated at 1–3% among the adult population and up to 5–8% of the paediatric population (Zeiger, 1990). Hazelnuts are considered some of the most common causes of food allergies among both children (Crespo *et al.*, 1995) and adults (André *et al.*, 1994) in Europe. Even near-fatal anaphylactic reactions to hazelnuts are described (Sampson *et al.*, 1992). Although cross-reactivities between hazelnut and tree pollen, such as

birch, alder, hazel, hornbeam, oak and mugwort are well documented (Halmepuro *et al.*, 1984; Calkoven *et al.*, 1987; Dreborg, 1988; Hirschwehr *et al.*, 1992; Caballero *et al.*, 1994; Asero, 1997; Caballero *et al.*, 1997), there are only a few investigations regarding the responsible allergenic proteins in hazelnuts (Hirschwehr *et al.*, 1992; Caballero *et al.*, 1997). By means of SDS-PAGE and immunoblotting, Hirschwehr *et al.* (1992) identified an 18 kDa protein as the major allergen in hazelnuts and additional IgE-binding proteins at 14, 37, 40, 46 and 69 kDa. Caballero *et al.* (1997) detected four main allergens at 42, 17 and two bands < 16 kDa. Although hazelnuts are used as ingredients of many foodstuffs and are marketed in many forms and many different varieties, very little is known about the influence of processing on the allergenicity and the heat stability of the allergens (Vieths *et al.*, 1997). The allergenicity of different hazelnut varieties has not been investigated either. The aim of the present study was to investigate the influence of heat treatment and storage on the allergenicity of the main allergens in hazelnuts by means of SDS-PAGE, immunoblotting and enzyme-allergosorbent test (EAST) inhibition. The differences between hazelnut varieties were assessed in a similar manner.

MATERIALS AND METHODS

Human Sera

A total of 18 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 patient selection was based solely on EAST test and medical history. Skin prick test or challenge test results were not available. All sera were EAST class ≥ 2 when tested for specific IgE against hazelnut extract by EAST test (Allergopharma, Reinbek, Germany). Pool-serum was collected using equal aliquots of 13 sera with specific IgE-binding against hazelnut. A serum from a non-atopic individual with no history of food hypersensitivity (EAST class 0) was used as negative control.

Hazelnuts

Three different varieties of whole hazelnuts (Runde Römer, origin Italy, Levantiner, origin Turkey and Neapler, origin Sicily) were obtained from M. Buenger Company, Oststeinbek, Germany. In addition the wild growing Contorta strain (origin Germany) was investigated. A further hazelnut variety (Barcelona, origin California, USA) used for the different heat treatments, storage and as a reference variety in EAST inhibition experiments was purchased at a local food store.

Applied Treatments

The ground hazelnuts of the Barcelona variety were conventionally heated for 15 min at temperatures between 100 and 185°C and for different times between 15 and 90 min at 100°C. In addition the ground hazelnuts were processed by 10 min microwave treatment at 630 W. Storage was carried out with ground nuts at room temperature for 1 week up to 19 weeks.

Allergen Extracts

The raw, peeled hazelnuts were frozen by liquid nitrogen, chopped into small pieces and then ground in acetone at -40°C (cooling by dry ice). The mixture was stored overnight at -20°C. The precipitates were washed three times with acetone (-20°C), filtered and lyophilized. Protein extracts were obtained by extracting 1 g of this acetone powder with 30 ml phosphate-buffered saline [PBS (0.01 M-potassium phosphate buffer, pH 7.4, 0.13 M-sodium chloride)] for 60 min by ice cooling. The suspension was centrifuged (4°C, 10 500 $\times$ g, 60 min) and the supernatant filtered. The solution was divided into aliquots of 2 ml, lyophilized and stored at -20°C until use. Heat treatment and storage was carried out with peeled and ground

hazelnuts. The processed or stored nuts were defatted three times with acetone, filtered and lyophilized. The proteins were extracted as described above. The protein contents of the extracts were determined by the method of Bradford (1976).

Enzyme Allergosorbent Test (EAST)

Commercial filter paper disks (6 mm diameter, Schleicher & Schuell, Dassel, Germany) were activated with cyanogen bromide according to the method of Ceska and Lundquist (1972). Proteins of the allergen extracts of hazelnuts of the Barcelona variety were coupled to these disks by a modified procedure described by Möller *et al.* (1997).

Specific IgE antibodies against hazelnuts in patients' sera were measured using a commercial test kit (Allergopharma, Reinbek, Germany) according to the manufacturer's instructions. In this indirect, non-competitive ELISA, IgE bound to allergens is immunodetected by a mouse monoclonal anti-IgE antibody labelled with alkaline phosphatase. The enzymatic activity is monitored by hydrolysis of *p*-nitrophenylphosphate (PNPP, Bio-Rad, Munich, Germany). After measuring the absorbances at 405 nm, the results were expressed in EAST classes (1–4) and specific IgE levels [in U ml⁻¹ (1U = 2.4 ng IgE)], respectively.

EAST Inhibition

For inhibition experiments a 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 raw and processed hazelnuts was prepared in five to six steps using the same incubation buffer. Non-specific inhibition was checked with ovalbumin (OVA). 50 µl of the diluted serum was mixed with 50 µl of the inhibitor solution and an 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, Reinbek, Germany), 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 (Möller *et al.*, 1997).

SDS-PAGE

SDS-PAGE was performed in 10% acrylamide gels (80 mm × 80 mm × 1.5 mm) using the 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 (Vieths *et al.*, 1992a) were boiled for 3 min. Applying about 20 µg protein per well, electrophoresis was performed at 200 V constant voltage for 0.5 h on the NuPAGE® electrophoresis system (Novex, San Diego, CA, USA) using a MultiDrive XL power supply (Pharmacia LKB, Uppsala, Sweden). Gels were stained by Coomassie Brilliant Blue R250 (0.07% in water:ethanol:acetic acid, 6:3:1) (Fling & Gergerson, 1986).

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 LKB, Uppsala, Sweden) according to Kyhse-Andersen (1984) with discontinuous buffer system as described by Vieths *et al.* (1992a). Afterwards the membrane was dried for 30 min and cut into strips of 2 mm.

Immunostaining of IgE was performed by the method of Vieths *et al.* (1992a) slightly modified as described previously by Möller *et al.* (1997). Briefly, dried membrane strips were blocked twice (15 min) with 5% skimmed milk powder and 0.1% (v/v) Tween 20 in PBS

TABLE 1. Characterization of patients' sera with IgE binding to hazelnut proteins

No.	Patient (sex, age)	Clinical symptoms	Specific IgE (U ml ⁻¹)	EAST class	Further sensitivities
1	St 27 (female, nk)	nk	nk	3	nk
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)		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	AT (female, nk)	nk	> 17.5	4	Apple, pear, potato, carrot, rye, wheat
8	PW (male, 51)	nk	nk	4	Birch pollen
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	UW (male, 26)	IM, SL, DB	4.24	3	Apple, potato, walnut, birch pollen, hazel pollen, alder pollen
17	BS 127 (nk, nk)	nk	6.04	3	nk
18	AS (male, 31)	nk	nk	4	nk
19	St 12 (male, nk)	nk	nk	2	nk

DB: difficulty in breathing, HB: heartburn, IM: itching in mouth, IT: itching in throat, SL: swelling of lips, nk: not known.

(Ahlroth *et al.*, 1995) in mini incubation trays (Bio-Rad, Munich, Germany). The strips were incubated overnight at room temperature with 100 µl of each serum diluted in a total volume of 1.5 ml of incubation buffer (0.3% (w/v) BSA and 0.1% (v/v) Tween 20 in PBS). Then strips were incubated consecutively with rabbit anti-human-IgE (1:4000, DAKO, Copenhagen, Denmark), and biotinylated goat anti-rabbit-IgG (1:6000, DAKO) for 1 h each and with streptavidin–horseradish peroxidase (1:20 000, Medac, Hamburg, Germany) for 20 min. 3,3',5,5'-tetramethylbenzidine and dioctylsodiumsulphosuccinate were used as substrates for staining. One strip was incubated with the serum of a non-allergic patient to check unspecific binding.

To check the efficiency of protein transfer, from each gel one strip including separated proteins and a molecular weight marker (Pharmacia LKB, Uppsala, Sweden) was stained with colloidal gold staining (Bio-Rad, Munich, Germany) according to Moeremans *et al.* (1985).

RESULTS

Characterization of Human Sera

The clinical information on the 19 hazelnut-allergic individuals in this study is summarized in Table 1. All patients with known anamnesis suffer from oral symptoms to hazelnuts, such as itching in the mouth or throat or swelling of the lips. One patient suffers from heartburn and two further individuals have difficulties in breathing after hazelnut consumption. None

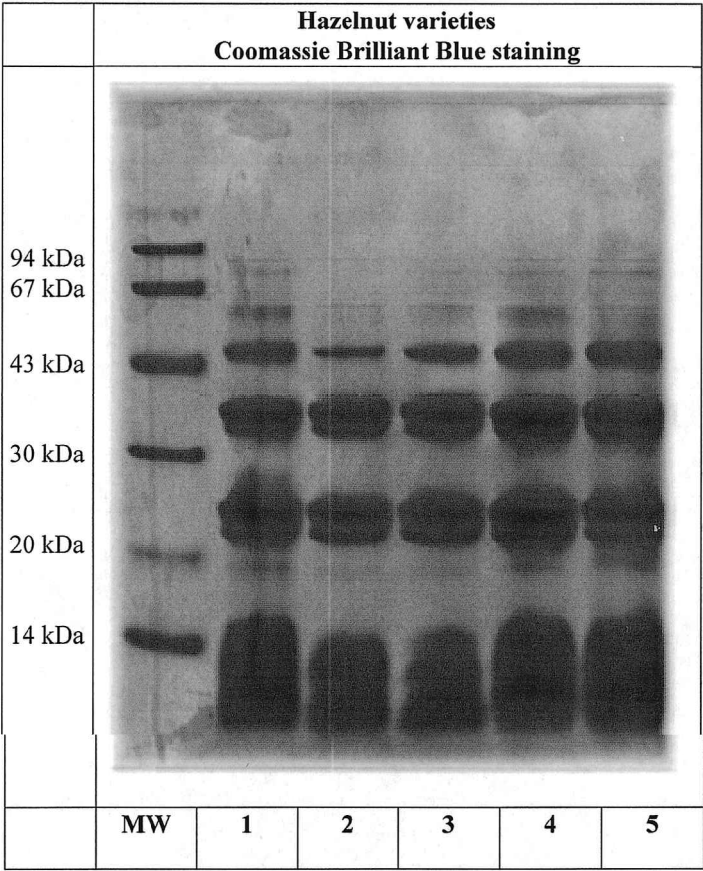


FIG. 1. Coomassie brilliant blue stained protein extracts of hazelnut varieties separated using SDS-PAGE. 1 = reference variety (Barcelona), 2 = Contorta variety, 3 = Levantiner variety, 4 = Runde Römer variety, 5 = Neapler variety.

of the patients with known anamnesis shows gastrointestinal symptoms or anaphylaxis. As Table 1 reveals many patients show further sensitivities to different fruits, nuts and tree pollen.

The sera of the 19 patients were examined for specific IgE antibodies against hazelnut. The IgE contents and the resulting EAST classes are also summarized in Table 1. The IgE contents vary from 0.76 U ml⁻¹ to > 17.5 U ml⁻¹ with EAST classes from 2 to 4. The pooled serum used for EAST inhibition experiments contains thirteen of the listed sera (3–6 and 9–17).

IgE Binding of Hazelnut Varieties

Figure 1 illustrates the Coomassie brilliant blue stained proteins in the extracts of the raw hazelnut varieties separated by SDS-PAGE. The molecular weight marker protein bands ranged from 14 to 94 kDa. The electrophoretic patterns for the four varieties and the reference variety are very similar but indicate slight differences in the relative concentrations of some proteins in the lower molecular weight range. Figures 2(a) and (b) show immunoblots of the different hazelnut varieties incubated with sera of eight allergic individuals. The serum from each patient exhibited similar IgE binding patterns in each variety with slight differences with

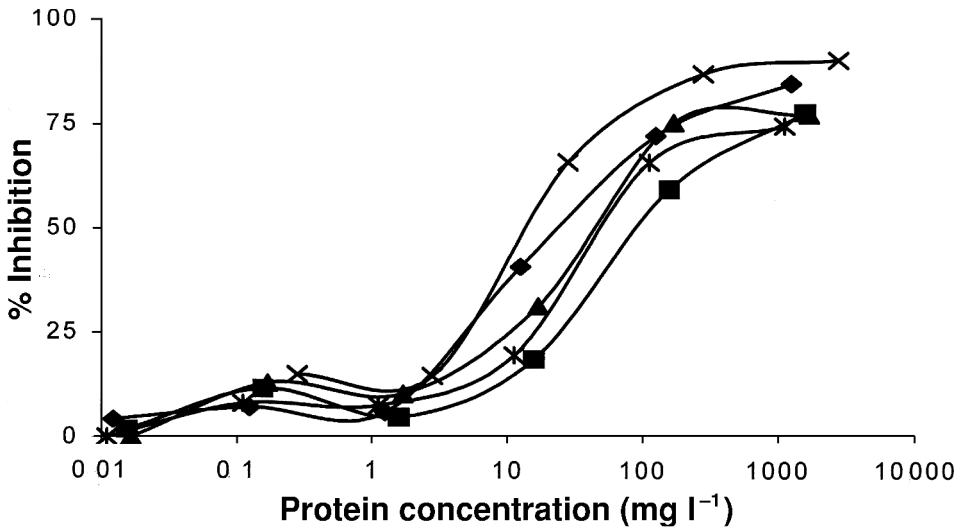


FIG. 3. Results of dose-related inhibition of IgE binding to allergen disks with reference hazelnut allergens. Extracts from native reference hazelnut (♦) and from the four varieties Contorta (×), Neapler (▲), Levantiner (*) and Runde Römer (■) were used as inhibitors.

regard to the intensity of detection. Whilst an 18 kDa protein was detected by almost each of the sera, sera 2 and 7 reacted strongly to additional proteins of approximately 40, 50 and < 14 kDa. In the unspecific protein staining using colloidal gold in each variety protein bands were detected between 10 and 100 kDa.

The EAST inhibition experiments, applied to compare the IgE binding potency of the different varieties, were carried out using the Barcelona variety as a reference on the solid phase and a pooled serum of 13 patients' sera of known allergen specificity (see Table 1). Figure 3 reveals the inhibition graphs of the four varieties and the homologous inhibition of the reference strain. Each of the varieties were allergenic as demonstrated by their ability to inhibit the binding of serum IgE to the solid-phase allergens. The following C_{50} -concentrations of hazelnut proteins responsible for a 50% inhibition of the IgE binding, which reflect the extracts' allergenic potencies, were obtained: the Contorta strain with a C_{50} -concentration of 14 mg l⁻¹ showed the highest inhibition ability followed by the Levantiner variety (45 mg l⁻¹), the Neapler variety (51 mg l⁻¹) and the Runde Römer variety (92 mg l⁻¹). The reference strain Barcelona revealed a 50% inhibition concentration of 26 mg l⁻¹. Controls with 1 mg ml⁻¹ of OVA resulted in 0% inhibition.

IgE Binding in Processed Hazelnuts

The influence of processing on the allergenicity of the hazelnut proteins was investigated using different heat treatments. The hazelnuts were heated conventionally by dry heat, varying temperature and heating time (15 min at 100°C, 155°C, 170°C and 185°C, 30 min, 60 min and 90 min at 100°C), and by microwave (10 min, 630 W). In addition the influence of storage (1–19 weeks at room temperature) on the allergenicity of the hazelnuts was investigated.

A selection of Coomassie Brilliant Blue stained protein extracts of the heat treated and stored hazelnuts separated by SDS-PAGE is shown in Figure 4. The electrophoretic patterns

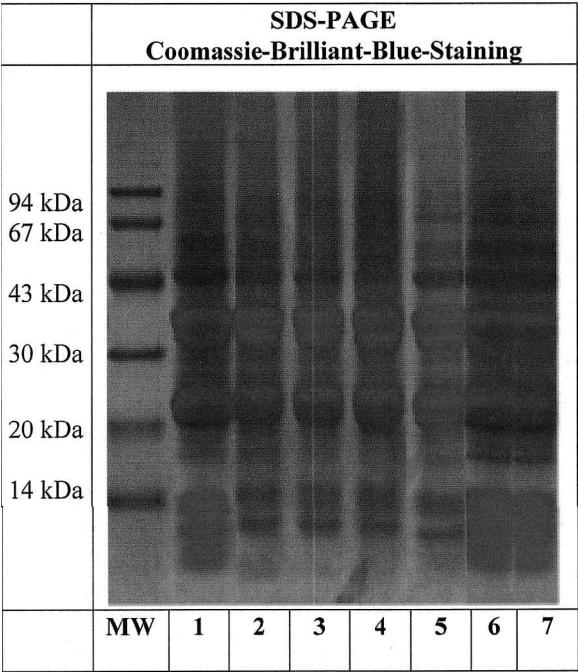


FIG. 4. Coomassie brilliant blue stained protein extracts of processed and stored hazelnuts separated by SDS-PAGE. MW = molecular weight marker, 1 = 90 min heated at 100°C, 2 = 15 min heated at 155°C, 3 = 15 min heated at 170°C, 4 = 15 min heated at 185°C, 5 = 19 weeks storage, 6 = 10 min microwave treatment at 630 W, 7 = native hazelnut extract (Barcelona variety).

of the conventionally heated extracts are similar, although the 18 kDa protein is not detected in the samples heated up to 170 and 185°C. The extract of the hazelnuts heated 90 min at 100°C shows no differences to the extract heated 15 min at the same temperature. The microwaved and the 19 weeks at room temperature stored hazelnut extracts are similar to the native reference extract.

The results of the EAST inhibition investigations of the allergen extracts of the processed and stored hazelnuts using the pooled serum of 13 allergic individuals (see Table 1) are illustrated in Figures 5 and 6. The inhibition graphs of the conventionally heated hazelnuts at 100°C show no differences with regard to the allergenic potency (not shown). The C₅₀ values hardly differ (18–22 mg l⁻¹) and are very similar to the C₅₀ value of the native hazelnut extract (26 mg l⁻¹). The allergenicity of the protein extracts apparently does not depend on heating time (15–90 min).

The inhibition graphs in Figure 5 were obtained by varying temperature at a constant heating time of 15 min. Whilst the C₅₀-concentrations did not increase from 100°C (18 mg l⁻¹) to 155°C (21 mg l⁻¹), they distinctly increased up to 175 mg l⁻¹ at 170°C and 2382 mg l⁻¹ at 185°C, which indicates a clearly recognizable decrease of the allergenic activity. OVA as an unspecific inhibitor inhibited 2.7%.

Figure 6 shows the inhibition curves of the extracts of stored and microwaved hazelnuts. The C₅₀-value after microwave treatment (25 mg l⁻¹) was similar to the native extract (26 mg l⁻¹). The 50% inhibition concentrations of the stored hazelnut extracts are nearly same (48 mg l⁻¹ after 1 week storage, 48 mg l⁻¹ after 3 weeks storage and 67 mg l⁻¹ after 19 weeks storage). Controls with 1 mg ml⁻¹ of OVA resulted in 2.7% inhibition.

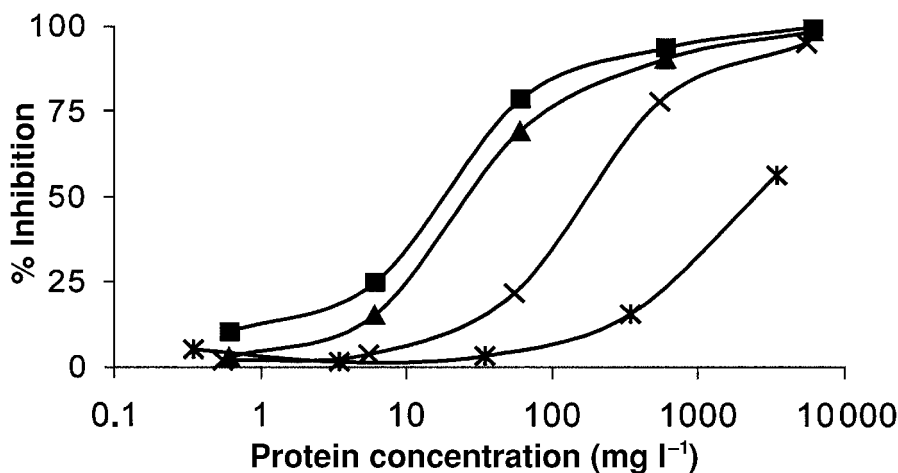


FIG. 5. Results of dose-related inhibition of IgE binding to allergen disks with reference hazelnut allergens. Extracts from 15 min heat treated hazelnuts were used as inhibitors. Applied temperatures: 100°C (■), 155°C (▲), 170°C (X) and 185°C (*).

The results of the immunoblot investigations of the allergen extracts of the processed and stored hazelnuts incubated with sera of seven allergic individuals (see Table 1) are illustrated in Figures 7(a) and (b). In the stored and the microwaved samples (Figure 7(a)) the sera detected the same proteins as in the native hazelnut extract (not shown). Figure 7(b) compares the immunoblots of the conventionally heated hazelnuts at 155 and 185°C. Whilst the allergen detection after heating at 155°C is comparable to the detection in the native extract, there is no detection of the 18 kDa major allergen and the 14 kDa protein in the extract heated at 185°C. After heating at this temperature only sera 2 and 7 detect allergen bands. A protein with a molecular weight of < 14 kDa appears to be very stable to heat treatment. Some allergens with molecular weights between 30 and 70 kDa seem to be heat stable as well.

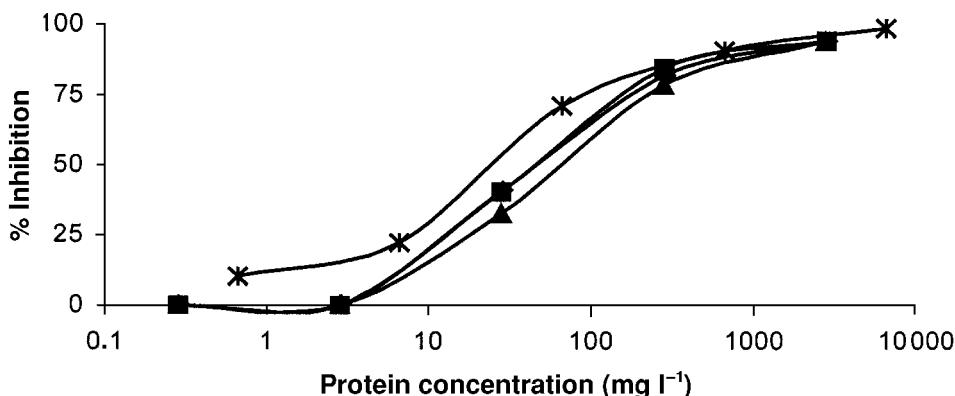


FIG. 6. Results of dose-related inhibition of IgE binding to allergen disks with reference hazelnut allergens. Extracts from hazelnuts stored at room temperature and from microwaved hazelnuts (10 min, 630 W) were used as inhibitors. Storing times: 1 week (◆); 3 weeks (■); and 19 weeks (▲). Microwaved extract (*1).

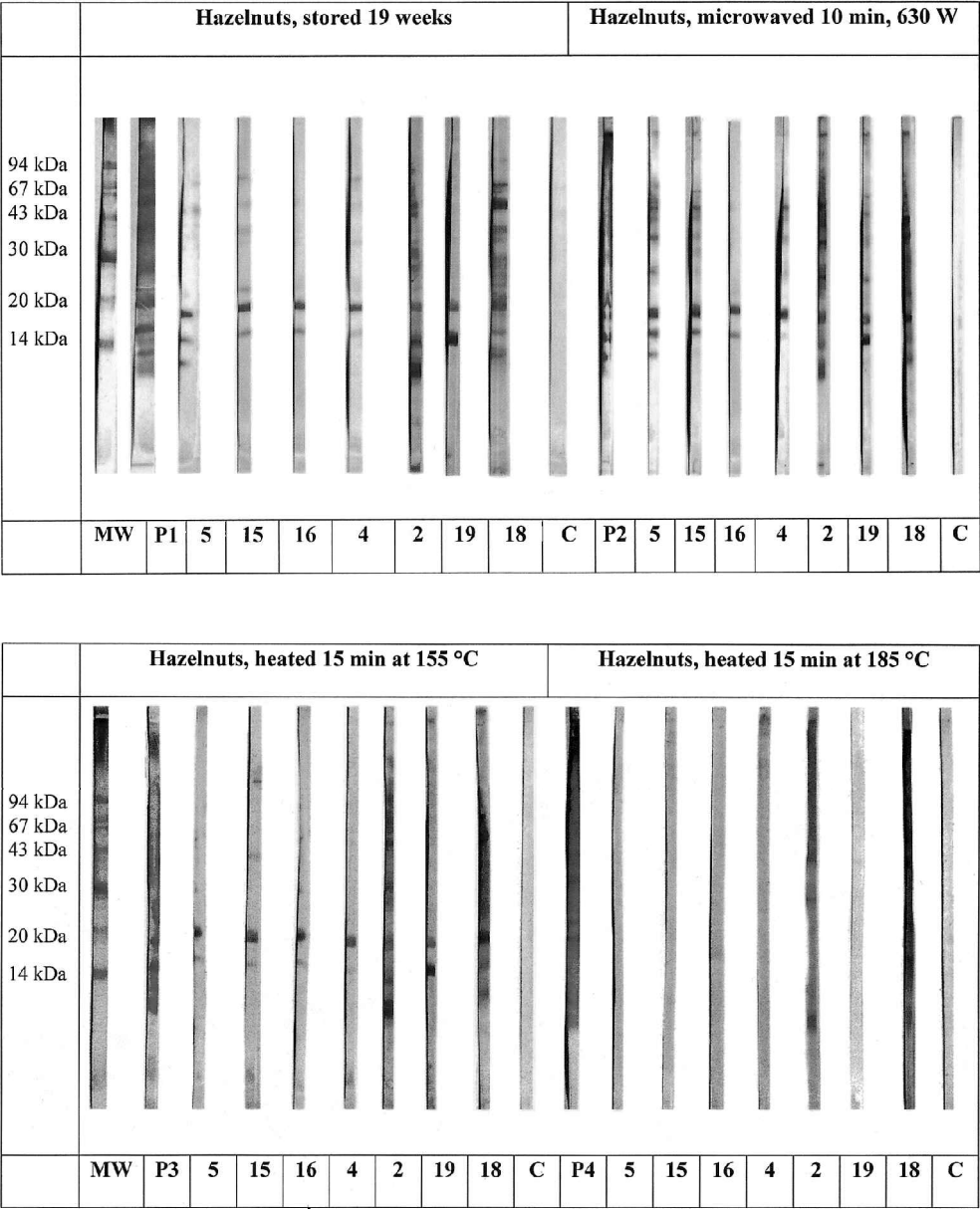


FIG. 7. (a) Immunoblot of 5 weeks stored and microwaved (10 min, 630 W) hazelnuts. Lanes marked represent incubation with sera from seven different hazelnut allergic individuals (see Table 1). C is a negative control and was incubated using a serum of a non-allergic individual. Molecular weight marker (MW) and protein extracts (P1 and P2) were colloidal gold stained. (b) Immunoblot of hazelnuts heated 15 min at 155°C and 185°C. Lanes marked represent incubation with sera from seven different hazelnut allergic individuals (see Table 1). C is a negative control and was incubated using a serum of a non-allergic individual. Molecular weight marker (MW) and protein extracts (P3 and P4) were colloidal gold stained.

DISCUSSION

Although many reports have been published during the last few years concerning the association between birch pollinosis and sensitization to hazelnuts and other fruits or vegetables (Roeser *et al.*, 1987; Dreborg, 1988; Gall *et al.*, 1994; Caballero & Martin-Esteban, 1998), there are only a few reports about the characterization of the proteins which are responsible for the allergenic reaction to hazelnuts. In recent studies, Hirschwehr *et al.* (1992) and Caballero *et al.* (1997) demonstrated a 17–18 kDa protein which shares common epitopes with the major birch pollen allergen Bet v I as the major allergen in hazelnuts. Each of the patients sera used in our immunoblot experiments detected this 18 kDa protein as well. In addition some of the sera detected allergens at approximately 40, 14 and < 14 kDa. These results are comparable with the results of Hirschwehr *et al.* (1992) and Caballero *et al.* (1997).

In our study we were able to show that the detection of the major hazelnut allergens only slightly depended on different varieties. Bargman *et al.* (1992), who carried out similar investigations on almond varieties, found no large differences in the IgE binding patterns, whilst the allergenic activity of different apple strains investigated by Vieths *et al.* (1994) clearly varied.

A further aim of this study was to investigate the influence of different heat treatments on the IgE binding activity of the main allergens in hazelnuts. Changes in IgE binding activity of food proteins and glycoproteins could be induced by various technological processes and treatments. The modification of epitopes by conformational changes, thermal or hydrolytic fragmentation of proteins is possible. In this study, heat processing was simulated by conventional dry heating for different periods and by microwaving. Immunoblot and EAST inhibition experiments revealed that the hazelnut allergens are, unlike different fruits and vegetables (Vieths *et al.*, 1992b), quite stable to storage. Even microwaving and conventional heating at 155°C did not reduce the allergenic activity of the hazelnut proteins. This distinguishes the hazelnut from many fruits and vegetables containing heat labile major allergens, such as for example apple or celery (Vieths *et al.*, 1992b; Jankiewicz *et al.*, 1997). The major celery allergen Api g 1 was destroyed by 10 min microwave treatment.

Although the hazelnut allergens appear to be quite resistant to heat treatment, there are differences in the stability of different allergens. The 18 kDa major allergen, which was detected by each serum used in this study, and the 14 kDa allergen are not resistant to temperatures > 170°C, whilst a protein of molecular weight < 14 kDa seems to be extremely heat stable. The results of our study are of great importance regarding the temperatures used in industrial roasting technologies. Perren (1996) discovered that the roasting temperature has a great influence on the oxidative stability of the hazelnuts. In order to avoid oxidation during roasting the temperature should not exceed 145°C. Therefore it is possible that the allergenic activity of the IgE binding hazelnut proteins is not eliminated by the temperatures applied in industrial roasting.

CONCLUSIONS

It has been demonstrated that the IgE binding activity of the main hazelnut allergens hardly depends on variety. The allergens are stable to storage, microwave treatment and conventional heating up to 155°C. There are proteins of low molecular weight < 14 kDa which are even stable at higher temperatures. To get more information about the allergenic activity of hazelnuts as a common food ingredient further studies are necessary to investigate the diversity of processed foodstuffs containing hazelnuts.

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