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Antibody binding to hazelnut (*Corylus avellana*) proteins: the effects of extraction procedure and hazelnut source

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The quality of an applied protein extract is important in both serological and *in vivo* diagnosis of allergy, and for allergen detection methods. In the present study the effects of the extraction procedure and hazelnut source on antibody binding to hazelnut (*Corylus avellana*) proteins were investigated. An overnight extraction procedure in tris–glycine buffer at 45°C was compared to a quick, vigorous extraction in a tris buffer at room temperature (RT). Antibody binding was studied by the use of polyclonal antiserum, a monoclonal antibody against the pollen-unrelated allergen Cor a 9, and human IgE from hazelnut allergic individuals. The extraction procedure, as well as the storage time and degree of processing of hazelnuts affected the protein constitution of the extract, as visualised by SDS–PAGE. Fresh and roasted (10 min at 170°C) hazelnuts generally contained a higher amount of intact hazelnut proteins than elder (>6 months) raw hazelnuts stored at RT. The time of storage of raw hazelnuts was inversely related to the amounts of Cor a 9 and IgE-binding proteins. In general, a rapid extraction procedure increased the efficacy of protein extraction and antibody-binding capacity to the proteins compared to an overnight extraction protocol. The present study indicates that for the production of hazelnut protein extracts, the use of fresh hazelnuts is important. A quick, vigorous extraction in a tris buffer might contribute positively, at least for extraction of Cor a 9.

Keywords: antibody; Cor a 9; extraction; hazelnut; IgE

Introduction

Hazelnut (*Corylus avellana*) is among the common tree nuts that cause food-induced allergic reactions. It is widely used in pre-packaged food, particularly in pastry and ice-cream products. The prevalence of hazelnut allergy is estimated to be between 0.1 and 0.5% in Europe (de Groot, de Jong, Vuijk, & van Wijk, 1996; Tariq et al., 1996). Allergy to hazelnuts is often associated with tree pollen allergy, although hazelnut allergy unrelated to pollen is also common. Patients with pollen-related hazelnut allergy generally express symptoms of the mild oral allergy syndrome, which are caused by cross-reaction between hazelnut allergens (Cor a 1.04 and Cor a 4) and tree pollen allergens. In contrast, in patients with pollen-unrelated hazelnut allergy, severe allergic reactions upon ingestion of hazelnuts have been reported. Allergens that have been related to these symptoms are Cor a 8, a lipid transfer protein (Schocker et al., 2004), Cor a 9, an 11S-globulin seed storage

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protein (Beyer, Grishina, Bardina, Grishin, & Sampson, 2002) and a minor allergen Cor a 11 (Lauer et al., 2004).

For the diagnosis of food allergy by serological testing and skin prick tests, and for *in vitro* methods for the determination of hazelnut proteins in foods, an adequate hazelnut extract is advantageous. Several protocols have been described for this purpose and these generally include buffers based on phosphate-based saline (PBS) for antibody-binding studies to allergens, or tris (Tris (hydroxymethyl) aminomethane) in detection methods. In our laboratory, the protein extraction procedure commonly used for detection methods is an O/N incubation of a homogenised (food) product in tris–glycine buffer at 45°C. This protocol was shown to be sufficiently adequate for a number of detection assays, including an assay for hazelnut proteins (Faeste, Holden, Plassen, & Almlı, 2006). However, when studies were performed with a monoclonal antibody against the hazelnut allergen Cor a 9, a low binding was observed towards the hazelnut extract. This prompted us to compare two extraction procedures by the use of antibody-binding studies with polyclonal, monoclonal and patient-derived IgE antibodies. In addition, the influence of duration of storage and roasting on the quality and antibody-binding capacity of the extract were included in the study.

Materials and methods

Materials

Hazelnuts of source A (raw hazelnuts) were obtained from a local store and originated from Iran (unknown variety). These hazelnuts were routinely used in our laboratory for the preparation of hazelnut proteins, for example, in a time-resolved fluoroimmunoassay for the determination of hazelnut proteins. The other hazelnut sources (B, C, D and E) were obtained as separate ingredients for a chocolate paste from a Norwegian manufacturer of chocolate products. The hazelnuts of these sources originated from Italy (variety Round Roman). The hazelnuts of sources B and C were raw. Source D consisted of roasted (10 min at 170°C), intact hazelnuts. Source E are roasted hazelnuts similar to source D that were homogenised into a hazelnut paste by the Norwegian manufacturer. The approximate storage times of the hazelnuts after arrival at the laboratory were: A, 5 years; C, 2 weeks and B, D and E, 6 months. An overview of the characteristics of the hazelnut sources is shown in Table 1.

Protein extractions

Two protein extraction procedures were used (Table 2). The first method has been described previously (Holden, Faeste, & Egaas, 2005). In brief, samples were homogenised with a blade homogeniser (Grindomic GM200, Retsch GmbH, Haan, Germany).

Table 1. Overview of the hazelnuts used throughout the study.

Batch	Condition	Country of origin	Time of storage	Storage temperature
A	Raw	Iran	Five years	–20°C
B	Raw	Italy	Six months	RT
C	Raw	Italy	Two weeks	RT
D	Roasted	Italy	Six months	RT
E	Roasted, paste	Italy	Six months	RT

Table 2. An overview of the two extraction procedures.

Extraction	Procedure 1	Procedure 2
Buffer	0.1 M tris–0.5 M glycine	0.05 M tris
pH	8.7	7.5
Duration	16 h	5 min
Temperature	45°C	RT
Treatment	Gentle shaking	vigorous mixing

Homogenised samples (2 g) were extracted with 10 mL of 0.1 M Tris–0.5 M glycine, pH 8.7, overnight at 45°C, centrifuged at 39,200 *g* for 25 min at 4°C and supernatants were filtered through a low protein-binding filter unit (0.45 µm, Millipore, Molsheim, France). The second procedure was based on a previously described method for the isolation of lupin globulins (Dooper, Holden, Faeste, Thompson, & Egaas, 2006). In short, hazelnuts were homogenised as described above and a portion (2 g) was defatted with hexane (10 ml) by vigorous mixing (5 min at room temperature, RT), centrifuged, and the pellet was air-dried. The proteins were dissolved in 50 mM Tris, pH 7.5 by vigorous mixing (5 min at RT). The samples were centrifuged at 39,200 *g* for 24 min at 4°C and the supernatant was filtered as described above.

Total protein concentrations were determined according to the Lowry method (DC protein assay; Bio-Rad, Hercules, CA, USA) using bovine serum albumin (BSA) as a standard. All protein extracts were stored in aliquots at –20°C. Each aliquot was only used once.

Time-resolved fluoroimmunoassay for the determination of hazelnut proteins

The fluoroimmunoassay for the determination of hazelnut proteins was performed as described by Faeste et al. (2006). In short, flat-bottom high protein-binding 96-well plates were coated with 5 mg/l of polyclonal anti-corylin antibody in 0.05 M carbonate–bicarbonate buffer (pH 9.8, Sigma-Aldrich, Oslo, Norway) for 16 h at 4°C. Blocking was performed with blocking/assay buffer (1% BSA in phosphate buffered saline, PBS) for 1 h at RT. The plates were then ready to use and stored at 4°C until further use. For the determination of hazelnut in foods, hazelnut protein standard, buffer blanks, matrix blanks, control samples and food samples were added and incubated for 1 h at 37°C. Proteins were detected with Eu3+–labelled polyclonal anti-corylin antibody (generated at our laboratory) for 1 h at RT under gentle shaking. The fluorescence signal was developed by DELFIA®-Enhancement solution (B118-100, PerkinElmer, Turku, Finland) and emission was measured at 615 nm. In between all steps, three washings were carried out in PBS, pH 7.6, containing 0.05% Tween-20 (PBST) using a programmable automatic plate washer (Skatron Instruments, Lier, Norway).

Preliminary indirect ELISA for the determination of Cor a 9

High-binding 96-well microplates (Corning Inc., Corning, NY, USA) were coated with 4 mg/l hazelnut protein extract in 0.05 M carbonate–bicarbonate buffer, pH 9.6 (Sigma-Aldrich) overnight at 4°C. Plates were blocked with PBST containing 1% BSA. The monoclonal antibody Lu11 used for the detection of Cor a 9 was originally developed in our laboratory for the detection of lupin proteins. This antibody cross-reacts strongly with

hazelnut Cor a 9, as determined by MS-analysis (Holden, Moen, Sletten, & Dooper, 2007 and unpublished results). For detection of Cor a 9, 0.1 mg/l Lu11 was diluted with PBST and incubated for 1 h at RT. Mouse IgG were detected with horseradish peroxidase (HRP)-conjugated rabbit anti-mouse Ig (DakoCytomatation, Glostrup, Denmark), diluted 1:3000, for 1 h at RT. Colour development was done with 75 µl K-Blue 3,3',5,5'-tetramethylbenzidine (TMB) substrate (Neogen, Lexington, KY) and stopped by addition of 50 µl/well of 2 M H₂SO₄. The optical density (OD) was read at 450 nm on a 1420 VICTOR (Tariq et al., 1996) multilabel plate counter (Wallac, Turku, Finland). In between all steps, three washings were carried out with PBST using a programmable automatic plate washer (Skatron Instruments, Lier, Norway).

Patients

Sera from five patients with hazelnut allergy and positive serum-specific IgE levels to hazelnut ($\geq$ level 4, ImmunoCAPTM 100, Phadia AB, Uppsala, Sweden) were received from The National Register of Severe Allergic Reactions to Food at the Norwegian Institute of Public Health, Oslo, Norway. The patients are referred to as P1 to P5 in the text, and their age, gender and specific serum IgE values for hazelnut are shown in Table 3.

Serum IgE indirect ELISA

High-binding 96-well microplates were coated with 4 mg/l protein in 0.05 M carbonate–bicarbonate buffer, pH 9.6 (Sigma) overnight at 4°C and blocked with PBST containing 1% human albumin (Calbiochem, La Jolla, CA, USA). Test and control sera, diluted with PBS containing 1% human albumin (1:30) were incubated for 1 h at RT with gentle shaking. For detection of IgE, wells were incubated with rabbit anti-human IgE (diluted 1:15,000 in PBST, DakoCytomation) followed by incubation with goat anti-rabbit IgG HRP conjugate (diluted 1:10,000 in PBST, Zymed). All washings and the final steps were performed as described above.

Electrophoresis and immunoblotting

Protein extracts (5 µg) were separated by SDS–PAGE under reducing conditions using 4–12% Bis–Tris pre-cast gels (Invitrogen, Carlsbad, CA, USA). Gels were either stained with SimplyBlue Safestain (Invitrogen) or the separated proteins were electrophoretically transferred from the gel onto nitrocellulose membranes. For studies with the polyclonal anti-hazelnut antibodies, membranes were blocked with tris-buffered saline containing 0.05% Tween-20 (TBST) and 1% BSA for 1 h, following incubation with the pAb (0.1 mg/l in TBST) overnight at 4°C. For detection of rabbit IgG, membranes were incubated with

Table 3. Clinical characteristics of the hazelnut allergic individuals.

Patient	Age (years)	Sex	ImmunoCap	Specific IgE hazelnut (kU/l)
P1	7	M	4	20.7
P2	22	F	4	35.0
P3	8	F	4	19.1
P4	3	M	4	4.7
P5	21	F	6	>100

goat anti-rabbit IgG HRP conjugate (1:5000, Zymed). For studies with the mAb Lu11, membranes were blocked as above, following incubation with Lu11 (0.1 mg/l in TBST) overnight at 4°C. For detection of mouse IgG, membranes were incubated with rabbit anti-mouse IgG–HRP conjugate (1:4000, DakoCytomation, Glostrup, Denmark). For studies with human serum IgE, membranes were blocked with TBST containing 3% horse serum for 1 h followed by incubation with diluted human serum (1:30 in TBST containing 3% horse serum) overnight at 4°C. For detection of human IgE, membranes were first incubated with rabbit anti-human IgE (1:6000, DakoCytomation, Glostrup, Denmark) and then with goat anti-rabbit IgG–HRP conjugate (1:5000, DakoCytomation), each for 1 h.

Antibody binding on all immunoblots was finally revealed with TMB substrate solution (Zymed, San Francisco, CA, USA). All washing and incubation steps were performed with TBST at RT with gentle shaking.

Results

Composition of protein extracts

In order to compare the protein composition of the different hazelnuts (Table 1), two extraction methods were used (Table 2) and SDS–PAGE was performed (Figure 1). Differences in protein composition were seen between the hazelnut sources extracted by

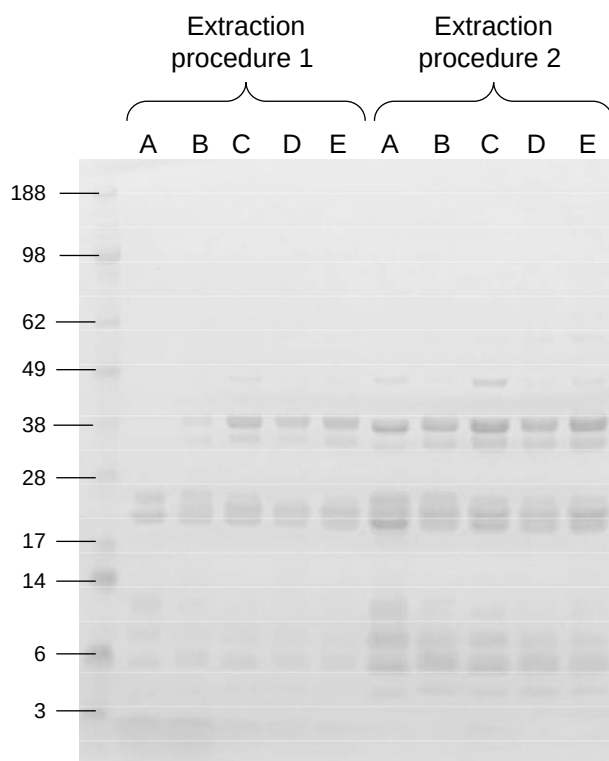


Figure 1. SDS–PAGE of hazelnut proteins extracted by two procedures (listed in Table 2). Letters A–E refer to the different hazelnut sources listed in Figure 1. Protein sizes (kDa) are indicated on the left side of the gel.

procedure 1. Hazelnuts of sources A and B (raw hazelnuts, stored for at least six months) contained considerably less proteins of approximately 38 kDa than the fresh (source C) or roasted hazelnuts (sources D and E). Furthermore, in hazelnut extracts of sources A, B and to a lesser extent C a protein smear smaller than 3 kDa in size was observed. When proteins were extracted by procedure 2, the differences between the hazelnut sources were less pronounced. These extracts contained three main protein clusters with protein sizes of approximately 38 kDa in the first group, between 20 and 28 kDa in the second group and proteins from 5 to 10 kDa in the third group. All extractions were performed at least twice, with similar results. Overall, the protein extracts obtained by extraction procedure 1 contained fewer and less intense proteins bands, especially of those proteins with highest and lowest molecular weights, as compared to the extracts obtained by procedure 2.

Measurement of hazelnut proteins by a time-resolved fluoroimmunoassay

The recovery of 1 µg of protein per extract was investigated in a time-resolved fluoroimmunoassay for the determination of hazelnut proteins. This assay was previously standardised and validated using a protein extract of hazelnut source A obtained by extraction procedure 1. The recovery of this protein extract (source A, procedure 1) was approximately 100% (Figure 2A). Remarkably, the hazelnuts of sources B, C and D all had a lower amount of proteins detected by the assay after extraction with procedure 1. The hazelnut extracts prepared by procedure 2 showed higher signals than procedure 1. The strongest signal was obtained with fresh, raw hazelnuts (source C), which was approximately seven times as high as the control proteins (source A, procedure 1).

An immunoblot was performed with the polyclonal antibody that was used for capture and detection in the assay. It showed binding towards proteins larger than 20 kDa and up to 70 kDa, with clear binding to proteins between 20 and 23 kDa in size in all extracts. Particular strong binding to larger proteins of approximately 60 kDa, that were weakly visible after gel electrophoresis (Figure 1), was seen in sources C, D and E, the fresh and roasted hazelnuts. Overall, extracts obtained by procedure 2 showed a stronger binding of the pAb than those obtained by procedure 1.

Measurement of Cor a 9

In order to investigate whether the differences in protein constitution of the extracts also influence the amount of Cor a 9, a preliminary, indirect ELISA was performed with a monoclonal antibody that binds to the allergen. A strong variation was observed in the amount of the Cor a 9. The duration of storage of the hazelnuts was inversely related to the amount of the allergen, while roasting of the hazelnuts seemed to maintain the amount of Cor a 9 (Figure 3A). Overall, the amounts of Cor a 9 were higher in the extracts obtained by procedure 2 than in the extracts of procedure 1. Similar findings were observed by immunoblotting with the monoclonal antibody (Figure 3B); a weak antibody binding was observed against hazelnut of sources A and B, while a stronger binding to fresh and roasted hazelnuts extracts (C, D and E) was seen.

IgE binding

Serum of five individuals with an allergy to hazelnuts was used to investigate differences in IgE binding between the hazelnut extracts. In line with previous results, the lowest IgE binding was observed towards the elder hazelnuts (sources A and B)

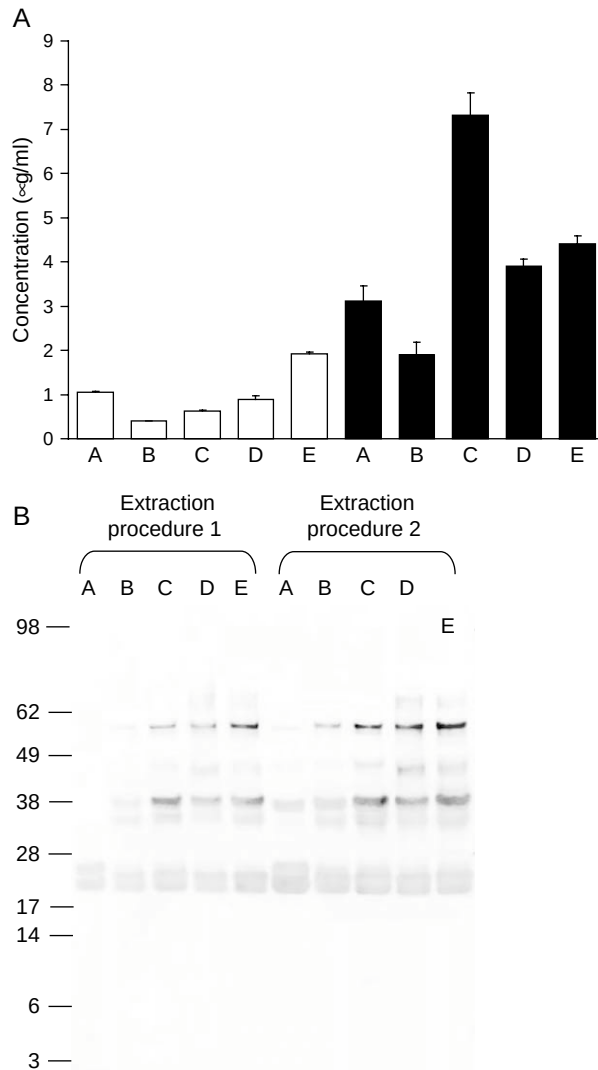


Figure 2. (A) The amount of hazelnut proteins in hazelnut extracts measured by time-resolved fluoroimmunoassay. White bars represent extraction procedure 1, black bars represent procedure 2. (B) Binding of polyclonal rabbit anti-hazelnut to hazelnut proteins shown by immunoblot. For figure legends A–E, see Table 1. Protein sizes (kDa) are indicated on the left side of the blot.

extracted by procedure 1 (Figure 4A). This was especially apparent in serum analysis of patients 1, 4 and 5. Overall, a somewhat stronger binding towards protein extracts from procedure 2 was observed by the indirect ELISA with patient sera. Similar differences between extraction procedures 1 and 2 were also clearly visible by immunoblot analyses (Figure 4B).

Discussion

The quality of a hazelnut extract is of primary importance for the outcome when used in diagnosis of hazelnut allergy or for the quantification of hazelnut proteins in food,

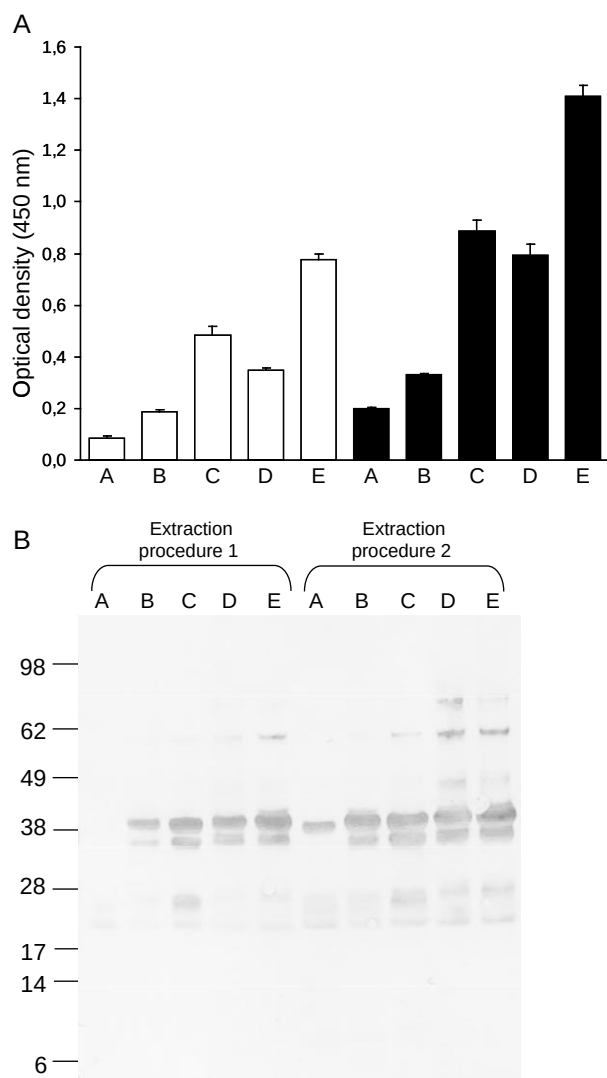


Figure 3. (A) The relative amounts of Cor a 9 in hazelnut extracts measured by ELISA using a monoclonal antibody. White bars represent extraction procedure 1, black bars represent procedure 2. (B) Western blot of the extracts, proteins were detected by the monoclonal antibody. For figure legends A–E, see Table 1. Protein sizes (kDa) are indicated on the left side of the blot.

A careful consideration of the correct source and storing conditions of the hazelnuts is therefore essential. In order to remain in a primary state of dormancy, hazelnuts are preferably stored at RT (Andriotis, Smith, & Ross, 2005). Chilling (0–15°C) of hazelnuts and subsequent returning to RT can alleviate dormancy and induce germination, and thereby activate proteolytic enzymes that can change the protein composition of raw hazelnuts (Ross, 1996). Although the raw hazelnuts of source B were stored at RT, a lower amount of intact proteins was observed in the extracts of these hazelnuts than the fresh hazelnuts (Source C). It therefore seems that in order to obtain a most optimal protein extract, it is important to use fresh hazelnuts for each protein extraction.

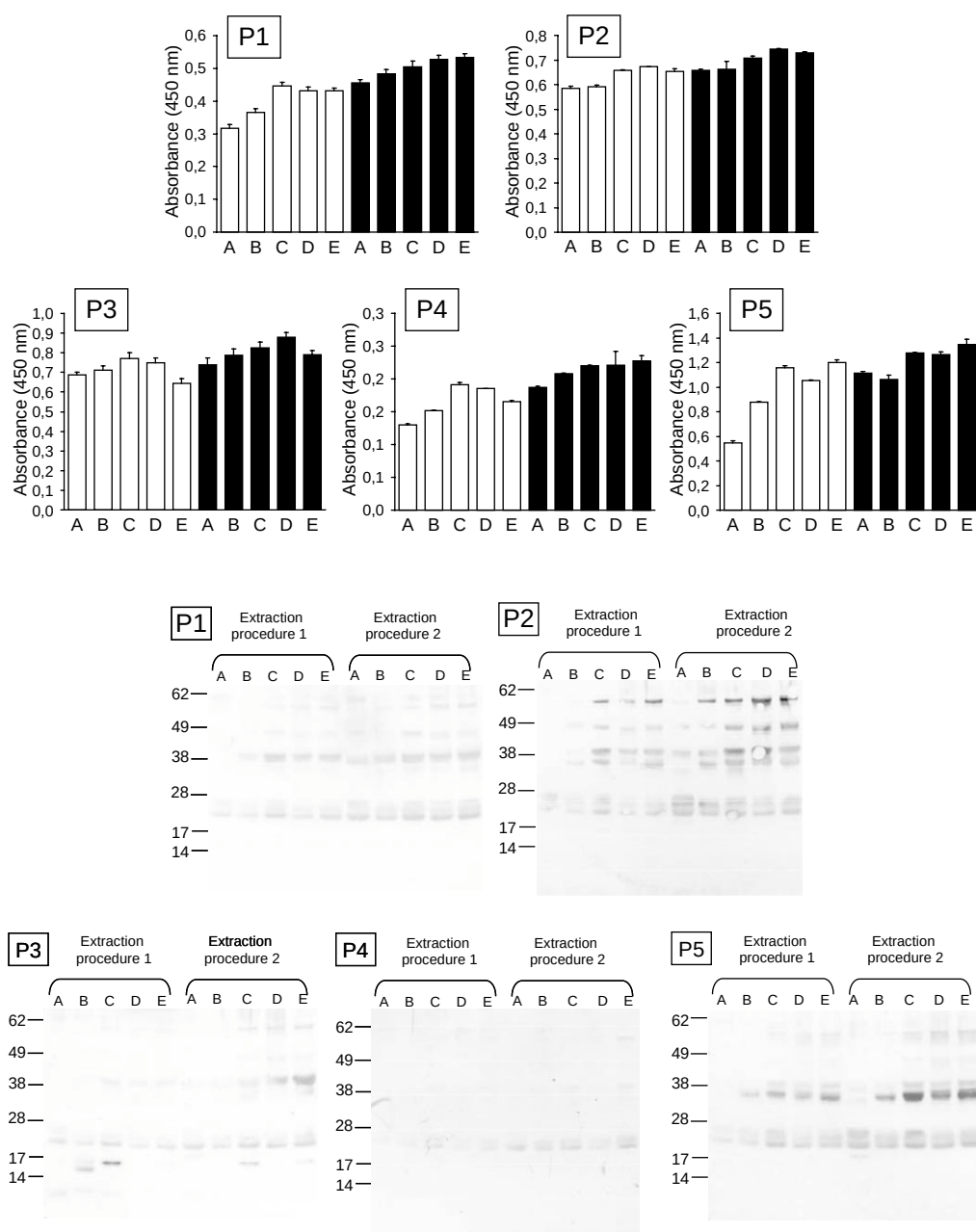


Figure 4. (A) IgE binding to hazelnut proteins studied by indirect ELISA. (B) Immunoblotting with serum from five hazelnut allergic individuals (P1–P5, listed in Table 3). For figure legends A–E, see Table 1. Protein sizes (kDa) are indicated on the left side of the blot.

Previous studies have reported a reduction in the allergenicity of hazelnuts after roasting (Hansen et al., 2003; Muller et al., 2000). The condition of roasting used in these studies was 140°C for 40 min, while in the current study the nuts were roasted at the manufacturers facility at approximately 170°C (temperature ranging from 150 to 180°C)

for 10 min. Preliminary tests in which fresh hazelnuts were roasted for 2, 5, 10, 20 and 30 min at 170°C indicated that the protein profile and amounts of the allergen Cor a 9 in the hazelnuts do not deteriorate until after 20 min of roasting (results not published). A shorter duration of roasting might therefore have prevented changes in the protein composition. Moreover, the (short) roasting of hazelnuts had a preserving effect on the protein constitution during storage, and consequently the antibody-binding capacity of hazelnut proteins, even after storage for at least six months.

The time-resolved fluoroimmunoassay for the detection of hazelnut protein has in our laboratory been successfully validated and applied for the detection of hazelnut traces in foods (Faeste et al., 2006). However, the use of hazelnuts of source A (older, raw nuts) extracted by procedure 1 as reference material might in this assay not be the most optimal choice, because the presented results indicate that this may lead to an overestimation in products containing fresh or roasted hazelnut proteins. We are therefore currently investigating whether the assay can be optimised further by inclusion of a standard derived from a fresh source of hazelnuts and/or a change in the extraction procedure of the assay.

Cor a 9 (corylin, 11S globulin) is a pollen-unrelated hazelnut allergen and the acidic chain of this protein was shown to be recognised by IgE of the majority of hazelnut allergic individuals that have systemic reactions to hazelnut (Beyer et al., 2002). In the present study, the amount of Cor a 9 was quantified by a preliminary method using a monoclonal antibody that was primarily developed against lupin (*Lupinus angustifolius*), but cross-reacts strongly with the 11S-globulin in hazelnut, and with almond proteins (Holden et al., 2007, unpublished results). The amounts of Cor a 9 in hazelnut extracts were, compared to results obtained from total hazelnut protein analysis, to a large extent influenced by the duration of storage of the hazelnuts and the means of protein extraction. The hazelnut source and extraction procedure might thus have strong effects on the outcome of diagnostic tests involving Cor a 9.

The two extraction procedures that were compared in the present study differed in many aspects, such as defatting, incubation time and buffer. Both procedures are routinely used in our laboratory. Procedure 1 is applied in ELISA for the determination of specific proteins (for example lupin, peanut, hazelnut and shrimp), based on a study in which several extraction buffers (based on citrate, high salt buffer, PBS, sodium carbonate, urea, sodium borate or tris-glycine) were compared for the extraction of lupin proteins (Holden et al., 2007). Procedure 2 has been used for the isolation of lupin globulins (Dooper et al., 2006). The extraction procedures that are generally applied for hazelnut proteins often include the use of a buffer based on PBS or Tris with conditions ranging from 1 h to O/N incubations and from 4°C to RT (Beyer et al., 2002; Enrique et al., 2005; Scheibe, Weiss, Rueff, Przybilla, & Gorg, 2001; Wigotzki, Steinhart, & Paschke, 2001). Vieths et al. (1998) compared a number of buffers (PBS, tris-trycine and ammonium-carbonate) and temperatures (4, 25 and 45°C) for the extraction of hazelnut proteins. They did not, however, observe clear differences in IgE cross-linking, in an enzyme allergosorbent test (EAST), using serum from immunised mice, and human serum from patients with pollen-related allergy to hazelnut. Later studies of the same group showed, however, that the extract was not optimal for IgE binding to the pollen-related allergen Cor a 1.04 and improved results were obtained with the recombinant allergen (Vieths et al., 2001). The fact that we found prominent IgE binding to all extracts produced by extraction procedure 2 indicates that this method might be suitable for further IgE-binding studies. However, it remains to be investigated whether binding to an individual pollen-related and pollen-unrelated allergen is optimal in the extracts obtained by this procedure.

In conclusion, the present study suggests that for the production of an adequate hazelnut protein extract, the use of fresh hazelnuts is important. A quick extraction procedure, such as procedure 2 described here, may be suitable for hazelnuts and especially for studies involving the pollen-unrelated allergen Cor a 9.

Acknowledgements

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