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Optimization of electrophoresis for the identification of low molecular mass allergens in hazelnuts

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Abstract

Conventional electrophoresis techniques used to identify food allergens are insufficient to separate low molecular mass proteins and peptides. In this paper we performed three different methods which provided an extended resolving power for small proteins. Applying the improved techniques, we were able to separate hazelnut proteins into distinct bands below 10 kDa.

Keywords: Food allergy; Optimization; Hazelnuts; Low molecular mass allergens

1. Introduction

It is well documented that hazelnut allergy is common in tree pollen allergic patients [1–9]. Recently, two prominent IgE binding proteins, a 17 kDa Bet v 1- and a 14 kDa Bet v 2 (Profilin)-analogous structure could be identified in hazelnuts which are responsible for the allergic cross-reactions between birch and hazel pollen and hazelnuts [2,4].

So far, no components have been found that belong to unique hazelnut allergens, unrelated to pollen allergens. In our investigations we analyzed the IgE-reactivity of a patient with severe anaphylactic reactions to hazelnuts without association to birch pollen allergy. We were particularly interested in small hazelnut proteins. Up to now, most identified food allergens belong to proteins with molecular masses of approximately 14 to 60 kDa. Actually, it is difficult to get access to small proteins by means of conventional methods such as sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) according to Laemmli [10] or Neville and Glossmann [11]. These techniques have an insufficient resolving for proteins below 10 kDa which is, however, a prerequisite to identify the allergens by immunoblotting and N-terminal sequencing. Therefore, we tested improved methods using the Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to Schägger and von Jagow [12], a modification of that technique developed by Westermeier [13] and NuPAGE™ Bis-Tris electrophoresis [14]. By this means, we were able to separate the low molecular hazelnut proteins and identified non pollen-related allergens with MWs of approximately 5 and 9 kDa.

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2. Experimentals

2.1. Patients' sera

Patient MS was a young woman with previous anaphylactic reactions after ingestion of hazelnuts, independently from birch pollen allergy [Hospital LMU, Dermatologische Klinik und Poliklinik, München, Germany (Dr. P. Thomas)]. Serum levels of specific IgE antibodies were determined by CAP FEIA test (Pharmacia, Uppsala, Sweden) showing CAP classes of birch pollen 0, hazelnut 6 and almond 5. One serum (patient BM) was chosen from a patient with birch pollen associated hazelnut allergy (CAP classes of birch pollen 6, hazel pollen 6, hazelnut 3).

A negative control serum was included from a nonatopic subject with no history of any allergies to inhalants or foods. The sera were diluted 1:20 in PBS, pH 7.4, containing 0.05% Tween for immunoblotting.

2.2. Allergen extracts

After mechanical homogenization the extract of native hazelnuts was incubated in a 0.1 *M* NH_4HCO_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 dialyzed against double-distilled water overnight. The extract was lyophilized and stored at 4°C until use. The peanut extract was prepared in the same way. For electrophoresis 20 μg hazelnut /peanut extract were applied per cm gel after reconstituting the freeze-dried extract in double-distilled water.

2.3. SDS–PAGE and peptide electrophoresis

Sodium dodecyl sulfate–polyacrylamid gel electrophoresis (SDS–PAGE) of the hazelnut extract was carried out according to Laemmli [10], with a polyacrylamid stacking gel (T=4%) on top of a homogeneous separation gel (T=15%). The sample buffer contained 20 m*M* Tris–HCl (pH 8.0), 2 m*M* 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 tracking dye. The electrophoresis was run under reducing conditions and performed as described by Laemmli.

Tricine–sodium dodecyl sulfate–polyacrylamid gel electrophoresis was carried out according to Schägger and von Jagow [12] in a vertical setup with slight modifications as described below. A 13% T, 3% C small-pore separating gel (the composition of the acrylamide–bisacrylamide mixture was calculated according to Hjerten [15]) and a 10% T, 3% spacer gel were polymerized together. After 12 h, it was overlaid by a 4% T, 3% C stacking gel. The protein samples were incubated for 10 min at 95°C in 2.3% SDS, 100 m*M* DTT, 100 m*M* Tris, 100 m*M* Tricine, 30% Glycerin (w/v) and 0.01% Serva blue G. For alkylation the samples were treated with 20% (w/v) iodacetamide directly after heating. The electrophoresis was run for 1 h at 30 V, 70 mA and 30 W and for 5 h at 600 V, 70 mA and 30 W.

A SDS electrophoresis of small peptides was performed as described by Westermeier [13]. This method was derived from the Tricine–sodium dodecyl sulfate–polyacrylamide gel electrophoresis according to Schägger and von Jagow and the PhastGel high density-method (Pharmacia LKB Biotechnology). Ethylene glycol was added to the polymerization solution. The stock solutions were composed according to Westermeier. The samples were diluted in Laemmli buffer and run under reducing conditions. The electrophoresis was carried out at 200 V, 70 mA and 18 W for 2 h and 45 min.

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 m*M* MES (2-(*N*-morpholino) ethane sulfonic acid), 50 m*M* Tris base, 3.5 m*M* SDS and 1 m*M* EDTA. The samples were reduced with NuPAGE™ sample buffer (0.293 *M* sucrose, 141 m*M* Tris base, 106 m*M* Tris HCl, 69.5 m*M* SDS, 0.51 m*M* EDTA, 0.22 m*M* Serva blue G 250, 0.175 m*M* Phenol red) and NuPAGE™ reducing agent according to the manufacturer's instructions [14]. Prior to sample application the samples were heated for 10 min at 70°C. Additionally, NuPAGE™ antioxidant was added to the NuPAGE™ MES SDS running buffer to prevent reduced proteins from reoxidizing during electrophoresis.

2.4. Electrophoretic blotting

For immunoblotting, the separated proteins were transferred from the separation gels onto nitrocellulose (NC) membranes (Schleicher & Schüll, Dassel, Germany) by semi-dry blotting (Khyse-Andersen, 1984) [16] at 0.8 $mA \cdot cm^{-2}$ for 30 min. After transfer, unspecific binding was blocked by incubation with PBS containing 0.05% Tween 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 m*M* Bicine, 25 m*M* Bis-Tris, 1.0 m*M* EDTA and 0.05 m*M* Chlorobutanol and 20% (v/v) methanol. For prevention of unoccupied protein binding sites, the membranes were also treated with 0.05% Tween in PBS.

For amino acid sequencing, electroblotting onto polyvinylidene difluoride (PVDF) was carried out using the NuPAGE™ transfer buffer system. Two different PVDF membranes were chosen differing in their pore sizes (Immobilon P membrane with 0.45 μm (Millipore, Eschborn, Germany) and Westran membrane with 0.2 μm (Schleicher & Schüll, Dassel, Germany)).

2.5. Protein staining and immunologic detection

Proteins were visualized by protein staining either directly in the gel or on NC membranes. The gels were stained with Coomassie Brilliant Blue according to Righetti and Drysdale [17]. For protein staining on the NC membrane, the blocked NC membrane was incubated in India ink (Pelican, Hannover, Germany) in PBS [18].

The PVDF membranes were washed in double-distilled water, stained by 0.1% (w/v) Coomassie Brilliant Blue R-250 (Serva, Heidelberg, Germany) in 50% methanol, destained in 50% methanol, and air dried.

For immunodetection the NC membrane was cut into strips and incubated on a shaker overnight at room temperature with patients' sera (diluted 1:20 in PBS, pH 7.4, containing 0.05% Tween). In order to detect bound IgE antibodies the strips were incubated with alkaline phosphatase-labelled anti-human IgE (Allergopharma, Reinbek, Germany; dilution 1:2000). The binding patterns were visualized by a substrate solution of 0.1 *M* Tris buffered saline (pH 9.5) containing 0.033% (w/v) nitro blue monotetrazolium chloride (NBT) and 0.017% (w/v) 5-bromo-4-chloro-3-indolyl phosphate (BCIP) (Serva, Heidelberg, Germany) [19].

3. Results

3.1. Identification of low molecular hazelnut allergens by immunodetection

Serum of patient MS who suffers from anaphylactic reactions to hazelnuts without association to tree pollen was investigated in a 15% SDS–PAGE immunoblot in order to identify non pollen-related hazelnut allergens. As indicated in Fig. 1 (lane 3),

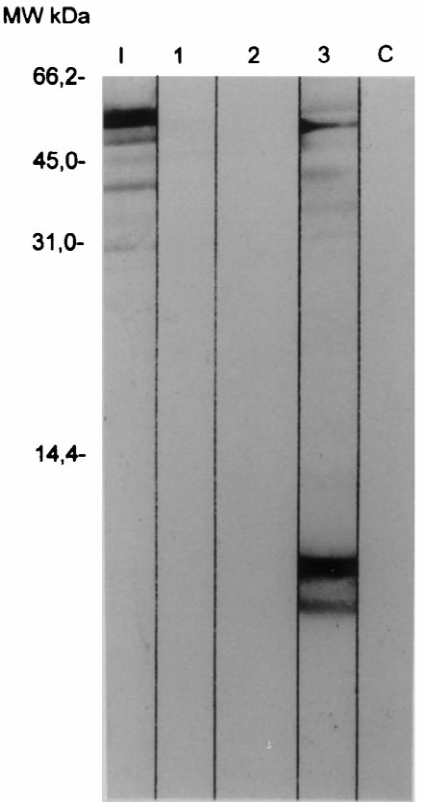

Fig. 1. Binding patterns of patients' IgE to hazelnut extract after Western blotting. I, protein staining by India ink; 1, serum of patient BM; 2, negative control serum; 3, serum of patient MS; C, second antibody control [20].

five dominating bands with the apparent MWs of 50, 42, 38, 9 and 7 kDa were found.

3.2. Electrophoretic separation of hazelnut proteins below 10 kDa with different electrophoresis techniques

To get access to the low molecular proteins which were detected by serum MS, we tested various electrophoresis techniques providing a better resolution of small proteins below 10 kDa. First, we performed a Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Schägger and von Jagow [12]. Among the various gel compositions tested a 13% T, 3% C separating gel overlaid by a 10% T, 3% spacer gel and a 4% T, 3% C stacking gel revealed the best resolution results. As shown in Fig. 2, the hazelnut proteins were separated into three bands in the low molecular range with MWs of approximately 12, 9 and 5 kDa.

Actually, the low molecular proteins were resolved into distinct bands which could not be achieved by running SDS–PAGE by Laemmli [10].

In Fig. 3 a modified gel electrophoresis according to Westermeier [13] is compared to the method of Schägger and von Jagow.

Using a 16% T, 6% C resolving gel and a 5% T, 3% C stacking gel with 30% ethylene glycol added, two distinct bands at 12 and 9 kDa became visible (lane 1). A 5 kDa hazelnut protein appeared as a weak band and did not focus as clearly as in the Tricine-sodium dodecyl sulfate-polyacrylamide gel electrophoresis according to Schägger and von Jagow (Fig. 2).

A sharply focussed separation of hazelnut proteins even below 5 kDa was obtained by running a 10% NuPAGE™ Bis-Tris gel.

By the means of this technique, four distinct low molecular mass bands could be detected at 11, 9, 5 and 3 kDa (Fig. 4).

With regard to the sharpness of the low molecular hazelnut proteins, these gels were preferably used.

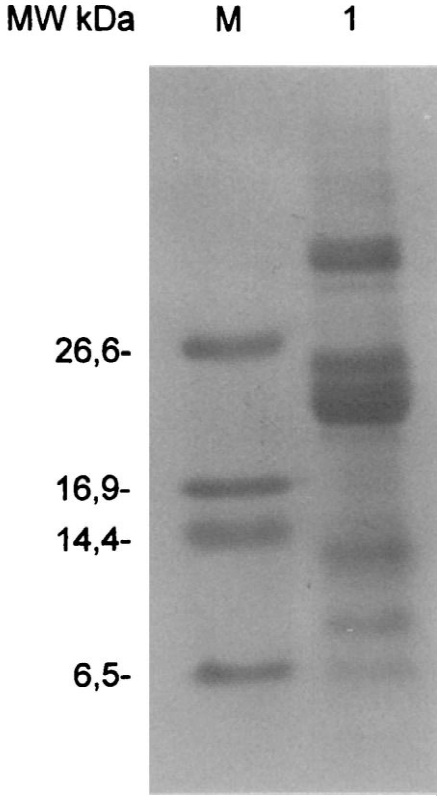

Fig. 2. Tricine-SDS–PAGE of hazelnut extract. M, Molecular weight marker; 1, protein pattern stained by Coomassie.

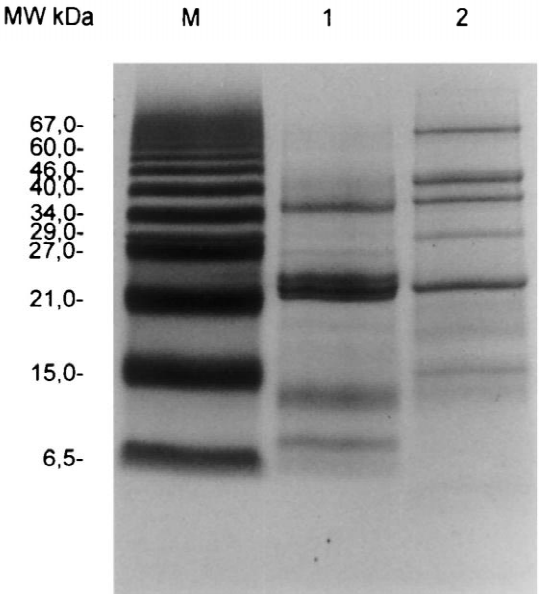

Fig. 3. SDS–PAGE for small proteins of hazelnut extract (according to Westermeier). *M*, Molecular weight marker; 1, hazelnut extract; 2, peanut extract; protein pattern stained by Coomassie.

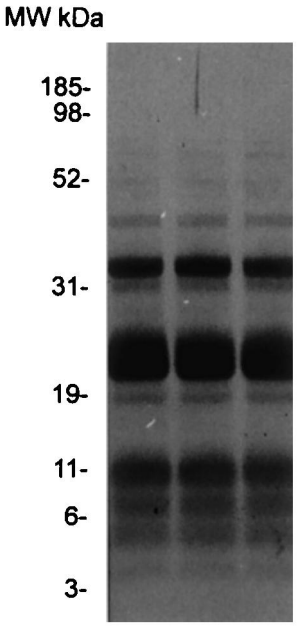

Fig. 4. Separation of hazelnut extract by NuPAGE™ Bis-Tris electrophoresis. Protein pattern stained by Coomassie.

Analyzing the IgE-reactivity of serum MS in a 10% NuPAGE™ Bis-Tris immunoblot, we finally located the hazelnut allergens at 5 and 9 kDa (data not shown).

In regard to immunoblotting for *N*-terminal amino acid sequencing, we found out that particularly the small proteins were very badly bound on conventional PVDF membrane (Immobilon P membrane). By the means of Westran membranes which offer a smaller pore size (0.2 μm in contrast to 0.45 μm of the current PVDF membrane used), the protein amounts were sufficient to analyze the protein probes.

Fig. 5 compares the binding efficiency of both membranes showing that the low molecular mass proteins cannot penetrate a second membrane during blotting when using the smaller pore size.

4. Discussion/Conclusion

The aim of this study was to get access to low molecular hazelnut allergens identified by a hazelnut allergic patient with severe allergic reactions to hazelnuts, independently from pollen allergy. We used Western blotting in a 15% SDS–PAGE and were able to detect two allergens in the low molecular mass range with MWs below 10 kDa at approximately 7 and 9 kDa. The SDS–PAGE system according to Laemmli is a well-established electrophoresis system yielding a resolution of polypeptides from 14 to 100 kDa and even higher. With a discontinuous buffer system, the proteins form sharp zones in the stacking gel which serves to increase resolution [10]. Depending on the MWs of the proteins under study, different pore sizes can be obtained by varying the total acrylamide concentration T and the degree of cross-linking C [15]. To get a higher resolution in the low molecular range, we used a 15% SDS–PAGE in our immunoblot experiments.

In our study, however, the separation of hazelnut proteins below 10 kDa was not sufficient in the Tris–glycin–HCl system according to Laemmli. These data are in line with findings of Schägger and von Jagow who focussed on the poor resolution of current techniques in regard to the separation of small proteins [12]. They optimized separations in the MW range less than 20 kDa by establishing a Tricine–SDS–polyacrylamide gel electrophoresis. Their experiments showed that proteins below 20 kDa were not separated from the bulk of SDS during electrophoresis. This was the reason for streaking effects of peptides when using higher acrylamide concentrations in separating gels according to Laemmli. They developed a new technique substituting the glycine for tricine as trailing ion. Using tricine, a stacking and destacking of small proteins was reached at the same pH as in the separating gel and at low acrylamide concentrations. The introduction of a spacergel and the increase of the molarity of the buffers improved the method likewise. For optimal resolution of hazelnut peptides, we used a gel composed of a 13% T, 3% C separating gel overlaid by a 10% T, 3% spacer gel and a 4% T, 3% C stacking gel. Fig. 2 shows the resolution of the hazelnut proteins in the Tricine–SDS–polyacrylamide gel electrophoresis according to Schägger and von Jagow which proved to be able to separate the low molecular proteins superior to the Laemmli system. In our experiments, we used a further SDS–

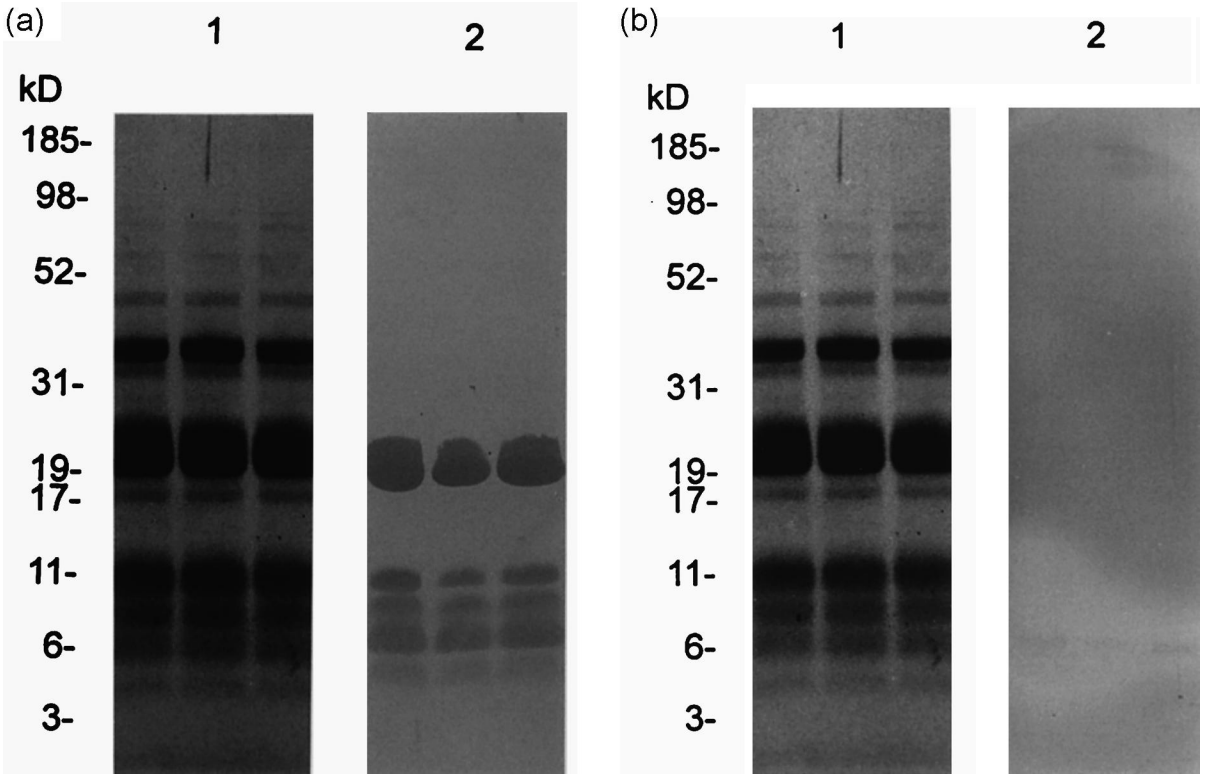

Fig. 5. Comparison of the binding efficiency of different PVDF membranes after NuPAGE™ Bis-Tris electrophoresis and blotting onto two layers of membranes. A. Immobilon P membrane; 1, first membrane; 2, second membrane. B, Westran membrane; 1, first membrane; 2, second membrane.

PAGE applicable for the separation of low molecular mass proteins. Westermeier derived this technique from the Tricine–SDS–polyacrylamide gel electrophoresis according to Schägger and von Jagow and Pharmacia's PhastGel high density-method [13]. Again, tricine was used instead of glycin. In contrast to the system of Schägger and von Jagow, 30% ethylene glycol was introduced to increase the resolution below 20 kDa. This additive decreases the size of the SDS–peptide-complexes, resulting in a better separation. Similar additives recommended in recent studies function in the same way, for instance urea (Swank and Munkres [21]) and glycerol (Wiltfang et al. [22]). In our investigations, this method was also superior to the 15% SDS–PAGE. By this means, we were able to separate the hazelnut proteins below 14 kDa into two clear bands of 12 and 9 kDa and a weaker band of 5 kDa.

We finally run a NuPAGE™ Bis-Tris gel, as shown in Fig. 4. This system is based upon a Bis-Tris–HCl buffered (pH 6.4) polyacrylamide gel. By using a MES (2-(*N*-morpholino) ethane sulfonic acid)-buffer system a wide molecular mass range is obtained in the low molecular range. Applying this method, the hazelnut proteins could be separated into four distinct low molecular mass bands of 3, 5, 9 and 11 kDa. When comparing the hazelnut protein pattern obtained by a 10% NuPAGE™ Bis-Tris gel (Coomassie-stained on a NC membrane) with the IgE-reactive bands of serum MS, the MWs of the IgE-reactive proteins could be determined more precisely at 5 and 9 kDa.

In conclusion, our study on various electrophoresis methods for small hazelnut proteins showed the benefit of the Tricine–SDS–polyacrylamide gel electrophoresis according to Schägger and von Jagow. Their technique allowed isolation of low molecular mass proteins which previously have been beyond

the limits of resolution of conventional methods. For hazelnut protein, the NuPAGE™ Bis-Tris gels led to the clearest peptide separation pattern.

The low molecular mass hazelnut peptides separated by employing this system may play an important role in high risk hazelnut allergic patients who suffer from anaphylactic reactions without relation to pollen allergy. For further studies, these separated peptides can now be isolated, purified or used to raise monoclonal antibodies in order to investigate the IgE-reactivity in more detail.

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