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Characterization of the 18-kDa Apple Allergen by Two-Dimensional Immunoblotting and Microsequencing

Key Words

Apple allergens
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Cross-reactivity
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Abstract

A low-temperature extract taken from Golden Delicious apples was separated by two-dimensional polyacrylamide gel electrophoresis. By means of two-dimensional immunoblotting with patients' serum containing IgE specific to *Bet v I*, a rabbit polyclonal antiserum raised against *Bet v I*, and two *Bet v I* specific monoclonal antibodies, epitopes cross-reactive to *Bet v I* were identified on an apple allergen with a molecular mass of 18 kDa and pI 5.5. Furthermore, certain antibody reactivities with 4 isoproteins of a molecular mass of 16 kDa and pIs ranging from 4.9 to 5.5 were observed, which may indicate the presence of *Bet v I* related epitopes on these proteins. Based on 26 amino acid residues, N-terminal sequencing of the 18-kDa apple allergen revealed 62% sequence identity between *Bet v I* from birch pollen and the apple allergen. Our results therefore support the view that both proteins express common as well as non-related IgE-reactive epitopes.

Introduction

Allergy to a variety of fresh fruits, nuts, as well as allergy to some vegetables and spices is highly associated with IgE-mediated hypersensitivity to birch pollen [1–4]. The observed clustering of hypersensitivities is due to cross-reactions of allergen-specific IgE antibodies [4–7]. Since its prevalence in birch-pollen-allergic patients is high, apple hypersensitivity has been studied as an immunopathologic model which represents the typical situation of the whole phenomenon [5, 8–10]. Ebner et al. [8] were the first to identify common structures of *Bet v I*, the major allergen of birch pollen, and a 17- to 18-kDa allergen from Golden Delicious apples by immunoblot and immunoblot-inhibition studies with pa-

tients' sera and monoclonal antibodies (MAbs) specific to *Bet v I*. Furthermore, they were able to detect homologous structures between *Bet v I* and an apple protein at the nucleic acid level by Northern blotting. At the protein level, we confirmed these results by immunological inhibition studies using isolated *Bet v I* and patients' sera as well as by immunoblotting with MAbs directed against *Bet v I* [10] which clearly revealed cross-reactivity between the two proteins. Up to now, no structural and functional data of the 17- to 18-kDa apple allergen are available. Therefore, we have further characterized the highly cross-reactive apple allergen by two-dimensional immunoblotting and N-terminal amino acid sequencing.

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Materials and Methods

Patients' Sera

The birch pollen serum pool Wn 13/32 ($n = 7$) had RAST classes of 3 to birch pollen and Golden Delicious apples. On one-dimensional (1-D) immunoblots, IgE antibodies of this serum reacted to *Bet v* I and a minor allergen of 35 kDa of birch pollen extract, and bound to proteins of 16, 18 and 37 kDa of separated Golden Delicious extract [7, 10]. Patient Bra was allergic to birch pollen, fresh apples and several other fruits, hazelnuts, celery, carrots and some spices and had RAST classes of 4 to birch pollen and Golden Delicious apples. On 1-D immunoblots of birch pollen extract, the IgE reactivity of this serum was restricted to *Bet v* I, whereas a 16- and a 18-kDa band were recognized with Golden Delicious extract.

Polyclonal Antiserum and MABs

A rabbit polyclonal antiserum raised against *Bet v* I and the anti-*Bet v* I 'MAB D' were kindly supplied by Allergopharma (Dr. B. Weber, Dr. R. Wahl, Reinbek, Germany). The *Bet v* I specific MAB 'BIP 1' was a gift from Prof. Dr. D. Kraft, Vienna, Austria. Culture supernatants of both MABs were used.

Apple Extracts

Apple extracts were prepared by a low-temperature 'acetone powder' method as previously described [11]. Briefly, pieces of mature fresh Golden Delicious apples were homogenized in acetone/dry ice at -60 to -65°C and the mixture was stored overnight and cooled with dry ice. The resulting precipitates were washed twice with acetone at -60°C and once with acetone/diethylether (1:1, v/v, -60°C), filtered and lyophilized. The dried acetone powders were stored at -20°C . Protein extracts were obtained by extracting approximately 2 g of acetone powder with 30 ml of 0.01 M potassium phosphate buffer pH 7.4, containing 0.15 M NaCl, centrifuged (5°C , 20,000 g, 45 min) and the supernatants were filtered through a fiber glass prefilter (No. 13400, Sartorius, Göttingen, Germany). The total amount of protein was determined by a modified Bradford assay as previously described [11] (typical protein contents: 0.1–0.4 mg/ml). The crude protein extracts were stored freeze-dried at -20°C until use. Prior to electrophoresis, the freeze-dried extracts were reconstituted, washed twice with double-distilled water and concentrated 3 times using disposable micro-concentrators (Centricon 3, cut-off: 3,000 D, Amicon, Witten, Germany). For two-dimensional polyacrylamide gel electrophoresis (2-D PAGE), 35 μg of total protein were applied to a final volume of 90 μl of 2-D PAGE sample buffer [12].

Two-Dimensional Electrophoresis

2-D PAGE was carried out by a procedure slightly modified from that previously described [12]. All polyacrylamide gels were prepared from a commercial stock solution containing 30% (w/v) acrylamide and 0.8% (w/v) methylene-bis-acrylamide (Rotiphorese Gel 30, Roth, Karlsruhe, Germany). The isoelectric focusing step in the first dimension was performed under native conditions in polyacrylamide rod gels (diameter 3.0 mm, $T = 4.8\%$ w/v, $C = 2.6\%$ w/v). Vertical DISC-SDS-PAGE (second dimension) was carried out under nonreducing conditions as described, with the exception that the resolving gel contained a lower total acrylamide concentration (13%, w/v instead of 15% w/v).

Semi-Dry Blotting

The blotting procedure was based on a modification of the system described by Khyse-Andersen [13]. For immunodetections, the separated proteins were transferred to nitrocellulose membranes (0.2 μm , Schleicher & Schüll, Dassel, Germany) using a Novablot semi-dry electroblotting apparatus (Pharmacia, Uppsala, Sweden) at 0.8 mA/cm² for 45 min, as described [12]. A stack of up to 6 polyacrylamide gels was blotted simultaneously. For microsequencing of the allergen, we employed semi-dry electroblotting onto polyvinylidene difluoride (PVDF) membranes (TransBlott, Bio-Rad, Munich, Germany) using a Pegasus blotter (Phase, Lübeck, Germany) fitted with a glass-carbon electrode, at 0.8 mA/cm² for 45 min. The transfer buffer was 10 mM 3-(cyclohexylamino)-1-propane-sulfonic acid, pH 11.0, containing 10% (v/v) methanol [14, 15].

Protein Staining and Immunodetection

The dried nitrocellulose membranes were blocked twice (15 min) in PBS containing 0.3% (v/v) Tween 20. Human sera and all immunoreagents were diluted in PBS containing 0.05% (v/v) Tween 20 and 0.1% (w/v) bovine serum albumin (incubation buffer). Nonspecific staining of the separated proteins was accomplished by incubating the nitrocellulose membrane in 0.1% (v/v) India ink (Pelikan, Hannover, Germany) [16]. In order to identify the cross-reactive apple allergen, immunodetections were performed with (1) patients antisera (1:25, 10 ml); (2) rabbit anti-*Bet v* I antiserum (1:7,000, 35 ml), and (3) the *Bet v* I specific MABs (1:175, 35 ml). Primary antibody incubations were carried out overnight and followed by a biotin/streptavidin-amplified immunodetection using horseradish peroxidase as the marker enzyme and 3,3', 5,5'-tetramethylbenzidine as the substrate [12]. Bound IgE antibodies were detected with (1) a rabbit polyclonal antihuman IgE (1:4,000, 1 h); (2) a goat polyclonal affinity-purified biotinylated anti-rabbit IgG (1:6,000, 1 h) (both from Dako, Copenhagen, Denmark), and (3) streptavidin/horseradish peroxidase (HRP) conjugate (1:20,000, 20 min) (Medac, Hamburg, Germany). Prior to each incubation step, the blots were washed three times with incubation buffer (10 min). For detection of rabbit anti-*Bet v* I antibodies, the anti-IgE incubation step was omitted. The bound MABs were detected with rabbit biotinylated affinity-purified anti-mouse IgG (1:3,000, 1 h) (Jackson, obtained from Danova, Hamburg, Germany), followed by streptavidin-HRP and the substrate as described.

Edman Degradation

The PVDF membrane containing the apple proteins separated by 2-D PAGE was washed with double-distilled water (30 min), followed by a total protein stain with 0.1% (w/v) Coomassie Brilliant Blue (Merck, Darmstadt, Germany) in 50% (v/v) methanol (10 min), destained in 50% (v/v) methanol, again washed with double-distilled water, and the membrane was air-dried. Protein spots containing the 18-kDa apple protein were cut out. Four protein spots were used for the microsequencing analysis. Microsequencing of the N-terminal amino acids was performed with a 473A pulsed liquid protein sequencer with an on-line phenylthiohydantoin amino acid analyzer (Applied Biosystems, Weiterstadt, Germany) [14, 15].

Results

Two-Dimensional Electrophoresis

The separation of the proteins of our Golden Delicious apple extract was accomplished by 2-D PAGE followed by semi-dry electroblotting. The isoelectric focusing (first dimension) was performed under native conditions. The SDS-PAGE of the second dimension was carried out under non-reducing conditions and no acetylating or alkylating agents were used. Protein staining of the 2-D nitrocellulose blot with India ink (fig. 1) revealed more than 50 protein spots with molecular masses between 14 kDa and approximately 80 kDa, most of them having pIs between 4.3 and 7.1. Only proteins in the molecular mass region of 40-kDa and one spot of approximately 15 kDa had pIs greater than 7.0 (7.1–9.0). Many isoprotein spots with identical molecular masses but different pIs occurred in the molecular mass region ≥ 30 kDa and in the low-molecular-mass region between 14 and 20.1 kDa. The presumed cross-reactive allergen consisted of one intense spot with a molecular mass of approximately 18 kDa and a pI of 5.5. Since some human sera (including serum of patient Bra) with IgE directed against *Bet v* I react with a double band of 18 and 16 kDa on 1-D immunoblots of our Golden Delicious extract [9, 12], four intense isoprotein spots with molecular masses of 16 kDa and pIs between 4.9 and 5.5 were suspected to be additional *Bet v* I-related allergens (fig. 1).

Immunodetection of the Allergen on Two-Dimensional Nitrocellulose Blots

In order to localize and identify the cross-reactive apple allergen, the 2-D nitrocellulose blots were incubated with the two human sera, the rabbit anti-*Bet-v* I antiserum, and the *Bet v* I-specific MAbs BIP 1 and MAb D. As can be seen from figure 2a–d, IgE antibodies from patient Bra as well as rabbit antibodies and the two MAbs recognized the 18-kDa allergen spot, but only MAb D bound exclusively to the 18-kDa allergen. BIP 1 showed additional binding to two of the four isoprotein spots of 16 kDa. The rabbit polyclonal antiserum reacted to all four 16-kDa isoallergens, and the IgE antibodies of patient Bra bound to three of the four isoallergen spots. IgE antibodies of the human serum pool Wn 13/32 bound to the 18-kDa allergen, three of the four 16-kDa isoallergen spots, and showed additional IgE binding in the molecular mass region of approximately 35 kDa (result not shown).

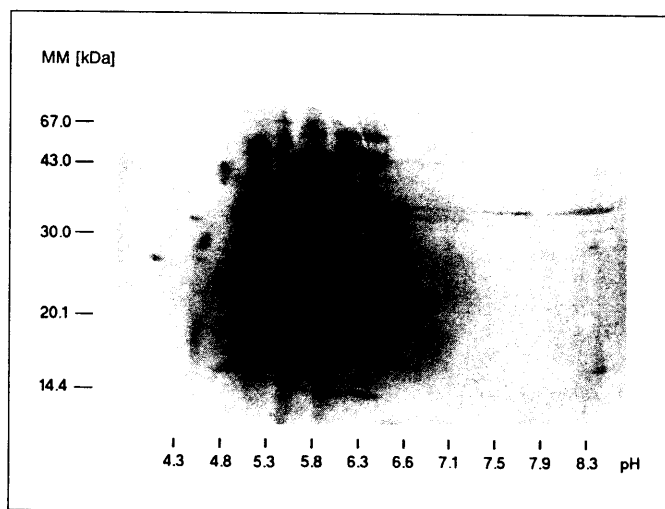


Fig. 1. Protein staining of Golden Delicious apple extract with 0.1% India ink after 2-D PAGE and semi-dry electroblotting onto nitrocellulose membranes.

Microsequencing Analysis

By microsequencing of four Coomassie Brilliant Blue stained protein spots of the 18-kDa allergen, 26 N-terminal amino acid residues could be clearly identified. Table 1 shows the N-terminal amino acid sequences of the apple allergen and of *Bet v* I (the latter from Breiteneder et al. [18]). 15/26 (62%) of the amino acid residues of the two allergens are identical. At position 9 of the apple protein, tyrosine and phenylalanine were simultaneously detected.

Discussion

Since the 18-kDa allergen spot was clearly recognized by all immunodetection systems on the 2-D PAGE immunoblots, the existence of *Bet v* I-related epitopes on this protein can be assumed. In addition, the reactivity of MAb 'BIP 1', the rabbit antiserum, and patients' IgE antibodies with the 16-kDa allergens may indicate the existence of epitopes cross-reactive to *Bet v* I on these proteins. Furthermore, microsequencing revealed similar amounts of phenylalanine and tyrosine at position 9 of the 18-kDa apple allergen (table 1). This result probably indicates the presence in our apple extract of two highly homologous isoforms of the 18-kDa protein having nearly identical pIs. According to our results, microheterogeneity of the apple allergen seems to be poorer compared to *Bet v* I of which more than 10 isoforms have been found [8, 12, 19]. Furthermore, sequence analysis revealed three consecutive proline residues in posi-

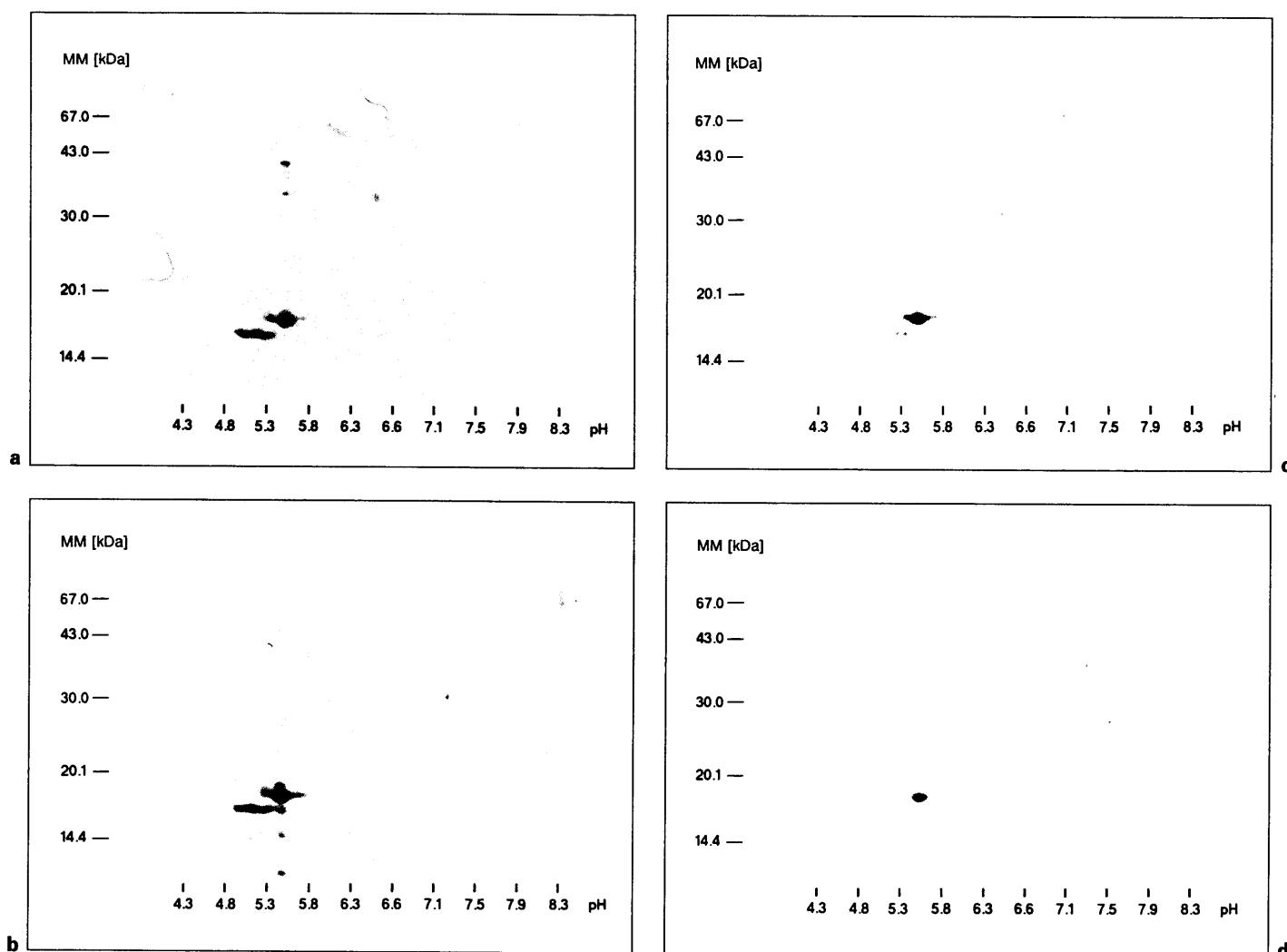


Fig. 2. **a.** Reactivity of IgE antibodies of serum from patient Bra with the 2-D PAGE. On 1-D SDS-PAGE immunoblots the IgE antibodies of patient Bra recognize exclusively *Bet v I* from birch pollen extract and the 18-kDa allergen from Golden Delicious apple extract. Serum dilution: 1:25. **b** 2-D PAGE immunoblot of Golden Delicious apple extract with rabbit polyclonal anti-*Bet v I* antiserum, diluted 1:7,000. **c** 2-D PAGE immunoblot of Golden Delicious apple extract with mouse monoclonal anti-*Bet v I* antibody BIP 1, culture supernatant diluted 1:175. **d** 2-D PAGE immunoblot of Golden Delicious apple extract with mouse monoclonal anti-*Bet v I* antibody MAb D, culture supernatant diluted 1:175.

tions 14–16, an uncommon sequence which is not present in *Bet v I*. However, confirmation analysis of an 18-kDa allergen from Granny Smith apples revealed the same sequence (result not shown). Cross-reactivity studies of birch pollen and plant food allergens which have been performed by RAST/EAST inhibition as well as by immunoblot and immunoblot inhibition suggest that all allergenic determinants of the vegetable foods are present in birch pollen but that only some determinants of the birch pollen allergens are present in fruits and vegetables [5–8, 10, 20]. In accordance with Ebner et al. [8] this view is further supported by our

MAb immunoblot studies: of seven anti-*Bet v I* MAbs tested so far, only two (BIP 1 from Prof. D. Kraft, Vienna, Austria, and MAb D from Allergopharma, Reinbek, Germany) recognized the 18-kDa allergen on apple immunoblots [10, and additional unpublished results].

On the basis of the 26 N-terminal amino acid residues, a sequence identity of 62% between *Bet v I* and the apple allergen was calculated. In addition, 3/26 residues of the two proteins are similar (pos. 3: Tyr/Phe, pos. 5: Phe/Tyr, pos. 23 Val/Ile). Therefore, identical as well as nonrelated epitopes may be present on the two allergens. Anamnesis data of the

Table 1. N-terminal amino acid sequences of the 18-kDa apple allergen and of *Bet v I*

	Position 1				Position 5					Position 10	
Apple	Gly	Val	Tyr	Thr	Phe	Glu	Asn	Glu	Tyr/Phe ¹	Thr	
<i>Bet v I</i> [18]	Gly	Val	Phe	Asn	Tyr	Glu	Thr	Glu	Thr	Thr	
	Position 11				Position 15					Position 20	
Apple	Ser	Glu	Ile	Pro	Pro	Pro	Arg	Leu	Phe	Lys	
<i>Bet v I</i>	Ser	Val	Ile	Pro	Ala	Ala	Arg	Leu	Phe	Lys	
	Position 21				Position 25						
Apple	Ala	Phe	Val	Leu	Asp	Ala					
<i>Bet v I</i>	Ala	Phe	Ile	Leu	Asp	Gly					

¹ Both amino acids were clearly detected in position 9, see text.

patients allergic to birch pollen who participated in our previous and current studies show that the sensitization patterns to various apple strains and to different kinds of fruits, nuts and vegetables are highly individual. Many different combinations of food hypersensitivities within the cluster of birch-pollen-related foodstuffs occur. Furthermore, our extended immunoblot and immunoblot inhibition studies with allergic patients' sera, rabbit antisera and MAb suggest that proteins which share common epitopes with *Bet v I* are widely distributed in plant foods (e.g. in pears, cherries, plums, hazelnuts, celery, carrots and some spices [7, 10, and additional unpublished results]). Other authors report similar results for immunoblot studies which partly deal with some different plant foods [4, 8, 22]. Most of the pollen-related food allergens have molecular masses between 14 and 20 kDa. To some extent, the observed cross-reactivity between various pollen species and plant foods is due to profilin which was recently identified as a novel plant pan-allergen [21, 23, 24].

The observations mentioned above support the assumption that a considerable part of the birch-pollen-related food hypersensitivities is caused by a family of low-molecular-mass allergens with a high degree of sequence identity to *Bet v I*, all of them probably belonging to a group of 'pathogenesis-related proteins' [8, 18]. Variation in the sensitization patterns of individual patients may be due to minor sequence variations between the *Bet v I*-related food allergens. Further work is in process to confirm this hypothesis.

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References

- 1 Eriksson NE, Formgren H, Svenonius S: Food hypersensitivity in patients with pollen allergy. *Allergy* 1982;37:437-443.
- 2 Dreborg S: Food allergy in pollen-sensitive patients. *Ann Allergy* 1988;61:41-46.
- 3 Thiel C: Nahrungsmittelallergien bei Pollenallergikern (sogenannte pollenassozierte Nahrungsmittelallergien). *Allergologie* 1988;11:397-410.
- 4 Calkhoven PG, Aalbers M, Koshte VL, Pos O, Oei HD, Aalberse RC: Cross-reactivity among birch pollen, vegetables and fruits is due to at least three distinct cross-reactive structures. *Allergy* 1987;42:382-390.
- 5 Björkstén F, Halmepuro L, Hannuksela M, Lahiti A: Extraction and properties of apple allergens. *Allergy* 1980;35:671-677.
- 6 Halmepuro L, Vuontela K, Kalimo K, Björkstén F: Cross-reactivity of IgE-antibodies with allergens in birch pollen, fruits and vegetables. *Int Arch Allergy Appl Immunol* 1984;74:235-240.
- 7 Vieths S, Brockmann S, Schöning B: Nahrungsmittelallergie gegen Obst und Gemüse: Eine aktuelle serologische Untersuchung zur Sensibilisierung von Pollenallergikern. *Allergologie* 1992;15:367-379.

- 8 Ebner C, Birkner T, Valenta R, Rumpold H, Breitenbach M, Scheiner O, Kraft D: Common epitopes of birch pollen and apples. Studies by Western and Northern blot. *J Allergy Clin Immunol* 1991;88:588–594.
- 9 Vieths S, Schöning B, Janciewicz A: Occurrence of IgE binding allergens during ripening of apple fruits. *Food Agric Immunol* 1993;5:93–105.
- 10 Vieths S, Schöning B, Aulepp H, Baltes W: Identifizierung kreuzreagierender Allergene in Pollen und pflanzlichen Lebensmitteln. *Lebensmittelchemie* 1993;47:49–53.
- 11 Vieths S, Schöning B, Brockmann S, Aulepp H: Untersuchungen zur Allergie gegen Lebensmittel pflanzlicher Herkunft: Herstellung und Charakterisierung von Obst- und Gemüseextrakten für serologische Untersuchungen. *Dtsch Lebensm Rdsch* 1992;88:239–243, 273–279.
- 12 Vieths S, Schöning B, Baltes W: Allergy to fruits and vegetables in pollen-sensitive patients: Allergen characterization by IgE immunoblotting and peroxidase staining. *Food Agric Immunol* 1992;4:181–197.
- 13 Khyse-Andersen J: Electrophoretic transfer of multiple gels: A simple apparatus without a buffer tank for rapid transfer of protein from polyacrylamide gels to nitrocellulose. *J Biochem Biophys Methods* 1984;10:203–209.
- 14 Petersen A, Becker WM, Schlaak M: Characterization of isoforms of the major allergen *Phl p V* by two-dimensional immunoblotting and microsequencing. *Int Arch Allergy Immunol* 1992;98:105–109.
- 15 Matsudaira P: Sequence from picomole quantities of proteins electrophoretically transferred onto polyvinylidene difluoride membranes. *J Biol Chem* 1987;262:10035–10038.
- 16 Hancock K, Tsang VCW: India ink staining of proteins on nitrocellulose paper. *Anal Biochem* 1983;133:157–162.
- 17 Jarolim E, Tejkl M, Rohac M, Schlerka G, Scheiner O, Kraft D, Breitenbach M, Rumpold H: Monoclonal antibodies against birch pollen allergens: Characterization by immunoblotting and use for single-step affinity purification of the major allergen *Bet v I*. *Int Arch Allergy Appl Immunol* 1989;90:54–60.
- 18 Breiteneder H, Pettenburger K, Bito A, Valenta R, Kraft D, Rumpold H, Scheiner O, Breitenbach M: The gene coding for the major birch pollen allergen *Bet v I* is highly homologous to a pea disease resistance response gene. *EMBO J* 1989;8:1935–1938.
- 19 Ipsen H, Klysner S, Nedergard Larsen J: Isoallergenic variation of the major allergen of birch pollen, *Bet v I*. *Allergy* 1993;48(suppl):125.
- 20 Lahti A, Björkstén F, Hannuksela M: Allergy to birch pollen and apple and cross-reactivity of the allergens studied with the RAST. *Allergy* 1980;35:297–300.
- 21 Valenta R, Duchene M, Vrtala S, Valent P, Silaber C, Ferreira F, Tejkl M, Hirschwehr R, Ebner C, Kraft D, Scheiner O: Profilin, a novel plant panallergen. *Int Arch Allergy Appl Immunol* 1992;99:271–273.
- 22 Helbling A, Lopez M, Schwartz HJ, Lehrer SB: Reactivity of carrot-specific IgE antibodies with celery, apiaceous spices, and birch pollen. *Ann Allergy* 1993;70:495–499.
- 23 Vallier P, Dechamp C, Valenta R, Vial O, Deviller P: Purification and characterization of an allergen from celery immunochemically related to an allergen present in several other plant species. Identification as a profilin. *Clin Exp Allergy* 1992;22:774–782.
- 24 Van Ree R, Voitenko V, van Leeuwen WA, Aalberse RC: Profilin is a cross-reactive allergen in pollen and vegetable foods. *Int Arch Allergy Appl Immunol* 1992;98:97–104.