

ORIGINAL PAPER

Cross-reactive and species-specific immunoglobulin E epitopes of plant profilins: an experimental and structure-based analysis

C. Radauer*, M. Willeroider†, H. Fuchs*, K. Hoffmann-Sommergruber*, J. Thalhamer†, F. Ferreira†, O. Scheiner* and H. Breiteneder*

*Department of Pathophysiology, Center of Physiology and Pathophysiology, Medical University of Vienna, Vienna, Austria and †Department of Molecular Biology, University of Salzburg, Salzburg, Austria

Clinical and Experimental Allergy

Summary

Background Profilins are cross-reactive plant allergens responsible for multiple pollen sensitization and pollen-associated food allergy. While it is assumed that profilins from different species are immunologically equivalent, some studies suggest partial or even lacking IgE cross-reactivity between certain profilins.

Objective We aimed to obtain a semi-quantitative assessment of the contributions of conserved and species-specific epitopes to IgE binding of plant profilins.

Methods We compared model structures of profilins from timothy, mugwort, celery and bell pepper with crystal structures of birch and latex profilins. We predicted potential conformational epitopes that consisted of contiguous patches of at least 20% surface-exposed residues. Celery and timothy profilins were purified from their natural sources, and profilins from birch, mugwort, bell pepper and latex were expressed in *Escherichia coli*. The structural integrity of all purified proteins was confirmed by circular dichroism spectroscopy. IgE ELISAs and ELISA inhibitions using sera from 22 profilin-sensitized allergic patients were carried out.

Results Peptide backbone conformations of all six profilins were highly similar. Nine variable epitopes and two containing high proportions of conserved residues were predicted. IgE from all sera bound to all tested profilins and the amounts were highly correlated. However, IgE inhibition experiments revealed that up to 60% of total IgE binding was mediated by species-specific epitopes. The extent of cross-reactivity among profilins from timothy, birch, latex and celery was greater than cross-reactivity to mugwort and bell pepper profilins. This pattern was mirrored by sequence similarities among one of the predicted variable epitopes. Patients with IgE to cross-reactive epitopes displayed allergic reactions to a greater number of plant foods than patients having IgE directed to species-specific epitopes.

Conclusion The large extent of cross-reactivity among plant profilins justifies using a single profilin for diagnosis. However, the fine specificity of IgE directed to variable epitopes may influence the clinical manifestation of profilin sensitization.

Keywords allergens, cross-reactions, epitopes, food allergy, immunoglobulin E, pollen allergy, profilin, protein structure

Submitted 31 January 2006; revised 27 March 2006; accepted 21 April 2006

Correspondence:

Heimo Breiteneder, Department of Pathophysiology, Center of Physiology and Pathophysiology, Medical University of Vienna, AKH-EBO 3Q, Waehringer Guertel 18-20, A-1090 Vienna, Austria.
E-mail: Heimo.Breiteneder@meduniwien.ac.at

Introduction

Patients allergic to pollen or latex frequently have allergic reactions towards several plant foods. This correlation is attributed to the cross-reactivity of pollen and latex allergen-specific IgE that reacts with homologous plant food proteins. Examples for such cross-reactive allergens are the major birch pollen allergen, Bet v 1, and its

homologues in fruits and vegetables, and class I chitinases that play a major role in the latex-fruit syndrome (reviewed in [1]).

Profilins are small, cytosolic proteins, which are found in all eukaryotic cells and take part in the regulation of the actin cytoskeleton. Plant profilins are minor allergens recognized by IgE from 10% to 20% of pollen-allergic patients (reviewed in [2]) and cause IgE cross-reactivity

not only among botanically unrelated pollen [3, 4] but also between pollen and food [5] as well as pollen and latex [6].

Most studies confirm the immunological equivalence of allergenic profilins, meaning that IgE from profilin-sensitized patients binds to profilins from different plant sources with similar affinity and IgE binding to profilins can be mutually inhibited [6, 7]. Hence, recombinant birch pollen profilin, Bet v 2, is now widely used as the allergen of choice in assays for profilin-specific IgE [8]. However, several reports suggest the existence of species-specific IgE epitopes on profilins, as exemplified by the lack of cross-reactivity between profilins from *Hevea brasiliensis* latex and *Mercurialis annua* pollen, although both plants belong to the family Euphorbiaceae [9]. Other studies showed only partial cross-reactivity between profilins from birch pollen, cherry, pear, celery and peanut [10, 11], as well as between *Parietaria* and grass pollen profilins [12].

This study was performed with the aim of obtaining semi-quantitative data on the proportions of species-specific and cross-reactive IgE epitopes of profilins and to assess differences in the repertoire of specific IgE among profilin-sensitized patients. We analysed known plant profilin structures and amino acid sequences with respect to potential linear and conformational IgE epitopes. Complementary to the structural approach, we performed IgE immunoassays using six purified profilins from different plant families: timothy pollen (Phl p 12, [13]), birch pollen (Bet v 2, [14]), mugwort pollen (Art v 4, [15]), celery tuber (Api g 4, [16]), bell pepper (Cap a 2, [17]) and *H. brasiliensis* latex (Hev b 8, [6]).

Methods

Sequence alignment

Sequences of 25 allergenic profilins from 18 different monocot and dicot families covering a wide spectrum of angiosperm taxa were taken from the Uniprot database (<http://www.ebi.uniprot.org/index.shtml>). The analysed profilins were as follows: Amb a 8 (*Ambrosia artemisiifolia*, plant family Asteraceae, Uniprot accession number Q64LH1), Ana c 1 (*Ananas comosus*, Bromeliaceae, Q94JN2), Api g 4 (*Apium graveolens*, Apiaceae, Q9XF37), Ara h 5 (*Arachis hypogaea*, Fabaceae, Q9SQI9), Ara t 8 (*Arabidopsis thaliana*, Brassicaceae, Q42449), Art v 4.01 (*Artemisia vulgaris*, Asteraceae, Q8H2C9), Bet v 2 (*Betula verrucosa*, Betulaceae, P25816), Cap a 2 (*Capsicum annuum*, Solanaceae, Q93YI9), Che a 2 (*Chenopodium album*, Chenopodiaceae, Q84V37), Cuc m 2 (*Cucumis melo*, Cucurbitaceae, Q7X9Q0), Cyn d 12 (*Cynodon dactylon*, Poaceae, O04725), Gly m 3 (*Glycine max*, Fabaceae, O65809), Hev b 8.0204 (*H. brasiliensis*, Euphorbiaceae, Q9LEI8), Lil 1 prof (*Lilium longiflorum*,

Liliaceae, Q9SNW7), Lit c 1 (*Litchi chinensis*, Sapindaceae, Q941H7), Mal d 4.0101 (*Malus domestica*, Rosaceae, Q9XF42), Mer a 1 (*M. annua*, Euphorbiaceae, O49894), Mus xp 1 (*Musa xp acuminata*, Musaceae, Q94JN3), Ole e 2.1 (*Olea europaea*, Oleaceae, O24169), Ory s 12 (*Oryza sativa*, Poaceae, Q9FUD1), Par j 3.0101 (*Parietaria judaica*, Urticaceae, Q9XG85), Phl p 12.2 (*Phleum pratense*, Poaceae, O24650), Pho d 2 (*Phoenix dactylifera*, Arecaceae, Q8L5D8), Pru av 4 (*Prunus avium*, Rosaceae, Q9XF39) and Zea m 12 (*Zea mays*, Poaceae, O22655).

A multiple sequence alignment was performed using the PileUp programme from the GCG package (Accelrys Inc., Munich, Germany). The alignment was used to calculate a variability plot, with variability defined as the number of different residues occurring at each position of the alignment divided by the frequency of the most common residue [18].

Homology modelling of profilin structures

Structures of profilins from mugwort pollen (Art v 4.01), timothy pollen (Phl p 12.2), celery tuber (Api g 4) and bell pepper (Cap a 2) were modelled using the Swiss-Model server (<http://swissmodel.expasy.org>, [19]). The structures of profilins from *A. thaliana* pollen (Ara t 8, PDB accession number 1AOK), birch pollen (Bet v 2, 1CQA) and *H. brasiliensis* latex (Hev b 8.0204, 1G5U) were used as templates.

Prediction of conformational epitopes on Hev b 8

The structure of *H. brasiliensis* latex profilin (Hev b 8.0204) was used to predict amino acid residues forming potential conformational epitopes on the surface of the protein. Solvent accessible surface areas (SASA) of each residue were calculated using the GETAREA 1.1 program [20] located at the University of Texas Medical Branch (http://www.scsb.utmb.edu/cgi-bin/get_a_form.tcl). Relative values were calculated in relation to the average SASA of the respective residue in the peptide GXG. The distances between residues were calculated using the Swiss-PDB Viewer [19].

Residues contributing to conformational epitopes were predicted by a structure-based algorithm, which is a modification of a previously published method [21]:

1. Protruding residues with relative SASA > 75% were chosen as centres of the epitopes. If two or more of these residues were closer to each other than 0.5 nm, they were combined to form a single epitope.
2. For each of these residues, all partially accessible residues (SASA > 20%) within 1 nm distance from the central residue(s) were included in the epitope. The chosen epitope radius reflects the typical size of an antigen-antibody contact surface (reviewed in [22]).

3. Finally, single residues located in the linear sequence between two residues or stretches that were already part of the epitope were also included. The rationale behind this step was the assumption that an amino acid will significantly affect the conformation of its neighbours.

Patients' sera

Sera from 22 profilin-sensitized allergic patients were included in this study. Sensitization to profilin was determined either based on specific IgE directed to recombinant Bet v 2 (CAP class ≥ 2) or, in cases where these data were not available, on positive IgE immunoblots with recombinant Bet v 2. The presence of typical symptoms of allergy to pollen and *H. brasiliensis* latex was confirmed by skin prick tests using commercial extracts (ALK-Abello, Hørsholm, Denmark). Food allergy was diagnosed solely based on symptoms reported by the patients.

Recombinant and natural profilins

Purified recombinant profilins from birch pollen (Bet v 2) and *H. brasiliensis* latex (Hev b 8.0204) expressed in *Escherichia coli* were obtained from Biomay (Vienna, Austria). Recombinant profilins from mugwort pollen (Art v 4.01) and bell pepper fruits (Cap a 2) were expressed in *E. coli* and purified by affinity chromatography on poly-L-proline sepharose [15, 17].

Profilins from celery tuber (Api g 4) and timothy pollen (Phl p 12) were purified from their natural sources. One gram of timothy pollen (Allergon, Ängelholm, Sweden) was stirred overnight at 4 °C in 10 mL of extraction buffer (20 mM Tris-HCl, 10 mM ethylenediamine tetraacetic acid (EDTA), pH 7.5, 1.5% (w/v) polyvinylpyrrolidone, 20 mM sodium diethyl dithiocarbamate, 5 mM dithiothreitol) supplemented with protease inhibitors (Complete EDTA free protease inhibitor tablets, Roche Molecular Biochemicals, Mannheim, Germany). One hundred grams of celery tuber was cut into pieces, frozen in liquid nitrogen, ground to a fine powder and stirred with 100 mL of extraction buffer overnight at 4 °C. Both extracts were cleared by centrifugation (40 000 g, 1 h, 4 °C) and applied to a column of 20 mg poly-L-proline (average molecular weight 5000, Sigma-Aldrich, St Louis, MO, USA) coupled to 1 g CNBr-activated sepharose 4B (Amersham Biosciences, Little Chalfont, UK). After washing the column with buffer A (20 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM dithiothreitol) and with buffer A containing 2 M urea, profilins were eluted with buffer A containing 6 M urea. Eluted profilins were refolded by stepwise dialysis using descending urea concentrations. The purity of proteins was checked by SDS-PAGE and staining with Coomassie Brilliant Blue R-250.

Circular dichroism

Secondary structures of purified profilins were checked using circular dichroism (CD) spectroscopy. The proteins were dialysed against 10 mM potassium phosphate, pH 7.5, and measured at concentrations of 0.1 mg/mL in a J-810 spectropolarimeter (Jasco, Easton, MD, USA) in a 0.2 cm quartz cuvette. Far UV spectra were recorded in the range between 190 and 260 nm at room temperature. Data of three measurements were accumulated.

Immunoglobulin E ELISA and ELISA inhibitions

For determination of profilin-specific IgE, 100 µL of purified profilins were immobilized overnight to 96-well MaxiSorp microtitre plates (Nunc, Roskilde, Denmark) at a concentration of 10 µg/mL in 25 mM sodium carbonate buffer, pH 9.5. After blocking non-specific binding sites with Tris-buffered saline containing 0.5% Tween 20 (TBST) and 2% non-fat milk powder, sera diluted 1 : 5 in TBST containing 0.5% bovine serum albumin were applied to the plates overnight at 4 °C. After applying a monoclonal anti-human IgE antibody conjugated to alkaline phosphatase (BD Pharmingen, Heidelberg, Germany) at a dilution of 1 : 1000 for 1 h, bound antibodies were detected by incubation with *p*-nitrophenyl phosphate (Sigma Fast *p*-nitrophenyl phosphate tablet sets, Sigma-Aldrich). Colour development was quantified by measuring at 405 nm. In all experiments, the sera of non-allergic individuals and buffer without serum were included as negative controls. All samples were analysed in duplicate. The cut-off for positive values was determined using OD values of five non-allergic patients' sera and calculated from their mean OD plus three standard deviations.

For IgE inhibition experiments, diluted sera were pre-incubated with purified profilins or recombinant Bet v 1a (Biomay) as a non-homologous negative control, before proceeding with the immunoassay as described above. A preliminary experiment was performed with three sera using inhibitor concentrations between 0.03 and 100 µg/mL. For the inhibition assay with 13 sera, the inhibitor concentration was 10 µg/mL, a value high enough to guarantee plateau inhibition, as determined in the pilot experiment. Inhibition values are given as percent reduction of bound IgE compared with the controls where no inhibitor protein has been added.

Statistical analyses

An IgE ELISA was performed with sera from 22 Bet v 2-sensitized patients to measure their IgE levels specific for profilins from birch pollen (Bet v 2), timothy pollen (Phl p 12), mugwort pollen (Art v 4), *H. brasiliensis* latex (Hev b 8), celery tuber (Api g 4) and bell pepper fruit (Cap a 2). Specific IgE values were subjected to a linear correlation analysis using the Pearson correlation coefficient.

In addition, an IgE inhibition assay using 13 sera was performed with Phl p 12 coated to the solid phase and six different profilins used as inhibitors. The results of this assay were compared with allergic symptoms of the patients. Inhibition values for single allergens as well as the average extent of inhibition (not including the self-inhibition with Phl p 12) were compared with the presence of allergic symptoms to birch, grass, mugwort, ragweed and plantain pollen, *Hevea* latex, or plant foods using the Mann-Whitney test. Correlation between the average amount of inhibition and the number of pollen or plant food sources, respectively, eliciting allergic reactions was tested using Spearman's rank correlation analysis. All statistical analyses were performed using SPSS (SPSS Inc., Chicago, IL, USA).

Results

Comparison of profilin sequences and structures

Amino acid sequences of 25 profilins from diverse plant families were aligned (Fig. 1). The high degree of similarity was reflected by this alignment: 54 of 134 residues (40%) were identical in all analysed sequences (Fig. 1a). Together with 25 alignment positions (19%) with an aberrant residue in a minority of the aligned sequences (variability less than 3), a total of 79 residues (59%) could be classified as conserved.

As antibodies bind to epitopes formed by the three-dimensional arrangement of amino acid residues on the protein surface, tertiary structures of profilins were compared. The three plant profilin structures found in the

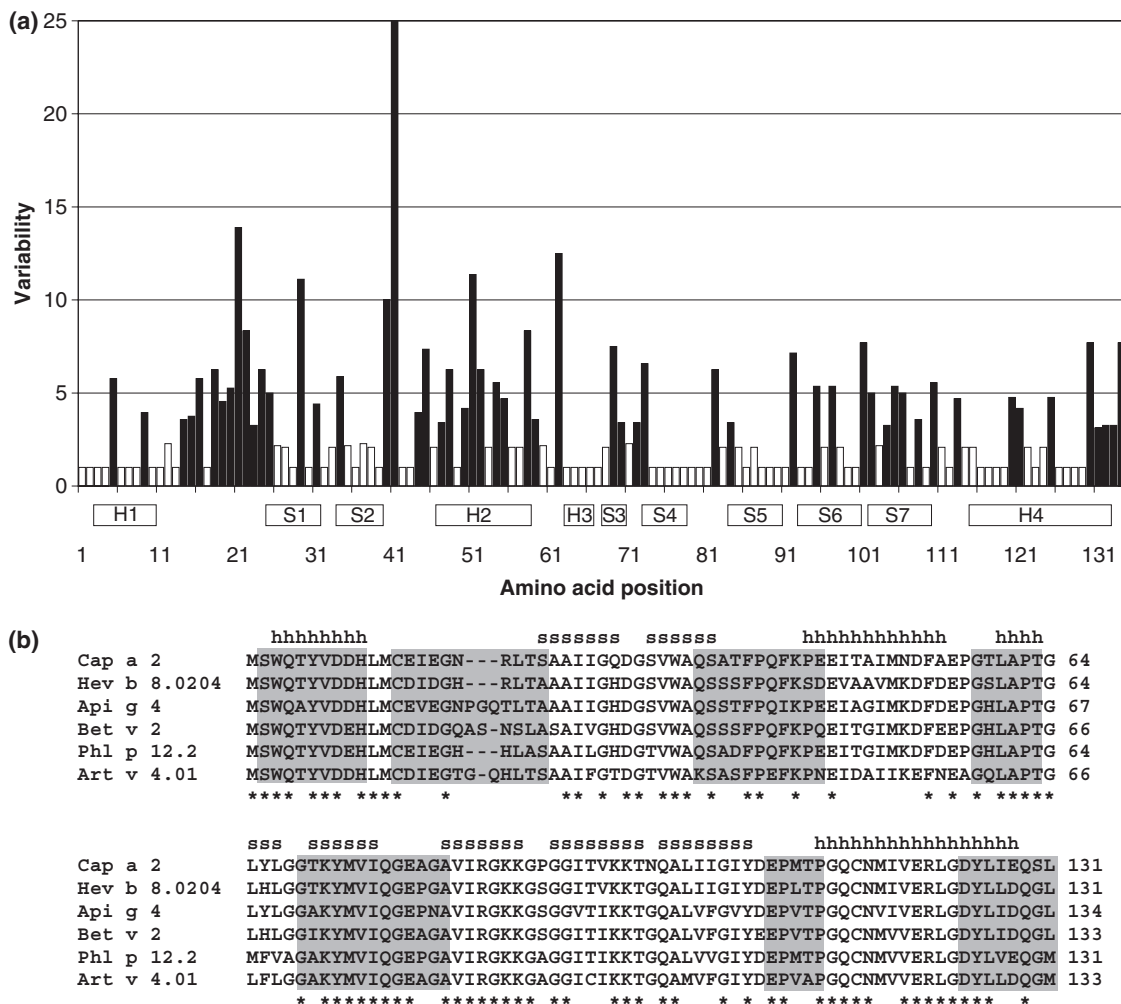


Fig. 1. Alignment of profilin sequences. (a) Variability plot generated from an alignment of 25 plant profilin sequences. White bars: conserved residues (variability < 3), black bars: variable residues. Variability is defined as the number of different amino acid residues divided by the frequency of the most common one [18]. Secondary structure elements are depicted below the chart (H, α -helix; S, β -sheet). (b) Alignment of six profilin sequences used in this study. Secondary structure elements are denoted in the top line. Asterisks designate residues identical in all six sequences. Linear stretches being parts of potential epitopes are shaded.

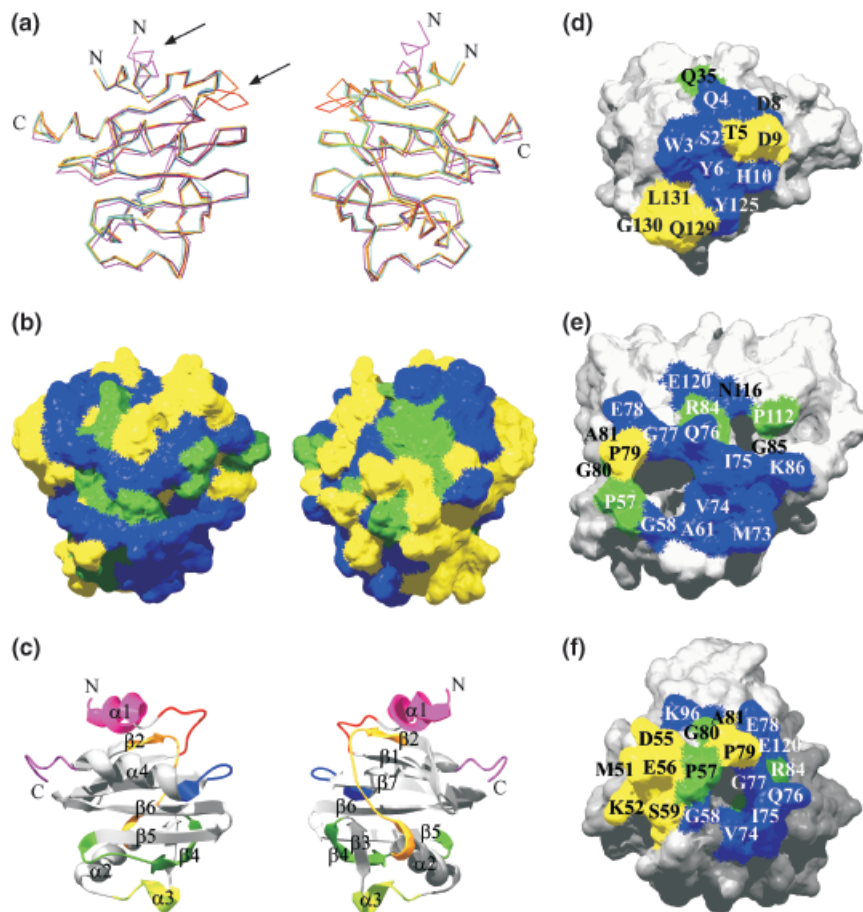


Fig. 2. Structural analysis of plant profilins. (a) α -carbon traces of profilins from celery tuber (Api g 4, red), mugwort pollen (Art v 4.01, brown), bell pepper fruit (Cap a 2, dark blue), timothy pollen (Phl p 12.2, green), *Arabidopsis thaliana* pollen (Ara t 8, orange), birch pollen (Bet v 2, purple) and *Hevea brasiliensis* latex (Hev b 8.0204, light blue). The N-terminal α -helix and the succeeding loop are marked by arrows. (b) Molecular surface of Hev b 8.0204 coloured by residue frequency: blue, residues identical in all analysed profilin sequences (variability = 1); green, conserved residues (variability < 3); yellow, variable residues (variability > 3). (c) Secondary structure of Hev b 8.0204 with linear stretches being parts of potential conformational epitopes: helix 1 (pink), the succeeding loop (red), the loop preceding helix 2 (orange), helix 3 (light green), strand 4 and the succeeding loop (dark green), the loop preceding helix 4 (blue) and the C-terminus (purple). (d–f) Conformational epitopes 1 (d), 7 (e) and 8 (f) predicted for Hev b 8. Amino acids are coloured as in (b). The right views in (a–c) are derived from the left ones by a 180° rotation around a vertical axis. All images were generated using the Swiss-PDB Viewer [19] and Pov-Ray 3.5.

Protein Data Bank (profilins from *A. thaliana* pollen, birch pollen and *H. brasiliensis* latex) and the models of four additional profilins showed nearly identical arrangements of the peptide backbone (Fig. 2a). The only major structural differences were observed with respect to the orientation of the N-terminal α -helix, which is unique in the birch pollen profilin structure, and the conformation of a highly solvent-exposed variable loop between the N-terminal α -helix and the first β -strand (marked by arrows in Fig. 2a). Thus, it is justified to attribute different antibody-binding capabilities of profilins to variations of solvent-exposed amino acid side-chains. Colouring of the Hev b 8 structure with respect to the variability coefficients obtained from the alignment of 25 profilin sequences showed that the surface was composed of a mosaic of conserved and variable residues (Fig. 2b). The

proportion of invariable and conserved residues within surface-exposed amino acids (SASA > 20%) was slightly lower than the value for the whole sequence (47% invariable or conserved, 53% variable).

Prediction of potential conformational epitopes on Hev b 8

Three structures of plant profilins are recorded in the PDB database: profilins from *A. thaliana* pollen, birch pollen and *H. brasiliensis* latex. As *A. thaliana* pollen has not been described as a significant allergen source and the structure of birch pollen profilin lacks the co-ordinates of a solvent-exposed loop, the structure of *H. brasiliensis* latex profilin (Hev b 8.0204) was chosen to predict potential conformational epitopes on its surface. Following the procedure outlined in 'Methods', 12 protruding

Table 1. Predicted conformational epitopes of Hev b 8. Residues identical or conserved in all analyzed profilin sequences are typed in boldface

	Central residue	Sequence	Residues total	Cons.	Var.
1	S2	2 SWQTYVDDH ₁₀ Q ₃₅ Y ₁₂₅ 129 QGL ₁₃₁	14	9	5
2	R19	8 DDH ₁₀ 13 CDIDGHRLT ₂₁ 37 SS ₃₈ 107 DEPLT ₁₁₁	19	6	13
3	S37+S38	Q ₄ D ₈ 19 RLT ₂₁ V ₃₂ 35 QSSSFPQ ₄₁ G ₆₉ D ₁₀₇	15	7	8
4	Q41	30 GSV ₃₂ 35 QSSSFPQFKSD ₄₅ H ₆₆ 69 GT ₇₀	17	7	10
5	S44+D45	28 HDGSV ₃₂ 40 PQFKSD ₄₅ 48 AAVMK ₅₂ T ₆₃ H ₆₆ G ₆₉	19	7	12
6	T63	D ₄₅ 48 AA ₄₉ K ₅₂ 58 GSLAPT ₆₃ H ₆₆ 69 GTKYMV ₇₄	17	9	8
7	Q76	57 PG ₅₈ A ₆₁ 73 MVIQGEPGA ₈₁ 84 RGK ₈₆ P ₁₁₂ N ₁₁₆ E ₁₂₀	18	15	3
8	P79	51 MK ₅₂ 55 DEPGS ₅₉ 74 VIQGEPGA ₈₁ R ₈₄ K ₉₆ E ₁₂₀	18	10	8
9	E108	17 GHRLT ₂₁ 86 KKGS ₈₉ 107 DEPLT ₁₁₂	15	8	7
10	M117	H ₁₀ 13 CDIDGH ₁₈ R ₈₄ 111 TPG ₁₁₃ 116 NM ₁₁₇ 120 ER ₁₂₁ 124 DY ₁₂₅	17	11	6
11	D128	Y ₆ 96 KTGQ ₉₉ 120 ER ₁₂₁ 124 DYLLDQGL ₁₃₁	15	8	7

residues were selected as centres of the epitopes. Two of their pairs (Ser37 and Ser38, Ser44 and Asp45) were closer than 0.5 nm to each other; thus, they were combined to form joint epitopes (Table 1, epitopes 3 and 5). The 10 overlapping potential epitopes defined by this procedure (Table 1, epitopes 1–9 and 11) included all solvent-exposed residues except Met117, which was subsequently chosen as the centre of an additional epitope (Table 1, epitope 10). The potential epitopes comprised between 14 and 19 residues distributed into two to four short stretches of two to 11 residues in length and up to five single residues dispersed over the linear sequence (Table 1). Although all potential epitopes were conformational, they contained linear stretches at least six residues in length (Figs 1b and 2c). All epitopes included both conserved and variable residues (Table 1). However, the central part of epitope 1 contained mainly conserved residues (Fig. 2d, Table 1). Epitope 7 consisted of conserved amino acids except a short stretch of three variable residues (Fig. 2e, Table 1). Hence, these epitopes can be classified as conserved.

Clinical data of profilin-sensitized patients

Twenty-two Bet v 2-sensitized patients were included in this study. Twenty of them (91%) had pollinosis caused by grass (20 cases), birch (16, 73%), mugwort (9, 41%), ragweed (9, 41%) and plantain pollen (6, 27%). Two patients without pollinosis had latex allergy with typical latex-associated fruit allergies (kiwi fruit and citrus fruits in one case, banana in the other). However, the amounts of profilin-specific IgE were very low in both sera. In total, nine patients (41%) had latex allergy; 11 patients (50%) were diagnosed with plant food allergy, with banana (4), walnut (4), kiwi fruit (3), stone fruits (3), hazelnut (3) and citrus fruits (2) as the most common offending foods.

Characterization of purified profilins

Six different recombinant (Art v 4.01, Bet v 2, Cap a 2, Hev b 8.0401) and natural profilins (Api g 4, Phl p 12) were

purified by affinity chromatography on poly-L-proline-sepharose columns. Purified proteins were at least 95% pure as determined by SDS-PAGE, followed by staining with Coomassie brilliant blue (data not shown). Correct folding of the purified profilins was checked using CD spectrometry (Fig. 3). All six profilins yielded similar spectra typical for folded proteins with mixed α - β structures.

Analysis of immunoglobulin E binding to different profilins

In order to compare the IgE-binding capacities of plant profilins with respect to different patients' sera, an IgE ELISA was performed with six different profilins adsorbed to the solid phase, and the amount of profilin-specific IgE from the sera of 22 allergic patients was determined. All sera contained IgE reactive with all six profilins, with the exception of one serum with very low amounts of profilin-specific IgE, whose value for Cap a 2 did not exceed the negative controls. Comparison of single patients' IgE-binding capacities to different profilins revealed a high degree of correlation between the ELISA values of all protein pairs. A nearly perfect linear correlation was found for all pairs of profilins, with Pearson's correlation coefficients ranging from 0.916 (Art v 4–Phl p 12) to 0.987 (Art v 4–Hev b 8, data not shown).

Patient-dependent immunoglobulin E cross-reactivity of profilins

A further investigation of the proportions of cross-reactive and species-specific IgE epitopes was performed using IgE ELISA inhibitions with sera from 13 patients (Table 2). As all tested patients were allergic to grass pollen, natural timothy pollen profilin (Phl p 12) was absorbed to the solid phase and IgE binding to this allergen was compared with the values obtained after pre-incubating the sera with six different profilins at a concentration of 10 μ g/mL. In an initial concentration-dependent inhibition experiment with

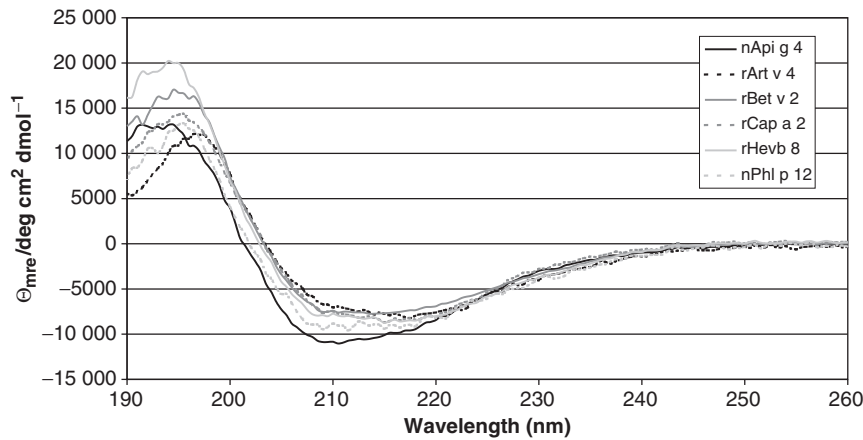


Fig. 3. Circular dichroism spectra of purified profilins. Spectra were recorded at a concentration of 0.1 mg/mL at room temperature. Data from three measurements were accumulated. The molar residue ellipticity (Θ_{mre}) is plotted.

Table 2. Immunoglobulin E inhibition data and allergic symptoms of profilin-sensitized patients

	Inhibition							Allergic symptoms					
Patient no.	Api g 4	Art v 4	Bet v 2	Cap a 2	Hev b 8	Phl p 12	Average*	Birch	Grass	Mugwort	Other pollen [†]	Latex	Plant Food [‡]
1	76	42	76	40	65	91	60	+	+	—	—	—	—
2	91	76	91	80	86	93	85	+	+	+	Ra	—	—
3	94	79	92	84	85	97	87	—	+	—	Pl, Ra	—	—
4	87	75	78	63	76	91	76	+	+	+	—	—	—
5	92	43	76	64	76	92	70	+	+	—	Pl, Ra	—	—
6	89	50	64	39	85	98	66	+	+	—	Ra	—	Po
7	81	89	99	95	96	99	92	+	+	+	Ra	—	Ha, Wa
8	92	65	87	60	64	94	74	+	+	—	Pl, Ra	+	—
9	82	60	51	40	40	99	55	+	+	—	Pl	+	—
10	93	92	90	84	90	97	90	+	+	—	—	+	Fi
11	96	60	80	93	90	99	84	+	+	—	—	—	—
12	91	70	77	90	86	94	83	—	+	+	Pl	+	Ba
13	99	93	85	98	97	98	94	—	+	+	Ra	+	Ha, Wa, Ba, Or, Pl, To

*Average extent of inhibition not including the self-inhibition with Phl p 12.

†Abbreviations: Pl, plantain; Ra, ragweed.

‡Abbreviation: Ba, banana; Fi, fig; Ha, hazelnut; Or, oregano; Pl, plum; Po, potato; To, tomato; Wa, walnut.

Sera were pre-incubated with profilins and assayed for IgE binding to Phl p 12 coated to the solid phase. Bold: inhibition values below 75%.

three sera, this inhibitor concentration was shown to exceed the concentration required for saturating inhibition by at least tenfold (data not shown).

Pre-incubation of the sera with different profilins inhibited IgE binding to Phl p 12 by 40–99%, depending on the serum and the inhibitor protein (Table 2). The values for the control inhibitions with Phl p 12 (self-inhibitions) were highest for all sera. The negative control pre-incubation with Bet v 1, a non-homologous allergen, yielded no inhibition (data not shown).

The extent of profilin cross-reactivity for the tested sera was compared with the presence of allergic symptoms to birch pollen, mugwort pollen and *Hevea* latex. No correlation between the inhibitory capacities of profilins from the respective sources and allergic symptoms could be estab-

lished (Table 2). In addition, the average amount of inhibition (not including the self-inhibition) was compared with allergic symptoms. No connection between the presence of allergic symptoms to latex or plant food or the number of pollen sources eliciting allergic reactions and the extent of profilin cross-reactivity could be established (Fig. 4a). However, the number of plants foods eliciting allergic reactions showed a significant correlation with the average amount of inhibition (Spearman's correlation coefficient: 0.558; $P = 0.047$; Fig. 4b).

Comparison of *in silico* epitope prediction with inhibition data

The examination of the inhibition data presented in Table 2 revealed two groups of sera: those containing IgE

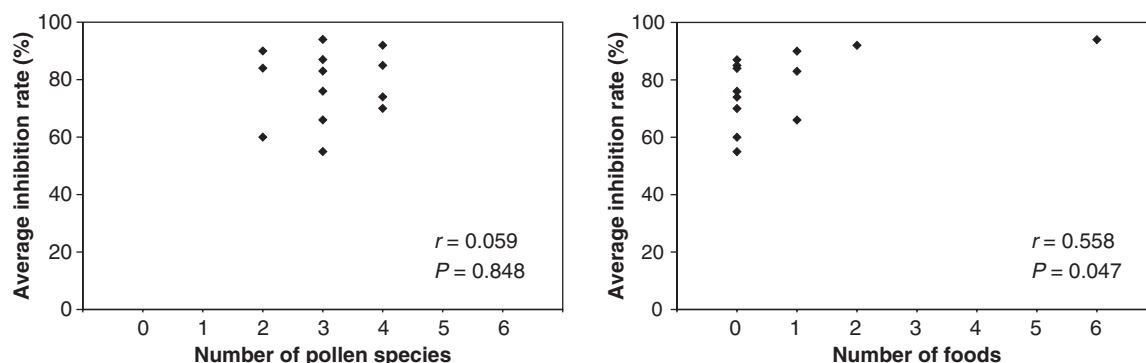


Fig. 4. Correlation of the average inhibition of IgE binding to Phl p 12 by other profilins with the number of pollen species (a) and plant food species (b) eliciting allergic reactions.

Table 3. Sequences of predicted epitopes 1, 7 and 8 compared with the sequence of Phl p 12.2

Epitope	Sequence alignment	% Identity to Phl p 12.2
1	Phl p 12.2 SWQTYVDEH Q Y QGM	
	Api g 4 --A----D- - - --L	79
	Art v 4.01 -----D- K - ---	86
	Bet v 2 -----D- - - --L	93
	Cap a 2 -----D- - - --SL	79
	Hev b 8.0401 -----D- - - --L	86
7	Phl p 12.2 PG A MVIQGEPGA RGK P N E	
	Api g 4 -- - -----N- - - - -	94
	Art v 4.01 A- - -----A- - - - -	89
	Bet v 2 -- - -----A- - - - -	94
	Cap a 2 -- - -----A- - - - -	94
	Hev b 8.0401 -- - -----A- - - - -	100
8	Phl p 12.2 MK DEPGH VIQGEPGA R K E	
	Api g 4 -- - -----N- - - - -	94
	Art v 4.01 I- N-A-Q -----A- - - -	72
	Bet v 2 -- E-----A- - - - -	89
	Cap a 2 -N A---T -----A- - - -	78
	Hev b 8.0401 -- - ----S -----A- - - -	94

Dashes denote residues identical to the corresponding residue in Phl p 12.

directed mainly to cross-reactive epitopes (patients 2, 3, 7, 10, 13), which is reflected by inhibition values above 75% for all profilins, and those recognizing variable epitopes to a significant extent. Two epitopes predicted to be highly cross-reactive by means of sequence alignment were epitopes 1 and 7 (Figs 2d and e, Table 3). Among the variable epitopes, epitope 8 (Fig. 2f, Table 3) best reflected the observed differences among profilins with respect to inhibitory capacity. IgE binding to Phl p 12 was nearly completely inhibited by Api g 4 (90% inhibition averaged over all sera), Bet v 2 (80%) and Hev b 8 (80%). Lower average inhibition values were observed for Art v 4 (69%) and Cap a 2 (71%). These numbers highly resemble the percent sequence identity of residues constituting epitope 8 to corresponding residues in Phl p 12 (Table 3).

Discussion

Profilins are highly cross-reactive plant allergens. IgE from profilin-sensitized allergic patients binds to profilins from a large number of botanically unrelated plants. In agreement with this, presence of profilin-specific IgE has been shown to elevate the risk of multiple pollen sensitization [4, 23] and pollen-related food allergy [24, 25]. Nevertheless, many profilin-sensitized patients do not exhibit symptoms of food allergy or react only to a small number of profilin-containing allergen sources. One reason for different clinical consequences of profilin-specific IgE could be differences in the patients' IgE repertoire resulting in a lack of sensitization to some species-specific epitopes. Therefore, we aimed to define the distribution of cross-reactive and species-specific IgE epitopes by IgE immunoassays and by *in silico* analysis of profilin structures.

Comparison of all surface-exposed residues of profilins from different plant families showed that the molecular surface was a mosaic of conserved and variable residues (Fig. 2b). Taking into account the typical size of antigen binding sites (about 2 nm in diameter, reviewed in [22]), 11 overlapping potential epitopes were identified (Table 1). These epitopes all contained conserved as well as variable residues. However, epitope 7 and the central part of epitope 1 can be classified as conserved (Figs 2d and e). Therefore, IgE specific for these epitopes can be assumed to be responsible for the high extent of cross-reactivity among profilins. Peptide mapping experiments using synthetic peptides or random fragments derived from the profilin sequence have previously shown IgE binding to peptides corresponding to the N-terminal and C-terminal helices of profilins, thus confirming possible involvement of epitope 1 in IgE binding [26, 27]. The linear stretch forming the central part of epitope 7 (M73-A81 in Hev b 8) has not been found to bind IgE in those experiments.

ELISA inhibition assays with 13 sera revealed that the inhibition of IgE binding at maximum inhibitor concentration, related to the percentage of epitopes shared

between timothy pollen profilin (Phl p 12) and the other five profilins used in this study, varied between 40% and 100%, depending on the serum and the proteins used (Table 2). A comparison of the average amount of inhibition of IgE binding to Phl p 12 caused by other profilins showed a significantly higher cross-reactivity between Api g 4, Bet v 2, Hev b 8 and Phl p 12 compared with the cross-reactivity to Art v 4 and Cap a 2. This pattern nicely fits the pattern of sequence similarities of residues forming epitope 8 in Phl p 12 to corresponding residues in other profilins (Table 3). Interestingly, a single chain antibody variable fragment selected for binding of Hev b 8 from a phage display library cross-reacted to Api g 4, Bet v 2 and Phl p 12, but not to Art v 4 and Cap a 2 [28]. Thus, epitope 8 is a good candidate for an important variable epitope on plant profilins. In a recent publication [29], the authors claimed that the extent of cross-reactivity among profilins could not be attributed to sequence similarity. However, this assertion was based on IgE inhibition ELISAs using various fruit and pollen extracts whose profilin content had not been quantified.

All surface-exposed patches defined in our study were composed of residues dispersed over the linear sequence (Table 1). A significant contribution of conformational epitopes to the IgE binding capacity of profilin has previously been shown by comparing frequencies of IgE binding with olive pollen profilin in native ELISA and denaturing immunoblot [30] and by EAST inhibition using soybean profilin and peptide fragments spanning the complete sequence [31]. A peptide mimotope with no similarity to linear profilin sequences selected from a phage display library by biopanning with purified profilin-specific patient's IgE accounted for 0–82% of total IgE binding [32]. Finally, fragments of melon profilin generated by acid hydrolysis did not bind IgE [29]. All these data taken together suggest only a limited value of denaturing methods such as IgE immunoblots for the quantitation of profilin-specific IgE. In addition, care must be taken when purifying recombinant or natural profilins by poly-L-proline affinity chromatography, a method that involves elution by 6 M urea, followed by *in vitro* refolding. Profilin preparations should be checked for successful refolding using CD or equivalent methods.

In accordance with the high extent of cross-reactivity among profilins, all tested sera bound to all analysed profilins in an ELISA using purified profilins from six different sources and sera from 22 profilin-sensitized patients. The amounts of profilin-specific IgE showed a strong linear correlation on comparing values of all patients for different profilins. No differences between the group of four recombinant profilins and the two natural profilins used in our study were detected. These results are in contradiction to the data obtained by Wensing et al. [33], who described a much lower IgE binding capacity of recombinant Bet v 2, obtained from

the same source as the protein used in our study, compared with natural Bet v 2 and Lol p 12 (ryegrass pollen profilin).

ELISA inhibition assays with 13 sera revealed that the extent of inhibition of IgE binding to timothy pollen profilin (Phl p 12) by the other five profilins used in this study varied between 40% and 100% depending on the serum and the proteins used (Table 2). However, binding patterns of profilin-specific IgE generally had no consequences for clinical symptoms of the patients, as neither the patterns of pollen allergy nor the presence of allergy to latex or plant food correlated with the outcome of the inhibition assay. In contrast, the number of plant foods but not the number of pollen sources eliciting allergic reactions was significantly higher for patients with a higher extent of profilin cross-reactivity (Fig. 4). The lack of correlation between profilin cross-reactivity and the extent of multiple pollen sensitization contradicts at first glance results from previous studies showing a connection between IgE reactivity or positive skin tests to profilin and multiple pollen sensitization [4, 23]. However, these studies determined an increased number of polysensitized patients among profilin-sensitized ones but did not determine the extent of cross-reactivity between profilins from different sources. Interestingly, patients with IgE directed mainly to cross-reactive profilin epitopes tend to develop allergic reactions to a greater number of plant foods, despite the fact that the connection between profilin sensitization and food allergy has frequently been disputed [33].

In conclusion, our data justify the use of a single profilin as a diagnostic marker for profilin sensitization. In addition, despite the high proportion of variable residues on the profilin surface and the relatively high fraction of IgE binding to species-specific epitopes, no strong connection between IgE binding patterns assessed by inhibition assays and clinical cross-reactivity could be established.

Acknowledgements

This study was supported by the Austrian Science Fund grant SFB F01802 (to H. B.) and grant 9313 from the 'Jubiläumsfonds der Oesterreichischen Nationalbank' (to C. R.). The authors thank Christine Hafner (Department of Dermatology, Medical University of Vienna, Austria), Christof Ebner (Allergieambulatorium am Reumannplatz, Vienna, Austria) and Randolph Brehler (Department of Dermatology and Venerology, University of Münster, Germany) for providing the sera and clinical data of allergic patients.

References

- 1 Breiteneder H, Radauer C. A classification of plant food allergens. *J Allergy Clin Immunol* 2004; 113:821–30.

- 2 Radauer C, Hoffmann-Sommergruber K. Profilins. In: Mills ENC, Shewry PR, eds. *Plant food allergens*. Oxford, UK: Blackwell Publishing, 2004; 105–24.
- 3 Valenta R, Duchene M, Ebner C et al. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992; 175:377–85.
- 4 Mari A. Multiple pollen sensitization: a molecular approach to the diagnosis. *Int Arch Allergy Immunol* 2001; 125:57–65.
- 5 van Ree R, Voitenko V, van Leeuwen WA, Aalberse RC. Profilin is a cross-reactive allergen in pollen and vegetable foods. *Int Arch Allergy Immunol* 1992; 98:97–104.
- 6 Ganglbauer E, Radauer C, Wagner S et al. Hev b 8, the *Hevea brasiliensis* latex profilin, is a cross-reactive allergen of latex, plant foods and pollen. *Int Arch Allergy Immunol* 2001; 125: 216–27.
- 7 Ebner C, Hirschwehr R, Bauer L et al. Identification of allergens in fruits and vegetables: IgE cross-reactivities with the important birch pollen allergens Bet v 1 and Bet v 2 (birch profilin). *J Allergy Clin Immunol* 1995; 95:962–9.
- 8 Rossi RE, Monasterolo G, Operti D, Corsi M. Evaluation of recombinant allergens Bet v 1 and Bet v 2 (profilin) by Pharmacia CAP system in patients with pollen-related allergy to birch and apple. *Allergy* 1996; 51:940–5.
- 9 Vallverdu A, Asturias JA, Arilla MC et al. Characterization of recombinant *Mercurialis annua* major allergen Mer a 1 (profilin). *J Allergy Clin Immunol* 1998; 101:363–70.
- 10 Kleber-Janke T, Cramer R, Scheurer S, Vieths S, Becker WM. Patient-tailored cloning of allergens by phage display: peanut (*Arachis hypogaea*) profilin, a food allergen derived from a rare mRNA. *J Chromatogr B Biomed Sci Appl* 2001; 756:295–305.
- 11 Scheurer S, Wangorsch A, Nerkamp J et al. Cross-reactivity within the profilin panallergen family investigated by comparison of recombinant profilins from pear (Pyr c 4), cherry (Pru av 4) and celery (Api g 4) with birch pollen profilin Bet v 2. *J Chromatogr B Biomed Sci Appl* 2001; 756:315–25.
- 12 Asero R, Mistrello G, Roncarolo D, Amato S. *Parietaria* profilin shows only limited cross-reactivity with birch and grass profilins. *Int Arch Allergy Immunol* 2004; 133:121–4.
- 13 Asturias JA, Arilla MC, Bartolome B, Martinez J, Martinez A, Palacios R. Sequence polymorphism and structural analysis of timothy grass pollen profilin allergen (Phl p 11). *Biochim Biophys Acta* 1997; 1352:253–7.
- 14 Valenta R, Duchene M, Pettenburger K et al. Identification of profilin as a novel pollen allergen; IgE autoreactivity in sensitized individuals. *Science* 1991; 253:557–60.
- 15 Wopfner N, Willeroidee M, Hebenstreit D et al. Molecular and immunological characterization of profilin from mugwort pollen. *Biol Chem* 2002; 383:1779–89.
- 16 Scheurer S, Wangorsch A, Hausteiner D, Vieths S. Cloning of the minor allergen Api g 4 profilin from celery (*Apium graveolens*) and its cross-reactivity with birch pollen profilin Bet v 2. *Clin Exp Allergy* 2000; 30:962–71.
- 17 Willeroidee M, Fuchs H, Ballmer-Weber BK et al. Cloning and molecular and immunological characterisation of two new food allergens, Cap a 2 and Lyc e 1, profilins from bell pepper (*Capsicum annuum*) and Tomato (*Lycopersicon esculentum*). *Int Arch Allergy Immunol* 2003; 131:245–55.
- 18 Wu TT, Kabat EA. An analysis of the sequences of the variable regions of Bence Jones proteins and myeloma light chains and their implications for antibody complementarity. *J Exp Med* 1970; 132:211–50.
- 19 Guex N, Peitsch MC. SWISS-MODEL and the Swiss-PdbViewer: an environment for comparative protein modeling. *Electrophoresis* 1997; 18:2714–23.
- 20 Fraczekiewicz R, Braun W. Exact and efficient analytical calculation of the accessible surface areas and their gradients for macromolecules. *J Comp Chem* 1998; 19:319–33.
- 21 Kolaskar AS, Kulkarni-Kale U. Prediction of three-dimensional structure and mapping of conformational epitopes of envelope glycoprotein of Japanese encephalitis virus. *Virology* 1999; 261: 31–42.
- 22 Laver WG, Air GM, Webster RG, Smith-Gill SJ. Epitopes on protein antigens: misconceptions and realities. *Cell* 1990; 61: 553–6.
- 23 Pauli G, Oster JP, Deviller P et al. Skin testing with recombinant allergens rBet v 1 and birch profilin, rBet v 2: diagnostic value for birch pollen and associated allergies. *J Allergy Clin Immunol* 1996; 97:1100–9.
- 24 Venuti A, Ricciuto GM, Romano A, Marsigli L, Stefanini GF, Gasbarrini G. Cross-reactivity between pollen and food allergens: the importance of Bet v 1 and Bet v 2 epitopes. *Int J Immunopathol Pharmacol* 1997; 10:71–5.
- 25 Daschner A, Crespo JF, Pascual CY. Specific IgE to recombinant vegetal panallergen (rBet v 2) and fruit allergy in pollinic patients. *Allergy* 1998; 53:614–8.
- 26 Fedorov AA, Ball T, Mahoney NM, Valenta R, Almo SC. The molecular basis for allergen cross-reactivity: crystal structure and IgE-epitope mapping of birch pollen profilin. *Structure* 1997; 5:33–45.
- 27 Asturias JA, Gomez-Bayon N, Arilla MC, Sanchez-Pulido L, Valencia A, Martinez A. Molecular and structural analysis of the panallergen profilin B cell epitopes defined by monoclonal antibodies. *Int Immunol* 2002; 14:993–1001.
- 28 Wagner S, Radauer C, Allwardt D, Wagner B, Scheiner O, Breiteneder H. Diagnosis and therapy of latex allergy with recombinant scFv antibody fragments. In: Bienenstock J, Ring J, Togias A, eds. *Allergy frontiers and futures*. Cambridge, MA: Hogrefe & Huber, 2004, (Suppl. 1) 252–4.
- 29 Sankian M, Varasteh A, Pazouki N, Mahmoudi M. Sequence homology: a poor predictive value for profilins cross-reactivity. *Clin Mol Allergy* 2005; 3:13.
- 30 Ledesma A, Rodriguez R, Villalba M. Olive-pollen profilin. Molecular and immunologic properties. *Allergy* 1998; 53: 520–6.
- 31 Rihs HP, Chen Z, Rueff F et al. IgE binding of the recombinant allergen soybean profilin (rGly m 3) is mediated by conformational epitopes. *J Allergy Clin Immunol* 1999; 104: 1293–301.
- 32 Leitner A, Vogel M, Radauer C et al. A mimotope defined by phage display inhibits IgE binding to the plant panallergen profilin. *Eur J Immunol* 1998; 28:2921–7.
- 33 Wensing M, Akkerdaas JH, van Leeuwen WA et al. IgE to Bet v 1 and profilin: cross-reactivity patterns and clinical relevance. *J Allergy Clin Immunol* 2002; 110:435–42.