

Cloning of the minor allergen Api g 4 profilin from celery (*Apium graveolens*) and its cross-reactivity with birch pollen profilin Bet v 2

S. SCHEURER, A. WANGORSCH, D. HAUSTEIN and S. VIETHS

Paul Ehrlich Institute, Department of Allergology, Paul Ehrlich Street 51–59, D-63225 Langen, Germany

Summary

Background Profilin is a panallergen that is recognized by IgE from about 20% of birch pollen- and plant food-allergic patients. A subgroup of celery-allergic patients shows IgE-reactivity with this minor allergen. To investigate the IgE-binding potential and cross-reactivity of celery profilin at the molecular level, this study was aimed at the cloning and immunological characterization of this allergen.

Objectives Cloning, expression and purification of profilin from celery tuber to characterize its immunological properties and its cross-reactivity with birch pollen profilin.

Methods Cloning of celery profilin was performed by polymerase chain reaction using degenerated primers and a 5'RACE method for the identification of the unknown 5'-end of the cDNA. Expression was carried out in *Escherichia coli* BL21 (DE3) using a modified vector pET-30a. The recombinant profilin was purified by affinity chromatography on poly L-proline coupled to sepharose. Immunological characterization was performed by immunoblotting, EAST and IgE-inhibition experiments.

Results The coding region of the cDNA of celery profilin was identified as a 399-bp open reading frame, coding for a protein of 133 amino acids with a calculated molecular weight of 14.3 kDa. The deduced amino acid sequence of the corresponding protein showed high identity with other plant profilins (71–82%) recently described as allergens. Celery profilin was isolated as highly pure nonfusion protein. The IgE-reactivity of celery profilin was similar to that of natural protein. Seven of 17 celery-allergic patients tested presented specific IgE-antibodies to the recombinant protein tested by immunoblotting. Inhibition experiments showed high cross-reactivity of IgE with both profilins from celery and birch pollen. Moreover, the biological activity of recombinant celery profilin was demonstrated by a histamine release assay.

Conclusions Celery profilin is an important allergenic compound in celery and shows high homology to birch pollen profilin, Bet v 2. According to the revised IUIS allergen nomenclature, we suggest naming the celery profilin Api g 4. In addition to the cross-reacting major allergens Api g 1 and Bet v 1, birch pollinosis and associated allergies to celery can therefore additionally be explained by the cross-reactivity between homologous profilins. Moreover, recombinant Api g 4 may be used for target-specific diagnosis and structural analyses.

Keywords: Api g 4, Bet v 2, birch pollen, celery, cross-reactivity, food allergy, IgE, minor allergen, profilin.

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Introduction

Birch pollinosis and related food allergies to fresh fruits and vegetables, for example celery tuber, are a well-known phenomenon in allergy as reviewed elsewhere [1,2,3]. Celery belongs to the most frequent allergenic foods in Switzerland [4]. The oral allergy syndrome affected 87% of 30 pollen-allergic patients who reported immediate-type adverse reactions after ingestion of native celery tuber [5]. In addition, celery-allergic patients are less often to suffer from gastrointestinal symptoms, urticaria or bronchial asthma. Eighty per cent of 30 celery-allergic patients showed IgE against the major allergen of celery Api g 1; 23% were sensitized against profilin and another group showed IgE-specific for carbohydrate moieties. Api g 1, the Bet v 1-related major allergen from celery has been cloned and sequenced [6]. Api g 1, displaying 40% of amino acid sequence identity with the major birch pollen allergen Bet v 1 [7] and celery profilin [8] were identified as cross-reactive molecules leading to type I allergy to tree pollen and associated celery allergy [9]. Recently, two potential allergens from celery, Api g 2 (accession number, ACC: Z75662), an Api g 1 homologue molecule, and Api g 3 (ACC: Z75663), a chlorophyll-binding protein were described.

Profilins belong to an ubiquitous family of actin- and phosphatidylinositol 4,5-bisphosphate-binding proteins [10,11] involved in signal transduction from the outer cell membrane to the inner cell and regulating the actin polymerization in nonmuscle cells [12]. Profilin was identified as minor allergen in birch pollen, Bet v 2 [13]. Because of their ubiquitous occurrence, profilins were described as panallergens [14]. The presence of profilin-specific IgE-antibodies is a reason for cross-reactivity to botanically unrelated allergen sources. As an example, IgE against grass pollen profilin was shown to cross-react with profilins from a wide range of vegetable foods [15]. Recently, allergenic profilins were identified and sequenced in pollen from olive tree, Ole e 2 [16] timothy grass, Phl p 11 [17] bermuda grass, Cyn d 12 [18] sunflower, Hel a 2 [19] and various foods such as soybean, Gly m 3 (ACC: O65809) and peanut, Ara h 5 (ACC: AF059616).

In the present study, the complete amino acid sequence of celery profilin was derived from the cDNA sequence. The recombinant nonfusion protein was produced in *Escherichia coli* (*E. coli*), purified and tested with sera from celery-allergic patients. Moreover, the cross-reactivity between celery extract, recombinant celery profilin and Bet v 2 was investigated by dose-related inhibition experiments.

Materials and methods

Patients' sera

Sera from 13 patients allergic to celery were selected for

this study. All patients showed clinical symptoms after double-blind placebo-controlled food challenge (DBPCFC) with celery. Sera were kindly supplied by Drs B. Wüthrich and B. Ballmer-Weber (National University Hospital, Allergy unit, Zürich, Switzerland). An additional group of eight patients was selected by a positive (at least class 2) CAP or EAST (Enzyme Allergo Sorbent Test) to birch pollen profilin (Bet v 2) from our in-house serum collection. Most of them reported clinical symptoms to birch pollen and oral allergy syndrome after ingestion of various plant foods, especially fruits. Four out of eight patients were allergic to celery, and the other four patients of this group had an unknown clinical history after ingestion of celery.

Recombinant allergens and protein extracts

Recombinant birch pollen profilin Bet v 2 and major celery allergen rApi g 1 were obtained from BIOMAY, Linz, Austria. Extract from celery tuber (*apium graveolens*) was prepared by a low temperature method as described elsewhere [20].

RNA purification and first-strand cDNA synthesis

Celery tuber (strain 'Prinz') was ground to a fine powder under liquid nitrogen. Total RNA was extracted from 100 mg powder using the RNeasy Plant Mini Kit (Qiagen, Hilden, Germany). First strand cDNA was synthesized from 1.6 µg total RNA using a degenerated 5'-T₁₆NN-3' primer (Eurogentec, Searing, Belgium) and the Moloney murine leukaemia virus reverse transcriptase (First-strand cDNA synthesis Kit, Pharmacia, Freiburg, Germany) as recommended by the manufacturer. The reaction was performed at 37 °C for 60 min followed by heat denaturation at 99 °C for 5 min.

Polymerase chain reaction

For cDNA amplification, a degenerated oligonucleotide primer Prof5(deg) 5'-ATGTCGTGGCARRCGTACGT-3' (ARK, Darmstadt, Germany) was designed according to the known N-terminal amino acid sequence of different plant profilins. For the reverse direction the 3'-primer 5'-T₁₆NN-3' was applied. The first-strand cDNA amplification was carried out in a final volume of 100 µL, containing 0.1 µmol of dNTPs (Pharmacia, Freiburg, Germany), 20 pmol of each primer and of 2 µL of first-strand cDNA synthesis product as template. After denaturation at 94 °C for 1 min 2.5 U Taq DNA polymerase (Pharmacia, Freiburg, Germany) was added. The sample was subjected to 30 cycles of each 1 min denaturation at 94 °C, 1 min annealing at 52 °C and 1 min extension at 72 °C, and a final extension for 5 min at 72 °C.

5'RACE (Rapid amplification of 5' cDNA ends)

For amplification of the unknown 5'-end of the celery profilin the 5'RACE method was used according to the manufacturer's instructions (5'-RACE System, GIBCO BRL, Eggenstein, Germany). First-strand cDNA synthesis was primed with an antisense gene specific primer CE3[211–230] 5'-ACC ATGTACTTCGCTCCGCC-3'. A homopolymeric dC-tail was ligated to the 3'-end of the purified cDNA by a terminal desoxynucleotidyltransferase reaction. The dC-tailed cDNA was amplified by polymerase chain reaction (PCR) using a nested second gene-specific primer CE3[187–203] 5'-AATCCAGTAGGTGCAAG-3' and an 'abridged anchor primer' (enclosed in the 5'-RACE system).

Cloning and sequencing of celery profilin cDNA

The purified profilin cDNA was ligated into pGEM-T (pGEM-T Vector System I, Promega, Wisconsin, WI, USA) and transformed in the *E. coli* strain XL1-Blue (Stratagene, Heidelberg, Germany). Positive clones were selected by a blue/white screening and PCR screening with the T7 promoter primer 5'-TAATACGACTCACTATAGG-3' and the SP6 primer 5'-GATTTAGGTGACACTATAGA-3'. DNA sequence analyses of the subsequently isolated plasmids were performed with an ABI 373 or 377 'stretch' automatic fluorescent sequencer (Perkin Elmer, Weiterstadt, Germany).

Expression of recombinant profilin

For ligation into the pET-30a expression vector Nde I and Hind III, restriction sites were introduced at the 5'- and 3'-ends of the celery profilin cDNA by PCR using the primers PROF5Nde 5'-GGAATTCCATATGTCGTGGCAGGCGTACGG TG-3' and CE3Hind 5'-CCCAAGCTTGGGTTACAGGCCCTGATCAATAAGG-3'. To obtain a nonfusion protein the N-terminal His-tag sequence of pET-30a was deleted by the use of Nde I and Hind III restriction enzymes. Initial cloning was done in *E. coli* strain JS5 (BioRad, Munich, Germany). Positive clones were selected by analytical restriction cuts using the enzymes Nde I and Hind III, PCR screening with the vector-specific primer T7 promoter and the gene-specific primer PROF3Hind, and by sequencing. For target gene expression the plasmid was transformed into *E. coli* BL21(DE3) cells. The cells were grown in LB-medium supplemented with 50 µg/mL kanamycin to an absorbance of 0.7 at a wavelength of 600 nm. Isopropyl-D-thiogalactopyranoside (IPTG) was added to a final concentration of 1 mM, and incubation was continued overnight at 30 °C. Cells were harvested by centrifugation and stored at –70 °C.

Purification of celery profilin

The pellet was resuspended in 20 mL of lysis buffer (50 mM

Tris-HCl, 100 mM NaCl, pH 7.5) and disrupted by sonication or homogenization. Insoluble material was removed by centrifugation at 15900 g for 45 min. Proteins from 1 litre of supernatant were applied overnight at 4 °C to a poly L-proline (PLP) column. Briefly, the column was prepared by coupling 34 mg PLP (ICN Biochemicals, Aurora, OH, USA) to 1 g freeze dried powder of CNBr-activated Sepharose 4B (Pharmacia, Freiburg, Germany). Coupling of PLP was performed at 4 °C overnight in 0.1 M NaHCO₃, 0.5 M NaCl, pH 8.3. Thereafter the supernatant was incubated overnight at 4 °C with the prepared PLP-Sepharose. The column was washed with 50 mL of lysis buffer. Finally the profilin was eluted with 6 M urea in lysis buffer. Fractions containing protein were pooled, dialysed three times within 30 h against lysis buffer containing decreasing amounts of urea (4 M, 2 M, and without urea, respectively) and lyophilized. After reconstitution in water, determination of the protein concentration was performed according to the Bradford method (Pierce, Rockford, IL, USA) with bovine serum albumin as standard.

SDS-gel electrophoresis and immunoblotting

Proteins from *E. coli* lysate and purified recombinant profilin were analysed by sodium dodecylsulphate polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions according to Laemmli [21]. Proteins were visualized by Coomassie Brilliant Blue R250 staining or immunoblotting. For immunoblot analysis recombinant profilin (0.7 µg/cm slot) was electrophoretically transferred onto nitrocellulose membranes (0.45 µm, Schleicher and Schuell, Dassel, Germany) by tank blotting. Immunodetection of recombinant celery profilin was performed with a polyclonal rabbit antiserum raised against celery profilin [8] (1 : 10000, overnight). A biotin-labelled goat antirabbit immunoglobulin antibody (1 : 6000, 1 h) (DAKO, Glostrup, Denmark) was used as the secondary antibody followed by incubation with streptavidin-conjugated alkaline phosphatase (1 : 3000, 1 h) (Medac, Hamburg, Germany). IgE-binding of patients' sera (1 : 6.7, overnight) was detected with an alkaline phosphatase (AP)-labelled mouse antihuman IgE monoclonal antibody (1 : 1000, 4 h) (Pharmingen, Hamburg, Germany). Antibody binding was visualized using the 'AP Conjugate Substrate Kit' (BioRad, Munich, Germany).

Immunoblot inhibition

For immunoblot inhibition of IgE-binding to recombinant celery profilin, 50 µL of patient serum was pre-incubated with 15 µg of rBet v 2, 100 µg of protein from celery extract and buffer as control for 2 h. Thereafter, samples were diluted to 1 mL and added to blot strips (3 mm width) of Api g 4. Bound IgE was detected with a rabbit antihuman

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ATGTCGTGGCAGGCGTACGTTGACGATCATCTTATGTGCGAGGTTGAAGGCAATCCAGGT 60 bp
M S W Q A Y V D D H L M C E V E G N P G 20 AA

CAAACCTAAGTCCCGCTATTATCGGCCACGACGGCAGCGTTGGGCTCAGAGCTCC 120
Q T L T A A A I I G H D G S V W A Q S S 40

ACTTTTCCTCAGATTAAGCCTGAAGAGATTGCTGGTATTATGAAAGACTTCGACGAGCCT 180
T F P Q I K P E E I A G I M K D F D E P 60

GGCCACCTTGCACCTACTGGATTGACCTTGGCGGAGCGAAGTACATGGTTATTCAGGA 240
G H L A P T G L Y L G G A K Y M V I Q G 80

GAGCCTAATGCTGTTATTCTGGCAAGAAGGGCTCTGGAGGTGTGACCATAAAGAGACG 300
E P N A V I R G K K G S G G V T I K K T 100

GGCCAAGCTCTAGTTTTCGGAGTCTATGATGAACCTGTACTCCAGGACAATGCAACGTG 360
G Q A L V F G V Y D E P V T P G Q C N V 120

ATTGTTGAGAGGCTGGGGATTACCTTATTGATCAGGGCTGTAA 402
I V E R L G D Y L I D Q G L * 134

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Fig. 1. Nucleotide sequence and deduced amino acid sequence of Api g 4 coding region. Numbers indicate amino acid residues (AA) and nucleotide positions (bp). The sequence of the degenerate 5'-primer is printed in bold letters. The nucleotide sequence reported has been submitted to GenBank/NCBI Databases under accession number AF129423.

IgE antiserum (1 : 4000, 1 h) (DAKO, Glostrup, Denmark), followed by a biotin-labelled goat antirabbit immunoglobulin antibody (1 : 6000, 1 h) (DAKO, Glostrup, Denmark) as the secondary antibody and streptavidin-labelled with alkaline phosphatase (1 : 3000, 1 h) (Medac, Hamburg, Germany). Visualization was accomplished as described above. For immunoblot inhibition of IgE-binding to natural celery profilin celery extract (20 µg of protein/cm of slot) was electrophoretically separated and transferred onto CNBr-activated NC (Dr W.-M. Becker, Forschungszentrum Borstel, Germany) by semidry blotting [22]. Patient serum was pre-incubated with 15 µg rApi g 4 for 3 h at room temperature. Thereafter samples (1 : 6.7 dilution) were incubated overnight with NC and immunodetection was performed by the AP-labelled antibody as described above.

Enzyme allergosorbent test inhibition

For determination of the IgE-binding capacity the allergens rApi g 1, rApi g 4 and rBet v 2, and celery extract were coupled to cyanogen bromide-activated filter paper disks (Hycor, Kassel, Germany) at a protein concentration of 3 µg/mL (for recombinant proteins) corresponding to 0.5 µg of protein per disk or at a protein concentration of 30 µg/mL (for celery extract) corresponding to 5 µg of protein per disk as previously described by Ceska and Lundkvist [23]. For Enzyme Allergosorbent Test (EAST)-inhibition a pooled patient serum (1 : 2.5 dilution) was pre-incubated with serial dilutions (10^2 – 10^{-3} µg/mL) of rApi g

1, rApi g 4, rBet v 2 and celery extract (protein content up to 10^3 µg/mL) for 15 min at room temperature. The EAST was performed with the 'Allergopharma Spez. IgE ELISA' (Allergopharma, Reinbek, Germany) according to the manufacturer's instructions with the exception that both antibody incubations were performed overnight. The average inhibition values were calculated from double determinations in relation to the uninhibited control.

Basophil histamine release

The assay was carried out on whole blood samples as described by Siraganian [24,25] and Siraganian and Brodsky [26], respectively.

Results

Cloning of celery profilin cDNA and sequence analysis

PCR amplification of celery cDNA using the degenerate 5'-primer Prof5(deg) and the 3'-primer T₁₆NN-3' resulted in a dominant fragment of approximately 700 bp. DNA sequencing revealed a 399-bp open reading frame coding for a protein of 133 amino acids (Fig. 1). The unknown 3'-end of the cDNA, obtained by the use of the degenerate primer was analysed by 5'RACE. The PCR resulted in a fragment of approximately 300 bp length with an overlap of 203 bp with the coding region of celery profilin. The complete deduced amino acid sequence comprised a protein with a calculated molecular weight of 14.3 kDa and a calculated pI of 4.54. The sequence analysis by PROSITE search [27] revealed two potential phosphorylation sites K₈₈KGS and T₉₅IK, a potential amidation site R₈₆GKK and a profilin consensus sequence S₁WQAYVD.

Comparison of the Api g 4 amino acid sequence with the SWALL Protein Database showed high identities (71–82%) with other plant profilins (Table 1). Sequence identity of 79.9% was obtained with Bet v 2 from birch pollen. Much lower identities of maximal 46% (slime mold, ACC: P26199) were found with nonplant profilins (not shown). Moreover, the sequence identity with human profilin (ACC: P35080), which has been described to cross-react with IgE against Bet v 2 is 33%. The data of eight profilins, all isolated from pollen, except for Gly m 3 from plant food and recently described as allergens according the allergen nomenclature list of the IUIS are shown in Table 1. Moreover, the size of all analysed profilins ranged from 131 to 134 amino acid residues.

Expression and purification of Api g 4 protein

Api g 4 was expressed as nonfusion protein in *E. coli* BL21 (DE3) after overnight induction with IPTG at 30 °C using a

Table 1. Amino acid identity (AA-ID) comparison of Api g 4 with profilins from different plants and plant foods. All profilins described as allergens according the IUIS-nomenclature are included in the table

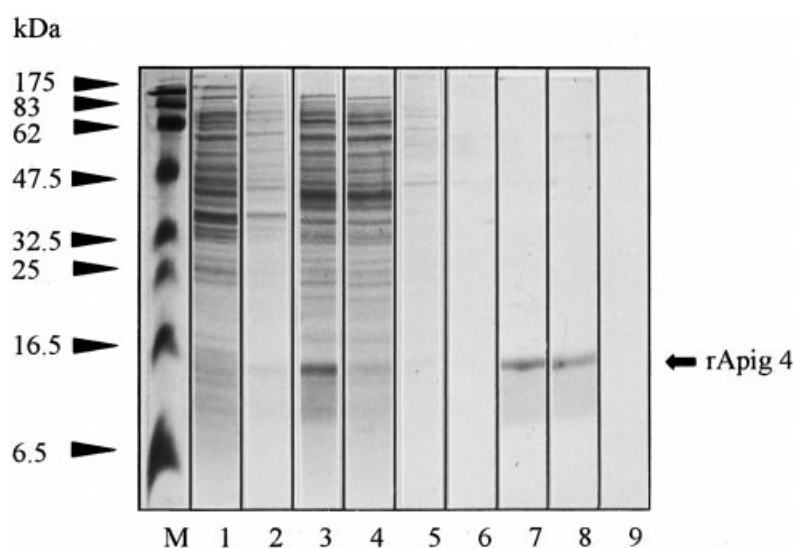
Profilin source	Gene	Allergen	AC	AA(<i>n</i>)	AA-ID(%)
Olive tree pollen	OLEEU	Ole e 2	O24170	134	82.1
Birch pollen	BETVE	Bet v 2	P25816	133	79.9
Mouse ear cress	ARATH		Q38905	134	79.9
Mercury pollen	MERAN	Mer a 1	O49894	132	79.7
Tobacco pollen	TOBAC		P41372	134	79.7
Bermuda grass pollen	CYNDA	Cyn d 12	O04725	131	78.4
Soybean	SOYBN	Gly m 3	O65809	131	78.2
Wheat	WHEAT		P49233	141	78.2
Timothy grass pollen	PHLPR	Phl p 11	O24650	131	76.9
Maize	MAIZE		O22655	131	76.9
Rubber tree pollen	HEVBR	Hev b 8	O65812	131	76.1
Tomato pollen	LYCES		Q41344	133	75.9
Sunflower pollen	O81982	Hel a 2	O81982	133	71.4

modified pET-30a vector. Most of the target protein was recovered as soluble material in the supernatant that was further purified on PLP-coupled Sepharose. After elution with 6 M urea, Api g 4 with an apparent molecular mass of 14 kDa was isolated with high degree of purity. Purification of the celery profilin was controlled by Coomassie Blue stained SDS-PAGE (Fig. 2). Approximately 1 mg of recombinant protein was purified from 1 L of bacterial culture.

Immunological characterization of recombinant Api g 4

IgE-reactivity to rApi g 4 was investigated by immunoblotting experiments (Fig. 3). Sera from 21 patients were selected for immunoblotting studies. Celery allergy of 13

patients (9840146, 98060710, 98060711, 98060712, 98070013, 98070752, 98070884, 98070932, 98080928, 98090451, 98091114, 98100149, 98110146) was confirmed by DBPCFC and an additional eight sera from patients (PEI39, PEI44, PEI61, PEI76, PEI99, PEI101, PEI127, PEI131) with IgE-reactivity to rBet v 2 were selected. All of the preselected patients were suffering from pollinosis and most of them, except for PEI39 and PEI44, reported oral symptoms after ingestion of various fruits. Patients PEI61, PEI76, PEI127 and PEI131 had a clear clinical history of celery allergy. Serum from a nonallergic patient PEI22, a rabbit serum directed against celery profilin, and a rabbit normal serum were used as controls. IgE from 10 sera [10/21] tested bound to rApi g 4. Three of 13 (23%)

**Fig. 2.** Purification of recombinant Api g 4 expressed in *E. coli*. Coomassie stained SDS-PAGE of *E. coli*-lysate before (1) and after IPTG addition (2), soluble fraction (3), PLP-sepharose column-nonretained fraction (4), wash (5) and elution fraction 1–4 (6–9).

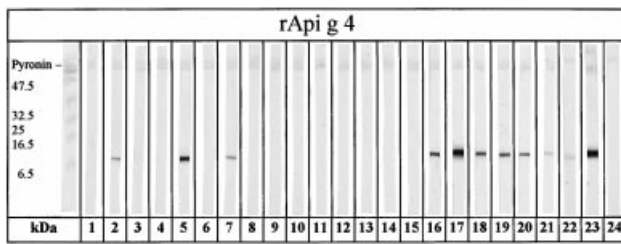


Fig. 3. IgE-immunoblot of purified rApi g 4 with sera from patients with confirmed allergy to celery (2–14: 9840146, 98060710, 98060711, 98060712, 98070013, 98070752, 98070884, 98070932, 98080928, 98090451, 98091114, 98100149, 98110146), an additional group of allergic patients with IgE-reactivity to birch pollen profilin (15–22: PEI39, PEI44, PEI61, PEI76, PEI99, PEI101, PEI127, PEI131), a control serum from a nonallergic donor (1), rabbit anticelery profilin serum (23) and a rabbit normal serum as control (24).

DBPCFC-positive patients exhibited IgE to recombinant celery profilin. Moreover, all patients preselected by IgE-binding to Bet v 2, except for patient PEI39 showed IgE-reactivity to rApi g 4. The serum of the nonallergic volunteer PEI22 presented no specific IgE to the recombinant molecule.

Inhibition experiments

IgE-immunoblot inhibition experiments were performed to investigate the cross-reactivity of IgE with rApi g 4 and with rBet v 2, and to compare the IgE-reactivity of the recombinant and the natural celery profilin (Figs 4 and 5). For IgE-immunoblot inhibition tests, all patients' sera with reactivity to rApi g 4, except for PEI131 were selected. IgE-binding to blotted rApi g 4 was completely inhibited after pre-incubation with celery extract (Fig. 4a). A clear, but only partially reduced IgE-binding was observed for most patients' sera tested after pre-incubation with rBet v 2, except for the sera 9807052 and PEI127, which showed a nearly complete inhibition of IgE-binding (Fig. 4b) in comparison to the uninhibited control (Fig. 4c). IgE-inhibition of natural celery profilin from celery extract was performed by pre-incubation of a pooled serum (consisting of all sera with reactivity to rApi g 4), two additional serum samples, PEI61 and PEI131 and serum from a nonallergic subject (not shown) with rApi g 4 (Fig. 5). Recombinant celery profilin showed the same IgE-reactivity as the natural homologue, demonstrated by a complete IgE-inhibition to the natural profilin at the solid phase when recombinant celery profilin was used as inhibitor. IgE-binding to the natural profilin was unaffected for PEI61 and appeared to be slightly reduced in case of the pooled serum. The IgE-reactivity with proteins with molecular masses greater than 30 kDa was not

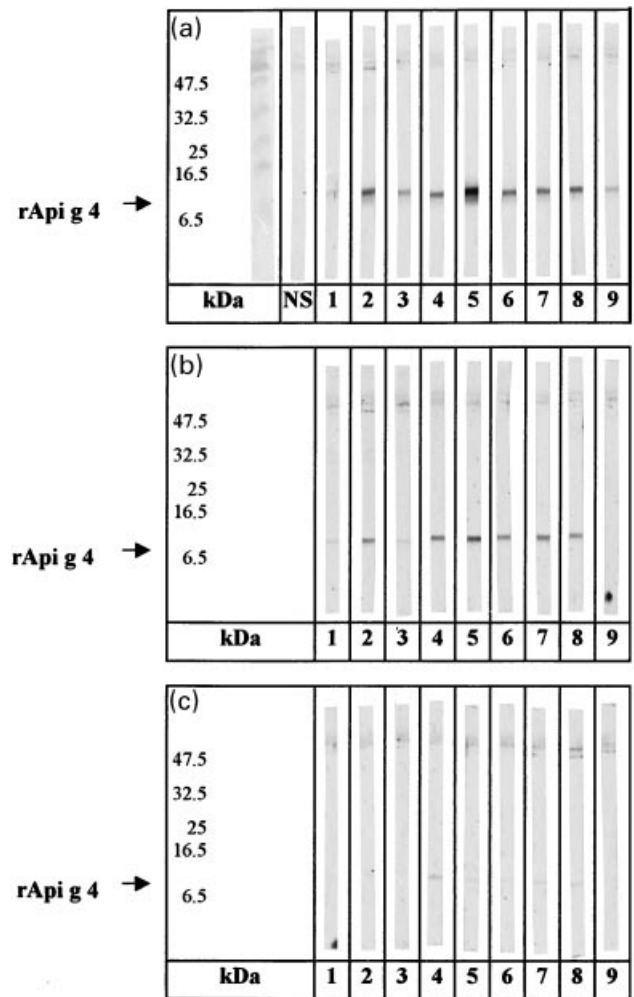


Fig. 4. IgE-immunoblot inhibition. Inhibition of IgE-binding to rApi g 4 after pre-incubation of nine patient sera [98040146 (1), 98060712 (2), 98070752 (3), PEI44 (4), PEI61 (5), PEI76 (6), PEI99 (7), PEI101 (8), PEI127 (9)] with (a) buffer as control; (b) 15 µg rBet v 2; (c) 100 µg celery protein extract and for negative control, a serum from a nonallergic donor (NS) was used.

reduced by rApi g 4 as inhibitor. Patient PEI131, with a known mono-sensitization to celery profilin, showed no IgE-reactivity with the major celery allergen.

Dose-dependent IgE-inhibition experiments were performed by EAST, to investigate the cross-reactivity between the celery allergens and Bet v 2 in more detail (Fig. 6). Inhibition of IgE-binding to celery extract (Fig. 6a) was performed after pre-incubation of a patient serum (PEI61) with celery extract, rApi g 1, rApi g 4 and a mix of equal amounts of rApi g 1 and rApi g 4, respectively. Inhibition of IgE-binding to rBet v 2 (Fig. 6b) and rApi g 4 (Fig. 6c) was performed after pre-incubation of a patients' serum pool (PEI61 and PEI131) with celery extract, rApi g 4 and rBet v 2. A complete inhibition of IgE-binding to celery extract

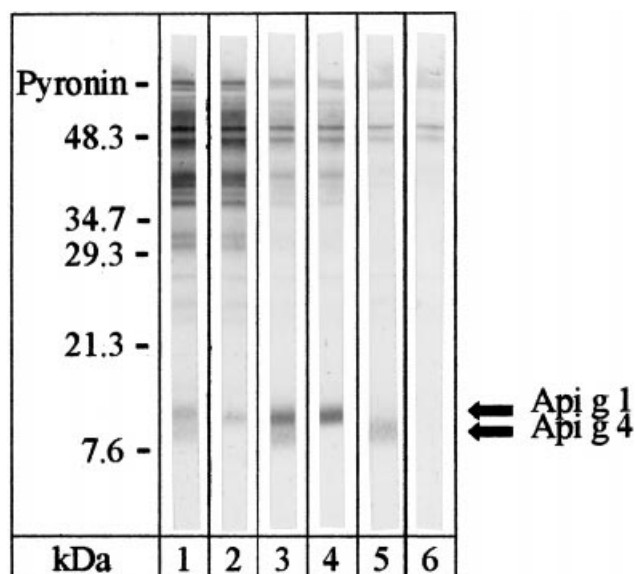


Fig. 5. IgE-immunoblot inhibition. Inhibition of IgE-binding to natural profilin in celery extract after pre-incubation of patient serum with 15 μ g rApi g 4. Pooled patient serum without (1) and with inhibitor (2), PEI61 without (3) and with inhibitor (4), PEI131 without (5) and with inhibitor (6).

was obtained by self-inhibition with celery extract (500 μ g/mL). Moreover, a maximal reduction of 40% and 50% of IgE-binding to celery extract was obtained using rApi g 4 and rApi g 1 as inhibitors, respectively. Pre-incubation of the serum with the mixture of both celery allergens (100 μ g/mL each) resulted in a maximal inhibition of IgE binding of 80% (Fig. 6a). Complete inhibition of IgE-binding to rBet v 2 was observed with rBet v 2 (10 μ g/mL), whereas 80% and 87% of reduction of antibody reaction were obtained with rApi g 4 and celery extract at the maximal inhibitor concentration of 100 μ g/mL for rApi g 4 and 1000 μ g/mL of protein for the extract (Fig. 6b). Moreover, self-inhibition with rApi g 4 resulted in an almost complete reduction of IgE-reactivity to rApi g 4 at the solid phase. Approximately 85% of IgE-inhibition to Api g 4 disks was obtained after pre-incubation with the highest concentration of celery extract (1000 μ g/mL) as inhibitor, whereas a maximal reduction of 80% was measured after pre-incubation with rBet v 2 (10 μ g/mL) (Fig. 6c).

Histamine release

To prove the allergenic property of rApi g 4 in the human system, basophils from patient PEI61 with strong IgE-binding to profilins from celery and birch pollen were triggered with rApi g 4 and rBet v 2 to induce mediator release. Patient PEI61 showed a maximal histamine release of about 70% for rApi g 4 and about 50% for rBet v 2 at an

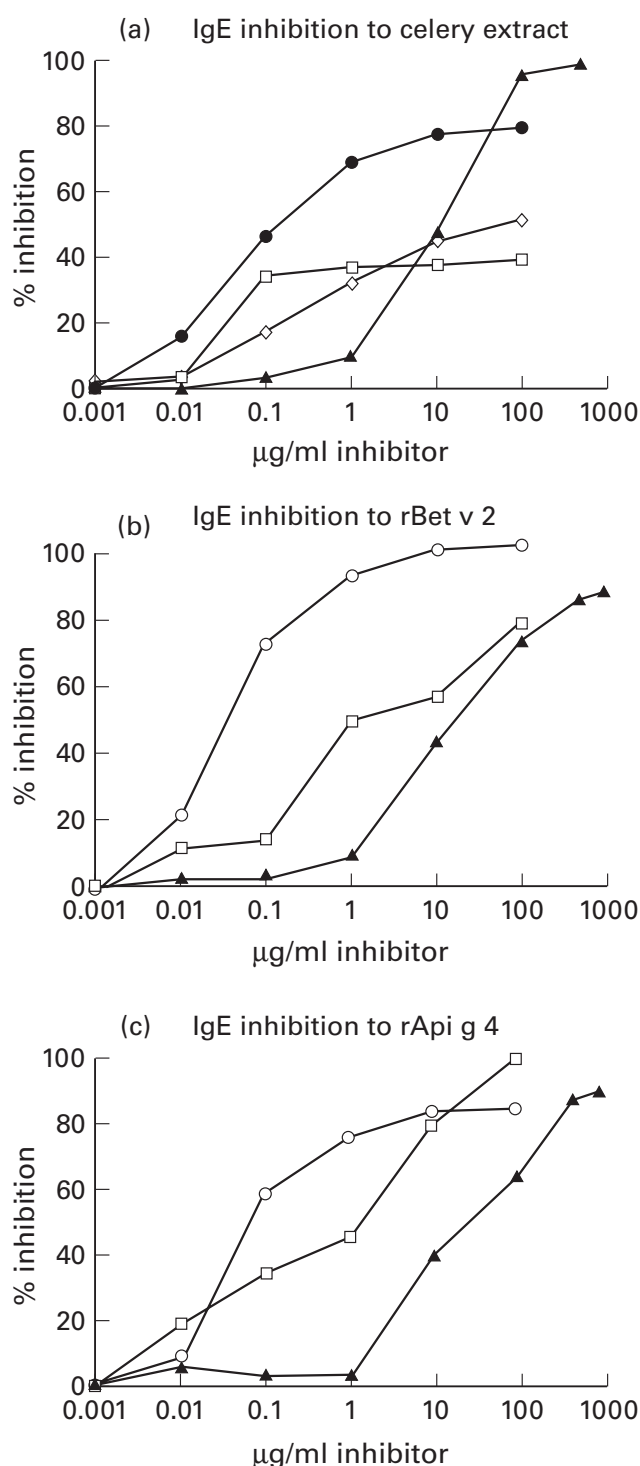


Fig. 6. IgE-EAST inhibition. Inhibition of IgE-binding to celery extract; (a) after pre-incubation of a patient sera with celery extract, rApi g 1, rApi g 4 and a mix of equimolar amounts rApi g 1 and rApi g 4. Inhibition of IgE-binding of a pooled patient serum to (b) rBet v 2 and (c) rApi g 4, after pre-incubation with celery extract, rApi g 4 and rBet v 2. (◇) rApi g 1; (□) rApi g 4; (●) rApi g 1/rApi g 4; (▲) celery extract; (○) rBet v 2.

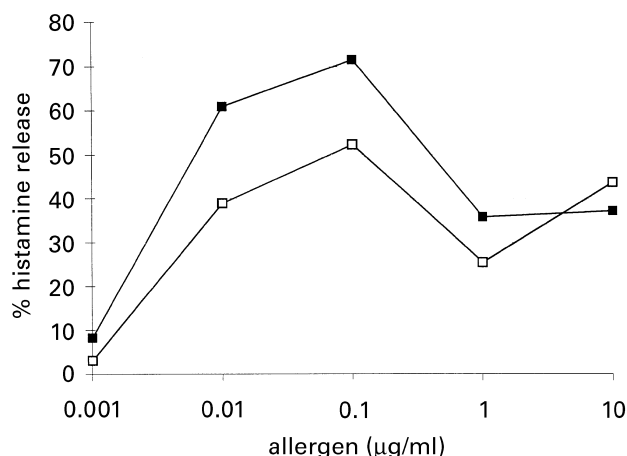


Fig. 7. Basophil histamine release assay with whole blood from patient PEI61 after stimulation with rApi g 4 (■) and rBet v 2 (□).

allergen concentration of 0.1 µg/mL for both allergens tested (Fig. 7). A control experiment was performed with a blood sample from a celery allergic patient without sensitization to profilins, where no mediator release was measured (not shown).

Discussion

Celery profilin Api g 4 was cloned by PCR strategy using a degenerated primer based on the codons of the highly conserved N-terminal amino acids of the profilin family. The primer sequence was confirmed by a subsequent 5'RACE. The coding region of the Api g 4 cDNA comprises 399 bp with a length of 133 amino acids and a calculated molecular weight of 14.3 kDa, correlating with the molecular characteristics to the known plant profilins. Celery profilin belongs to the conserved family of plant profilins, which was confirmed by high sequence identities (71–82%) to known allergenic plant profilins. The existence of profilins with different apparent molecular weights in the same plant source reported by some authors [8,17,28] may be explained by potential post-translational modification sites, as for phosphorylation and for amidation, present in the Api g 4 sequence.

Only weak protein expression in comparison to the noninduced cells was observed in *E. coli* by Coomassie Blue stained SDS-PAGE. A higher expression level, resulting in up to 3 mg of recombinant protein was obtained when protein synthesis was performed at 37 °C overnight. Highly purified Api g 4 was chromatographically isolated from the soluble fraction as a nonfusion protein according a modified method described by Lindberg [29].

Immunological characterization showed 10/21 (48%) of allergic patients' sera with a specific IgE-reactivity to recombinant Api g 4. This frequency of IgE-reaction is not representative for the average celery-allergic population, since a

subgroup of patients' sera were selected by a positive IgE-reaction to rBet v 2. Moreover, no data about a history of celery allergy were available for four of these patients. In contrast to an IgE prevalence of 23% (3/13) in the DBPCFC-positive patient group, 7/8 of the preselected patients showed IgE-binding to rApi g 4. In concordance with the known sensitization frequency of 23% of celery allergic patients against profilin [5] celery profilin was classified as minor allergen and named Api g 4 according to the revised allergen nomenclature [30]. Moreover, the frequency of IgE-reactivity to profilin has been considered to be 20% of all patients allergic to pollen [14]. Recent results indicate that profilin sensitization within the group of birch pollen allergic patients in Italy (38%) is more important than in Sweden (7%) [31]. Moreover, profilin from *Mercurialis annua*, Mer a 1, is the first profilin described as major allergen [32].

Profilins were described as panallergens in botanical-related [33] and -unrelated plants [8,15]. Cross-reaction of IgE against Bet v 2 with these homologue molecules are one explanation for birch pollen-associated food allergies and the birch–mugwort–celery syndrome [34,35,36]. In this study, the cross-reaction of purified rApi g 4 with natural Api g 4 and rBet v 2 was investigated by IgE-inhibition experiments. The results clearly indicated that recombinant Api g 4 comprises IgE-epitopes of its natural homologue. IgE-immunoblotting revealed a complete inhibition of IgE-binding to rApi g 4 after pre-incubation with celery extract. Moreover, the blotting experiments showed a complete inhibition of IgE-binding to natural celery profilin after pre-incubation with the recombinant molecule, whereas IgE EAST experiments showed an inhibition of 85% by celery extract at the maximal protein concentration of 1000 µg/mL. In this case the incomplete IgE-inhibition to rApi g 4 may be explained by the low profilin content in the allergen extract. Celery profilin is capable of binding a high proportion of celery-specific IgE, as indicated by an IgE-inhibition of 40% to celery extract for patient PEI61. Api g 4 shares IgE-epitopes with rBet v 2, as demonstrated by a positive reaction to Api g 4 in 7/8 sera from preselected patients with an IgE-binding to rBet v 2, by IgE-immunoblot inhibition and, in more detail, by dose-dependent IgE-inhibition in EAST. Pre-incubation with birch profilin resulted in a nearby 80% inhibition of IgE-binding to celery profilin at the solid phase. Therefore slight differences in the IgE-epitopes may be present on the two molecules. This view is also supported by the observation that IgE from one Bet v 2-positive patient (PEI39) did not react with Api g 4. On the other hand the existence of further isoforms with different IgE-reactivities may explain this effect. The occurrence of differences in the IgE-epitopes of plant profilins of the same taxonomically family was reported recently [32]. The allergenic properties of Api g

4 and the birch pollen profilin Bet v 2 were compared by a histamine release assay. For both profilins a maximal mediator release was measured at identical concentrations, indicating a similar allergenic potency. However, the reactivity of the cells from this patient was somewhat higher when Api g 4 was used for cross-linking. The observed strong overall cross-reactivity between plant profilins [37] and a partial cross-reactivity with mammalian profilin [13] is most probably based on the common tertiary structure [38]. Secondary structure prediction of Api g 4 and Bet v 2 using the program 'predict protein' revealed almost identical structural elements (not shown), which were in accordance with the experimentally-defined crystal structure of Bet v 2 [39,40]. The predicted structure of Api g 4 comprises three α -helices and seven β -strands suggesting an identical tertiary structure shared with Bet v 2.

In conclusion the recombinant celery profilin Api g 4 can serve as a tool for more accurate diagnostic measures and further immunological characterization of the cross-reactivity between plant profilins. Investigation of the tertiary structure may be helpful to identify cross-reactive epitopes.

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References

- Caballero T, Martin-Esteban M. Association between pollen hypersensitivity and edible vegetable allergy: a review. *Invest Allergol Clin Immunol* 1998; 1:6–16.
- Jankiewicz A, Aulepp H, Baltes W, Bögl KW, Dehne LI, Zuberbier T, Vieths S. Allergic sensitization to native and celery root in pollen-sensitive patients investigated by skin test and IgE-binding. *Int Arch Allergy Immunol* 1996; 111:268–78.
- Bircher AJ, Van Melle G, Haller E, Curty B, Frei PC. IgE to food allergens are highly prevalent in patients allergic to pollen, with and without symptoms of food allergy. *Clin Exp Allergy* 1994; 24:367–74.
- Wüthrich B. Zur Nahrungsmittelallergie: Häufigkeit der Symptome und der allergieauslösenden Nahrungsmitteln bei 402 Patienten – Kuhmilchallergie und Neurodermitis atopica. *Allergologie* 1993; 16:280–7.
- Jankiewicz A, Aulepp H, Altmann F, Fötisch K, Vieths S. Serological investigation of 30 celery-allergic patients with particular consideration of the thermal stability of IgE-binding celery allergens. *Allergo J* 1998; 7:87–95.
- Breiteneder H, Hoffmann-Sommergruber K, O'Riagain G, Susani M, Ahorn H, Ebner C, Kraft D, Scheiner O. Molecular characterization of Api g 1, the major allergen of celery (*Apium graveolens*), and its immunological and structural relationships to a group of 17-kDa tree pollen allergens. *Eur J Biochem* 1995; 233:484–9.
- 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 1 is highly homologous to a pea disease resistance response gene. *EMBO J* 1989; 8:1935–8.
- Vallier P, Dechamp C, Valenta R, Vials 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; 22:774–82.
- Ebner C, Hirschwehr R, Bauer L, Breiteneder H, Valenta R, Ebner H, Kraft D, Scheiner O. 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.
- Goldschmidt-Clermont PJ, Machesky LM, Baldassare JJ, Pollard TD. The actin-binding profilin binds to PIP₂ and inhibits its hydrolysis by phospholipase C. *Science* 1990; 247:1575–7.
- Goldschmidt-Clermont PJ, Kim JW, Machesky LM, Rhee SG, Pollard TD. Regulation of phospholipase C- γ 1 by profilin and tyrosine phosphorylation. *Science* 1991; 25:1231–3.
- Machesky LM, Pollard TD. Profilin as a potential mediator of membrane-cytoskeleton communication. *Trends Cell Biology* 1993; 3:381–5.
- Valenta R, Duchene M, Pettenburger K, Sillaber C, Valent P, Bettelheim P, Breitenbach M, Rumpold H, Kraft D, Scheiner O. Identification of profilin as a novel pollen allergen; IgE auto-reactivity in sensitized individuals. *Science* 1991; 253:557–60.
- Valenta R, Duchene M, Ebner C, Valent P, Sillaber C, Deviller P, Ferreira F, Tejkl M, Edelmann H, Kraft D, Scheiner O. Profilins constitute a novel family of functional plant pan-allergens. *J Exp Med* 1992; 175:377–85.
- 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.
- Asturias JA, Arilla MC, Gomez-Bayon N, Martinez J, Martinez A, Palacios R. Cloning and expression of the panallergen profilin and the major allergen (Ole e 1) from olive tree pollen. *J Allergy Clin Immunol* 1997; 100:365–72.
- 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). *Biochem Biophys Acta* 1997; 1352:253–7.
- Asturias JA, Arilla MC, Gomez-Bayon N, Martinez J, Martinez A, Palacios R. Cloning and high level expression of *Cynodon dactylon* (Bermuda grass) pollen profilin (Cyn d 12) in *Escherichia coli*: purification and characterization of the allergen. *Clin Exp Allergy* 1997; 27:1307–13.
- Asturias JA, Arilla MC, Gomez-Bayon N, Aguirre M, Martinez A, Palacios R, Martinez J. Cloning and immunological characterization of the allergen Hel a 2 (profilin) from sunflower pollen. *Mol Immunol* 1998; 35:469–78.

- 20 Vieths S, Schöning B, Petersen A. Characterization of the 18-kDa apple allergen by two-dimensional immunoblotting and microsequencing. *Int Arch Allergy Immun* 1994; 104: 399–404.
- 21 Laemmli UK. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* 1970; 227:680–5.
- 22 Khyse-Andersen J. Electrophoretic transfer of proteins from polyacrylamide to nitrocellulose. *J Biochem Biophys Meth* 1984; 10:203–9.
- 23 Ceska M, Lundkvist U. A new and simple radioimmunoassay method for the determination of IgE. *Immunochemistry* 1972; 9:1021–30.
- 24 Siraganian RP. An automated continuous-flow system for the extraction and fluorometric analysis of histamine. *Anal Biochem* 1974; 57:383–94.
- 25 Siraganian RP. Refinements in the automated histamine analysis system. *J Immunol Meth* 1975; 7:283–90.
- 26 Siraganian RP, Brodsky MJ. Automated histamine analysis for in vitro allergy testing. I. A method utilizing allergen-induced histamine release from whole blood. *J Allergy Clin Immunol* 1976; 57:525–40.
- 27 Bairoch A, Bucher P, Hofmann K. The PROSITE database, in status 1997. *Nucl Acids Res* 1997; 25:217–21.
- 28 Vieths S, Jankiewicz A, Wüthrich B, Baltes W. Immunoblot study of IgE binding allergens in celery roots. *Ann Allergy, Asthma Immunol* 1995; 74:48–55.
- 29 Lindberg U, Schutt CE, Hellsten E, Tjäder AC, Hult T. The use of poly L-proline-sepharose in the isolation of profilin and profilactin complexes. *Biochimica Biophysica Acta* 1988; 967:391–400.
- 30 King TP, Hofmann D, Loewenstein H, Marsh DG, Platt-Mills TAE, Thomas W. Allergen nomenclature. *Int Arch Allergy Immunol* 1994; 105:224–33.
- 31 Elfman L, Svensson M, Lindholm J, Pauli G, Valenta R. Different profiles in specific IgE to rBet v 1 and rBet v 2 in patients allergic to birch pollen from six countries. *Int Arch Allergy Immunol* 1997; 113:249–51.
- 32 Vallverdu A, Asturias JA, Arilla C, Gomez-Bayon N, Martinez A, Martinez J, Palacios R. Characterization of recombinant *Mercurialis annua* allergen Mer a 1 (profilin). *J Allergy Clin Immunol* 1998; 101 (3):363–70.
- 33 Van Ree R, Fernandez-Rivas M, Cuevas M, Van Wijngaarden M, Aalberse RC. Pollen-related allergy to peach and apple: an important role for profilin. *J Allergy Clin Immunol* 1995; 95:726–34.
- 34 Jensen-Jarolim E, Santer B, Leitner A, Grimm R, Scheiner O, Ebner C, Breiteneder H. Bell peppers (*Capsicum annuum*) express allergens (profilin, pathogenesis-related protein P23 and Bet v 1) depending on the horticultural strain. *Int Arch Allergy Immunol* 1998; 116:103–9.
- 35 Bauer L, Ebner C, Hirschwehr R, Wüthrich B, Pichler C, Fritsch R, Scheiner O, Kraft D. IgE-cross-reactivity between birch pollen, mugwort pollen and celery is due to at least three distinct cross-reacting allergens. Immunoblot investigations of the birch–mugwort–celery-syndrome. *Clin Exp Allergy* 1996; 26:1161–70.
- 36 Wüthrich B, Stäger J, Johansson SGO. Celery allergy associated with birch and mugwort pollinosis. *Allergy* 1990; 45:566–71.
- 37 Vrtala S, Wiedemann P, Mittermann I, Eichler HG, Sperr WR, Valent P, Kraft D, Valenta R. High-level expression in *Escherichia coli* and purification of recombinant plant profilins: comparison of IgE-binding capacity and allergenic activity. *Biochem Biophys Res Commun* 1996; 226 (1):42–50.
- 38 Thorn KS, Christensen HEM, Shigetani R, Huddler D, Shalaby L, Lindberg U, Chua N-H, Schutt CE. The crystal structure of the major allergens from plants. *Structure* 1997; 5 (1):19–32.
- 39 Federov 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.
- 40 Federov AA, Ball T, Valenta R, Almo SC. X-ray crystal structures of birch pollen profilin and Phl p 2. *Int Arch Allergy Immunol* 1997; 113:109–13.