

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*)

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Key Words

Tomato profilin · Bell pepper profilin · Allergen · cDNA cloning · Purification · Non-fusion protein · IgE binding

Abstract

Background: Profilins are recognised by IgE of about 20% of patients allergic to birch pollen and plant foods. They are ubiquitous intracellular proteins highly cross-reactive among plant species. Therefore, they were called panallergens and are made responsible for cross-sensitisation between plant pollen and food. **Objectives:** The aim of the present study was to clone the cDNAs encoding profilins from bell pepper and tomato, to produce and purify the recombinant proteins and to compare their IgE-binding capacities to those of the natural proteins. **Methods:** cDNA clones coding for profilin were obtained by RT-PCR from total RNA of tomato and bell

pepper fruits, sequenced and expressed as non-fusion proteins in *Escherichia coli*. The recombinant profilins were subsequently purified and tested for IgE-binding and inhibition capacity with sera from 34 food-allergic patients. Possible oligomerisation of recombinant profilins was investigated by HPLC analysis and its influence on IgE binding assayed by ELISA. **Results:** The open reading frame from both profilins encompasses 393 bp with a predicted molecular mass of 14,184 kD and a pI of 4.44 for bell pepper profilin (Cap a 2) and 14,257 kD and a pI of 4.46 for the profilin from tomato (Lyc e 1). The two protein sequences display 91% identity, whereas tomato profilin from pollen shares only 75% identity with tomato fruit profilin. Eleven out of 34 food-allergic patients (32%) display IgE binding to both purified profilins. Preincubation of a serum pool with either purified rCap a 2 or rLyc e 1 nearly abolished IgE binding to natural Cap a 2 and Lyc e 1, respectively. In addition, purified recombinant Cap a 2 was able to inhibit IgE-binding to rLyc e 1 by approximately 50%, whereas rLyc e 1 completely blocked IgE-binding to rCap a 2 in cross-inhibition assays. HPLC analysis showed that in solution Cap a 2 and Lyc e 1 can be found predominantly as dimers, which can

M.W. and H.F. contributed equally. The complete cDNA sequences coding for the bell pepper and tomato profilin are available from the EMBL/GenBank/DBJ database under the accession numbers AJ417552 for bell pepper profilin and AJ417553 for tomato profilin.

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be partially reduced to monomers by addition of dithiothreitol (DTT). In ELISA DTT-treated Lyc e 1 displayed a clearly lower IgE-binding capacity than untreated profilin. **Conclusions:** Purified rCap a 2 and rLyc e 1 proved to be valuable tools for studying cross-reactivity to profilins in patients allergic to pollen and food.

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Introduction

Type 1 allergic reactions to food in atopic humans range from mild symptoms such as oral allergy syndrome to life-threatening anaphylactic reactions [1]. Especially, pollen-allergic patients frequently display allergic symptoms against plant-derived food, like fruits, vegetables and spices. Profilins are a family of ubiquitous cytosolic proteins that control actin polymerisation and interact with poly-*L*-proline, proline-rich proteins and membrane polyphosphoinositides, with an important role in cell signalling and morphological processes [2, 3].

Profilins are recognised by IgE of about 20% of birch pollen- and plant food-allergic patients. Due to their high conservation among plant species, profilins are well known to be cross-reactive allergens not only in different pollen but also in vegetable food extracts [4] and were therefore termed panallergens [5]. They are responsible for cross-reactivities found between fruit, pollen and latex [6], in the birch-mugwort-celery syndrome [7–9] or among spices [10]. Profilins were postulated to be partly responsible for IgE cross-reactivities between tomato fruit or bell pepper and pollen allergens [10, 11].

van Toorenenbergen et al. [12] compared IgE-binding patterns in patients with occupational allergy against pollen and fruit from bell pepper and tomato to IgE-binding patterns in food-allergic patients. They found profilin-specific IgE only in food-allergic patients but not in pollen-allergic patients. Recently, Groenewoud et al. [13] reported occupational allergy to bell pepper pollen in 35.4% of 472 greenhouse workers in the Netherlands. These greenhouse workers suffered predominantly from work-related symptoms of the upper and lower airways. But none of these studies reported related food allergies upon consumption of bell pepper or tomatoes. At present, there is no evidence that sensitisation to occupational allergens from bell pepper or tomato (via the nasal route) leads to consecutive food allergy.

IgE immunoblot inhibition studies have already identified structures homologous to profilin in potato, tomato and bell pepper. In bell pepper, profilin was detected as an

IgE-binding protein in several horticultural strains; however, no IgE binding to profilin could be found in paprika powder [14]. Recently, some allergens of tomato fruits were identified [15–17]. A certain isoform of tomato profilin was found to be pollen specific, whereas other isoforms of profilin were expressed constitutively in all tissues of the plant [18]. Tomato profilin was also identified as a relevant allergen in a group of patients allergic to birch pollen with adverse reactions to tomatoes [17]. However, a detailed molecular characterisation of tomato fruit or bell pepper profilin has not been performed so far.

Here we report the cloning, expression and molecular characterisation of recombinant profilin from tomato (*Lycopersicon esculentum*) and bell pepper (*Capsicum annuum*) and their IgE-binding capacity.

Materials and Methods

RNA Isolation, cDNA Synthesis and RT-PCR Amplification

Total RNA was extracted from 100 g bell pepper and tomato fruits by the acidic guanidinium method with mercapto-ethanol followed by cesium chloride gradient centrifugation and isopropanol precipitation [19]. Briefly, the plant material was frozen in liquid nitrogen and ground to powder in an electric blender. Subsequently, the powder was suspended in 15 ml of 7.5 M guanidine hydrochloride/25 mM sodium citrate /0.5% sodium-N-lauryl-sarcosinate/0.1 M β -mercapto-ethanol. The suspension was centrifuged at room temperature for 15 min at 6,000 g. The supernatant was filtered through sterile Nylon filters and transferred to fresh tubes and overlaid with 5.7 M cesium chloride solution and centrifuged at 18,000 g, 20°C for 24 h. Then the RNA pellet was dissolved in di-ethylpyrocarbonate-treated water, further purified by precipitation after addition of 3 M sodium acetate (10% v/v) and isopropanol (100% v/v). After centrifugation, the pellet was again dissolved in RNase-free water. For reverse transcription 1.2 μ g of total RNA, an oligo-dT primer (T₂₅NN; MWG Biotech AG, Ebersberg, Germany), 50 units of M-MuL V reverse transcriptase, 5 mM magnesium chloride, and a mix of nucleotides, and RNA inhibitor (all MBI Fermentas, Vilnius, Lithuania) were used. The reaction was performed at 42°C for 60 min followed by a heat denaturation step at 95°C for 5 min using a Perkin-Elmer thermocycler. Gene-specific primers were synthesised according to the already known N-terminal sequence from tomato pollen profilin (accession No. Q41344). Subsequently, PCR was performed using a 5'-primer matching the N-terminal sequence of tomato pollen profilin [18] (5'-ATG TCG TGG CAA ACA TAT GTC GAT G-3'; MWG Biotech AG) and an oligo-dT primer (T₂₅NN). Six microlitres of cDNA product were used for the reaction. After a hot start (5 min at 95°C) Taq DNA Polymerase (MBI Fermentas) was added and 35 cycles (denaturation step: 93°C for 45 s; annealing step: 59°C for 30 s, an elongation step at 72°C for 1 min and for 7 min after the last cycle) were performed.

Cloning and Sequence Analysis

PCR products were separated by electrophoresis on 1.2% agarose gel. DNA fragments of ~600 bp in length were isolated (QIAEX,

Qiagen, Santa Clara, Calif., USA), ligated into the pGEM vector (Promega Madison, Wisc., USA) and propagated in *Escherichia coli* XL-1 Blue cells. Inserts were sequenced using the Thermo Sequenase fluorescent-labelled primer cycle sequencing kit (Amersham, Biosciences, Little Chalfont, UK) and analysed on an automatic sequencer (Licor Sequencer 4000L, Li-COR, Biosciences GmbH, Bad Homburg, Germany).

The complete sequences coding for the bell pepper (*C. annuum*) and tomato (*L. esculentum*) profilin were obtained. The sequences were analysed and compared to already known profilin sequences from the EMBL/Genbank and Swiss Prot databases using Fasta and PileUp programs from the GCG sequence analysis package (Genetics Computer Groups, Madison, Wisc., USA) and were designated Cap a 2 from bell pepper and Lyc e 1 from tomato according to the guidelines of the Allergen Nomenclature Committee.

Cloning of Cap a 2 and Lyc e 1 as a Non-Fusion Protein in pMW175

Afl III and *Eco* RI restriction sites were introduced to the complete coding sequences of Cap a 2 and Lyc e 1 via PCR (Cap a 2 and Lyc e 1 forward primer: 5'-CAC CAC ATG TCG TGG CAA ACA TAT GT-3', *Afl* III site underlined, Cap a 2 reverse primer: 5'-CCC GAA TTC TTA GAG ACT CTG TTC AAT GA-3' (*Eco* RI site underlined; Lyc e 1 reverse primer: 5'-CCC GAA TTC CTA GAG ACT CTG TTC TAT AA-3' (*Eco* RI site underlined; both primers: MWG Biotech AG), respectively. PCR-amplification was conducted with a Pfu-Polymerase (MBI Fermentas) according to standard protocols. PCR products were ligated via *Nco* I and *Eco* RI restriction sites into the high level expression plasmid pMW175 [20].

Expression and Purification of Cap a 2 and Lyc e 1

For heterologous expression of the bell pepper and tomato profilin, respectively, competent *E. coli* BL21[DE3] cells were transformed with either Cap a 2-pMW175 or Lyc e 1-pMW175. Single transformant colonies were used to inoculate 300 ml Luria-Bertani medium containing 0.1 mg/ml ampicillin and 2 mmol/l MgCl₂. After overnight incubation in flasks, cells were diluted in 9 litres of medium of the same composition and transferred to a 10-litre fermenter (New Brunswick, Bioflow 3000, Edison) and grown under constant shaking (100 rpm), aeration (2.5 litres/h) was checked by an oxygen electrode and held at 18% saturation. Protein expression was induced by addition of 0.4 mmol/l isopropyl-β-D-thiogalactopyranoside (IPTG) at an A₆₀₀ of 1.0. After 4 h of incubation, cells were harvested by centrifugation. Pellets were dissolved in 50 mmol/l Tris-Cl, 0.1% Triton X-100, 1 mmol/l β-mercapto-ethanol, and lysozyme was added at 0.1 mg/g cell pellet. The cells were disrupted by three cycles of freezing-thawing and homogenised using an Ultraturrax, while NaCl was added to the lysate to a final concentration of 200 mmol/l during the last homogenising step. The profilin in the supernatant was enriched by fractionated ammonium sulphate precipitation to a final concentration of 45%. The pellet was dissolved in 20 mmol/l imidazole buffer at pH 7.4, 2 mmol/l β-mercapto-ethanol and NaCl 200 mmol/l (buffer A). This solution was subjected to poly-(L-proline)-CNBr-activated Sepharose (PLP-Sepharose; Amersham Biosciences) column [21]. A linear gradient of increasing buffer B (20 mmol/l imidazole, pH 7.4, 6 mol/l urea) over buffer A was established to elute the recombinant profilins. The fractions containing profilin were pooled and subjected to a DEAE-Sepharose column. The column was equilibrated with 20 mmol/l imidazole, pH 7.4, 2 mmol/l β-mercapto-ethanol, 0.1 mmol/l ethylenediamine tetra-acetic acid. Recombinant pro-

filin was eluted by an NaCl gradient. The fractions containing Cap a 2 or Lyc e 1 were pooled and dialysed against 5 mmol/l ammonium hydrogen carbonate buffer, respectively. Subsequently, purified rCap a 2 and rLyc e 1 were analysed for their N-terminal sequences. Therefore, 10 µg of each protein was absorbed onto a ProSorb sample preparation cartridges (Applied Biosystems, Foster City, Calif., USA), administered onto an automated Edman-protein sequencer (Procise 491 sequencer, Applied Biosystems), and the first ten amino acid residues determined. In order to determine bacterium-derived endotoxin levels, purified rCap a 2 and rLyc e 1 were subjected to a limulus amoebocyte lysate assay according to the manufacturer's instructions (Bio Whittaker, Verviers, Belgium).

Size Exclusion HPLC

Possible oligomerisation of bell pepper and tomato profilin was investigated by size exclusion chromatography using an HP 1100 HPLC system (Agilent Technologies, Palo Alto, Calif., USA). A G-SW 2000 column (Tosohas, pore diameter 10 µm) was equilibrated with phosphate-buffered saline. In separate runs, the retention times of BSA (molecular mass 67,000 kD), ovalbumin (molecular mass 43,000 kD), chymotrypsinogen (molecular mass 25,000 kD), and ribonuclease A (molecular mass 13,700 kD) were used as molecular mass standards. UV absorbance was monitored at 280 and 214 nm. Afterwards, 20 µg of either recombinant purified bell pepper or tomato profilin were loaded onto the column at a flow rate of 0.2 ml/min. In another set of experiments, samples of both recombinant profilins were pretreated with 10 mmol/l dithiothreitol (DTT) for 2 h, 37°C with PBS as buffer before loading into the G-SW 2000 column.

Patients and Patients' Sera

Thirty-four food-allergic patients were selected on the basis of a history of an allergic reaction to bell pepper. Total serum IgE was measured by either RAST or a Pharmacia UniCap device (Pharmacia, Uppsala, Sweden). In most of the cases, specific IgE for birch and grass pollen was measured. Skin prick tests with birch pollen extract and grass pollen extract were performed as well (Soluprick ALK, Horsholm, Denmark). For inhibition studies, sera No. 1, 2, 4, 5, and 6 were pooled. The serum of a non-allergic person (NHS = normal human serum) served as a negative control. Sera were stored at -20°C.

IgE Immunoblots and Inhibition Assays

Aqueous protein extracts were prepared from ripe bell pepper (Red Marras) and tomato fruits (Tocredo) according to standard protocols [22]. Proteins were separated on either 12% SDS-PAGE or gradient gels (7.5–20%) under reducing conditions [23]. Proteins were visualised by staining with Coomassie Brilliant Blue R-250. For immunoblot analysis, protein extracts (50 µg total protein per 0.5 cm lane of 1 mm gels) and purified rCap a 2 and rLyc e 1 (1.5 µg per 0.5 cm gel), respectively, were separated by SDS-PAGE and subsequently transferred to nitrocellulose membranes [24]. The IgE-binding ability of natural protein extracts and recombinant proteins was detected applying sera from food-allergic patients (1:4 diluted in 50 mmol/l sodium phosphate buffer/0.5% Tween 20/1% BSA pH 7.5) according to established protocols [22]. Bound IgE was detected by ¹²⁵I-labelled anti-human IgE antibodies (1:20 dilution; MALT Allergy System Isotope Reagent, IBL, Hamburg, Germany) and visualised by autoradiography. For inhibition studies, the serum pool was preincubated with either purified rCap a 2 (50 µg/ml) or purified

rLyc e 1 (50 µg/ml) overnight at 4°C. Afterwards, the remaining IgE binding to natural profilin from bell pepper extract or tomato extract was tested in IgE immunoblot as described above.

Additionally, the serum pool was preincubated with either purified rCap a 2 or rLyc e 1 (50 µg/ml serum) overnight at 4°C. Subsequently, IgE binding to rLyc e 1 was tested and vice versa. In parallel, a positive control (without preincubation) was performed.

ELISA Experiments

In order to compare IgE binding to reduced and non-reduced profilin, rCap a 2 and rLyc e 1 10 µg/ml were dissolved in Tris-buffered saline (TBS) with 10 mM DTT. The sample was heated to 37°C for 2 h. For control, protein was dissolved in TBS without DTT. Subsequently, the proteins were diluted (1:2, 1:4, 1:8, and 1:16) in TBS-DTT, the control proteins in TBS, and used for coating a microtiter plate (Nunc Maxisorp, Nalge Nunc International, Roskilde, Denmark) overnight at 4°C. After blocking of unspecific binding sites with TBS/0.05% (v/v) Tween 20/2% milk powder, serum No. 5 was applied onto the coated wells and incubated overnight at 4°C. After washing, the plate was incubated with alkaline-phosphatase-conjugated mouse anti-human IgE (BD-Biosciences Pharmingen, San Diego, Calif., USA) for 2 h at room temperature. The amount of bound enzyme was determined by colour development using 0.1% *p*-nitrophenyl-phosphate substrate and measured at 405 nm in an ELISA reader (Spectra Max Plus 384; Molecular Devices GmbH, Munich, Germany). NHS and TBS were used as negative controls.

Results

Patients

Thirty-four patients (12 male/22 female) aged 17–58 years (mean 35 years $\pm$ 11) with allergy to bell pepper according to their case history were included in this study. Out of those, 23 reported food-allergic symptoms when eating tomatoes. Total serum IgE ranged from 10 to 980 kU/l; median: 278.5 kU/l. Positive CAP/RAST against birch pollen and grass pollen were obtained from 16 and 14 patients, respectively (table 1). In skin prick tests, 20 patients scored positive for birch pollen and 19 for grass pollen. The reported clinical symptoms ranged from allergic rhinoconjunctivitis in 17 cases (50%), oral allergy syndrome in 13 cases (38%), urticaria in 11 cases (32%), allergic asthma in 8 cases (24%), diarrhea in 2 cases (6%), angio-oedema in 1 case (3%), cephalgia in 2 patients (6%), and exacerbation of atopic eczema dermatitis in 1 case (3%).

Additional allergic symptoms were caused by apple/strawberry/stone fruits in 16 patients (47%), fig/avocado/banana/in 7 patients (21%), cucumber/melon/zucchini in 6 patients (18%), nuts in 6 patients (18%), carrot/celery in 5 patients (15%), kiwi in 5 patients (15%), spices in 4 patients (12%), cheese/egg in 3 patients (9%) and potato in 3 patients (9%). In addition, soy, orange, and pepper-

mint also evoked allergic reactions in individual patients. Patients with allergy to fig, avocado, and banana also displayed allergic symptoms against latex and ficus (patients No. 4, 32–34; table 1).

Amplification and Sequence Analysis of Bell Pepper and Tomato Profilin Sequences

PCR amplification of cDNAs complementary to Cap a 2 and Lyc e 1 transcripts revealed products of approximately 600 bp in length. Several PCR-products were cloned and sequenced, consisting of an open reading frame of 393 bp for each profilin.

The 3'-non-coding region (3'-UTR) is composed of 218 bp for bell pepper profilin and 185 bp for tomato profilin. The deduced amino acid sequences encompass 131 amino acid residues with calculated molecular masses of 14,184 kD and a pI of 4.44 for bell pepper profilin and 14,257 kD and a pI of 4.46 for the corresponding tomato protein according to the GCG sequence analysis package (fig. 1). Sequence comparisons on the amino acid level revealed high similarities between both profilins from bell pepper and tomato fruits (91% identity), whereas tomato profilin from pollen [18] shares only 75% identity with tomato fruit profilin, which encompasses 133 amino acid residues (fig. 1). A search of Swiss-Prot databases exhibited significant identities to other plant profilins (table 2). The complete cDNA sequence coding for the bell pepper and tomato profilin is available from the EMBL/GenBank/DBJ databases under accession No. AJ417552 for bell pepper profilin and AJ417553 for tomato profilin, respectively.

Expression and Purification of Recombinant Bell Pepper and Tomato Profilin

Cap a 2 and Lyc e 1 were expressed as recombinant proteins in *E. coli* yielding 9.7 mg purified Cap a 2 and 8.2 mg Lyc e 1 per litre culture medium, respectively. Purification of the recombinant profilins by PLP-Sepharose affinity chromatography followed by anion exchange chromatography resulted in highly pure protein fractions. The purified fractions were analysed on gradient SDS-PAGE and showed single bands of each recombinant protein with an apparent molecular mass of 14 kD which corresponds to the calculated mass of both profilins (fig. 2). Purified proteins were further analysed for their correct N-terminus by Edman degradation, showing that the initiating methionine was cleaved off according to the side chain length of the penultimate amino acid [25]. The following ten amino acid residues revealed the same peptide sequence SWQTYVDDHL for Cap a 2 and SWQTYV-

Fig. 1. Amino acid sequence from bell pepper profilin (Cap a 2), tomato fruit profilin (Lyc e 1) and tomato pollen profilin (Pro1_lyces). Identical sequences are underlaid in grey, the N-terminal sequence verified by Edman is underlined.

	1				50
Cap a 2	<u>MSWQTYVDDH</u> LMCEIE..GN RLTSAAIIGQ DGSVWAQSAT FPQFKPEEIT				
Lyc e 1	<u>MSWQTYVDEH</u> LLCENE..GN HLTSAAIIGQ DGTVWAQSAN FPQFKPEEIT				
Pro1_lyces	<u>MSWQTYVDDH</u> LMCDIEGTGH HLSSAAILGF DGSVWAQSPN FPKFKAEEIT				
	51				100
Cap a 2	AIMNDFAEPPG <u>TLAPTGLYL</u> G GTKYMWIQQE AGAVIRGKKG PGGITVKKTN				
Lyc e 1	GIMNDFAVPG <u>TLAPTGLYL</u> G GTKYMWIQQE PEAVIRGKKG PGGITIKKTN				
Pro1_lyces	NIMKDFDEPG <u>HLAPTGLFL</u> A GTKYMWIQQE PGAVIRGKKG PGGITIKKTA				
	101				133
Cap a 2	<u>QALIIGIYDE</u> PMTPGQCNI VERLGDYLIE QSL				
Lyc e 1	<u>QALIIGIYDE</u> PMTPGQCNI VERLGDYLIE QSL				
Pro1_lyces	<u>QALIFGVYEE</u> PVTGQCNI MV EKIGDYLVD QGY				

Table 1. Clinical data of bell-pepper-allergic patients

No.	Age	Sex	Total IgE kU/l	sIgE ¹ birch pollen	sIgE ¹ grass pollen	SPT birch pollen	SPT grass pollen	Symptoms ²	Anamnesis		
									bell pepper	tomato	other foods
1	23	M	/	/	/	+	+	OAS, U	+	+	carrot, nuts, apple, celery, potato
2	17	M	/	/	/	+	+	RC	+	+	cucumber, apple, nuts, carrot, zucchini
3	50	F	/	-	-	-	-	U, D	+	-	/
4	31	F	/	/	/	+	+	OAS, U	+	+	stone fruits, apple, latex
5	22	M	480	3.5 (R)	3.2 (R)	+	+	RC	+	+	apple, cucumber, melon, banana
6	43	M	160	2.4 (R)	3.5 (R)	+	+	RC	+	+	cucumber
7	39	F	/	2 (C)	2 (C)	/	/	RC, OAS, AB	+	+	/
8	34	F	980	4.1 (R)	4.6 (R)	+	+	RC	+	not eaten	cucumber, watermelon
9	49	F	/	/	/	+	+	/	+	+	strawberry, peach
10	23	F	340	-	4.8 (R)	-	+	RC	+	+	cucumber, kiwi, avocado
11	38	F	122	4 (C)	3 (C)	+	+	OAS, RC	+	+	stone fruits, maize, zucchini, apple, banana, plum
12	18	F	680	4.5 (R)	-	+	+	AB	+	+	carrot
13	55	F	50	2.8 (R)	/	+	/	AB, U	+	-	cherry, peach
14	29	M	90	-	3.1 (R)	-	+	RC, OAS, AB	+	+	spices
15	28	M	190	3.7 (R)	-	+	-	RC	+	-	apple
16	35	M	140	-	-	-	-	OAS	+	-	/
17	22	F	210	4.8 (R)	-	+	-	OAS	+	+	apple, peach, kiwi
18	36	F	40	2.5 (R)	2.6 (R)	+	+	RC, U	+	+	carrot, hazelnut, kiwi, stone fruits
19	28	F	10	-	-	-	-	C	+	-	nuts, strawberry, kiwi
20	34	F	130	/	/	+	+	D	+	-	egg
21	17	M	170	3.4 (R)	2.2 (R)	+	+	U, AB	+	-	nuts, apple, kiwi, cherry
22	26	F	/	-	3 (C)	-	+	U	+	+	celery, potato
23	35	M	80	3.8 (R)	-	+	-	RC, OAS, AB	+	+	apple, nuts, cherry, strawberry
24	53	F	10	/	/	-	-	RC, C	+	-	cheese, eggs
25	40	F	140	3.7 (R)	2.1 (R)	+	+	AB, OAS	+	-	stone fruits, spices, peppermint
26	27	F	/	/	/	/	/	AD, OAS	+	+	/
27	37	M	/	-	-	-	-	OAS	+	-	/
28	58	M	/	-	-	-	-	A, U	+	+	cheese, stone fruits, spices
29	43	F	/	3.1 (R)	-	+	-	OAS, RC	+	+	apple, soy, orange, potato
30	51	F	/	-	2.4 (R)	-	+	/	+	+	/
31	34	M	/	2.6 (R)	-	+	-	OAS, RC	+	+	spices
32	29	F	115	-	3 (C)	-	+	RC, U, AB	+	+	avocado, banana, ficus, latex
33	38	F	118	3 (C)	3 (C)	+	+	RC, U	+	+	avocado, banana, ficus, latex, fig
34	41	F	273	-	-	-	-	RC, U	+	+	avocado, ficus, latex

¹ C = CAP; R = RAST; - = negative; / = not done.

² OAS = Oral allergy syndrome; RC = rhinoconjunctivitis; C = cephalgia; AB = asthma bronchiale; U = urticaria; AD = atopic dermatitis; A = angio-oedema; D = diarrhoea.

Fig. 2. Gradient SDS-PAGE (7.5–20%) of recombinant Cap a 2 and Lyc e 1 in *E. coli*. **a** Bacterial protein lysate from BL21[DE3] (lane 1), Lysate from *E. coli* (BL21) expressing rCap a 2 (lane 2) and purified rCap a 2 (lane 3) were electrophoresed and subsequently stained with Coomassie. **b** Lane 1: *E. coli* (BL21) lysate; lane 2: Lysate from *E. coli* (BL21) expressing rLyc e 1; lane 3: purified rLyc e 1; molecular mass standards are marked.

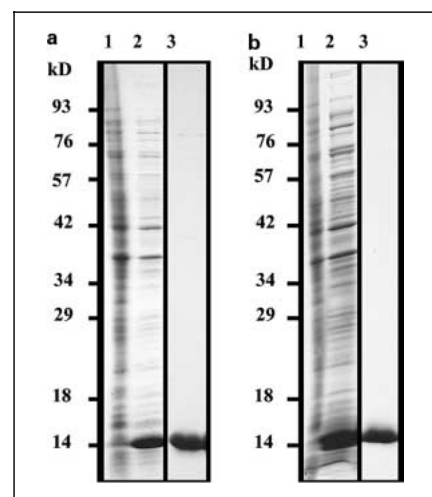


Table 2. Amino acid sequence comparisons of selected plant profilins to rCap a 2 and rLyc e 1

Plants (sources)	Allergen name	Accession No.	Identity, %	
			to Cap a 2	to Lyc e 1
<i>Hevea brasiliensis</i> (para rubber tree; pollen)	Hev b 8	O65812	88.5	84.7
<i>Glycine max</i> (soybean)	Gly m 3	O65809	86.9	83.8
<i>Prunus avium</i> (sweet cherry)	Pru av 4	Q9XF39	87.0	79.4
<i>Phleum pratense</i> (common timothy; pollen)	Phl p 11	O24650	79.4	80.9
<i>Arabidopsis thaliana</i> (mouse-ear cress; ubiquitous)	Ara t 8	Q42449	77.1	78.6
<i>Olea europaea</i> (common olive; pollen)	Ole e 2	O24169	79.9	73.9
<i>Hordeum vulgare</i> (barley; leaf)	–	P52184	77.1	77.9
<i>Lycopersicon esculentum</i> (tomato; pollen)	–	Q41344	76.5	75.0
<i>Betula verrucosa</i> (white birch; pollen)	Bet v 2	P25816	76.7	73.7
<i>Nicotiana tabacum</i> (common tobacco; pollen)	–	P41372	75.9	72.2

DEHL for Lyc e 1, respectively (fig. 1). Analysis for endotoxin levels yielded 4 pg lipopolysaccharide (LPS)/10 µg protein for purified rCap a 2 (= 0.05 endotoxin units; EU) and approximately 500 pg LPS/10 µg protein for purified rLyc e 1 (= 6 EU).

Immunoblot Analysis of Bell Pepper and Tomato Extracts

By immunoblot analysis, 34 sera from persons allergic to bell pepper were tested on natural red bell pepper extract. All 34 sera displayed IgE binding to a number of bell pepper proteins in a molecular mass range from 14 to 90 kD. Eleven sera (32%; sera No. 1–11; table 1) displayed IgE-binding to the 14-kD protein (= natural profilin; fig. 3a, upper panel). The same 11 selected sera were further used to perform an IgE-immunoblot on tomato

extract (fig. 3b, upper panel). All of them (11/11) exhibited IgE reactivity to a 14-kD protein from tomato extract as well. As these patients have reported allergic reactions upon eating tomatoes, with the exception of patient 8, who claimed not to consume any tomatoes.

Immunoblots of Recombinant Profilin, Inhibition Assays and Cross-Reactivity

The purified recombinant profilins from bell pepper and tomato were tested for their IgE-binding capacity in immunoblot assays (fig. 3a, b, lower panels). Both 14-kD proteins were recognised by the same set of 11 profilin-sensitised sera. Preincubation of the serum pool with purified rCap a 2 (50 µg/ml) nearly abolished all the IgE binding to natural bell pepper profilin (fig. 4). In addition, the same blocking of IgE binding of the serum pool to nat-

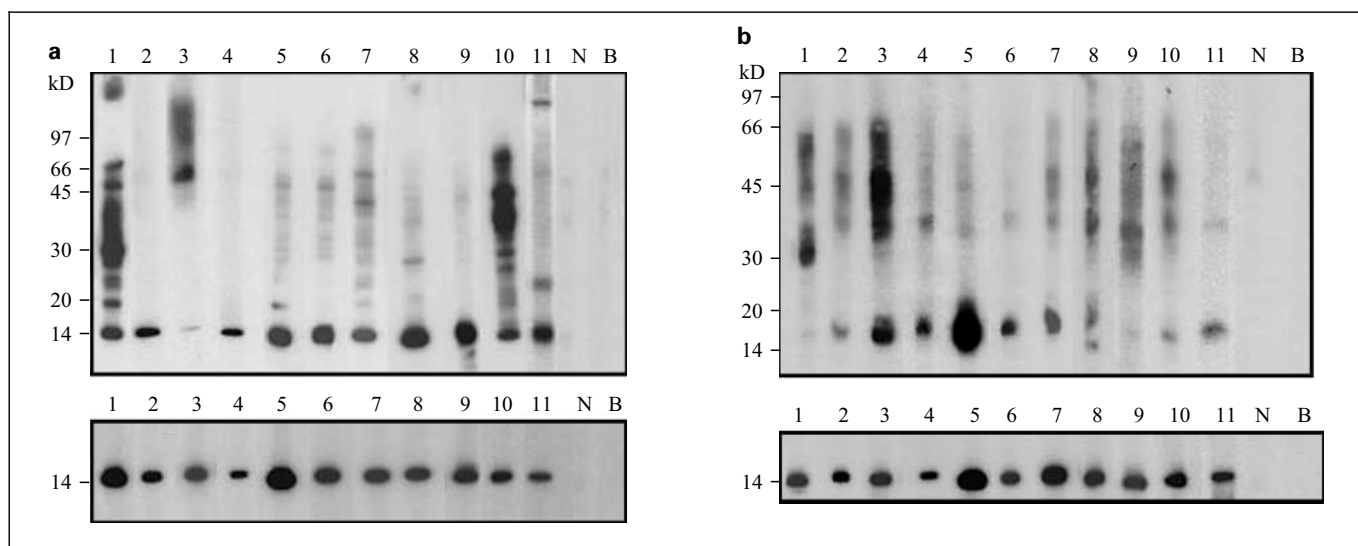
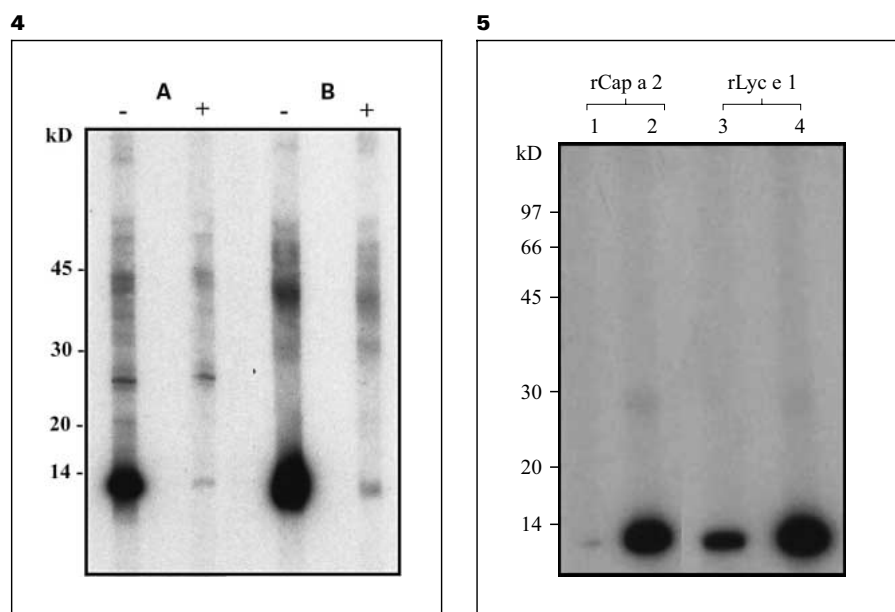


Fig. 3. a Immunoblot analysis of bell pepper extract (upper panel) and purified rCap a 2 (lower panel). Extract separated by 12% SDS-PAGE: 11 sera from bell-pepper-allergic patients were tested for IgE binding to bell pepper protein extract and rCap a 2. Bound IgE was

detected by ^{125}I -labelled anti-IgE. NHS (N) and buffer (B) were used as controls. **b** Immunoblot analysis of tomato extract (upper panel) and purified rLyc e 1 (lower panel). The same set of sera was tested for IgE binding to tomato proteins and rLyc e 1.

Fig. 4. Inhibition assay: A serum pool of bell-pepper-allergic patients ($n = 4$) was tested for IgE binding to bell pepper proteins with or without (+/-) pre-incubation of purified rCap a 2 (50 $\mu\text{g}/\text{ml}$; panel A). In parallel, the same serum pool was tested for IgE binding to tomato proteins (panel B) after pre-incubation (+/-) of purified rLyc e 1 (50 $\mu\text{g}/\text{ml}$). The remaining IgE binding was visualised by autoradiography using ^{125}I -labelled anti-human IgE.

Fig. 5. Cross-inhibition: a serum pool of profilin-sensitised patients ($n = 4$) was tested for remaining IgE binding to rCap a 2 after pre-incubation with rLyc e 1 lane 2. Reversely, the same serum pool was tested for IgE binding to rLyc e 1 after incubation with rCap a 2 (lane 3). Positive controls (without inhibitor protein) were run in parallel (lane 2: rCap a 2; lane 4: rLyc e 1).



ural tomato profilin was detected in an immunoblot after preincubation with purified rLyc e 1 (50 $\mu\text{g}/\text{ml}$; fig. 4). In addition, cross-reactivity of rCap a 2 and rLyc e 1 was investigated by mutual inhibition experiments with the same serum pool. Purified rCap a 2 was able to inhibit IgE binding to rLyc e 1 by approximately 50%, but reversely

rLyc e 1 completely blocked IgE binding to rCap a 2 (fig. 5). The sequences coding for profilin from tomato and bell pepper were submitted to the Allergen Nomenclature Committee and received the allergen names Cap a 2 for bell pepper profilin and Lyc e 1 for tomato profilin. According to results obtained from the immunoblot anal-

yses, these two food allergens are ranked as minor allergens.

ELISA Experiments

IgE binding of serum 5 to reduced (DTT-treated) rCap a 2 was compared to IgE binding to non-reduced rCap a 2 in ELISA. No significant difference in IgE binding to both forms of rCap a 2 could be observed within the used range of dilutions (fig. 6). In contrast, DTT-treated rLyc e 1 displayed a clearly lower IgE-binding capacity than non-reduced recombinant tomato profilin when using the same serum.

HPLC Analysis

Size exclusion chromatography showed that rCap a 2 is found predominantly in a dimeric form in solution which can be partially dissociated to a monomer by addition of DTT (fig. 7a). The chromatogram of purified rLyc e 1

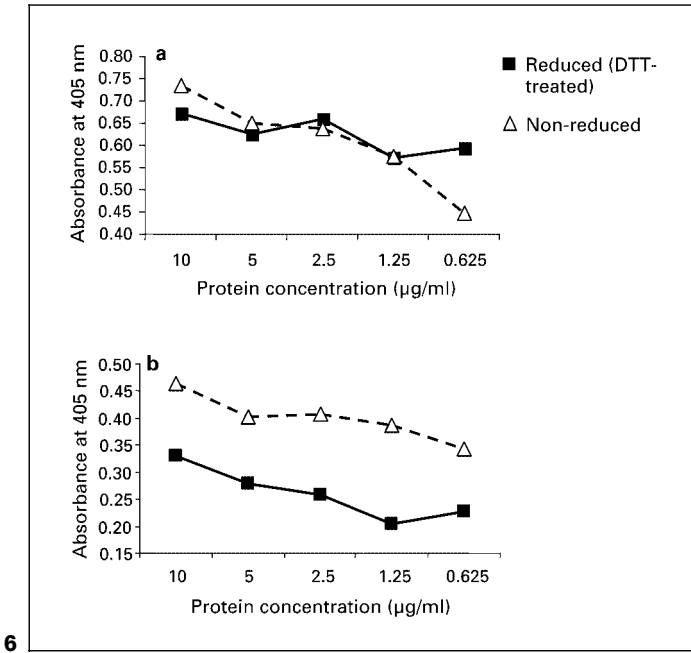
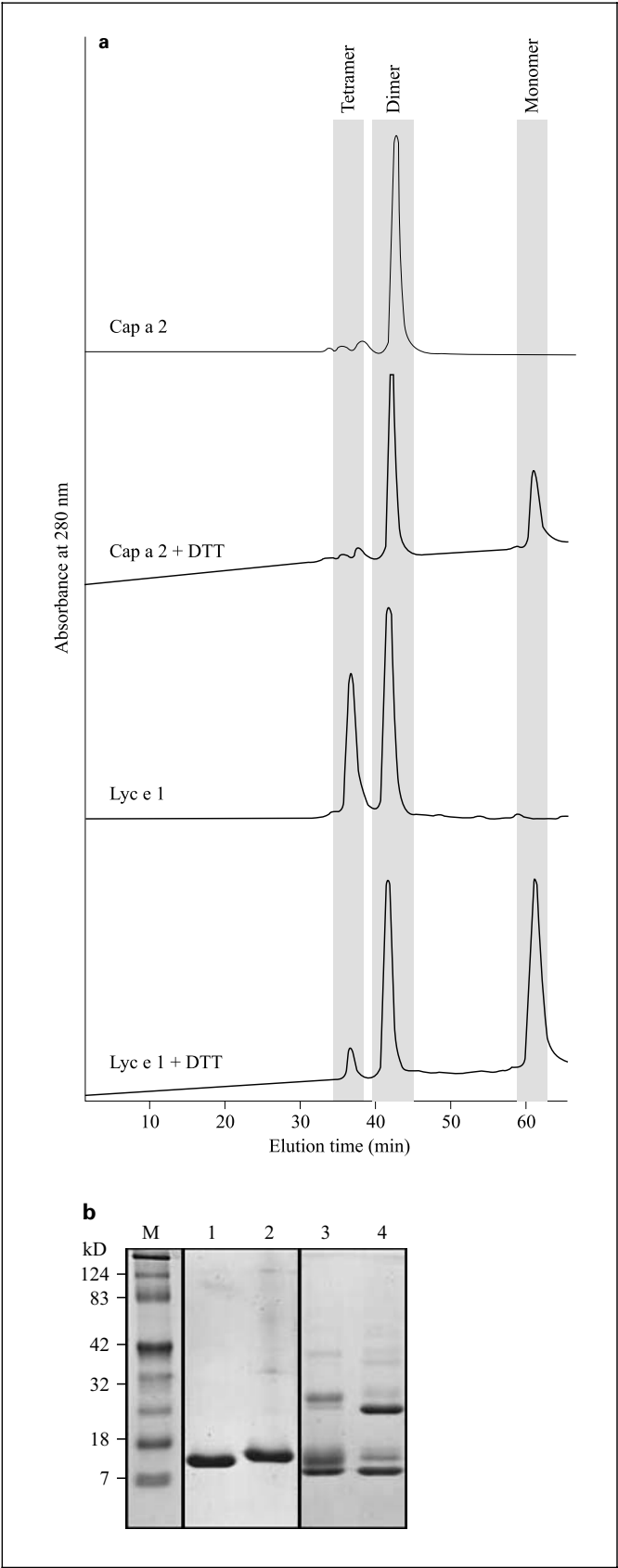


Fig. 6. IgE-ELISA of purified rCap a 2 (**a**) and purified rLyc e 1 (**b**). Serum 5 was tested for IgE binding to non-reduced and reduced purified recombinant bell pepper profilin and tomato profilin.

Fig. 7. a HPLC analysis of purified rCap a 2 and rLyc e 1. Both recombinant profilins display oligomer forms (dimers or tetramers) in solution, which can be partially reduced to monomers by addition of DTT. **b** Gradient SDS-PAGE analysis: purified rCap a 2 and rLyc e 1 were electrophoresed under reducing conditions (lane 1: rCap a 2, lane 2: rLyc e 1 and non-reducing conditions, respectively (lane 3: rCap a 2, lane 4: rLyc e 1).



revealed the presence even of higher-order oligomers (tetramers) which can be partially reduced to presumably monomers by DTT (fig. 7a). In parallel, gradient SDS-PAGE analysis revealed that under reducing conditions, both profilins migrate as monomers (fig. 7b, lanes 1, 2). Gradient SDS-PAGE performed under non-reducing conditions displayed a significant percentage of oligomer conformation of rCap a 2 (fig. 7b, lane 3) and rLyc e 1 (fig. 7b, lane 4).

Discussion

In the recent past, several profilins were characterised as food allergens, thus partially explaining the pollen-food cross-reactivity at the molecular level. Vallier et al. [26] described celery profilin as the first allergenic profilin from foods. Pollen-allergic patients with adverse reactions to birch and mugwort also frequently display symptoms when eating celery, various spices and vegetables or fruits from the Solanaceae family, such as tomato and bell pepper. In addition, tomato also evokes allergic reactions in patients allergic to grass pollen [11]. The prevalence of sensitisation to profilin ranges from 8 to 25% in hazelnut [27, 28]-, 17–30% in celery [8, 9, 29]-, 20% in carrot [30]-, to 57% in peach-allergic patients [31].

However, a reliable diagnosis of allergy always depends on the quality of the test solutions applied. Especially commercially available plant food extracts for skin prick tests have been reported to be of low sensitivity [32]. Therefore, well-defined and well-characterised allergens should represent alternative diagnostic tools of higher quality. In the present study, cDNAs coding for profilin have been isolated, cloned and sequenced from bell pepper fruits (*C. annuum*) and tomatoes (*L. esculentum*), respectively. The bell pepper profilin consists of 131 amino acid residues with a predicted molecular mass of 14,184 kD designated Cap a 2. Lyc e 1, the tomato profilin also encompasses 131 amino acid residues with a predicted molecular mass of 14,257 kD (fig. 1). The two sequences are highly similar to each other (91% identity). Comparison of these two new profilin sequences to other already known plant profilins revealed significant sequence identities. Pairwise alignments of Cap a 2 and Lyc e 1 revealed highest sequence identity with profilin from *Hevea latex* profilin (89 and 85%). Among others, significant identity was observed with cherry profilin (87 and 84%), *Phleum pratense* profilin (79 and 81%), and *Betula verrucosa* profilin (77 and 74%) (table 2). Lyc e 1 shares only 75% sequence identity with tomato pollen profilin.

Similar sequence differences have already been observed between *Arabidopsis thaliana* profilin isoforms and *H. latex* profilins from leaves and pollen [33, 34]. It has been shown that several profilin isoforms are expressed in plants. They can be subgrouped into pollen-specific profilins and constitutively expressed profilins detectable in all tissues. These data suggest that two groups of profilin sequences must have been separated in early phylogeny.

Cap a 2 and Lyc e 1 were expressed in *E. coli* and purified to near homogeneity with a yield of 8–10 mg recombinant protein per litre culture medium (fig. 2). The purified recombinant profilins were tested for bacterium-derived endotoxin levels. The LPS contamination differed between rCap a 2 (0.5 EU/10 µg protein) and rLyc e 1 (6 EU/10 µg protein); however, these values are still below the values allowed for human therapeutics for parenteral application (e.g. Austrian threshold level: 8 EU/ml). The correct N-terminal sequence was verified by Edman degradation without the initiating methionine according to the side chain length of the penultimate amino acid (fig. 1) [25]. The purified recombinant profilins were tested for their IgE binding capacity in immunoblots, inhibition assays and ELISAs. Thirty-four patients with food allergy to bell pepper were enrolled in this study (table 1). Among them 23 also had food allergy to tomatoes. They all displayed IgE binding to proteins from bell pepper extracts and tomato extracts, respectively. Proteins of 14 kD from bell pepper extract and tomato extract were recognised by a profilin antiserum and therefore identified as profilins (data not shown). Eleven patients displayed IgE binding to these two 14 kD proteins (fig. 3). This represents IgE binding to profilin in 34% of patients allergic to bell pepper and 48% in patients allergic of tomato. The same set of patients also showed IgE binding to the recombinant profilins from bell pepper, Cap a 2, and tomato, Lyc e 1 (fig. 3). Furthermore, the purified recombinant proteins were also tested in inhibition assays, and ELISA verifying the IgE binding capacity of both purified recombinant profilins (fig. 4–6). Preincubation of a serum pool with purified rCap a 2 inhibited IgE binding to natural bell pepper profilin up to 90% and in parallel, IgE binding to natural tomato profilin was blocked by preincubating the serum pool with purified rLyc e 1 (fig. 4). In cross-inhibition assays, rLyc e 1 completely abolished the IgE binding to rCap a 2 from a serum pool. Reversely, rCap a 2 reduced IgE binding by 50% (fig. 5). Whether this effect is based upon the primary sensitisation to tomato profilin followed by cross-sensitisation to bell pepper profilin remains unclear. The patients included in this study were mostly polysensitised: nearly

half of them had IgE directed against inhalant grass and/or birch pollen allergens, some of them were allergic to latex and ficus, and most of the patients displayed food-allergic symptoms against a range of fruits, vegetables, and spices.

Again, the route of sensitisation against profilin remains unclear and it has to be carefully investigated whether cross-reactivity patterns based on profilin can be predicted in inhalant- and food-allergic patients [32]. The clinical relevance of IgE binding to profilin has to be distinguished from cross-reactive IgE binding without evoking allergic symptoms in patients based on a well-performed diagnosis of food allergy, as pointed out in several papers [8, 9, 32].

HPLC analysis and subsequent SDS-PAGE analysis revealed that rCap a 2 and rLyc e 1 tend to form oligomers in solution, which can be dissociated to monomers by adding reducing agents. In IgE-ELISA experiments performed with a selected serum, oligomeric rLyc e 1 displayed higher IgE-binding capacity, in contrast to rCap a 2, which did not significantly change its IgE-binding activity upon reduction. However, the effect of oligomer formation of Cap a 2 and Lyc e 1 will be further investigated in future experiments using a representative number of sera. Oligomeric forms of profilin may represent a more allergenic form with possible additional epitopes relevant for IgE-mediated reactions compared with monomers. For human and maize profilin it has been shown that they are able to form oligomers [35] which bound stronger to specific antibodies in immunoblots. Especially, oligomers of maize profilin, obtained under non-reducing conditions displayed a stronger IgE-binding capacity compared to the monomers [36]. Oligomerisation of profilin does not represent an artefact due to

recombinant production and purification. Recently, Wopfner et al. [37] reported that natural profilin, purified from mugwort pollen, was identified as well in a stable oligomeric state and monomers of natural profilin were obtained only after harsh treatment with reducing agents and heat.

Profilin-sensitised patients usually tend to develop allergies not only to multiple pollen species, but also to a number of pollen-related allergenic food. In general, these patients suffer from rather mild allergic symptoms [32]. However, due to the high cross-reactivity among different profilins, the predictive value of a profilin sensitisation as a diagnostic marker is still controversial and the presence of cross-reactive IgE antibodies directed against profilin does not necessarily result in clinically relevant food allergies [32]. Therefore, the diagnostic use of well-characterised individual members of the profilin family, such as rCap a 2 and rLyc e 1, instead of total food extracts may help to better define the clinical significance of profilin sensitisation. It will also be possible to identify the primary sensitising agent in a group of multiple-food-allergic patients using purified recombinant allergens with defined physicochemical characteristics in future experiments.

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