

Lipid Transfer Protein: A Pan-Allergen in Plant-Derived Foods That Is Highly Resistant to Pepsin Digestion

Riccardo Asero^a Gianni Mistrello^b Daniela Roncarolo^b Sacco C. de Vries^c
Marie-Françoise Gautier^d Caroline L.F. Ciurana^e Eddy Verbeek^e
Tamimount Mohammadi^e Vlasta Knul-Brettlova^f Jaap H. Akkerdaas^e
Ingrid Bulder^e Rob C. Aalberse^e Ronald van Ree^e

^aAmbulatorio di Allergologia, Ospedale Caduti Bollatesi, Bollate, ^bLofarma SpA, Milan, Italy;

^cDepartment of Molecular Biology, Agricultural University of Wageningen, The Netherlands;

^dUnité de Biochimie et Biologie Moléculaire des Céréales, INRA, Montpellier, France; ^eDepartment of Allergy, CLB and Laboratory for Experimental and Clinical Immunology, Academic Medical Centre, University of Amsterdam, ^fPrinsengracht Hospital, Department of Allergology, Amsterdam, The Netherlands

Key Words

Food allergy · Cross-reactivity · Lipid transfer protein · Protein stability · Recombinant allergens

Abstract

Background: Lipid transfer proteins (LTPs) are small molecules of approximately 10 kD that demonstrate high stability. They have recently been identified as allergens in the Rosaceae subfamilies of the Prunoideae (peach, apricot, plum) and of the Pomoideae (apple). They belong to a family of structurally highly conserved proteins that are also present in non-Rosaceae vegetable foods. **Objective:** The aim of this study was to investigate the cross-reactivity to non-Rosaceae LTPs, and to study the role of protein stability in allergenicity. **Methods:** Thirty-eight patients with a positive SPT to Rosaceae fruit extracts enriched for LTP were characterized by interview and SPT. To investigate IgE cross-reactivity between Rosaceae and non-Rosaceae LTPs, RAST and RAST inhibition as well as ELISA and ELISA inhibition were performed,

using whole food extracts and purified LTPs. Both purified natural LTPs (peach, carrot and broccoli) and *Pichia pastoris* recombinant LTPs (carrot and wheat) were included. Pepsin digestion was used to address the role of stability in the allergenicity of LTPs. **Results:** IgE antibodies to Rosaceae LTPs reacted to a broad range of vegetable foods, including Gramineae (cereals), Leguminosae (peanut), Juglandaceae (walnut), Anacardiaceae (pistachio), Brassicaceae (broccoli), Umbelliferae (carrot, celery), Solanaceae (tomato), Cucurbitaceae (melon), and Actinidiaceae (kiwi). Binding and inhibition studies with purified natural and recombinant LTPs confirmed their role in this cross-reactivity. Many of these cross-reactivities were accompanied by clinical food allergy, frequently including systemic reactions. Antibody binding to LTP was shown to be resistant to pepsin treatment of whole extract or purified LTP. **Conclusion:** LTP is a pan-allergen with a degree of cross-reactivity comparable to profilin. Due to its extreme resistance to pepsin digestion, LTP is a potentially severe food allergen.

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Introduction

Food allergy to fruits of the family of the Rosaceae is frequently linked to birch pollinosis [1–7]. Symptoms are usually restricted to the oral cavity (oral allergy syndrome, OAS) [8], most likely because the responsible food allergens are very labile and do not survive digestion in the gastrointestinal tract [9, 10]. The most important of these labile food allergens belong to a family of pathogenesis-related proteins together with their pollen homologue, the major birch pollen allergen Bet v 1 [5]. Their lability is also the reason why commercial skin test preparations for fruits like apple and peach are usually not very sensitive for the diagnosis of birch-pollen-related fruit allergies [11–13]. In recent studies on several hundreds of subjects with Rosaceae-induced OAS, it was reported that patients with positive skin tests to commercial fruit extracts had several common characteristics [14, 15]. They frequently had systemic symptoms next to their OAS, they showed a low prevalence of respiratory allergy, and they often recognized a 10-kD allergen on peach immunoblot. For a group of patients with a similar clinical history, it was demonstrated that the typical birch-pollen-related cross-reactive allergens, Bet v 1 and profilin, were not involved but that a 10-kD allergen in apple extract played an important role [16]. In 1994, Pastorello et al. [17] showed that allergenic cross-reactivity among members of the Prunoideae subfamily is due to a common 13-kD allergen (by SDS-PAGE) that is not found in grass or birch pollen. Recently, this allergen was identified as a 9-kD lipid transfer protein (LTP) in peach and apple [18, 19], most likely corresponding to the major peach allergen originally described by Lleonart et al. [20]. For both peach and apple, it was reported that the allergen is present predominantly in the peel of the fruit [16, 20].

Nonspecific plant LTPs have a molecular mass of 9–10 kD. Their exact function is not yet completely clear, but they have been implicated in transport of fatty acids, both intracellularly and extracellularly [21–23]. A role in the transport of cutin monomers to the cuticular layer of leaves and fruits could explain their abundance in the peel of fruits [16, 20] and in the wax layer of broccoli leaves [24]. The three-dimensional structure of several LTPs has been elucidated and it consists of a random coil C-terminal tail and 4 α -helices surrounding a hydrophobic cavity that can harbor fatty acids for transport [25–32]. LTPs have also been reported to act as plant defense proteins against bacterial and fungal infections [33–38]. They have been detected in many botanically unrelated vegetable foods, such as peach, apple, tomato, rape, carrot, barley,

corn, wheat, rice, sorghum, broccoli, onion and grapevine [18, 19, 24, 29, 31, 38–47]. Their primary structures demonstrate moderate to high degrees of sequence homology (35–95%). Finally, LTPs have been described as extremely stable molecules.

The aim of this study was to investigate whether the ubiquitous and conserved nature of LTPs justifies their designation as pan-allergen, and whether their stability influences their allergenicity.

Materials and Methods

Patients

Patients in this study were selected from 498 subjects (age > 12 years) with Rosaceae allergy as judged by skin prick test (SPT) with fresh fruits and (in most cases) clinical history. The majority had OAS (>97%), and only a minority (approximately 10%) showed urticaria/angioedema, gastrointestinal symptoms, food-induced rhinitis, asthma, or anaphylaxis. Selection was made on the basis of a positive SPT with both a commercial plum extract (1:20 w/v; Dome/Hollister-Stier, Spokane, Wash., USA) and a peach peel extract (see below). The rationale behind this approach was to select for LTP-reactive patients, since both skin test materials were shown to contain LTP, but were virtually devoid of Bet v 1- and Bet v 2-like structures [15]. Forty-nine (approximately 10%) patients were positive to both LTP-enriched SPT preparations; sera from 37 patients (16 males/21 females) were available for follow-up studies. One serum (pf194) of a Dutch subject allergic to grass pollen and apple was included in the study. This patient recognized fruits and nuts exclusively on the basis of LTP; no IgE antibodies to pollen-vegetable food cross-reactive structures (Bet v 1, Bet v 2 and carbohydrate structures) were detected.

Clinical Investigations

Patients underwent a thorough interview aiming to ascertain all episodes of food allergy (OAS, gastrointestinal symptoms, urticaria, angioedema, asthma, rhinitis, anaphylaxis) following the ingestion of vegetable foods. Inhalant allergies were diagnosed by interview and confirmed by SPT with commercial extracts (Allergopharma, Reinbeck, Germany) of the main endemic airborne allergen sources. All patients were subjected to SPT with the following commercial extracts of non-Rosaceae vegetable foods (Dome/Hollister-Stier): wheat, rye, barley, corn, oat and rice (cereals), peanut, green pea, soybean and white bean (legumes), celery, carrot, walnut, potato, tomato, banana, garlic, onion, and orange. SPT for hazelnut was carried out with fresh hazelnut by the prick-prick technique. Skin tests were performed on the volar side of the forearm using 1-mm standard disposable prick lancetters (Dome/Hollister-Stier). Readings were taken after 15 min, and skin reactivity was evaluated by comparison with the wheal induced by histamine (10 mg/ml); wheals with a diameter below 50% of the positive control were considered negative. Positive skin tests were divided into three classes: 2+ (50–100%), 3+ (100%) and 4+ (>100%) [48].

Food Extracts

Source materials for the preparation of food extracts were obtained from local food stores. Extracts were prepared essentially

according to Björkstén et al. [9]. In brief, foods were homogenized at 10% w/v in 0.1 mol/l phosphate buffer pH 7.0, containing 2% polyvinylpyrrolidone, 7 mmol/l diethyldithiocarbamate, 2 mmol/l ethylenediaminetetraacetic acid disodium salt (EDTA) and 2.6 mmol/l NaN₃. After stirring for 1 h, particulate matter was removed by centrifugation at 18,000 g for 30 min. The supernatant was dialyzed against distilled water and lyophilized. For application in RAST, 40 mg dry weight was coupled to 1 g of CNBr-activated Sepharose 4B (Pharmacia, Uppsala, Sweden). In case of peach peel extract, the supernatant was dialyzed against 5 mmol/l ammonium bicarbonate pH 8. This peach peel extract was either diluted in an equal volume of glycerol/0.04% phenol for application in SPT, or lyophilized and used for the purification of peach LTP.

Cross-Reactive Allergenic Structures from Birch and Grass Pollen

Natural Bet v 1 and profilin (Lol p 12) were purified from birch pollen and *Lolium perenne* grass pollen extract, respectively, by means of affinity chromatography as described elsewhere. In case of Bet v 1, a Sepharose-coupled Bet v 1-specific monoclonal antibody (5H8) was used [49]. For profilin, the affinity of profilin for poly(L-proline) was exploited [50]. Both Bet v 1 and profilin were coupled to Sepharose (100 µg to 100 mg) for application in RAST. As source of birch-pollen-derived IgE-binding cross-reactive carbohydrate determinants (CCD), a proteinase-K-digested birch pollen extract was used with <1% residual protein as judged by SDS-PAGE/silver staining, immunoblotting and specific Bet v 1 and profilin measurements [51]. Proteinase K digestion was carried out as reported earlier [52]. For RAST, the digest was coupled to 100 mg Sepharose at a concentration equivalent to 2.5 mg undigested extract.

Purification of Peach, Broccoli and Carrot LTP

Peach LTP was purified from peach peel extract. Lyophilized extract was resuspended in 1/10 of the original volume and submitted to gel permeation chromatography on a Superdex 75 column (Pharmacia) equilibrated in PBS/0.05% NaN₃ (bed volume: 150 ml). Fractions were analyzed by SDS-PAGE in conjunction with silver staining (not shown). Fractions exclusively containing the 9-kD LTP band were pooled, dialyzed against 5 mmol/l ammonium bicarbonate and lyophilized. This preparation was used for ELISA.

Purification of LTP from the wax layer of broccoli (*Brassica oleracea*) was performed essentially according to the method described by Pyee et al. [24]. In brief, broccoli was dipped in a 2/1 (v/v) mixture of chloroform and methanol for 10 s. The chloroform/methanol mixture was completely evaporated in a waterbath at 55°C under reduced pressure in a rotary evaporator. The resulting residue was then dissolved in 100 ml of chloroform, transferred to a separatory funnel, and vigorously shaken with 50 ml of water. Both fluid phases were allowed to separate for 1 h. Then the aqueous phase was separated from the organic phase and lyophilized. SDS-PAGE analysis revealed a single 9-kD band (not shown). Purified broccoli LTP (1.7 mg) was coupled to 100 mg of Sepharose for application in RAST.

Carrot LTP was purified by ion-exchange chromatography and gel filtration as described elsewhere [41]. For application in RAST, 100 µg of purified carrot LTP was coupled to 100 mg Sepharose.

Expression of Carrot and Wheat LTP in *Pichia pastoris*

For expression in *P. pastoris*, carrot LTP-specific cDNA was transferred from a pBluescript vector containing cDNA encoding carrot LTP [42] to the *P. pastoris* expression vector pPICZαA. To

this end, a *Xho*I and a *Not*I restriction site as well as a Kex2 cleavage site were added to the LTP-specific sequence. In addition, a stop codon was introduced at the 3' end of the LTP-specific sequence to avoid expression of a *myc* epitope and a polyhistidine tag, both present in the vector. The following primers were used for PCR:

5'-GGG CTC GAG AAA AGA ↓ **GTC CTC ACA TGC GGG CAG** (sense strand)

5'-GGG GGC GGC CGC TCA **CAC CAC CCT GTT GCA ATC** (antisense strand).

The sequences in bold represent carrot LTP specific sequences. The arrow indicates the Kex2 cleavage site, and the underlined sequences are the *Xho*I and *Not*I restriction sites, respectively. The stop codon introduced at the 3' end of the carrot LTP sequence is shown in italics. The PCR product was ligated into the pPICZαA vector and sequenced. The obtained sequence was identical to the published carrot LTP sequence [42]. After transformation of *P. pastoris* GS115 with the carrot LTP containing pPICZαA by electroporation, the yeast was grown in BMGY (0.1 M potassium phosphate, pH 6.0, 1% yeast extract, 2% peptone, 1.34% yeast nitrogen base, 4 × 10⁻⁵% biotin, 1% glycerol) in shake flasks at 30°C. At an A_{600 nm} of approximately 9, the culture was diluted in BMMY (=BMGY +0.5% methanol instead of glycerol) to A_{600 nm} 1. The addition of methanol results in induction of recombinant protein production. After 24 h, 0.5% of methanol was added. The culture supernatant was harvested after 48 h by centrifugation and filtration. Production of LTP was demonstrated by immunoblot analysis with a monospecific polyclonal rabbit antiserum against natural carrot LTP (not shown). For application in RAST(inhibition) culture supernatants were concentrated 10 times by ultrafiltration over a YM1 filter (Amicon Corp, Danvers, Mass., USA) with a 1-kD cut-off.

Wheat LTP was also expressed in strain GS115 and purified from the culture supernatant as described elsewhere [53]. For application in RAST, 50 µg purified recombinant wheat LTP was coupled to 100 mg of Sepharose.

ELISA/ELISA Inhibition

LTP from peach peel was reconstituted in 50 mmol/l sodium bicarbonate pH 9.6 and coated overnight onto polystyrene microtiter plates (100 ng/well). After washing, plates were saturated with 5% non-fat dry milk in 0.15 mmol/l Tris buffer pH 7.2 for 1 h. After washing, serum samples were added (50 µl serum plus 50 µl Tris buffer) and incubated for 1 h. Bound IgE was detected with peroxidase-conjugated anti-human IgE serum (Sigma, Milan, Italy) and tetramethylbenzidine as substrate. The enzyme reaction was stopped after 10 min by the addition of 1 mol/l sulfuric acid, and absorbance was read at 450 nm. IgE binding was expressed as absorbance (OD); values <300 were considered negative. ELISA inhibition was performed with various food extracts at a protein concentration of 0.6 mg/ml. To this end, 50 µl of serum was preincubated for 1 h with 50 µl of food extract prior to addition to the coated plate. Peach peel extract was used as positive, ovalbumin as negative control.

Radio-Allergosorbent Test (RAST) and RAST Inhibition

RAST was performed as described previously [54]. Briefly, serum (50 µl) was incubated overnight with 0.5 mg of Sepharose-coupled allergen in a final volume of 300 µl PBS, 0.3% BSA, 0.1% Tween-20 (PBS-AT). After washing away unbound serum components, radiolabeled sheep antibodies (CLB, Amsterdam, The Netherlands) di-

Table 1. Reported offending foods

Patient No.	Pollinosis	Offending foods (symptoms)
1	N	peach (UG) cherry (U) plum (U) apricot (U) almond (U) tomato (O)
2	GB	peach (OS) walnut (O)
3	N	apple (OU) peach (OU) cherry (OU) almond (O) walnut (U) hazelnut (O) <i>soybean (OU)</i> corn (O)
4	N	pear (U) peach (OU) walnut (O) hazelnut (OU) <i>stringbean (X)</i> fennel (U) kiwi (O) cabbage (U) mustard (O)
5	GBM	peach (G) walnut (U) melon (U)
6	MR	apple (O) pear (O) plum (O) walnut (O) hazelnut (O) <i>soybean (O)</i> <i>peanut (O)</i> fig (O) dates (O) mustard (O)
7	GR	peach (O) apricot (O) walnut (O) chestnut (O) corn (O) orange (O) melon (O) watermelon (O) kiwi (O) banana (O) grapes (O) persimmon (O) chocolate (O)
8	G	apple (OU) peach (OU) cherry (OU) plum (OU) apricot (OU) almond (O)
9	P	apple (U) pear (U) peach (U) plum (U) apricot (U) almond (U) walnut (U) hazelnut (U)
10	N	peach (C) walnut (UR)
11	GBMRP	peach (U) hazelnut (U) <i>peanut (U)</i>
12	N	apple (UG) pear (UG) peach (UG) cherry (UG) plum (UG) apricot (UG) almond (UG) walnut (UG) hazelnut (UG) <i>lentils (UG)</i> kiwi (UG) fig (UG) strawberry (UG)
13	N	peach (OC) <i>lentils (U)</i> <i>lupine (O)</i> orange (C)
14	N	apple (O) pear (O) peach (O) apricot (O) hazelnut (U)
15	G	apple (U) peach (O) walnut (U) hazelnut (O) <i>peanut (O)</i> <i>stringbean (U)</i>
16	GB	apple (O) peach (O) melon (O)
17	N	peach (U) walnut (O) hazelnut (O) <i>peanut (O)</i> pineapple (C) mustard (O)
18	GP	apple (UG) peach (U) <i>lupine (U)</i> <i>peanut (U)</i>
19	M	tomato (U)
20	G	apple (X) peach (X) banana (O) potato (C)
21	M	peach (O) plum (U) apricot (O) almond (O) walnut (O) hazelnut (O) pistachio (O) <i>white bean (OU)</i> <i>chickpea (OU)</i> <i>lentils (OU)</i> <i>peanut (O)</i> celery (O) fennel (O) corn (U) lemon (U) fig (O) tomato (O) chocolate (U)
22	G	almond (O) walnut (O) banana (O)
23	G	peach (GR) walnut (US) hazelnut (US) watermelon (S) grapes (GR)
24	N	apple (O) pear (O) peach (O) cherry (O) plum (O) pineapple (O) strawberry (O)
25	GP	fennel (U) pesto sauce (U)
26	N	strawberry (U) walnut (U) hazelnut (U)
27	N	apple (U) peach (O) cherry (O) apricot (O) almond (O) hazelnut (O) <i>peanut (O)</i>
28	N	almond (O)
29	G	walnut (G) hazelnut (G) celery (U) fennel (U) kiwi (U) grapes (G) mustard (X)
30	GRP	peach (UC)
31	G	apple (G) peach (U) cherry (U) apricot (O) <i>peanut (O)</i>
32	GBMR	apple (G) peach (G) apricot (G) beer (U)
33	GBMRP	peach (U) walnut (U) wheat (U) corn (U)
34	GM	apple (O) peach (O)
35	GBMR	peach (O) walnut (O)
36	N	peach (O) beer (U)
37	GP	apple (O) peach (U) cherry (G) plum (G) walnut (O) hazelnut (O) <i>peanut (O)</i> celery (O) fennel (O) corn (U) fig (O) onion (U) eggplant (O)

The clinical histories of the 37 patients in this study are summarized. Column two lists the pollen allergies observed in the patients.

Food allergies (column 3) are grouped in **bold** (Rosaceae), **bold italic** (treenuts), *italic* (legumes), and normal type (the rest).

G = Grass; B = birch; M = mugwort; R = ragweed; P = parietaria; N = no pollinosis; O = OAS; U = urticaria;

C = contact urticaria; G = gastrointestinal symptoms; X = anaphylaxis; S = asthma; R = rhinitis.

rected to human IgE (in 500 µl PBS-AT plus 4.5% bovine and 0.5% sheep serum (v/v): PBS-AT+BS) were added (approximately 20,000 cpm per test). After overnight incubation and washing, bound radioactivity was measured. Results were expressed as international units IgE per milliliter (IU/ml). Calculation was performed

by means of a standard curve that was obtained by RAST with a dilution series of a chimeric monoclonal IgE antibody against the major housedust mite allergen Der p 2 and Sepharose-coupled mite extract [55]. A result of >0.3 IU/ml was regarded as positive. One IU is 2.4 ng IgE.

Table 2. Skin reactivity to non-Rosaceae food

	Patient No.																																					
Food	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	
Walnut																											N											
Hazelnut										N								N																				
Green pea										N																	N											
White bean										N																												
Soybean										N																	N											
Peanut										N																												
Celery																																						
Carrot										N																												
Wheat										N																												
Oat										N																												
Rye										N																												
Barley										N																												
Rice										N																												
Corn										N																												
Orange										N																	N											
Banana										N																	N											
Potato										N																	N											
Tomato										N																	N											
Onion										N																	N											
Garlic										N																	N											

SPT results: white = negative; light gray = 2+; dark gray = 3+; black = 4+. Some patients were not tested with all foods; this is indicated with N.

For RAST inhibition, serum was pre-incubated with (serial) dilutions (in 50 µl PBS-AT) of inhibitor (carrot rLTP or food extract), prior to addition of Sepharose-coupled LTP or food extract. For the uninhibited value, serum was preincubated with 50 µl PBS-AT. Subsequent steps were identical to those described for the RAST. Results were either expressed as percentage inhibition, or as IU/ml.

SDS-PAGE/Immunoblotting

Apple extract was separated by SDS-PAGE using 12.5% polyacrylamide gels (protein: 10 µg/cm). After separation, protein was transferred (semi-dry) to nitrocellulose on a Novablot electrophoretic transfer apparatus (Pharmacia). After blocking with PBS, 1.8% (w/v) BSA, 0.1% (v/v) Tween 20, the blots were cut into strips prior to immunoprobng with 150 µl human serum. Radiolabeled sheep antibodies against human IgE (in 5 ml PBS-AT + BS) were used for detection of bound IgE.

Competitive RIA

Recombinant carrot LTP and a pepsin-digest thereof were compared in a competitive RIA [56] with polyclonal antiserum against natural carrot LTP. Binding of radiolabeled purified carrot LTP to this antiserum was inhibited with both recombinant samples. To this end, 0.2 µl of antiserum was incubated for 2 h with serial dilutions of carrot recombinant (r)LTP or of its digest in a final volume of 100 µl PBS-AT. Then, 0.5 mg Protein A Sepharose (Pharmacia) and 20,000 cpm ¹²⁵I-labeled natural carrot LTP was added in a final volume of 900 µl PBS-AT. After overnight incubation by end-over-end

rotation, samples were washed and bound radioactivity was measured. Results were expressed as percent binding of radiolabeled LTP.

Pepsin Digestion

Pepsin digestion was carried out by dissolving peach extract or diluting LTP preparations (carrot rLTP in culture supernatant or radiolabeled natural carrot LTP) in 10 mM HCl and adding pepsin to a final concentration of 0.35% (w/v) and pH 2. Digestion was performed at 37°C for 1 h. Digestion was stopped by addition of 0.5 volume of 0.5 mol/l K₂HPO₄. Efficacy of the digestion was monitored by peach RAST inhibition with serum of a typical birch-fruit-allergic patient with Bet v 1-related cross-reactivity. After pepsin treatment >99% of the inhibitory potency of peach extract was lost [57]. Peach extract was also incubated for 1 h in 10 mM HCl without pepsin. This had no effect on the binding of cross-reactive anti-Bet v 1 IgE.

Results

Clinical Observations

Thirty-seven patients suspected of food allergy to Rosaceae fruits were included in this study (table 1). Peach was identified most frequently as an offending Rosaceae food (in 30/37 patients), followed by apple (16/37), apri-

cot (11/37), cherry (9/37), plum (8/37), almond (7/37) and pear (6/37). Four patients did not have any clinical symptoms related to Rosaceae fruits, despite a positive SPT with commercial plum extract and peach peel extract. All 37 patients were interviewed for allergies to other foods. Walnut and hazelnut were most frequently reported as offending foods (19/37 and 15/37, respectively), closely followed by peanut (9/37). For the other 33 different foods, the number of patients reporting symptoms ranged from 1 to 5. These foods were found among all major groups of vegetable foods, including cereals (corn, wheat), legumes (soybean, string bean, white bean, chick pea, lentils, lupine), Solanaceae (potato, tomato, eggplant) Brassicaceae (cabbage, mustard), Umbelliferae (celery, fennel), Rutaceae (lemon, orange), and several other plant families. On average, individual patients reported allergy to 3 non-Rosaceae foods. When Rosaceae were included, the average number of offending foods was 5. OAS was the most frequent manifestation of food allergy ($n = 107$), followed by urticaria ($n = 87$). Gastrointestinal symptoms were reported 26 times. Contact urticaria and anaphylaxis were both observed 4 times.

Most patients were skin tested with a panel of 20 non-Rosaceae food extracts (table 2). Six patients did not show any positive SPT in addition to Rosaceae fruits (No. 1, 8, 13, 24, 30 and 31). Of a total of 709 skin tests, 246 were positive. Only 18% of these positive results were concordant with the reported symptoms. Legumes were responsible for 89 positive SPTs, of which only 9 were true positives. Only in case of walnut and hazelnut, was a majority of the positive skin tests concordant with the reported symptoms (10/14 and 11/18, respectively). For all other foods, the fraction of true-positive skin tests was below 30%. In contrast, 95% of all negative SPT results were true negatives. Only three foods showed >10% false-negative results: walnut (8/22), hazelnut (5/17) and peanut (2/11).

IgE Reactivity to Purified Natural and Recombinant LTPs

Sera of 34 patients (all but No. 12, 27, 28 and 32) were tested by RAST for IgE against natural carrot and broccoli LTP as well as recombinant wheat LTP. Carrot LTP was recognized by 21, broccoli LTP by 18, and wheat LTP by 4 sera. Sera with wheat-LTP specific IgE were No. 7, 33, 35 and 36. Sera of 29 patients (all but No. 11, 18, 19, 23, 24, 26, 29, 33 and pf194) were available for measurement of specific IgE against natural peach LTP. Twenty-one sera were positive. None of the peach-LTP-negative sera demonstrated a positive RAST to the other LTPs tested.

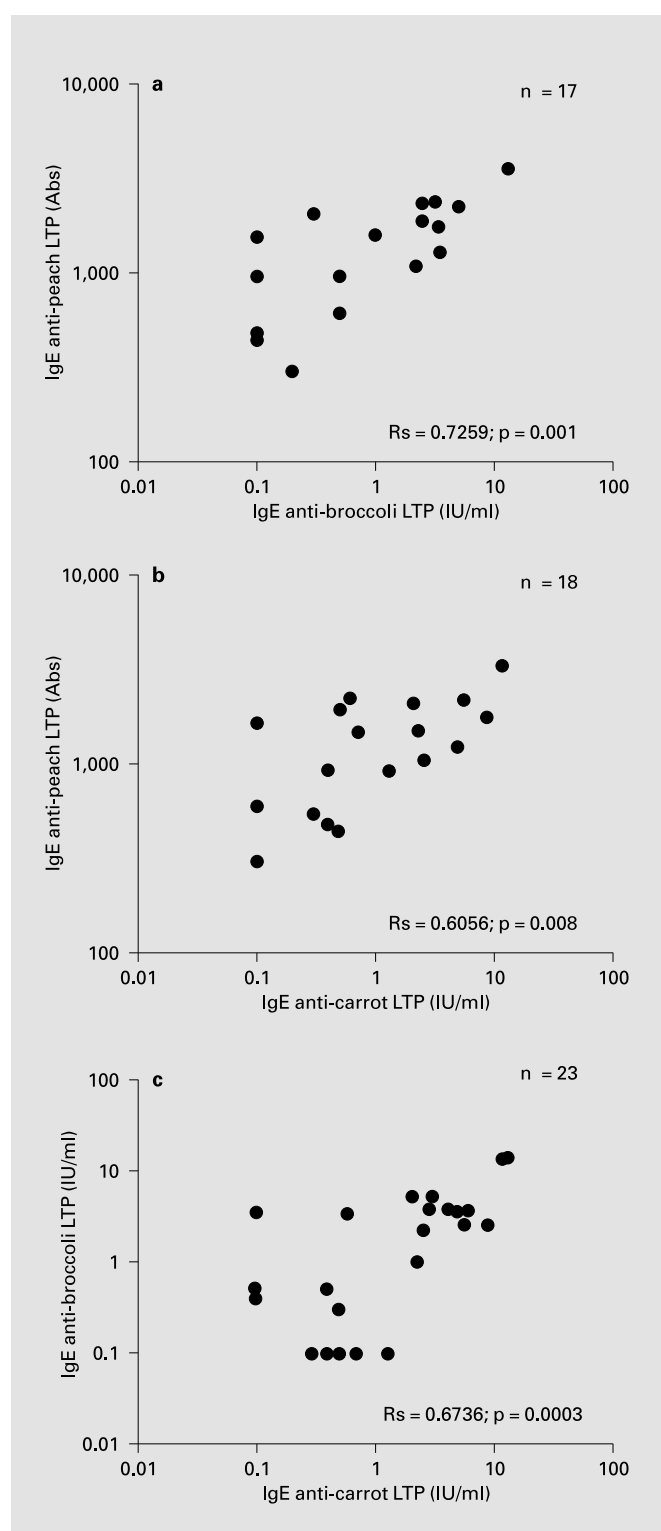


Fig. 1. RAST for purified LTPs. Correlations are shown between IgE responses to three different purified natural LTPs: broccoli versus peach (**a**), carrot versus peach (**b**), and carrot versus broccoli (**c**). Results are expressed in IU/ml of IgE for broccoli and carrot. For peach, the absorbance (Abs) at 450 nm is used.

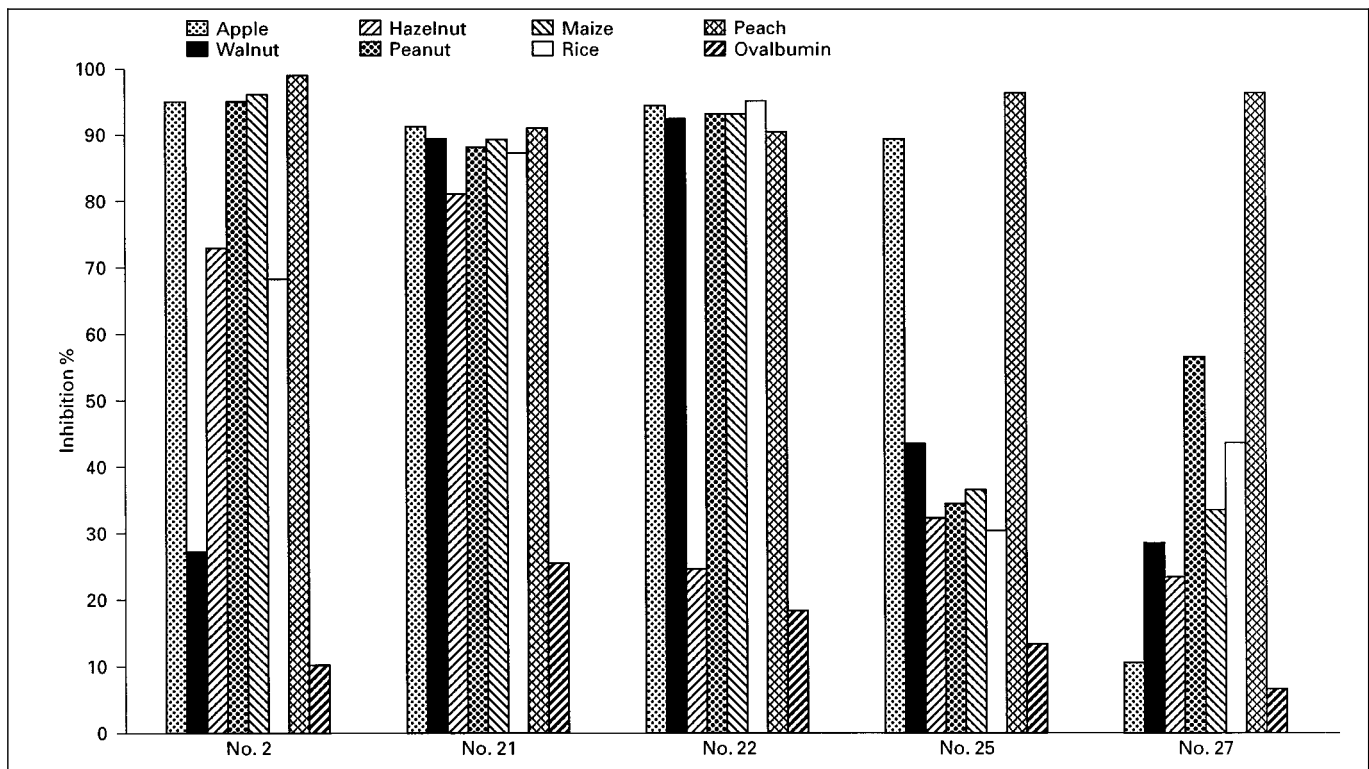


Fig. 2. Peach LTP ELISA inhibition. Binding of IgE to peach LTP was inhibited with different food extracts (0.6 mg/ml). Results are shown for 5 sera to illustrate the different patterns of cross-reactivity. Uninhibited absorbances in the peach LTP ELISA were 1,756, 1,888, 1,586, 1,294 and 551, respectively (from left to right). The results are expressed in percentage inhibition.

Table 3. IgE against pollen-derived cross-reactive allergens

Patient No.	Bet v 1	Profilin	CCD
2	4.9	4.1	0.5
5	2.1	neg	neg
7	neg	32.0	0.4
9	NT	neg	NT
11	neg	26.7	neg
15	neg	0.4	neg
16	2.3	0.7	neg
18	neg	1.4	0.7
20	neg	5.9	neg
22	neg	7.3	1.0
23	neg	NT	0.8
29	neg	0.8	neg
33	neg	4.0	neg
34	neg	neg	1.5
35	54.7	28.5	1.5
36	0.7	neg	0.3
37	neg	neg	0.6

Results are expressed in IU/ml of IgE. Results ≤ 0.3 are negative (neg). NT = not tested.

At the same time, peach-LTP-positive sera frequently did not show IgE against other LTPs. This was observed in 4/18 sera for carrot, 6/18 broccoli and 15/18 wheat. IgE responses to the 3 natural LTPs did, however, demonstrate strong correlation as judged by Spearman rank correlation analysis (fig. 1).

IgE Antibodies against Pollen-Derived Cross-Reactive Structures

Allergy to vegetable foods is frequently caused by pollen-vegetable food cross-reactive IgE. Sera of 34 patients (all but No. 12, 27, 28 and 32) were tested for IgE against the three cross-reactive structures involved: Bet v 1, profilin (from *L. perenne* grass pollen) and pollen-derived carbohydrate structures (table 3). Seventeen sera were negative for all 3 structures tested. Five sera had specific IgE against Bet v 1 (15%), 11 against profilin (29%) and 9 against proteinase-K-resistant structures (27%).

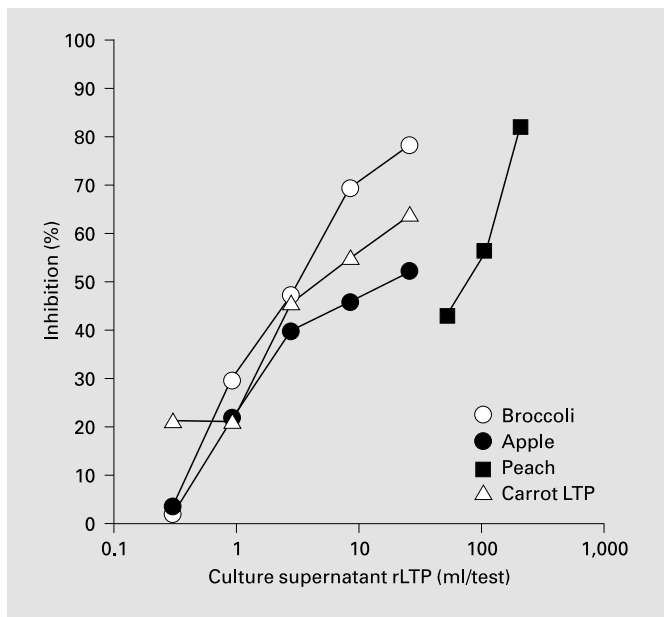


Fig. 3. RAST inhibition with carrot rLTP. IgE binding of pf194 to broccoli, apple, peach and natural carrot LTP was inhibited with *P. pastoris* culture supernatant, containing carrot rLTP. The results are expressed in percent inhibition.

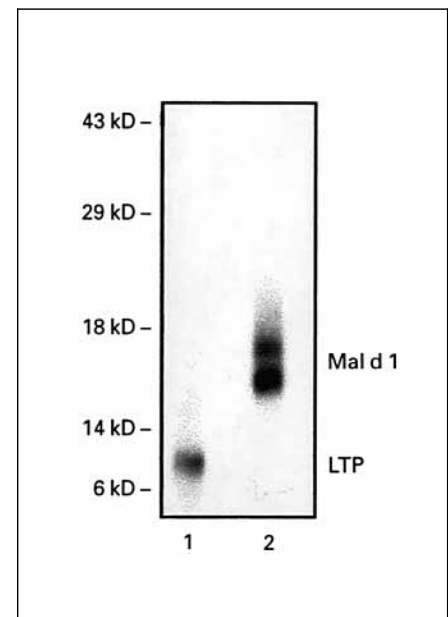


Fig. 4. Apple immunoblot analysis. Apple immunoblot was tested for IgE reactivity with serum pf194 (lane 1) and serum reactive with the major apple allergen Mal d 1 (lane 2). MW markers are indicated on the left side.

Anti-LTP IgE Is Highly Cross-Reactive

A close correlation between IgE responses to different LTPs is no direct proof of cross-reactivity. Therefore, the peach LTP ELISA was inhibited with 6 different food extracts: apple, walnut, hazelnut, peanut, maize and rice ($n = 21$). The mean percentage inhibition ($\pm$ SD) were 74 ± 30 , 57 ± 32 , 51 ± 25 , 71 ± 22 , 71 ± 26 and $67 \pm 25\%$, respectively. Peach extract was used as a positive control ($94 \pm 4\%$) and ovalbumin as a negative control ($15 \pm 10\%$). Though peach LTP demonstrated a high degree of cross-reactivity to all 6 foods tested, different patterns of cross-reactivity were observed (fig. 2). To confirm the cross-reactive nature of IgE antibodies against LTP, a RAST inhibition was performed with rLTP from carrot as inhibitor. IgE binding of serum pf194 to peach, apple, broccoli and natural carrot LTP was inhibited with the recombinant protein (fig. 3). This serum is negative in a RAST for Bet v 1, profilin and carbohydrates and exclusively recognizes LTP on vegetable-food immunoblots, as is shown for apple extract in figure 4. To further illustrate the extreme cross-reactivity of IgE antibodies against LTP, IgE binding of serum pf194 to 15 different vegetable foods was inhibited with apple extract (fig. 5). The mean percentage inhibition was $88 \pm 17\%$.

Influence of Pepsin Treatment on LTP

To test the stability of LTP as an allergen under the acidic and proteolytic conditions of the stomach, carrot rLTP was incubated for 1 h at 37°C in 0.35% pepsin/10 mmol/l HCl. Treated rLTP inhibited the binding of radiolabeled carrot LTP to a monospecific rabbit antiserum as well as the untreated control (fig. 6a). Radiolabeled rLTP was also treated with pepsin and compared to untreated rLTP by SDS-PAGE in conjunction with autoradiography. No change in MW was observed (fig. 6b).

To investigate whether IgE binding is equally resistant to pepsin treatment, peach extract was treated and used to inhibit IgE binding (pf194) to purified broccoli LTP and to peach extract. No significant difference was observed between the inhibitory potency of treated and of untreated peach extract (fig. 6c). In contrast, binding to peach extract of IgE from a typical birch-fruit-cross-reactive subject was not inhibited by digested peach extract, illustrating the lability of the Bet v 1 homologue under these conditions.

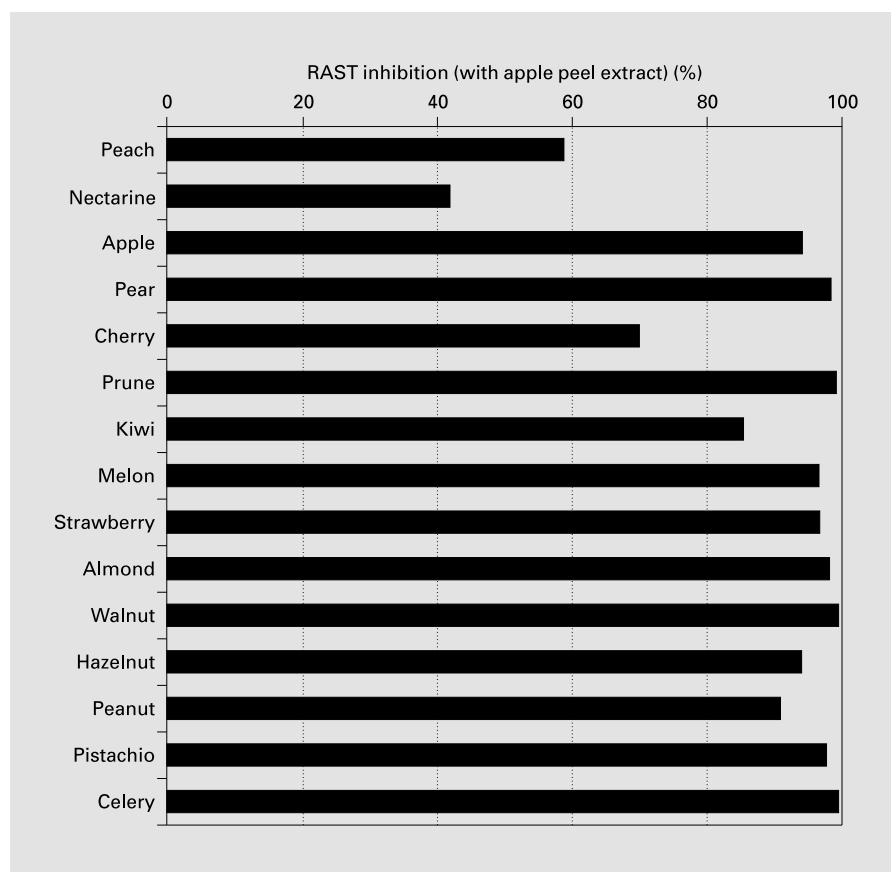


Fig. 5. RAST inhibition with apple peel extract. IgE binding of pf194 to 15 different vegetable food extracts was inhibited with apple peel extract (2.3 mg/ml). Results are expressed in percent inhibition.

Discussion

By the application of purified natural and recombinant LTPs from Rosaceae and non-Rosaceae foods, it was demonstrated that LTP is a genuine pan-allergen. The potential spectrum of cross-reactivity is comparable to that reported earlier for profilin [58, 59]. Sensitization to profilin was shown to be caused by exposure to pollen leading to IgE cross-reactivity to profilins in foods [6, 60, 61]. In contrast, IgE reactivity to LTPs is not exclusively observed in pollen-allergic subjects, suggesting that sensitization occurs by the oral route. Since peach LTP was most frequently recognized and selective recognition of other food LTPs was not observed, peach is a likely candidate for primary sensitization in this group of patients. Yet cross-reactivity to homologous structures in pollen seems to exist. IgE binding of serum pf194 to apple and hazelnut was inhibited with mugwort pollen extract by 35 and 90%, respectively (not shown). Grass pollen and birch pollen extract did not inhibit IgE binding with these Rosaceae LTPs, similar to what was reported earlier for

Prunoideae LTPs by Pastorello et al. [17]. Another indication for the presence of related allergens in weeds is the homology shared by the major *Parietaria judaica* allergen, Par j 1, with LTPs [62–65]. So far, however, no proof for cross-reactivity to foods was reported.

As was earlier described for Bet v 1-related food allergens and for profilins, patterns of cross-reactivity differed considerably between individual patients [57]. This implies that it is not possible to design diagnostic tests on the basis of a single LTP (e.g. peach LTP) that will predict reactivity to a defined panel of foods. Among LTP molecules, most likely, a group of cross-reactive epitopes with different patterns of cross-reactivity is present. The selection of these epitopes recognized as well as the affinity of the interaction will then determine the range of foods to which the individual will be reactive.

Seven patients with clinical peach allergy had no significant levels of IgE against peach LTP, although the majority (5/6 tested) had specific IgE against peach as judged by CAP analysis (not shown). Only two of these patients had IgE against profilin, and one against Bet v 1. Therefore,

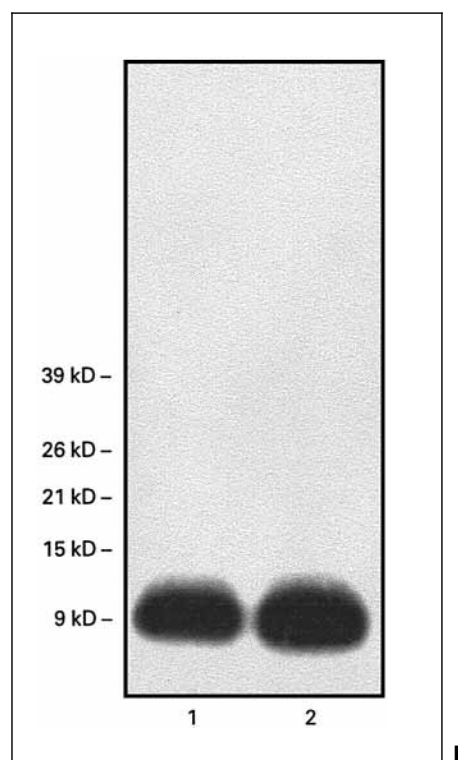
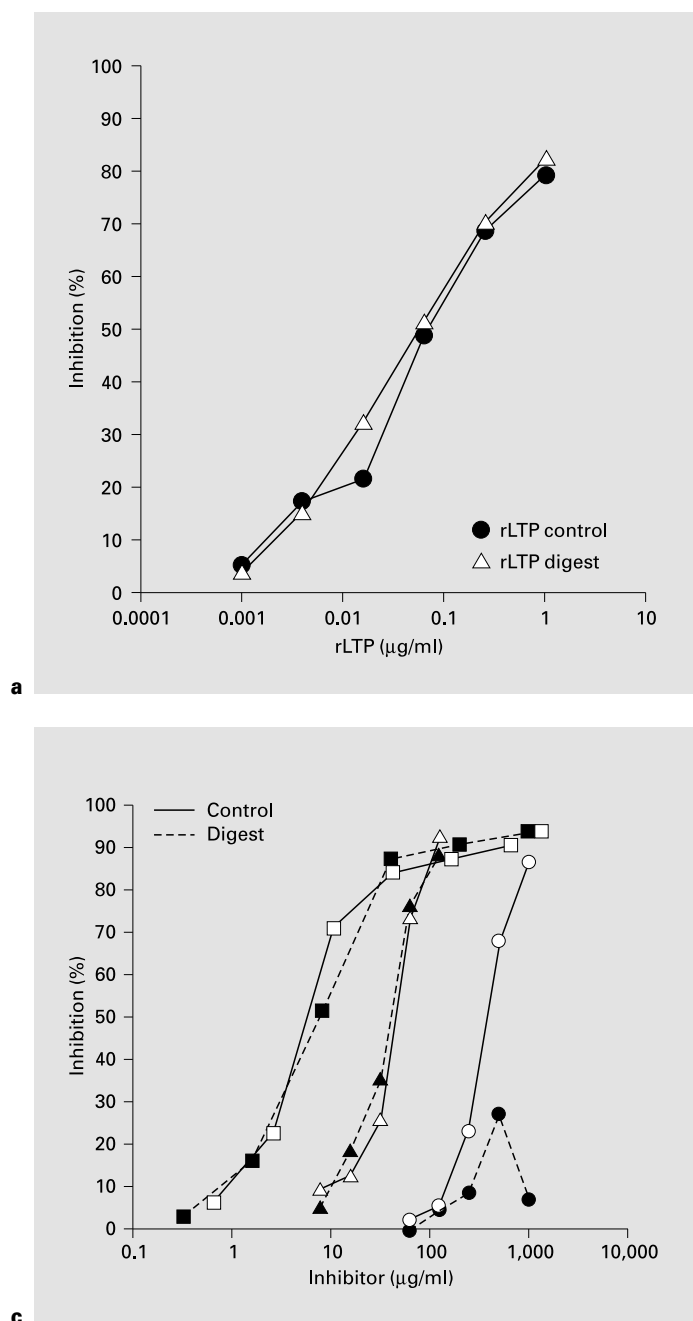


Fig. 6. Influence of pepsin treatment. **a** Binding of radiolabeled natural carrot LTP to rabbit anticarrot LTP was inhibited with pepsin-treated and untreated rLTP. Results are expressed in percent inhibition. **b** SDS-PAGE analysis of untreated (lane 1) and pepsin-treated (lane 2) radiolabeled carrot rLTP. **c** Binding of IgE (pf194) to purified broccoli LTP (□, ■) and to peach extract (△, ▲) was inhibited with pepsin-treated and untreated peach extract in a RAST inhibition. No differences were observed between control and digest. As positive control for the pepsin treatment, the same assay was performed with a serum reactive with Bet v 1-related peach allergen Pru p 1 (○, ●). In this case, no significant inhibition was observed with peach digest. Results are expressed in percent inhibition.

other allergens than LTP and these pollen-related cross-reactive structures are likely to be involved. Over the past years, several cross-reactive allergens in fruits and other vegetable foods were identified that could play a role. Among these are thaumatin, chitinases and 2S-albumins [66–73]. At the same time, we have to realize that clinical histories in this study were not confirmed by double-blind placebo-controlled food challenges. It is, therefore, possi-

ble that some subjective reports would not be supported by such a challenge, although the clinical histories were convincing.

Selection of patients from a group of patients allergic to Rosaceae foods by positive SPT to a commercial plum extract and a peach peel extract resulted in a subgroup of patients with significantly more systemic reactions (besides OAS) and with a lower frequency of pollinosis [14,

15]. In agreement with this, only 15% of the selected patients had specific IgE antibodies against Bet v 1, and 29% against profilin. For the typical pollen-fruit-allergic patients with OAS, these numbers are much higher [3, 6, 60]. Recognition of peach LTP in this group was much higher: >70%. Together, these observations suggest that IgE against LTP is correlated with severer symptoms of food allergy. A likely explanation for this phenomenon, earlier suggested for some other food allergens [74, 75], is the extreme stability of LTP under the acidic and proteolytic conditions of the gastrointestinal tract. The pepsin digestion experiments support this hypothesis: IgE binding to LTP is insensitive to pepsin treatment of the aller-

gen. In contrast, Bet v 1 loses its IgE-binding capacity within 15 s under similar conditions [57].

In conclusion, LTP has been shown to be a genuine pan-allergen that derives its high allergenicity from its extreme resistance to digestion. Purified and recombinant LTPs will prove to be useful tools to distinguish between potentially severe and mild vegetable food allergies [57].

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