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Respiratory allergy to peach leaves and lipid-transfer proteins

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Summary

Background Several lipid-transfer proteins (LTPs) have been identified as important food allergens, especially in fruits of the *Rosaceae* family. The major peach (*Prunus persica*) allergen has been identified, sequenced and designated Pru p 3.

Objective To present Pru p 3 as an aeroallergen able to induce occupational asthma.

Methods A thorough investigation was performed in a fruit grower with occupational asthma. Skin prick–prick tests with peach leaves and prick tests with perennial respiratory allergens and pollens, fruits and peach leaf extracts were done. Serum-specific IgE was tested for peach leaf, peach fruit, peach skin and respiratory allergens that were positive in skin prick tests. Specific bronchial provocation tests (BPTs) with extracts of peach leaf were also done. Before and 24 h after the BPT, BPTs with methacholine and sputum induction were done. The IgE reactivity pattern to peach leaf and fruit extracts and to Pru p 3 was identified by using SDS-PAGE and immunoblotting. Blotting inhibition of peach leaf extract by Pru p 3 was also performed. The putative allergen was quantified in leaf and fruit skin extracts with ELISA based on an anti-Pru p 3 antibody.

Results Skin tests were positive for peach leaf and fruit. The BPT was positive, with immediate and delayed response. This test induced a decrease in PD_{20} (dose of agonist that induces a 20% fall in FEV_1) methacholine and an increase in eosinophils and eosinophil cationic protein in sputum. Peach leaf extract contained concentrations of Pru p 3 similar to those found in peach skin. Specific IgE immunodetection showed that patient's sera reacted with Pru p 3, and with a single major band from the peach leaf extract fully inhibited by Pru p 3.

Conclusion Pru p 3 from peach leaves can act as a respiratory allergen and cause occupational rhinoconjunctivitis and asthma.

Keywords lipid-transfer proteins, occupational asthma, peach, Pru p 3, respiratory allergy
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Introduction

Plant lipid-transfer proteins (LTPs) are a family of around 9 kDa polypeptides composed of 90–95 amino acids, with eight cysteine residues that form four disulphide bridges [1]. Currently, their main activity *in vivo* is thought to be related to plant defences against pathogens, and they are, therefore, classified as belonging to group 14 of the pathogenesis-related proteins (PR) [2]. As befits this function, these proteins are located mainly in the outer layers of plant organs [1].

Several LTPs have been identified as important food allergens, especially in fruits of the family *Rosaceae* [3, 4]. Pru p 3, the major peach (*Prunus persica*) allergen, is probably the best characterized, being fully sequenced at the protein level [5], as well as cloned and expressed as recombinant protein [6]. Pru p 3 is highly homologous to allergens of other fruits of the subfamily *Prunoideae* such as apricot (Pru ar 3) [7], cherry (Pru av 3) [8] and plum [9], a feature responsible for the high degree of clinical cross-reactivity between these fruits [10]. The degree of homology with the major apple allergen Mal d 3 is also high [6]. LPTs that act as food allergens have also been identified in other phylogenetically distant plant foods [11].

The first LTP known to be potentially allergenic was Par j 1 [12], the main allergen of *Parietaria judaica* pollen. This respiratory allergen, which is widespread in the Mediterranean region, shows less homology with other allergenic LTPs but is highly similar to Gly m 1 (previously designated soybean hydrophobic protein), a soybean hull allergen identified as the main cause of epidemic outbreaks of asthma in Spain [13]. Another LTP known to be an important respiratory allergen is Ole e 7, of *Olea europaea* (olive) pollen, which is recognized by 60% of patients allergic to olive pollen [14]. There is no proof that sensitization to Par j 1 is responsible for the clinical cross-reactions to fruits in patients diagnosed as being allergic to *Parietaria* pollen. However, it has been shown that in patients allergic to olive and

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vegetables, sensitization to Ole e 7 is more frequent among patients sensitized to *Rosaceae* allergens with clinical manifestations of anaphylaxis than among those with oral allergy syndrome [15]. Cross-reactivity has also been found, both *in vitro* and *in vivo*, for the LTPs of *Artemisia vulgaris* pollen, Pru p 3 and Mal d 3 in patients allergic to *Rosaceae* fruits [11, 16].

Because of their wide distribution in the plant kingdom and in different plant organs (roots, stems, leaves, pollen and fruits), LTPs could be implicated both in food and in respiratory allergy. This is the case of Asp o 1.1 and Asp o 1.2 recently identified as relevant allergens in patients with asthma, anaphylaxis and/or contact urticaria after asparagus ingestion or exposure [17]. We present data from the study of a patient with occupational asthma that clearly support that Pru p 3 can act as an aeroallergen as well.

Methods

Patient

A patient was referred to the Sección de Alergología of the Hospital Virgen del Camino (Pamplona, Spain) because of suspected respiratory allergy. A detailed history was obtained, and physical examination and basal spirometric tests were complemented by the tests described below. As a control for specificity, the same tests were done in a patient with suspected contact allergy to peach leaves, but who did not have respiratory symptoms.

The patient was 27-year-old man who worked as a farmer and who was a non-smoker. He reported a 7-year history of symptoms of rhinoconjunctivitis, cough, dyspnea and wheezing when he was in the peach grove when the trees bore leaves (from May to September). The exposure period needed for symptoms to appear had become steadily shorter, and at the time of consultation was 1 h. Symptoms appeared immediately and dyspnea persisted for several hours. He tolerated exposure to peach fruit in the warehouse and handling. During the first 18 months after developing respiratory symptoms the patient tolerated peach ingestion and then he started to suffer oropharyngeal itching after peach ingestion. He also reported oropharyngeal itching when he ate kiwis. Physical examination and basal spirometric values were normal.

The control was a 51-year-old man who worked as a farmer reported outbreaks of itchy, weals in exposed areas of the skin, which appeared immediately upon contact with peach leaves. He had no respiratory symptoms and was able to eat peaches with no problems.

Peach extracts

A 10% w/v extract of freeze-dried peach leaves or of peach skin in phosphate-buffered saline (PBS) buffer (0.1 mmol/L sodium phosphate (pH 7.0) and 0.15 mmol/L NaCl) was obtained and centrifuged. The supernatant was dialysed against PBS buffer and filtered (0.2 μm pore diameter), and the concentration of protein was determined with the Lowry method to be 4.35 and 2.5 mg/mL, respectively.

In vivo *study*

Skin tests Prick tests were done with a battery of inhalants (ALK-Abelló, Madrid, Spain) that included perennial allergens (house mites, warehouse mites, *Tetranichus*, moulds, *Ficus*, latex, cat and dog) and pollens (*Alnus glutinosa*, *A. vulgaris*, *Corylus avellana*, *Cupressus arizonica*, *Cynoden dactylon*, *Chenopodium album*, *P. judaica*, *Phragmites communis*, *Pinus* spp, *Platanus acerifolia*, *Populus nigra*, *Quercus ilex*, *Rumex acetosella*, *Secale cereale*, *Tilia cordata*, *Ulmus campestris*, *Urtica dioica*, *Mercurialis annua*, *Phleum pratense*, *Salsole kali*, *O. europaea* and *Plantago lanceolata*). Also tested were the fruits peach (ALK-Abelló), strawberry, orange, melon, apple, pear, fig, prune, dark grapes, white grapes, cherry, banana, kiwi and blackberry. Skin tests were done with the prick–prick method for peach leaf, and with the prick method for peach leaf extract. As positive and negative controls we used histamine chlorhydrate at 10 mg/mL and 0.9% saline solution, respectively (ALK-Abelló).

Bronchial provocation All functional tests were done with the same spirometer (CPR, Medical Graphics Corp., St Paul, MN, USA). Before provocation tests were done a basal value of forced expiratory volume in 1 s (FEV_1) $\geqslant$ 70% was required. Patients did not receive anti-asthma medication except for short-acting β-adrenergic agonists as needed, but not for 8 h before the bronchial provocation tests (BPTs). All tests were done with the dosimetric method (Mefar dosimeter with output 10 μL per inhalation; Mefar SpA, Bovezzo, Italy).

For unspecific bronchial provocation, methacholine (Provocholine, Roche, Nutley, NJ, USA) was used as the agonist. Solutions of methacholine of 5–25 mg/mL in 0.9% saline solution were used and a standardized abbreviated method was used [18]. It was performed under basal conditions and 24 h after allergen bronchial challenge.

The specific BPT was done with peach leaf extract. An initial concentration of 10% w/v was used to prepare 2-fold dilutions in 0.9% saline solution, to enable selection of a safe initial dose. Spirometric values were recorded 10 min after five inhalations of each solution until FEV_1 had decreased by 20% or more, or until the highest concentration of the extract had been inhaled. After this time spirometric tests were done 20, 30, 60 and 90 min, then peak expiratory flow was recorded hourly for the following 12 h.

Induced sputum test Sputum samples were obtained with a technique reported previously [19] the day before and 24 h after the allergen bronchial challenge. Cell differential counts and levels of eosinophil cationic protein (ECP) in the supernatant of induced sputum (Pharmacia CAP System, Uppsala, Sweden) were recorded.

In vitro *studies*

Specific IgE Serum-specific IgE was measured with the CAP System for respiratory allergens that were positive in the skin prick test, and by RAST for peach skin and leaves. Paper discs previously activated with BrCN were sensitized with one of the two extracts alternatively [20], and the discs were incubated with 50 μL of the patient's serum and then with approximately 100 000 c.p.m. of [^{125}I]anti-human IgE mAb HE-2 [21]. As a reference we used discs sensitized with *Lollium*

perenne and four dilutions of a mixture of sera from patients allergic to grass pollens. The mixture was calibrated with the Pharmacia Phadebas RAST system (Uppsala, Sweeden).

IgE immunodetection and inhibition tests Peach leaf and peach skin extracts and rPru p 3, were analysed by SDS-PAGE (15% acrylamide) under non-reducing conditions. The proteins in the PAGE were transferred electrophoretically to nitrocellulose strips [22]. The strips were saturated with 5% bovine serum albumin (BSA) in PBS for 1 h at room temperature and incubated for 18 h with the patient's serum at a dilution of 1 : 5. To inhibit immunodetection, the serum was pre-incubated for 1 h with 4 μg of Pru p 3. As a negative control a nitrocellulose strip with peach leaf and fruit skin proteins was incubated with diluent (1% BSA and 0.1% Tween-20 in PBS). The strips were washed with 0.1% Tween in PBS and incubated for 2 h at room temperature with a 1 : 2000 dilution of the biotinylated anti-human IgE mAb HE-2 anti-IgE [21], and then incubated in streptavidin-peroxidase (Amersham, Piscataway, NJ, USA). As a final step the strips were washed and the IgE-binding proteins were detected with a chemoluminescence method (ECL-Amersham, Piscataway, NJ, USA) according to the manufacturer's instruction.

Pru p 3 quantification The peach allergen was measured using a monoclonal antibody, as recently reported [23].

Results

Skin test with leaf as is and with leaf extract were positive both in patient and control, but only in patient's serum IgE to peach leaf and skin was detected (Table 1). In the patient BPT with peach leaf extract was positive, with a dual response (Fig. 1), and induced an increase in bronchial reactivity to methacholine, in eosinophil count and in levels of ECP in induced sputum (Table 2). In the control subject, specific bronchial challenge was negative and no change in reactivity to methacholine was detected.

Figure 2 shows the results of IgE-immunodetection studies. The patient's IgE recognized mainly a protein of approximately 10 kDa present in both the peach leaf extract and fruit skin extracts, and also recognized rPru p 3. Recognition of the protein in the leaf extract was fully inhibited by pre-incubating the patient's serum with rPru p 3.

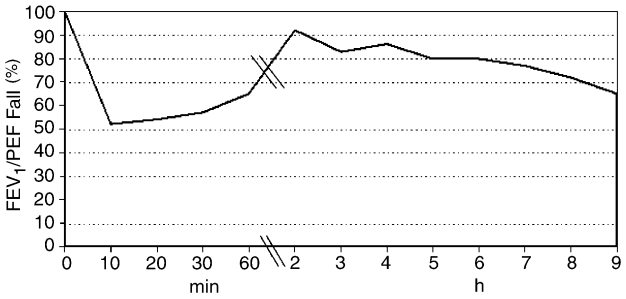

Fig. 1. Specific bronchial provocation test with peach leaf extract.

The amount of LTP in leaf extract (Fig. 3) was similar (approximately 700 μg/g raw matter) to that detected in the peach skin extract. The binding curves of the anti-Pru p 3 monoclonal antibody to LTP were similar in leaf and fruit skin extracts, with no significant differences in the slope of the curves.

Discussion

The main source of plant aeroallergens is pollen – both the pollen grains *per se* and submicronic particles created by osmotic shock of the grains. Parts of the plant other than pollen can also express pollen allergens and give rise to airborne pauci or submicronic particles [24]. In addition, increasing attention is being given to the allergenicity of ornamental plants such as *Ficus benjamina* [25], whose leaves produce non-pollen aerosol allergens from sap.

Until now, the respiratory allergenic potential of LTPs expressed in non-pollen structures such as leaves has not been documented. In the present article we report that peach leaves can act as a source of a protein that acts as a respiratory allergen. This conclusion is supported by the immediate and delayed functional responses to specific bronchial provocation, and by the observation that exposure induces a specific inflammatory response in the airways, confirmed by biological (increased sputum eosinophil count and sputum ECP) and functional changes (increased bronchial sensitivity to methacholine) that are completely compatible with the clinical manifestations. The specificity of these findings was confirmed by the negative results of tests in the control patient, who had contact urticaria and sensitization to peach leaves

Table 1. Results of skin tests and serum specific IgE

	Skin tests (mm diameter)					Serum specific IgE		
		Prick				RAST (classes)		
	Prick–prick Leaf	Leaf extr.	rPru p 3	Peach	Other	Leaf extr.	P. skin	CAP (kU/L)
Patient	8 × 8	7 × 7	5 × 5	5 × 5	D. p. 5 × 5 A. v. 4 × 4 Kiwi 8 × 6	1	2	Peach 4.01 D. p. 1.03 A. v. 1.27 Kiwi 0.41
Control	10 × 6	5 × 4	–	–	D. p. 4 × 4	–	–	D. p. 0.44

Leaf extr., peach leaf extract; D. p., *Dermatophagoides pteronyssinus*; A. v., *Artemisia vulgaris*; P. skin, peach skin; –, negative.

Table 2. Results of bronchial provocation tests and analysis of induced sputum

	Pre-BPT			BPT	Post-BPT		
	PD_{20}-m	EOSs	ECPs	PD_{20}-a	PD_{20}-m	EOSs	ECPs
Patient	12.8	0	14.9	20.8	2.46	19	37.9
Control	–	ND	ND	–	–	ND	ND

BPT, specific bronchial provocation test; PD_{20}-m, PD_{20} methacholine (μmol); EOSs, eosinophils in sputum (%); ECPs, eosinophil cationic protein in sputum (μg/L); PD_{20}-a, PD_{20} allergen (cumulative units); PD_{20}, dose of agonist that induces a 20% fall in FEV_1; –, not detectable; ND, not done.

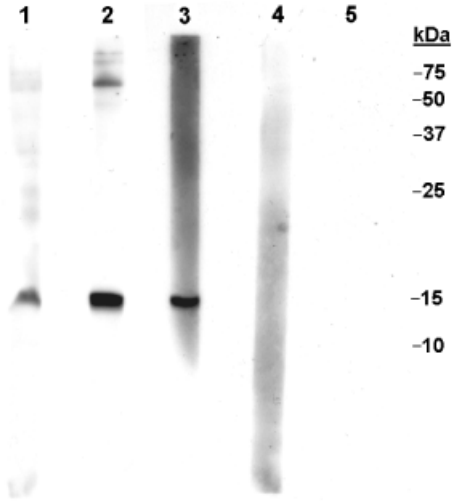

Fig. 2. IgE immunodetection. Lane 1, peach leaf extract; lane 2, peach fruit skin extract; lane 3, rPru p 3; lane 4, immunodetection in peach leaf extract inhibited by rPru p 3; lane 5, negative control. The molecular weight pre-stained markers, run in parallel, are indicated on the right.

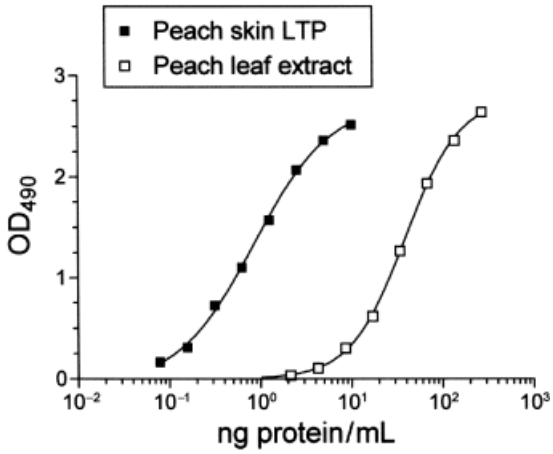

Fig. 3. ELISA curves obtained with anti-Pru p 3 monoclonal antibody in peach fruit skin (reference preparation) and leaf extracts.

but no bronchial asthma. The causative protein, shown by SDS-PAGE to have a molecular size of approximately 10 kDa, is also present in the skin of peach fruit, and pre-incubation of the patient's serum with Pru p 3 inhibited the IgE recognition of the protein. Specific IgE immunodetection showed that patient's serum also reacted with purified Pru p 3. Moreover, the affinity of the anti-Pru p 3 monoclonal antibody for LTP in peach leaf and fruit skin was similar, as illustrated by the parallel slopes of the curves obtained by ELISA.

These findings suggest that the protein that acted as a respiratory allergen in our patient was the Pru p 3 from peach leaf. The ability of Pru p 3 to cause sensitization and trigger symptoms when ingested is well-documented [5, 7, 15, 16]. However, its ability to act as a respiratory allergen has not been clearly established. Patients who have been sensitized via respiratory exposure would be expected to show symptoms of food allergy to peach given the stability of Pru p 3. A recent report described a patient with antecedents of food allergy to peach (itching) who also had occupational asthma due to inhalatory exposure to peach. However, the allergen responsible was not identified [26]. The evolution of the clinical symptoms of our patient suggests an inverse scenario, inhaled exposure to peach LTP being the primary sensitization route.

Evidence for the role of LTPs as occupational allergens is limited. A single case of occupational sensitization to *Triticum spelta* LTP [27], manifested as contact dermatitis on the hands and simultaneous food allergy to cereals and *Rosaceae* fruits, was reported. Moreover we have described LTPs from asparagus have been described as relevant allergens in patients with asthma, rhinoconjunctivitis and/or contact urticaria after occupational exposure to asparagus [17]. Nevertheless, to date Pru p 3 has not been identified as the cause of occupational asthma.

We conclude that LTPs from non-pollen structures such as leaves can act as occupational respiratory allergens, and that our findings are evidence of the importance of these proteins as plant panallergens.

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