

Lipid Transfer Proteins and Allergy to Oranges

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Key Words

Cross-reactivity · Lipid transfer protein · Orange allergy · Pru p 3 · Recombinant allergen

Abstract

Background: Lipid transfer proteins (LTPs) are relevant fruit allergens, recently proposed as model plant food allergens. No citrus fruit allergen has been characterized to date. We sought to identify and isolate citrus fruit LTPs and to explore their relevance in orange allergy. **Methods:** Twenty-seven patients, showing mainly oral allergy syndrome after orange ingestion, as well as positive prick responses and serum-specific IgE levels to orange, were selected. Natural orange and lemon LTPs, as well as a recombinant orange LTP isoform expressed in *Pichia pastoris*, were isolated by chromatographic methods and characterized by N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization mass spectrometry, and DNA sequencing of the corresponding cDNA in the case of the recombinant allergen. Specific IgE determination, immunodetection, ELISA-inhibition assays and in vivo skin prick tests (SPTs) were performed with all three purified allergens and with

the major peach LTP allergen, Pru p 3. **Results:** The natural allergens purified from orange (nCit s 3) and lemon (nCit l 3) showed very similar N-terminal amino acid sequences (18 out of 20 identical residues), typical of LTPs, and molecular masses of 9,610 and 9,618 Da, respectively. The recombinant orange isoform (rCit s 3) expressed in *P. pastoris* (16 out of 20 residues identical to its natural counterpart in the N-terminal region) presented 92 amino acid residues and 9,463 Da, and 67% sequence identity with rPru p 3. Of the 27 sera analyzed, specific IgE to the purified allergens was found in 54% for nCit l 3, 48% for nCit s 3, 46% for rCit s 3 and 37% for rPru p 3. Positive SPT responses were obtained in 7 out of 26 patients tested for nCit s 3, 3 out of 8 for nCit l 3 and 10 out of 26 for nPru p 3. ELISA-inhibition assays showed an equivalent IgE-binding pattern for the natural and recombinant orange LTPs, and IgE cross-reactivity among the purified orange, lemon and peach LTP allergens. **Conclusions:** Members of the LTP allergen family are involved in allergy to oranges, displaying positive in vitro and in vivo reactions in 30–50% of the patients studied. Both orange and lemon allergens show cross-reactivity with the major peach allergen Pru p 3.

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Introduction

Orange (*Citrus sinensis*) is the most largely produced and consumed citrus fruit included as fresh fruit or derived products (e.g. juice and jam) in the European and American diet. However, studies on orange allergy are very limited [1–7], and most of them have dealt with non-orange allergens (enzymes used in fruit processing [4] or from citrus mites [5]), minor non-protein components of citrus peel oil [3] or non-IgE-mediated reactions [6]. Accordingly, no orange fruit allergen has been isolated and characterized to date. In fact, a single published report [7] describes a preliminary search of these allergens carried out using sera from 6 pediatric patients with well-documented allergy to oranges, with the oral allergy syndrome (oropharyngeal pruritus and erythema with/without perioral urticaria, with/without lip angioedema) being the most frequent manifestation. Apart from an uncharacterized 25-kDa IgE-binding protein which reacted with all sera and a putative profilin response in 4 of them, the sera from the only 2 patients with systemic reactions revealed an unknown protein band of around 13 kDa. Both its molecular size and clinical picture led to speculate that the 13-kDa reactive component could be a lipid transfer protein (LTP).

Plant LTPs are basic polypeptides of approximately 9 kDa (91–95 amino acid residues) that have been identified as relevant allergens in several foods and pollens [see ref. 8–10 for review]. LTPs are highly resistant to both gastric digestion and heat treatment, and seem to be linked with severe and systemic symptoms and to induce sensitization by the oral route in fruit-allergic patients without associated pollinosis [9–12]. Therefore, LTPs have been claimed as model allergens of true food allergy [9]. High levels of sensitization to this allergen family occur in the Mediterranean area, but not in central and northern Europe [10]. Thus, skin prick test (SPT) responses to the major peach LTP allergen Pru p 3 have been found positive in 60–90% of Spanish patients with allergy to this fruit [12, 13]. On the other hand, extensive cross-reactivity among allergenic LTPs from different foods is now well established, both in vitro and in vivo [10, 14].

Based on the above-presented background, we have explored here the sensitization to LTPs in a group of 27 patients with orange allergy. To reach this objective, a reactive natural member of the LTP family was identified and purified from orange fruit, and a recombinant isoform produced in yeast. Furthermore, homologous fruit allergens from taxonomically close (lemon) and distant

(peach) species were also isolated. The four purified allergens were tested both in vitro and in vivo. The new citrus allergens have been designed as nCit s 3, rCit s 3 and nCit l 3, and included in the IUIS list as Cit s 3.0101, Cit s 3.0102 and Cit l 3, respectively (see results for assigned nomenclature).

Material and Methods

Patients and Sera

Twenty-seven patients with adverse reactions after ingestion of orange fruit and/or juice were selected among subjects referred to the Servicio de Alergia of the 'Hospital Universitario Niño Jesús' and the 'Fundación Jiménez Díaz' (Madrid, Spain). Selection was based on the following criteria: (1) a clinical history of orange allergy, confirmed by positive open food challenge responses to this fruit; (2) a positive prick-by-prick test response (mean wheal diameter of at least 3 mm) to fresh orange peel and/or pulp, and (3) a specific IgE level to oranges greater than 0.35 kU/l, determined using the Pharmacia CAP System FEIA (Pharmacia Diagnostics, Uppsala, Sweden). Clinical data of the selected patients are summarized in table 1. All patients except one (No. 21) were also sensitized to pollen, mainly grass and olive pollen. Nineteen of the 27 selected patients showed positive prick-by-prick test to peach, and 18 of them had allergic reactions to this fruit according to clinical history. Written informed consent was obtained from patients or from their parents.

A serum pool (including the sera from the 27 study patients or those indicated) or individual sera from each of the selected patients were used in the IgE immunodetection and ELISA assays. A pool of sera from 5 atopic subjects with allergy to house dust mites but not to plant foods or pollen was used as negative control.

Citrus Fruit Extracts

Fresh orange (*Citrus sinensis* var. Navel Late) and lemon (*Citrus limon* var. Verna) fruits were peeled, the resulting pulps liquified using a blender (non-filtered juice), and then peels and juices were freeze-dried separately. Non-filtered citrus juices were extracted with 1.8% NaCl (1 × 1: 10 w/v, 90 min, 4°C), centrifuged (16,300 g, 30 min, 4°C), dialyzed (cut-off point, 3.5 kDa) against H₂O and freeze-dried. Citrus peels were ground, defatted with acetone (2 × 1:5 w/v, 1 h, 4°C), dried, and then extracted with PBS buffer (100 mM sodium phosphate, pH 7.0, 150 mM NaCl). After centrifugation (16,300 g, 30 min, 4°C), the supernatants were dialyzed against H₂O and freeze-dried. Protein concentration was determined according to the method of Bradford [15].

Isolation and Characterization of Allergenic Citrus LTPs

To isolate natural allergenic LTPs from oranges and lemons, the corresponding crude peel extract was resuspended in H₂O (1:10 w/v) and centrifuged at 20,200 g (30 min, 4°C). The precipitate, enriched in LTP as shown by immunodetection with anti-Pru p 3 polyclonal antibodies, was dissolved in 20 mM formic acid, and then fractionated by means of cation exchange chromatography on an Accell Plus CM WatersTM SepPak^R cartridge (Waters, Milford, Mass., USA). Elution was performed with 20 mM formic acid, pH 4.0, and the retained material (enriched in LTP) was then eluted

Table 1. Clinical data of selected patients allergic to oranges

Patient No.	Age/sex	Symptoms to oranges	Total IgE kU/l	Specific IgE (kU/l) to			Prick by prick (mm) to oranges		Other plant food allergies
				oranges	lemons	peaches	peel	pulp	
1	12/F	OAS	71	1.02	1.86	–	4	5	p, a, pe, wm, k
2	12/F	OAS	109	0.47	2.05	–	5	3	p, a, pe, c, t, l, al
3	19/F	OAS/EA	290	1.07	3.06	5.26	4	5	p, a, pe, ch, m, k, t, l, b
4	16/M	OAS	164	1.88	2.8	–	8	7	wm, m, k, t, l, b
5	13/M	OAS	99	2.59	6.96	1.67	–	6	p, m, k, t, av
6	12/F	OAS	632	6.65	12.00	2.67	5	6	pe, b, pi
7	18/M	OAS/S	120	3.39	7.92	17.4	4	5	p, k, b, s
8	15/M	OAS	1,309	49.9	81.3	14.1	4	6	p, pe, k, b, wl
9	16/F	OAS	150	1.1	3.42	–	–	4	k, t, al, wl, h, ch
10	10/F	OAS	ND	0.38	1.34	ND	–	5	wm, m
11	15/M	OAS/G/V	285	6.1	15.70	1.67	5	5	p, a, ap, b, st
12	13/M	OAS	263	0.87	3.28	ND	4	4	p, b, pn, g
13	9/F	OAS/U	251	1.21	2.69	6.42	6	–	p, t, bl, mu, wl, h
14	14/M	OAS	729	11.9	ND	5.54	–	6	p, k, b
15	12/F	OAS/TT	598	6.18	15.5	4.48	5	6	p, a, k, b, wl, h, r, co
16	15/F	G/V	4,963	2.48	2.71	ND	5	3	pe, a, t
17	12/F	OAS/AD	809	4.91	8.13	ND	3	3	k, wl, h, le
18	14/M	OAS	244	0.84	1.75	3.59	6	7	p, a, m
19	7/M	OAS	739	6.86	21.9	10.5	6	5	a, pe, wm, m, k, t, b, pi, h, st, pn, gr
20	9/M	OAS	2,103	20.6	40.6	54.5	3	5	p, k, c, b, al, ch, e, ck, sy
21	0.5/F	OAS/V	ND	0.37	–	–	3	–	b
22	16/M	OAS	197	0.72	1.33	5.83	8	4	p, m, wl, h
23	11/M	OAS	699	3.67	11.2	1.16	3	3	p, a, pe, wm, m, t, b
24	16/M	CT	1,708	25.7	40.1	16.8	6	4	p, wm, m, k, c, b, le, ck, wl, h
25	12/M	OAS	1,204	2.07	6.34	–	3	3	p, m, b, wl
26	19/F	OAS	399	5.49	6.34	–	–	6	p, m, al, h
27	19/F	OAS	66	0.39	–	–	7	5	c, l, b, po

OAS = Oral allergy syndrome; EA = eyelid edema; S = sneezing; G = epigastralgia; V = vomiting; U = generalized urticaria; TT = throat swelling; AD = atopic dermatitis; CT = chest tightness. ND = not determined; – = specific IgE <0.35 kU/l or prick by prick <3 mm; p = peach; a = apple; pe = pear; wm = watermelon; k = kiwi; c = carrot; t = tomato; l = lettuce; al = almond; ch = cherry; m = melon; b = banana; av = avocado; pi = pineapple; s = sunflower; wl = walnut; h = hazelnut; ch = chestnut; ap = apricot; st = strawberry; pn = peanut; g = green bean; bl = blackberry; mu = mustard; r = rice; co = corn; le = lentil; gr = grape; ck = chickpea; sy = soybean; po = potato.

with 750 mM NaCl in the same buffer (1 ml/min), dialyzed against H₂O and freeze-dried. The LTP-enriched preparation was further separated by reverse-phase HPLC on a Nucleosil 300-C4 column (8 × 250 mm; particle size 5 µm; Scharlau Science, Barcelona, Spain), eluting with a linear gradient of acetonitrile in 0.1% trifluoroacetic acid (10% in 5 min and 10–100% in 150 min; 1 ml/min).

The recombinant allergenic orange LTP was purified from the supernatant of the corresponding *Pichia pastoris* culture medium. After dialysis against 100 mM ammonium acetate and freeze-drying, the preparation (6 mg of protein) was fractionated by means of gel filtration on a Superdex HR 75 16/26 column (Amersham Pharmacia Biotech, Uppsala, Sweden), eluting with 100 mM ammonium acetate (1 ml/min). LTP-enriched fractions were dialyzed against H₂O and freeze-dried, and further separated by means of reverse-phase HPLC using the same column and conditions described above for natural citrus LTPs. The natural and recombi-

nant forms of Pru p 3, the major peach allergen and LTP, were isolated as previously reported [16].

Fractions containing allergenic LTPs in the chromatographic profiles were detected by immunodetection with a rabbit polyclonal antibody against nPru p 3, after SDS-PAGE separation. Purified proteins were quantified by means of the commercial bicinchoninic acid test (Pierce, Cheshire, UK).

SDS-PAGE was performed according to Laemmli [17] on Bio-Rad Miniprotein II System (Bio-Rad Laboratories, Hercules, Calif., USA) gels (15% polyacrylamide).

N-terminal amino acid sequencing was carried out using standard methods with a 477A gas-phase sequencer (Applied Biosystems, Foster City, Calif., USA).

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI) analysis was performed using standard methods with an Axima CFR spectrometer (Kratos Analytical, Manchester, UK).

Isolation of cDNAs and Heterologous Expression of Allergenic Orange LTP

Total RNA from orange peel was obtained by the method of Chang et al. [18]. The reverse transcription was performed using primer and conditions described previously [16]. The cDNA obtained was amplified by polymerase chain reaction (PCR) using the oligonucleotide 5'-primer 5'-ATAACATGTGGGCAGGT-GACTGGC-3' designed from the N-terminal amino acid sequence of natural allergenic orange LTP and the 5'-nucleotide sequence of a cDNA encoding a putative LTP of natural orange abscission zones [19] (accession No. AF369931). For reverse direction, the 3'-primer 5'-TCACCTGACCTTAGAGCAGTCAGT-3' (complementary to that used for the reverse transcription) was applied.

Amplification was carried out with Ampli-Taq Gold DNA polymerase (Perkin-Elmer, Branchburg, N.Y., USA), as recommended by the manufacturer. The PCR program was 10 min of denaturation at 95°C, followed by 35 cycles each of 30 s of denaturation at 95°C, 30 s of annealing at 65°C and 1 min of extension at 72°C, and final extension for 7 min at 72°C. The PCR products were cloned into pGEM T-Easy vector (Promega, Madison, Wisc., USA), and then constructs used to transform *Escherichia coli* XL1-Blue cells. The DNAs from five clones were sequenced using the ABI PRISM Dye Terminator Kit and ABI 3100 sequencer (Perkin-Elmer Biosystems, Warrington, UK).

Expression of the cDNA encoding the orange LTP isoform in GS115 *P. pastoris* cells was achieved by using essentially the conditions previously described for rPru p 3 [16]. Briefly, the coding region of the Cit s 3.0102 cDNA was amplified by PCR with a sense primer 5'-CCTCCTCGAGAAAAGAGAAATAACATGTGGGCAG-3' (*Xho* I site underlined) and an antisense primer 5'-CCTC-GAATTCTCACCTGACCTTAGAGCA-3' (*Eco*R I site underlined). The PCR product was purified, digested with *Xho* I/*Eco*R I restriction enzymes, and inserted into the same sites of the pPIC9 plasmid to transform *E. coli* XL1-Blue cells. The DNA from several clones was sequenced to confirm the proper arrangement of the construct allowing the extracellular expression of Cit s 3.0102 with an extra glutamic acid residue at the N-terminus. The pPIC9/Cit s 3.0102 DNA was digested with *Sac* I restriction enzyme, and the larger purified fragment was used to transform GS115 *P. pastoris* cells by electroporation. Positive clones selected by patching the colonies in replica-plating on minimal glucose versus minimal methanol plates were cultured in buffered minimal glycerol medium, and expression of the recombinant allergen was induced by resuspension of collected cells in buffered minimal methanol medium. Maximum expression was achieved after 4 days, and the medium supernatant was used as starting material to purify rCit s 3.0102.

Immunodetection Assays

Samples (20 µg of protein of crude extracts from orange or from *P. pastoris* medium supernatants; 3 µg of purified allergens) were fractionated by SDS-PAGE and then electrotransferred onto nitrocellulose membranes as described by Towbin et al. [20]. After blocking with 5% BSA in PBS buffer, membranes were incubated overnight with a pool of sera from orange-allergic patients (1:7 dilution in 0.5% BSA, 0.05% Tween-20 in PBS), washed with 0.05% Tween-20 in PBS and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid [21] (1:3,000 dilution) for 2 h. After washing, a rabbit anti-mouse IgE peroxidase-conjugated antibody (Dako, Glostrup, Denmark; 1:5,000 dilution) was added and the mem-

branes incubated for 1 h. Detection of IgE-binding components was carried out by enhanced chemiluminescence, following the manufacturer's instructions (ECL-Amersham Biosciences, Little Chalfont, UK).

Alternatively, replica gels were electrotransferred onto polyvinylidene difluoride membranes and immunodetected with rabbit polyclonal antibodies to peach allergen nPru p 3 (1:1,000 dilution), then treated with alkaline-phosphatase-conjugated anti-rabbit IgG (Sigma, St. Louis, Mo., USA; 1:500 dilution), and finally revealed with a 5-bromo-4-chloro-3-indolylphosphate/nitroblue tetrazolium solution as previously reported [16].

Specific IgE Determination and ELISA Inhibition

Specific IgE binding to nCit s 3.0101 and nCit l 3, and to rCit s 3.0102 and rPru p 3 purified allergens, was carried out as previously described for rPru p 3 [22], using 1:2 dilution of 27 individual sera from orange-allergic patients (table 1) and 3 µg/ml of each purified allergen. PBS buffer with 1% BSA was tested as negative control, as well as a serum pool from patients allergic to house dust mites but not to plant foods or pollen (optical density below 0.05 units when tested toward each purified citrus allergen or rPru p 3). All tests were performed in triplicate.

ELISA inhibition assays were carried out by the same method, using 3 µg/ml of an isolated allergen as the solid phase and a selected individual serum (1:2 dilution), previously preincubated with 3 µg/ml of the purified allergen, as inhibitor. Comparison of nCit s 3.0101 and rCit s 3.0102 was carried out in the same conditions, using a serum pool (patients 7, 13, 15, 16, 18, 19 and 20; table 2) and serial dilutions of each inhibitor (range, 3 to 3×10^{-4} µg/ml). All tests were performed in triplicate.

Skin Tests

SPTs with the purified LTP allergens were carried out at 20 µg/ml of protein, following standard procedures [13, 23], on the volar side of the forearm, using a solution of 0.9% NaCl, 50% glycerol and 0.4% phenol as diluent. Histamine dihydrochloride (10 mg/ml) and saline solution were used as positive and negative controls, respectively. Duplicates of each sample were assayed. A mean wheal diameter of 3 mm or greater (15 min after puncture) compared with that produced by the negative control was considered a positive response.

Results

Putative LTPs present in crude extracts from orange pulp/juice or peel were searched by immunodetection using a previously obtained rabbit polyclonal antiserum to the peach LTP allergen Pru p 3 [16] (fig. 1, lanes Pe and Pu). A protein band with an apparent molecular size of around 10 kDa was recognized in both extracts (fig. 1). This band was also detected in both extracts by IgE from a serum pool from orange-allergic patients (fig. 1). No IgE-binding band was detected in the pulp/juice or peel extract by the negative control serum pool. The reactive component was isolated from the peel extract by cation

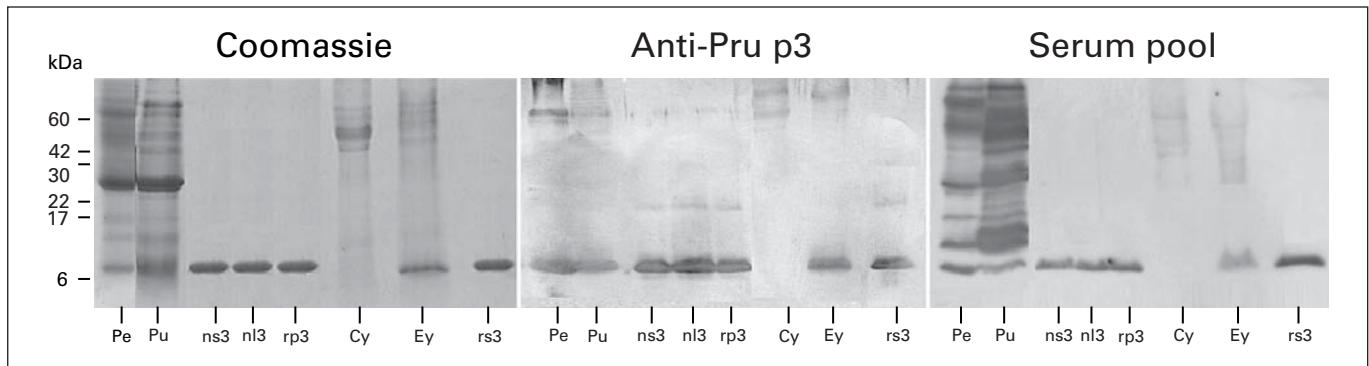


Fig. 1. Protein staining (Coomassie) and immunodetection with anti-Pru p 3 rabbit polyclonal antibodies and IgE of a serum pool from patients allergic to oranges of the following samples: crude extracts from orange peel (Pe) and pulp/juice (Pu), and from control (Cy) and transformed *P. pastoris* yeast expressing Cit s 3.0102 (Ey), and purified natural Cit s 3.0101 (ns3) and Cit l 3 (nl3) and recombinant rPru p 3 (rp3) and rCit s 3.0102 (rs3) allergens.

Table 2. Specific IgE and SPT responses to purified LTP allergens

Patient No.	Specific IgE, OD units				SPT, mm		
	nCit s 3	rCit s 3	nCit l 3	rPru p 3	nCit s 3	nCit l 3	nPru p 3
1	–	0.13	0.21	–	–	ND	–
2	–	–	–	–	–	ND	–
3	0.53	ND	ND	0.28	–	ND	6
4	–	0.21	–	–	–	ND	–
5	–	–	–	–	–	ND	–
6	0.11	–	0.35	–	–	–	–
7	1.13	0.91	1.16	1.14	7	ND	10
8	0.68	–	0.46	–	–	ND	–
9	–	–	–	–	–	ND	–
10	–	–	–	–	–	ND	–
11	–	–	0.23	–	–	–	–
12	–	–	0.37	–	–	ND	–
13	1.55	0.40	1.26	0.79	4	ND	6
14	0.15	–	–	–	–	ND	–
15	0.19	0.35	–	0.35	4	ND	6
16	0.88	1.33	0.51	1.38	ND	ND	ND
17	–	0.42	0.31	–	–	ND	–
18	0.20	0.27	0.10	0.22	–	–	4
19	1.59	0.38	1.66	1.30	3	4	5
20	2.60	1.39	2.44	2.56	5	ND	6
21	–	–	–	–	–	ND	–
22	0.12	0.13	–	0.10	8	6	9
23	–	–	0.84	–	–	ND	–
24	0.13	0.15	0.16	0.71	3	ND	9
25	–	–	–	–	–	–	–
26	–	–	–	–	–	4	6
27	–	–	–	–	–	–	–
Positive responses	13/27	12/26	14/26	10/27	7/26	3/8	10/26

ND = Not determined; – = optical density (OD) <0.10 or SPT <3 mm. Values are means (n = 3 for specific IgE and n = 2 for SPT; SE being <10% in all cases).

Fig. 2. Alignment of the determined N-terminal amino acid sequences for the natural allergen Cit s 3.0101 and Cit l 3, and those deduced from cDNA clones encoding Cit s 3.0102 (accession No. AJ783335) and rPru p 3 (accession No. AJ277163). Heterogeneity found at position 8 of (G/A) is indicated. The experimentally determined N-terminal amino acid sequence for rCit s 3.0102 is underlined. Sequential IgE epitopes of Pru p 3 previously described [24] are in boxes. The additional N-terminus (E) of the recombinant allergens was added for their proper expression in *P. pastoris*. Bold letters indicate differences with respect to the rCit s 3.0102 sequence.

ALLERGEN	AMINO ACID SEQUENCE
nCit s 3.0101	ITXGQVTGSLAPCIAFLRTK
nCit l 3	ITCGQVT ^G _A SLAPCIPFLRTG
rCit s 3.0102 (E)	<u>ITCGQVTGSLAPCIVYLRSGGP</u> I PVPCCNGVRSLNAAARTTPDRQTAC
rPru p 3	(E)ITCGQVSSS <u>LAPCIPYVRGG</u> GAVPPACCNG <u>IRNVNNLARTT</u> PDRQAAC
rCit s 3.0102	NCLKQAAGS I PNLNPNNAVGLPRACGVSIPIYKIS I STDCKSVR
rPru p 3	NCLKQ LSASVPGVNP NNAAL P <u>GKCGVSIPIYK</u> I SASTNCATVK

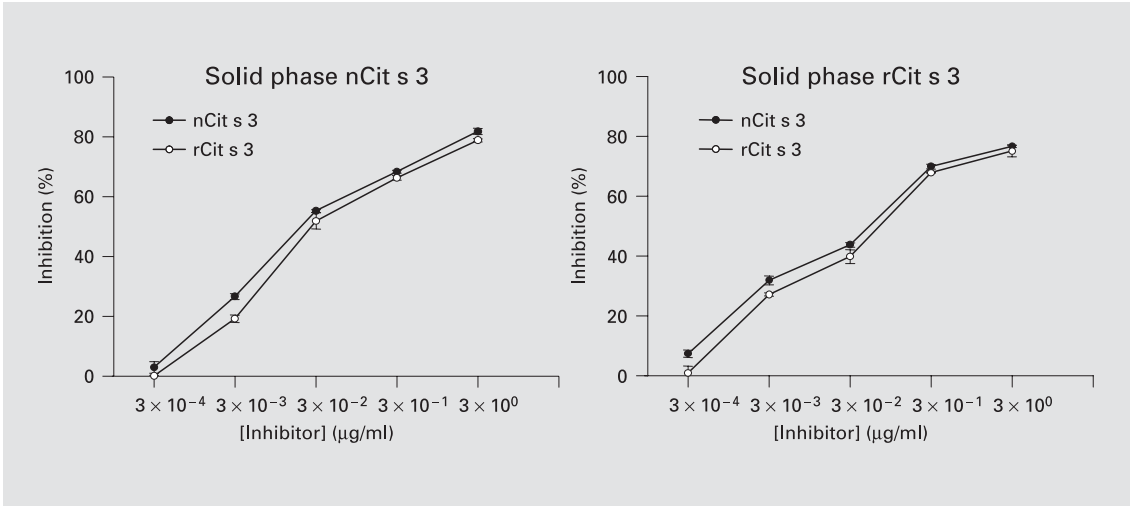


Fig. 3. ELISA inhibition with nCit s 3.0101 or rCit s 3.0102 in the solid phase and the same allergen isoforms as inhibitors. A serum pool from patients allergic to orange was used. Means (n = 3) and SE (bars) are represented.

Table 3. ELISA inhibition (% inhibition of IgE binding) with purified LTP allergens

Patient No.	nCit s 3 ¹				rCit s 3 ¹			
	nCit s 3 ²	rCit s 3 ²	nCit l 3 ²	rPru p 3 ²	nCit s 3 ²	rCit s 3 ²	nCit l 3 ²	rPru p 3 ²
7	97 ± 1.2	65 ± 2.4	77 ± 1.2	97 ± 1.0	51 ± 1.0	75 ± 2.2	55 ± 0.8	79 ± 1.0
8	95 ± 1.3	91 ± 0.2	77 ± 1.5	90 ± 1.5	no IgE binding to solid phase			
16	99 ± 2.3	83 ± 1.3	76 ± 1.6	98 ± 1.2	76 ± 1.5	83 ± 1.5	59 ± 2.4	76 ± 1.5

% inhibition <15%. Values are means (n = 3) ± SE.
¹ Solid phase.
² Inhibitor.

exchange chromatography followed by reverse-phase HPLC (not shown). The purified allergen, designated as Cit s 3.0101 (herein nCit s 3) according to the proposal of the International Committee of Allergen Nomenclature, behaved as a single band in SDS-PAGE and was recognized by both anti-Pru p 3 antibodies and IgE of sera from orange-allergic patients (fig. 1, lane ns3). Its molecular mass of 9,610 Da, determined by MALDI analysis, and N-terminal amino acid sequence (fig. 2) (accession No. P84161, Swiss-Prot database; 12 of 20 identical residues to peach Pru p 3) fully confirmed the inclusion of nCit s 3 in the LTP panallergen family.

Two different experimental lines have enabled to further explore the allergenic LTP family of citrus fruits. Firstly, the same isolation protocol used for nCit s 3 allowed to purify its homologous protein from lemon peel extract. The allergen, designated as Cit l 3 (herein nCit l 3) by the International Committee of Allergen Nomenclature, showed a single band in SDS-PAGE and a similar reactivity to that of Cit s 3 against anti-Pru p 3 antibodies and IgE of the serum pool (fig. 1, lane nl3). nCit l 3 had a molecular mass of 9,618 Da (MALDI analysis), and its homology to nCit s 3 was confirmed by N-terminal amino acid sequencing. The determined sequence (accession No. P84160, Swiss-Prot database) presented heterogeneity at position 8 (G/A) and 18 of 20 identical residues to nCit s 3 (fig. 2).

Secondly, cDNA clones encoding an isoform of the orange LTP allergen were isolated by PCR amplification with primers based on the N-terminal amino acid sequence of nCit s 3. Sequencing of five independent cDNAs revealed an identical 273-bp open reading frame (see accession No. AJ783335, EMBL database, for the nucleotide sequence), coding for a 91-amino acid protein (rCit s 3.0102 in fig. 2). The N-terminal region deduced from the cDNA shared 16 of 20 residues with that deter-

mined for nCit s 3, and the full amino acid sequence showed 67% identity with that previously reported for rPru p 3 [16]. To produce the recombinant allergen, *P. pastoris* GS115 cells were transformed with pPIC9 expression plasmid carrying the Cit s 3.0102 cDNA. Maximum extracellular expression of the recombinant allergen (herein rCit s 3) was achieved 4 days after induction with methanol (fig. 1, lane Ey; compared with control lane Cy). rCit s 3 was isolated from the supernatant of transformed *P. pastoris* culture medium by gel filtration chromatography followed by reverse-phase HPLC (not shown). Proper expression and purification were confirmed by the presence of a single band in SDS-PAGE and recognition of rCit s 3 by anti-Pru p 3 antibodies and IgE from sera of orange-allergic-patients similar to that of nCit s 3 (fig. 1, lane rs3). Identity of the recombinant allergen was further supported by determination of its N-terminal amino acid sequence (EITCGQVTGS), which fully corresponded to the nine N-terminal residues deduced from the cDNA Cit s 3.0102 (fig. 2), preceded by a glutamic acid that was added for proper extracellular expression of the protein [14]. MALDI analysis revealed a molecular mass of 9,463 Da for rCit s 3, which was in agreement with that calculated from its amino acid sequence (9,472 Da). A 91% amino acid sequence identity was found between Cit s 3.0102 and a putative LTP from orange abscission zones reported previously [19]. The equivalence in IgE-binding capacity of the natural and recombinant Cit s 3 isoforms was confirmed by ELISA inhibition experiments (fig. 3), which showed similar inhibitory patterns for both allergen variants.

The three purified citrus allergens described above, together with peach Pru p 3 selected as a model of fruit allergenic LTPs, were tested in vitro and in vivo in the group of patients with allergy to orange (table 1). Interestingly, rPru p 3 was clearly recognized by IgE of the serum

Table 3 (continued)

nCit l 3 ¹				rPru p 3 ¹			
nCit s 3 ²	rCit s 3 ²	nCit l 3 ²	rPru p 3 ²	nCit s 3 ²	rCit s 3 ²	nCit l 3 ²	rPru p 3 ²
73 ± 1.4	52 ± 1.3	74 ± 0.2	72 ± 0.6	–	17 ± 0.4	21 ± 0.8	90 ± 1.0
67 ± 1.7	43 ± 1.4	84 ± 0.5	47 ± 2.2	no IgE binding to solid phase			
86 ± 0.5	80 ± 1.2	94 ± 0.4	76 ± 0.6	89 ± 0.5	86 ± 0.5	86 ± 0.1	99 ± 1.1

pool from these patients in immunodetection assays (fig. 1, lane rp3). In vitro analysis was performed by direct ELISA to determine specific IgE to each purified allergen in each individual serum (table 2). Specific IgE (at least twice the optical density of the negative control) to nCit 1 3 was found in 54% of the sera tested, to nCit s 3 in 48%, to rCit s 3 in 46% and to rPru p 3 in 37%. Eight sera (30%) showed no recognition to any allergen, whereas 7 sera (26%) detected all of them.

In vivo reactivity was demonstrated by SPT, using only purified natural allergens (table 2). Positive SPT responses were obtained in 38% of the 26 patients tested for nPru p 3, and in 27% for nCit s 3. A subgroup of these patients (n = 8) was also tested with nCit 1 3, with 3 of them (37%) being reactive. Specific IgE to a particular allergen was found in all cases of positive SPT responses, except for patient No. 26.

The data summarized in table 2 suggested that the orange, lemon and peach LTP allergens were partially cross-reactive. This presumption was confirmed by ELISA-inhibition assays using each purified allergen both as solid phase and inhibitor (table 3) at levels (3 µg/ml) of maximum inhibition, according to the results presented in figure 3. Three individual sera were selected for these assays, two of them (No. 7 and 16) with specific IgE to the four purified LTP allergens, and a third one (No. 8) with IgE to only nCit s 3 and nCit 1 3. The inhibition values obtained in most cases (>60%) indicated the presence of common IgE epitopes in the four allergenic LTPs. On the other hand, significant differences in inhibition capacity were found with a particular serum and solid phase, such as serum 7 and rPru p 3 or serum 8 and nCit 1 3, strongly suggesting that IgE epitopes specific for a particular allergen (e.g. rPru p 3 for serum 7) were also present.

Discussion

This study was based mainly on two different, but potentially complementary scenarios: the rise of LTPs as plant food allergens, particularly in fruits and in the Mediterranean area [9, 10], and the almost complete lack of data on citrus fruit allergens despite the wide consumption of oranges, tangerines and lemons. Therefore, a group of 27 patients with allergic symptoms after orange ingestion was recruited, with 16 of them presenting only the oral allergy syndrome. Additionally, citrus fruit LTPs were isolated and tested in vitro and in vivo in this group of patients. The identification of the three purified allergens as members of the LTP panallergen family was con-

firmed by their reaction to fruit LTP (Pru p 3) antibodies and the determination of their N-terminal amino acid sequences and molecular sizes (MALDI analysis), both typical of LTPs.

Two isoallergens of orange fruit were obtained. Several LTP isoforms, encoded by a multigene family, have been detected in most plant species analyzed [8, 10]. The major allergenic variants from Rosaceae fruits, such as Pru p 3, are mainly located in peel [10], thus supporting that peach peels show a higher allergenic potency than pulps [25]. The results presented in figure 1 suggest an accumulation of nCit s 3 both in orange pulps and peels, but the latter is stronger reactive. These data, together with the fact that citrus fruits are usually eaten peeled, could be clinically relevant for both the low prevalence and the mild symptoms in allergic responses after ingestion of these fruits. A recombinant form, rCit s 3, was produced in *P. pastoris* cells transformed with a cDNA isolated by PCR amplification using specific primers for orange LTPs. rCit s 3 shared 67% sequence identity with peach Pru p 3, but several non-conservative amino acid changes were detected in the regions corresponding to the sequential IgE epitopes recently defined for the peach allergen [24]. A third citrus allergenic LTP, nCit 1 3, was isolated from lemon peel. Its close relationship with nCit s 3 was supported by their strong similarity in both molecular size and N-terminal amino acid sequence. On the other hand, the presence of specific IgE to peach in most (15 of 23) sera from the selected patients with orange allergy, together with the partial divergence between rCit s 3 and Pru p 3, led to include the peach allergen in this study. Moreover, the three purified citrus fruit allergens, as well as Pru p 3, were recognized in IgE immunodetection assays by a pool of sera from the selected patients.

Interestingly, ELISA-inhibition assays using a serum pool from 7 patients with specific IgE to both isolated orange isoallergens indicated the equivalent IgE-binding properties of nCit s 3 and rCit s 3. Moreover, there is a significant correlation ($r_s = 0.677$, $p = 0.001$) between the IgE binding data to nCit s 3 and rCit s 3 in ELISA (table 2). Thus, the recombinant form can be a new and useful tool for the diagnosis of citrus fruit allergy. Similar results have been reported for nPru p 3 and rPru p 3 [22]. A similar experimental approach, using individual sera, was designed to search for potential cross-reactions among the orange, lemon and peach LTP allergens. The inhibition values determined, although based on a limited number of sera, pointed to the presence of common IgE epitopes in all purified LTPs tested, but also indicated that the IgE of some individual sera reacted also to epitopes

specific for a particular allergen. In this context, sequential differences between rCit s 3 and Pru p 3 in regions harboring IgE epitopes have been detected (see above and fig. 2).

The presence of specific IgE to each of the purified allergens in the individual sera from the selected patients varied from 54% (Cit 1 3) to 37% (rPru p 3). Positive SPT responses were found in 27% of the patients for nCit s 3, and around 35% for nCit 1 3 and nPru p 3. Considering the presence of specific IgE and/or positive SPT responses to nCit s 3 and/or rCit s 3 sensitization to orange LTPs were detected in 16 out of the 27 (59%) patients. A higher proportion (7 of 9; 77%, 95% confidence interval: 0.40–0.97) of patients with systemic symptoms (No. 3, 7, 11, 13, 15, 16, 17, 21 and 24; table 1) compared to those presenting only the oral allergy syndrome (9 of 18; 50%, 95% confidence interval: 0.26–0.74) showed such sensitization, although the difference is not statistically significant. These data led to consider LTPs as true and relevant allergens of oranges, at least in the group of patients studied. This role is in line with that of other fruit LTPs in the Mediterranean population [10], particularly that of the major peach allergen Pru p 3, which showed positive in vitro and in vivo reactions in 60–90% of Spanish subjects with allergy to Rosaceae fruits [12, 13]. Furthermore, Pru p 3 seemed to be linked with the development of severe and systemic allergic reactions [10, 12]. Interestingly, in these subjects, the prevalence (tested in vitro) of other

panallergens usually associated with pollinosis and mild clinical symptoms, such as profilin, was significantly lower (around 35%) [12]. Our patient group has not only been tested with allergenic LTPs, but also with other not yet described orange allergens, i.e. Cit s 1 (a germin-like protein [Ahrazem et al., unpublished]) and profilin Cit s 2 [7, and Lopez-Torrejon et al., unpublished]. A high prevalence (>65%) to orange profilin has been revealed according to the specific IgE levels and positive SPT responses obtained. These data could explain the association with pollen allergy found in most of the orange-allergic patients. Similar results have been obtained for melon profilin in pollinic subjects allergic to melon and showing the oral allergy syndrome [26]. Overall, the results presented here indicate that, as has been previously described in other plant foods [10], LTPs are relevant allergens in citrus fruits.

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