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Germin-like protein Cit s 1 and profilin Cit s 2 are major allergens in orange (*Citrus sinensis*) fruits

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Oranges are clinically relevant allergenic foods. To date, orange allergens have not been characterized in detail. The study is aimed at analyzing the sensitization profile in orange-sensitized subjects with and without clinical allergy, and to identify orange allergens. Fifty-six sensitized subjects with self-reported reactions to orange were grouped into reactors (anaphylaxis or multiple episodes of immediate reactions and/or positive challenge tests) and non-reactors (negative open food challenge tests). Allergens were characterized by IgE immunoblotting, N-terminal sequencing, IgE-inhibition assays, and mediator release assays were performed to determine the allergenic potency of orange profilin. Of 56 subjects, 23 were classified as orange allergic showing mainly an oral allergy syndrome. Of 23 subjects classified as orange allergic, 22 were sensitized to profilin, Cit s 2. In patients with monosensitization to profilin *in vitro* histamine releases up to 75% from basophils were induced using orange extract and purified plant profilins. Of the allergic patients 78% were sensitized to germin-like protein, Cit s 1. Both allergens showed retained IgE reactivity in heat-processed orange juice. Interestingly, subjects with and without clinical allergy showed a comparable sensitization profile. Profilin and germin-like proteins are major orange allergens. The potential clinical relevance of orange profilin was indicated by its strong capacity to release histamine from basophils. However, a predominant sensitization to both allergens in subjects without symptoms also indicates a high frequency of clinically insignificant sensitization.

Keywords: *Citrus sinensis* / Germin-like protein / Isoflavone reductase-like protein / Orange allergy / Profilin

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1 Introduction

Orange (*Citrus sinensis*) fruits and juice are frequently consumed in Europe and North America. Total annual citrus production was estimated at over 105 million tons in the period from 2000 to 2004 (http://r0.unctad.org). Although in the daily clinical practice, orange allergy is obviously rarely observed in Central Europe some early studies suggested oranges being an important allergenic food. For example, citrus fruits have been described belonging to the ten major offenders among allergenic foods [1]. Studies performed in Finland revealed citrus fruits as common cause of allergy, especially in children, with a frequency of about 3% at the age of 3 years [2, 3]. Approximately 10% of atopic asthmatic children with food allergy reported symptoms after ingestion of orange [4]. The frequency of adverse reactions caused by ingestion of oranges was 17% in 100 adult patients from Italy with a history of an oral allergy syndrome after ingestion of fruits and vegetables [5]. Moreover, case reports described patients who developed food-dependent exercise-induced anaphylaxis after ingestion of oranges [6, 7]. In addition to the importance in immediate allergic reactions, oranges were suspected of deteriorating the course of dermatoses and the induction of skin lesions [8–10]. However, studies confirming the clinical relevance of food allergy to orange by challenge tests are limited [8]. Initial studies addressing the identification of allergens in orange fruits described a 35-kDa IgE-reactive orange protein with homology to a birch pollen minor

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Abbreviations: DBPCFC, double blind placebo controlled food challenge; **GLP**, germin-like protein; **HMW,** high molecular weight; **IFRL,** isoflavone-reductase-like; **LTP**, lipid transfer protein; **MUXF**, Manα1–6(Xylβ1-2)Manß1-4GlcNAcβ1-4(Fucα 1-3)GlcNAc) glycopeptide; **OFC**, open food challenge

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allergen [11]. Later, this protein was identified as isoflavone-reductase-like (IFRL) protein, Bet v 6 from birch [12–14]. Cross-reacting homologues were found in several plant foods, including orange and pear [15]. Recently, Ibanez *et al*. [16] analyzed the sensitization pattern of six pediatric patients from Spain with allergic reactions after ingestion of fruit juice, positive skin-prick test responses and oral provocation tests. All patients were sensitized to an orange protein with a molecular mass of 25 kDa, which had not identified so far. Moreover, some patients showed IgE reactivity to high molecular weight (HMW) proteins and profilin, recently identified by Lopez-Torrejon *et al*. as major allergen in orange [17]. Two patients had IgE against a low-molecular weight protein, which was identified as a lipid transfer protein (LTP), Cit s 3, from orange [16]. IgE specific to nCit s 3 prepared from peel was found in 48% out of 27 sera from orange allergic patients indicating LTP as minor allergens in orange [18].

In this study, we evaluated a group of 82 patients from Spain, who were sensitized to orange, confirmed the presence of clinical food allergy in a subgroup by oral challenge tests, and analyzed the allergen profile of all patients. In addition to profilin, Cit s 2, germin-like proteins (GLP), Cit s 1, were identified as a new class of major allergens in orange fruit allergy. Moreover, we found a strong histamine release capacity of Cit s 2 in profilin mono-sensitized patients, suggesting its potential clinical relevance in orange allergy. At the same time, clinical tolerance to orange in sensitized patients was found to be a common phenomenon.

2 Materials and methods

2.1 Patients and sera

By serological screening, 82 patients with IgE specific to orange were identified and included in this study. Levels of orange-specific IgE antibodies ranged from 0.38 to 17.5 kUA/L (median = 1.96, interquartile range = 1.2–3.83), as determined by the ImmunoCAP assay (Pharmacia Diagnostics, Uppsala, Sweden). A specific IgE level of 0.35 kUA/L was used as cut-off for positive results. Fifty-six (68%) of the 82 patients reported adverse reactions to orange and were further evaluated by allergy testing and oral provocations, whereas no further clinical information was available for 26 patients. Skin-prick tests were performed by the prick-prick technique with fresh orange, and, in addition, with inhalant allergen extracts (ALK-Abelló, Hørsholm, Denmark) from pollen, mammals and moulds (Table 1). A skin test result was recorded positive when the mean wheal diameter was at least 3 mm greater than that of the wheal induced by the negative control (saline solution). After providing written informed consent, actual clinical reactivity was evaluated in 24 patients by open food challenge (OFC) and double blind placebo controlled food challenge (DBPCFC). OFC was performed with a piece of

Table 1. Results of skin prick testing with inhalant allergen extracts (ALK-Abelló) in 56 patients reporting adverse reactions to orange and correlation with IgE binding to orange allergens

	Skin prick testing			*Correlation of results of skin prick testing with inhalant allergens and IgE-binding to orange allergens*		
	N	Positive (%)	Negative (%)	*Cit s 2*	*Cit s 1*	*HMW*
POLLEN						
Grass	45	42 (93.3)	3 (6.7)	*n.s.*[a)]	*n.s.*	*n.s.*
Olea europaea	45	40 (92.9)	5 (11.1)	*n.s.*	*p = 0.047*	*n.s.*
Plantago lanceolada	40	32 (80.0)	8 (20.0)	*n.s.*	*n.s.*	*n.s.*
Ulmus americana	25	20 (80.0)	5 (20.0)	*n.s.*	*n.s.*	*n.s.*
Platanus acerifolia	39	31 (79.5)	8 (20.5)	*p = 0.022*	*n.s.*	*n.s.*
Fraxinus americana	27	21 (77.8)	6 (22.2)	*n.s.*	*n.s.*	*n.s.*
Oak	34	25 (73.5)	9 (26.5)	*n.s.*	*n.s.*	*n.s.*
Taraxacum vulgare	26	19 (73.1)	7 (26.9)	*n.s.*	*n.s.*	*n.s.*
Chenopodium album	36	26 (72.2)	10 (27.8)	*n.s.*	*n.s.*	*n.s.*
Artemisia vulgaris	37	26 (70.3)	11 (29.7)	*n.s.*	*n.s.*	*n.s.*
Cupressus arizonica	38	24 (63.2)	14 (36.8)	*n.s.*	*n.s.*	*n.s.*
Parietaria judaica	35	16 (45.7)	19 (54.3)	*n.s.*	*n.s.*	*n.s.*
Cupressus sempervirens	26	10 (38.5)	16 (61.5)	*n.s.*	*p = 0.008*	*n.s.*
OTHER ALLERGENS						
Mammals (cat, dog)	37	18 (48.6)	19 (51.4)	*n.s.*	*n.s.*	*n.s.*
Mites (*D. pteron., D. farinae*)	37	11 (29.7)	26 (70.3)	*n.s.*	*n.s.*	*n.s.*
Moulds (*Aspergillus, Alternaria, Cladosporium*)	36	8 (14.3)	28 (77.8)	*n.s.*	*n.s.*	*n.s.*

a) *n.s.*: $p > 0.05$

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orange (~180 g). For DBPCFC, performed in 4 out of 24 patients, ~180 g orange was masked in a vehicle containing a mix of chocolate ice cream, sugar, wheat flour, and lemon flavoring. Subjects were challenged randomly with either food or placebo (vehicle), divided in five doses given every 15 min. The verum and placebo challenges were performed in 2 separate days. Oral provocations were not carried out in 15 patients with self-reported multiple immediate reactions after ingestion of isolated oranges, in 1 patient diagnosed with orange-dependent exercise-induced anaphylaxis, and in 16 patients who refused this procedure.

2.2 Orange extract

Fresh oranges (200 g) with and without peel were cut in small pieces and mixed with 500 mL of ammonium carbonate buffer, 0.05 M pH 8 plus ascorbic acid (10 mM) and triturated by using a Minipimer apparatus (Waring Products Division, New Hartford, USA). Extraction was performed during 4 h at 4°C with stirring. Oxidation was preserved using a vase with hermetic cup. Samples were centrifuged (4800 × g) for 20 min at 4°C. Supernatant was dialyzed against a total volume of 15 L of glycine 10 mM (three changes of 5 L each) for 18 h at 4°C using dialysis tubing with a cut-off of 3.5 kDa. After centrifugation (17 300 × g) for 20 min at 4°C, the supernatant was filtrated (0.45-mm pore size) and freeze-dried. The protein content was determined with a Bradford dye binding assay (Bio-Rad, Laboratories, Hercules, CA, USA) and BSA as standard.

A commercial available orange juice (Vaihinger, Lauterecken, Germany), pasteurized at 98°C for 30 s, was purchased in a local supermarket. Orange juice (300 mL) was filtrated, lyophilized and re-dissolved in distilled water (approximately 12 mL) with a protein concentration of 325 μg/mL.

2.3 SDS-PAGE, immunoblotting and IgE-inhibition assay

Orange extract with and without peel (10 μg protein/cm) was separated by SDS-PAGE according to Laemmli [19] on a MINIPROTEAN 3 system (Bio-Rad) (T = 12.5%; C = 0.33%). Proteins were transferred onto NC (0.2 μg, Schleicher & Schuell, Dassel, Germany) by tank blotting with a Mini-Trans-Blot-Cell (150 V, 42 min, Bio-Rad).

Detection of patient's IgE (1 : 10) was performed by a rabbit anti-human IgE antibody (1 : 4000, DAKOCytomation, Hamburg, Germany) or by a goat anti-human IgE antibody coupled to biotin (1 : 750 or 1 : 1500, KPL, Gaithersburg, MD, USA). For the detection of profilins a cross-reactive antibody from rabbit raised against ragweed profilin (provided by P. Deviller, Lyon, France) was applied (1 : 20 000). A rabbit anti-peach LTP antibody (1 : 20 000, provided by D. Barber, Madrid, Spain) and a rabbit normal serum (1 : 20 000) were applied as controls. The secondary antibody was a goat anti-rabbit IgG coupled to Biotin (1 : 6000, DAKOCytomation). Antibody binding was visualized by streptavidine coupled to alkaline phosphatase (1 : 3000, Caltag Lab., Burlingame, CA USA) and nitroblue-tetrazolium/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) (Bio-Rad) as substrate.

Inhibition of IgE-binding to orange extract on the solid phase was performed by preincubation of patient serum (1 : 10) with 10 μg recombinant profilin from sweet cherry, rPru av 4 [20], 20 μg MUXF [Manα1–6(Xylβ1-2)Manß1-4GlcNAcβ1-4(Fucα 1-3)GlcNAc) glycopeptide] from bromelain or 20 μg mannose-mannose-(Manα1-6(Manα1-3)Manβ1-4GlcNAcβ1-4 GlcNAc) peptide derived from fibrin (provided by F. Altmann, Vienna, Austria), 20 μg recombinant IFRL-protein from pear, rPyr c 5 [15] and birch pollen, rBet v 6 [13, 21], respectively.

2.4 N-terminal sequencing of the 25-kDa orange allergen

An IgE-reactive protein with a molecular mass of approximately 25 kDa from orange pulp extract was partially purified by preparative SDS-PAGE (PrepCell, Bio-Rad), (T = 12.5%; C = 0.33%). Fractions were screened by immunoblotting with pooled sera (n = 12) from patients preselected by reactivity to the 25-kDa orange allergen. Fractions containing the allergen were pooled and transferred onto a PVDF membrane (Immobilon™, 0.2 μm, Millipore Corporation, Bedford, MA, USA). After staining with CBB the protein band was excised and subjected to N-terminal sequencing by an Applied Biosystems 492 Procise sequencer (Applied Biosystems, Foster City, CA, USA).

2.5 *In vitro* histamine-release assay

Mediator-release assay was performed by a method described previously [22]. Briefly, peripheral blood was drawn from a non-allergic donor and peripheral blood mononuclear cells were purified by Ficoll-Histopaque® (Sigma, Taufkirchen, Germany) centrifugation. After stripping of IgE [23], basophils were passively sensitized with serum from orange allergic patients with mono-sensitization to orange profilin. Subsequently, cells were incubated dose-dependently with orange extract (0.01–10 μg protein/mL), profilins from birch pollen, nBet v 2 (Allergopharma, Reinbek, Germany) and sweet cherry, rPru av 4 [20] (0.0001–0.1 μg/mL, respectively). Passively sensitized cells without antigen and BSA (10 μg/mL) served as nega-

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tive controls. Released histamine was measured by a competitive enzyme immunoassay (histamine kit, Immunotech, Marseille, France). After subtraction of the spontaneous release, the allergen-induced histamine release was calculated as percent of the total amount of histamine determined after lysis of the basophils by twofold freezing and thawing of the cells. A histamine release of more than 10% was considered positive. Duplicate determinations were performed in all cases.

3 Results

3.1 Multiple IgE-binding proteins detected in orange

The IgE-binding profile of all individual sera (n = 82) was screened by immunoblotting of extracts derived from fresh oranges without peel (Fig. 1). Most of the tested subjects (78%, 64/82) were sensitized to a protein with a molecular weight of approximately 13 kDa, identified as profilin, Cit s 2 (see below). Interestingly, 20% (16/82) of the sera exclusively reacted with this allergen. Fifty-four (66%) out of 82 serum donors were sensitized to a protein with an apparent molecular mass of 25 kDa, subsequently identified as GLP, Cit s 1 and 56 (68%) of the subjects revealed IgE binding to HMW (>50 kDa) proteins, 5 sera bound IgE to a 35-kDa protein from orange pulp. IgE-reactivity to these HMW bands was caused by cross-reactive carbohydrate determinants, as investigated by inhibition of IgE-binding with a MUXF-glycopeptide derived from bromelain (not shown). In contrast, IgE binding to Cit s 1 was not significantly reduced after preincubation of a pooled patient's serum with the same MUXF-glycopeptide (not shown). Immunoblotting analysis of extract from whole orange including peel revealed an additional, but weak IgE-reactive protein of approximately 10 kDa (not shown). There were concordant reactivities to Cit s 1 and HMW proteins (kappa = 0.694, $p < 0.005$), while reactivity to Cit s 2 was strongly associated to pollen allergy ($p = 0.017$, not shown).

3.2 Clinical characteristics and reactivity of patients with self-reported allergy to orange

IgE-sensitization to orange in patients with self-reported symptoms was strongly associated (>90%) with positive skin-prick testing to grass and olive pollen. Interestingly, a significant correlation was observed between skin-prick test reactivity to plane pollen and Cit s 2 and between sensitization to pollen from olive and cypress pollen and Cit s 1 (Table 1).

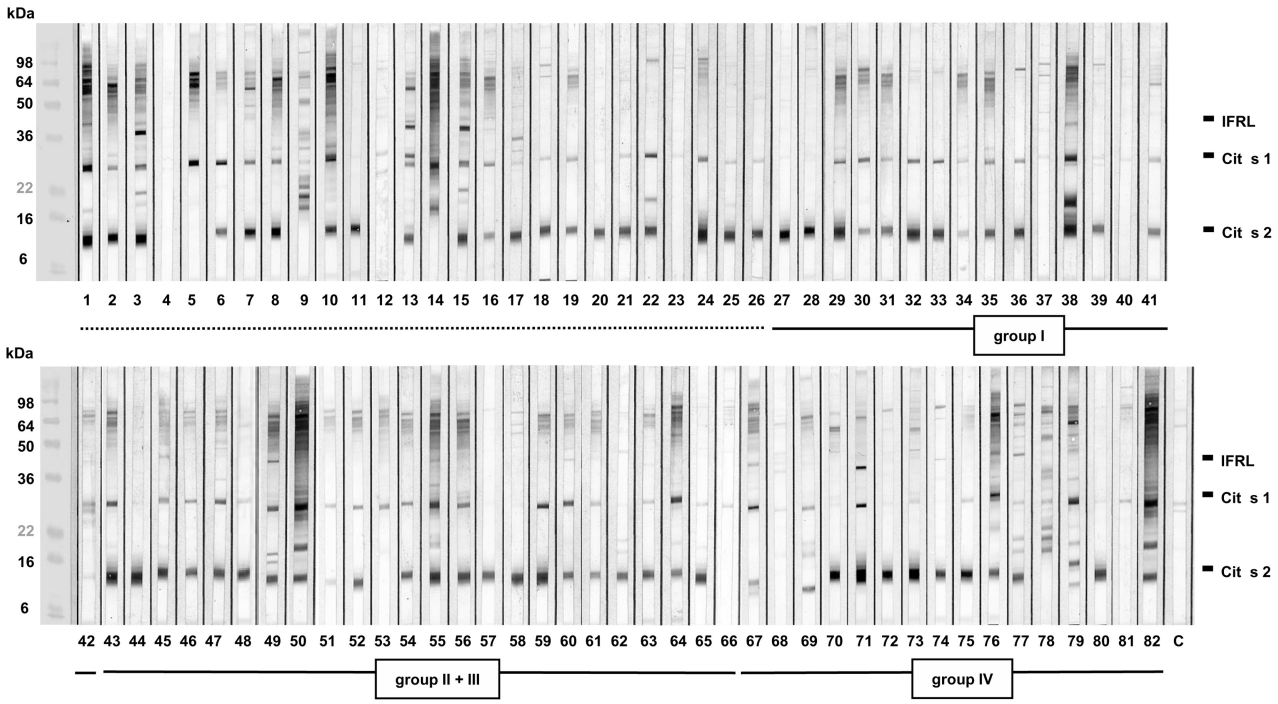

Figure 1. IgE-binding pattern of 82 sera from patients with sensitization to orange pulp extract from orange (dashed line: patients without clinical data; group I: non-reactors; group II: clinically reactive patients; group III: patient no. 66 with exercise-induced anaphylaxis; group IV: indefinite clinical reactivity). Cit s 1 and Cit s 2 are indicated, C (non-allergic control).

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Table 2. Characteristics of 56 subjects with self-reported reactions to orange[a)]

CHARACTERISTIC	I (n = 16)	II (n = 23)	III (n = 1)	IV (n = 16)	Overall (n = 56)
Age at onset					
Mean (yr.)	20.2	20.5	14	14.1	18.8
Self-reported symptoms					
OAS[b)] (%)	12 (75)	18 (78)	0	11 (69)	41 (73)
Other (%)	4 (25)	5 (22)	1 (100)	5 (31)	15 (27)
Prick-by-prick					
Median (mm)	4	4.5		0	3.5
Orange-specific IgE					
Median (KU_A/L)	1.1	1.7		3.3	1.7
Reactivity to orange allergens					
Cit s 1 (GLP, 25 kDa) (%)	11 (69)	18 (78)	0	8 (50)	37 (66)
Cit s 2 (Profilin, 13 kDa) (%)	12 (75)	22 (96)	0	11 (69)	45 (80)
HMW, > 50 kDa (%)	11 (69)	17 (74)	0	10 (63)	38 (68)
Pollen allergy	16 (100)	22 (96)	0	14 (88)	52 (93)

a) I: Negative open orange challenges; II: Positive oral provocation or multiple episodes of immediate symptoms after isolated ingestion of oranges; III: Orange-dependent exercise-induced anaphylaxis; IV: Actual clinical reactivity not defined.
b) Oral allergy syndrome.

Table 2 summarizes the clinical features and reactivity of 56 patients with self-reported orange allergy. Actual clinical reactivity was evaluated in 24 patients by OFC, yielding positive results in 8 patients. Four of them underwent a DBPCFC, all eliciting positive reactions. Patients were categorized as non-reactors when open provocation was negative (Group I, n = 16); reactors, if patients reacted in the oral challenges or reported multiple episodes after ingestion of orange (Group II, n = 23); orange-dependent exercise-induced anaphylaxis (Group III, n = 1); and indefinite clinical reactivity (Group IV, n = 16). There were no significant differences between group I and II in respect to the age of onset of reported reactions (20.2 *vs.* 20.5 years), type of symptoms reported (oral allergy syndrome, 75 *vs.* 78%), wheal size induced by skin testing with fresh orange (4 *vs.* 4.5 mm; median), levels of orange-specific IgE (1.1 *vs.* 1.7 kUA/L; median), and the frequency of pollen allergy (100 *vs.* 96%).

IgE from 12 (75%) of the 16 non-reactive patients (group I) and 22 (96%) of 23 reactive patients (group II) bound to Cit s 2. Sera from 5 (22%) of 23 clinically reactive patients bound only to this allergen (Fig. 1; patients 44, 57, 58, 62, 65). Moreover, the frequency of IgE binding to Cit s 1 and HMW proteins was 69%, respectively, in non-reactive patients (group I) and 78 (74%) in reactive patients (group II). The sensitization pattern to the respective allergens showed no significant difference between patients (n = 82) with sensitization to orange (profilin Cit s 2 78%, GLP Cit s 1 66% and HMW proteins 68%, Fig. 1) and patients with self-reported reactions and patients of group I and II (Table 1). The serum from a reactive patient (Fig, 1; patient 66) diagnosed of orange-dependent exercise-induced anaphylaxis (group III) did not bind any of the proteins detected in immunoblotting.

3.3 Identification of germin-like protein Cit s 1 and profilin Cit s 2 as major allergens in orange (*Citrus sinensis*) fruits

To identify the predominant IgE-reactive protein with a molecular mass of approximately 25 kDa orange pulp extract was separated by preparative SDS-PAGE (PrepCell, Bio-Rad). Fractions containing the 25-kDa protein were selected by pooled serum (n = 12) from patients with sensitization to the 25-kDa orange allergen and pooled. After a second SDS-PAGE and transfer to PVDF-membrane, the 25-kDa band was subjected to N-terminal sequencing. The N-terminal peptide comprising 15 amino acids (TDPGHLQDVXVAIND) completely matched to the N-terminal sequence of GLP from orange, Cit s 1 (P84159) and showed high amino acid sequence identities (9/15) with an allergenic GLP from pepper (*Capiscum annuum*, AY391748) [24]. The 13-kDa IgE-reactive protein from orange pulp extract was recognized by an antibody raised against ragweed profilin (Fig. 2). Moreover, pre-incubation of a serum from a patient with mono-sensitization to the 13-kDa allergen with cherry profilin rPru av 4 [20] completely inhibited the IgE binding to the orange allergen. Therefore, the 13-kDa allergen was identified as orange profilin Cit s 2, recently described as a predominant IgE-binding protein in orange allergic children [16] and adolescents [17]. In addition to the identification of Cit s 1 and Cit s 2

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as major allergens in fresh fruit, both orange allergens were identified in orange juice (Vaihinger) obtained from the market. Patient sera selected by the IgE reactivity to Cit s 1 or Cit s 2, or to both allergens were reactive with the respective allergens in extract from orange juice (Fig. 3). In addition, 5 patients had IgE directed against a protein with a molecular weight of approximately 35 kDa. Since IgE binding to the 35-kDa protein in orange extract was completely inhibited by pre-incubation of patient sera with allergenic IFRL-proteins from pear, rPyr c 5 and birch pollen, rBet v 6, it is very likely that this band represents a homologous protein in orange (not shown).

Moreover, patients' sera were frequently reactive with a 10-kDa protein, probably LTP Cit s 3, in whole orange extract including peel. The accumulation of Cit s 3 exclusively in orange peel was shown by a cross-reactive polyclonal antibody raised against peach LTP (not shown).

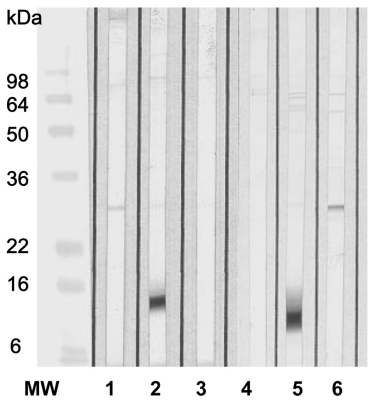

Figure 2. Inhibition of IgE-binding to orange extract by preincubation of a serum from a patient with mono-sensitization to the 13-kDa allergen Cit s 2 with cherry profilin rPru av 4 (3), without inhibitor (2), non-allergic control (1). Immunoblotting of orange extract with a rabbit serum raised against ragweed profilin (5), anti-peach LTP (4) and with a rabbit normal serum (6).

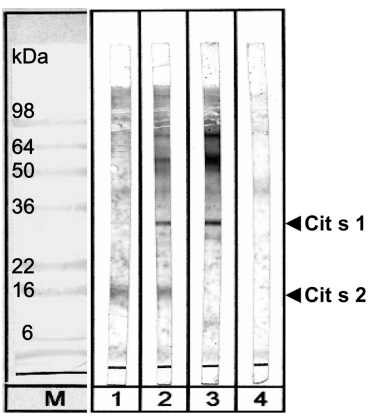

Figure 3. IgE-binding to extract from commercial orange juice with pooled sera from patients with mono-sensitization to Cit s 2 (lane 1), with IgE-reactivity to Cit s 1 and Cit s 2 (lane 2), and mono-sensitization to Cit s 1 (lane 3). Non-allergic control (4).

3.4 Profilin Cit s 2 shows strong basophil activation capacity

Sera from three patients with mono-sensitization to orange profilin were selected to evaluate biological activity of Cit s 2 for eliciting type I reactions. An *in vitro* mediator-release assay was performed in a dose-dependent manner with orange extract as antigen. By cross-linking of IgE a maximum histamine releases of 35% (patient 57), 70% (patient 80), and up to 75% (patient 27) were obtained with protein concentration of 10 μg/mL (patient 27 and 57) and 1 μg/mL (patient 80). In addition, purified profilins from birch pollen, nBet v 2 and from sweet cherry, rPru av 4, were capable of inducing histamine release in the same order of magnitude. HR50% values were calculated at $2.5-10\times10^{-1}$ μg/mL for orange extract, $1-2.5\times10^{-4}$ μg/mL for rPru av 4 and $5-25\times10^{-4}$ μg/mL for nBet v 2. Therefore, in comparison to orange extract a protein concentration of approximately 1/1000 of the purified profilins was sufficient to induce a comparable mediator release (Fig. 4).

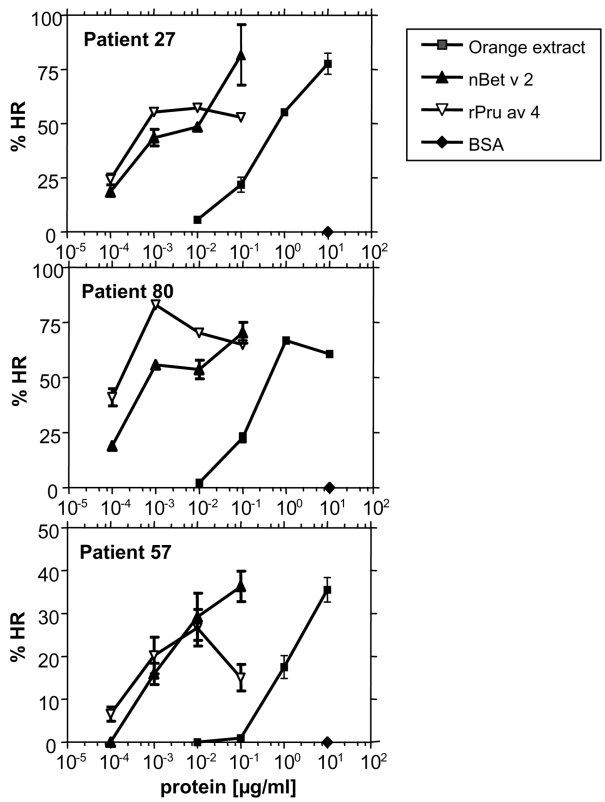

Figure 4. Biological potency of profilin Cit s 2 shown by *in vitro* histamine release assay with orange extract, profilins from birch pollen, Bet v 2 and sweet cherry, Pru av 4 in 3 orange allergic patients with mono-sensitization to orange Cit s 2.

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4 Discussion

Oranges are considered common allergenic fruits in Europe, Northern America and China and may induce severe food allergy in sensitized individuals [16, 17]. Although orange peel and seeds are consumed as well, and seeds have been reported to contain highly potent allergens [25], this study focused on the sensitization to orange pulp, which is the main edible part of oranges. To identify relevant orange allergens we used a large collection of sera from patients with IgE specific for orange proteins, most of them with self-reported reactions.

Our data indicate on the one hand that a very high percentage of sensitization to orange is not accompanied by clinical reactivity to this fruit because only one third of self-reported reactions were confirmed by oral provocations. OFC was performed with a maximum amount of 180 g of orange, because most of the patients reported allergic symptoms after ingestion of one or two slices or half of an orange, whereas only some of the patients developed symptoms after ingestion of a whole orange. However, we cannot fully exclude that a few patients would have shown clinical reactions if a higher amount of orange were used in the food challenge experiments. No difference was observed between results of OFC and DBPCFC that was performed to confirm the open challenges in four out of eight patients. In agreement with the study by Lopez-Torrejon *et al*. [17] we found profilin to be the major IgE-binding protein in all sensitized subjects. Moreover, our data are in accordance with recent results describing a relatively high frequency of profilin sensitization in fruit sensitized subjects without clinical allergy [26]. On the other hand it is also evident that clinical orange allergy exists and our study is one of the very few investigations in which orange allergy was confirmed by challenge tests [7, 16, 17].

The potential relevance of profilin as orange allergen, Cit s 2, has initially been addressed by Ibanez *et al*. [16] in a very small number ($n = 4$) of patients. Profilins known as cross-reactive panallergens in pollen, fruits and vegetables [14], were described as minor allergens in birch pollen-related food allergies. The strong association between birch pollen profilin hypersensitivity in patients without sensitization to Bet v 1 and allergy to citrus fruits has been shown in a recent study [27]. Moreover, a higher frequency of sensitization occurs in allergies to foods that are not typically related to birch pollinosis, *e.g.* melon (100%), banana (44%), pineapple (42%) and lychee (70%) [14, 28, 29]. In Spain where birch trees are of low abundance, others [17] and we identified profilin Cit s 2 as a predominant allergen in orange, the results supporting the data from Asero *et al*. [27] suggesting that allergy to citrus fruits can be used as a marker for profilin hypersensitivity. In accordance to the study by Lopez-Torrejon *et al*. [17] describing a prevalence of IgE sensitization up to 87% to purified Cit s 2, IgE from 80% of our patients' panel with adverse reactions to orange bound to Cit s 2. Moreover, our study addressed the cross-reactivity, stability and biological potency of Cit s 2. Sensitization to Cit s 2 was strongly associated with sensitization to plane tree pollen. Recently, an allergenic profilin was described in plane pollen [30]. Therefore, it is tempting to speculate that sensitization to profilin is responsible for a potential IgE cross-reactivity between plane pollen and orange. Strong cross-reactivity of IgE directed against Cit s 2 was shown with cherry profilin Pru av 4 and by Pru av 4 and profilin from birch, Bet v 2, both capable of inducing *in vitro* histamine release in Cit s 2-sensitized patients. Interestingly, in 22% of clinically reactive patients (group II) profilin was the only orange protein recognized by specific IgE, providing evidence that profilin may indeed be responsible for eliciting orange allergy in a subgroup of our patients, despite the fact that an IgE-response to profilin is considered to be of low clinical significance [31–33]. In addition to the fact that several mono-sensitized subjects were present in the orange allergic patient group, further evidence that profilin may be responsible for symptom elicitation results from *in vitro* mediator-release experiments. When sera from such patients exclusively sensitized to Cit s 2 in orange were used, orange extract and purified profilins as antigens had a strong potency of triggering histamine release. It is noteworthy, that extremely low concentrations of purified profilins (approximately 10^{-4} to 10^{-3} μg/mL) and as well a very low concentration of orange extract (<1 μg/mL) were sufficient to trigger half-maximal histamine release. In comparison to profilins in the present study similar concentrations for HR50% mediator release have been reported for the major birch pollen allergen Bet v 1 [33], supporting the view that sensitization to Cit s 2 may result in clinical symptoms after ingestion of orange. Interestingly, in the study by Bolhaar *et al*. [33] birch pollen profilin triggered almost no histamine release in a subject with systemic allergic reactions to Sharon fruit and strong IgE response to profilin. In this subject, co-sensitization to a Bet v 1 homologue was the likely reason of the reaction to fruit. In our study, histamine release curves with purified profilins and orange extract had almost identical shapes, indicating that the whole allergenic potency of the extract was represented by profilin. Concentration differences between extract and purified protein are due to the fact that profilin represents a minor protein constituent in plants, for example, 0.015 to 0.02% of total protein in various pollens [34]. Therefore, we conclude that it is very likely that Cit s 2 has the potential to trigger clinical symptoms in orange allergy while at the same time cross-reactivity not correlated with clinical food allergy is also common and may reflect low affinity binding to orange profilin of IgE from subjects sensitized to profilins from various pollen sources.

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Recently, an IgE-binding orange protein with a molecular weight of 25 kDa was described by Ibanez *et al*. [16] and was supposed to correspond to β-1,3-glucanases or thaumatin-like proteins. We were not able to confirm the presence of thaumatin-like proteins in orange extract by rabbit antiserum directed against Mal d 2, the thaumatin-like protein from apple (provided by H. Breiteneder, Vienna, Austria) as well as by inhibition of IgE binding after preincubation of patient sera with nPru av 2, the thaumatin-like protein from cherry (provided by H. Breiteneder) (data not shown). Therefore, the 25-kDa protein was partially purified and subsequently identified as germin-like protein Cit s 1 by N-terminal sequencing. Although germin-like protein has been recently denominated as Cit s 1 in the IUIS allergen database no information describing the identification and clinical relevance of this allergen has been published so far. We identified Cit s 1 as the major allergen in our orange allergic patients' group. So far, a GLP from pepper has been described as allergen in 93% of patients with mugwort-birch-celery-spice syndrome [24]. GLP are members of the cupin superfamily comprising allergenic globular storage proteins such as legumins (11S) and euvicilins (7S) [35]. GLP are glycoproteins involved in the germination process as well as in plant defense [36]. Deglycosylation experiments indicated that the glycan moieties contribute to the IgE binding [37]. Interestingly, by preincubation of the patients' sera with the MUXF-glycopeptide IgE binding to Cit s 1 was almost not reduced (not shown). This finding was in agreement with an observation of Jensen-Jarolim *et al*. [37] who reported that a carbohydrate-specific patient's serum was not capable of detecting glycosylated germin, possibly due to the presence of glycan residues distinct from these glycan moieties. The biological activity of germin from wheat (*Triticium aestivum*) and GLP from *Arabidopsis thaliana* was shown by histamine-release assay and skin-prick tests [37]. Several patients reported allergic symptoms after ingestion of one glass of orange juice. Interestingly, IgE-reactive Cit s 1 and Cit s 2 were retained in heat-processed orange juice. Therefore, we speculate that both allergens contribute to the allergenic potency of orange juice.

Moreover, only 5 subjects were sensitized to a 35-kDa allergen, which was identified as a cross-reactive IFRL-protein (not shown). In contrast to birch and pear this protein seems to have a minor importance as an allergen in orange. LTP are the major fruit allergen in the Mediterranean area. Although the presence of orange LTP, Cit s 3, has been described in pulp [18], it was unexpected that none of our study subjects showed IgE binding to a 9 – 10-kDa band in orange pulp extract. Expanding the testing to whole orange extract (including peel) revealed that approximately half of the tested sera had a weak IgE reactivity with a 10-kDa protein, which could be suspected to be Cit s 3 [18]. The moderate IgE reactivity might be explained by the low concentration of LTP in whole extracts prepared from pulp and peel and by an inadequate buffer with basic pH for the extraction of LTP. However, by a rabbit serum raised against the peach LTP Pru p 3 we confirmed the presence of an allergenic LTP in orange peel using PBS as extraction buffer.

In conclusion, according to the criteria of the IUIS allergen nomenclature subcommittee Cit s 1 and Cit s 2 were identified as major allergens in orange fruits. Both major allergens show persistent IgE-binding capacity during industrial preparation of orange juice, which included pasteurization, and may thus contribute to allergic reaction after ingestion of orange fruits and heat-processed juice. However, we also observed a predominant sensitization to Cit s 1 and Cit s 2 in sensitized subjects without clinical allergy to orange, indicating a high frequency of clinically insignificant sensitization.

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