

Orange Germin-Like Glycoprotein Cit s 1: An Equivocal Allergen

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Key Words

Orange · Allergen · Complex glycan · Germin-like protein

Abstract

Background: Orange allergens are virtually unknown, in spite of the large consumption of this fruit. Germin-like proteins, together with vicilins and legumins, form the cupin superfamily of plant proteins, which includes many seed allergens. **Methods:** Twenty-nine patients with allergy to oranges were studied. A major IgE-binding protein from orange extracts was isolated by means of a two-step cation-exchange chromatographic protocol. The allergen was characterized by N-terminal amino acid sequencing and MALDI analysis, and its reactivity explored by specific IgE determination in individual sera, ELISA inhibition assays and in vivo skin prick tests (SPT). Chemical deglycosylation of the purified allergen was achieved by trifluoromethylsulfonate acid treatment. **Results:** The 24-kDa purified allergen, designated Cit s 1, was identified as a germin-like glycoprotein, based on its N-terminal amino acid sequence, molecular size and recognition by rabbit anti-complex N-linked glycan antibodies. Specific IgE to Cit s 1 was detected in 62% of 29 individual sera from orange-allergic patients, whereas positive SPT responses to the purified allergen were ob-

tained in only 10% of such patients. Deglycosylation of Cit s 1 resulted in a loss of its IgE-binding capacity. Moreover, the unrelated plant glycoprotein horseradish peroxidase inhibited over 70% the IgE-binding to Cit s 1. **Conclusions:** Over 60% of patients with allergy to oranges show specific IgE to Cit s 1. However, the purified allergen exerts a low in vivo reactivity. Complex N-linked glycans seem to play a prominent role in the IgE-binding capacity of Cit s 1. This characteristic of Cit s 1, as well as of other orange glycoproteins, could lead to false positives if the diagnosis of allergy to oranges is mainly based on in vitro specific IgE determination.

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Introduction

Oranges are largely consumed worldwide and appear among the main allergenic plant food in public perception surveys of food allergy [1]. However, its major allergens are virtually unknown. Only two orange allergens have been identified so far: Cit s 2, a profilin [2, 3], and Cit s 3, a new member of the lipid transfer protein family [4]. Besides, an unidentified major 25-kDa IgE-binding protein band from orange extract, which was recognized by all 5 sera from children allergic to oranges analyzed in that study, has been recently described by Ibáñez et al. [2].

We report the isolation, characterization and in vitro and in vivo reactivities of this major 25-kDa IgE-binding component. It has been identified as a germin-like protein, named Cit s 1, according to the proposal of the International Committee of Allergen Nomenclature. Germins and germin-like proteins (GLPs) are around 25-kDa ubiquitous plant glycoproteins, which seem to be involved in defense mechanisms against pathogens, as well as in several developmental processes [5]. Some of them act as oxalate oxidase and/or superoxide dismutase enzymes [5]. Regarding food allergy, only two members of this protein family, namely wheat germin [6] and black pepper GLP [7], have been identified as potential allergens. Interestingly, germins and GLPs, together with the main seed storage proteins of legumes, vicilins and legumins, have been grouped in the cupin superfamily of plant proteins [8]. Several important allergens from legumes (i.e. peanut Ara h 1) and nut seeds are vicilins or legumins, and are therefore structurally related to GLPs [9].

Material and Methods

Patients and Sera

Twenty-nine patients with a clinical history of allergy to oranges, confirmed by controlled challenge tests, were recruited by Servicios de Alergia of Hospital Universitario Niño Jesús and Fundación Jiménez Díaz (Madrid). All selected patients had specific orange IgE levels higher than 0.35 kU/l (Pharmacia CAP System, Uppsala, Sweden) and a positive prick-by-prick test to fresh oranges (mean wheal diameter of at least 3 mm). Demographic and clinical data of the selected patients are summarized in table 1. Moreover, most patients (26 out of 29) were also sensitized to pollens, mainly grasses and/or olive. Four control healthy subjects were also selected for skin prick tests (SPT) with purified Cit s 1. Written informed consent was obtained from all patients.

A serum pool from all selected patients was used for IgE immunodetection and each individual serum for ELISA assays. A pool of sera ($n = 5$) from subjects with allergy to house dust mites, but not to plant sources, was tested as negative control.

Isolation and Characterization of Cit s 1

Fresh oranges (*Citrus sinensis* var. Navel Late) were peeled, and liquified pulps and peels freeze-dried separately. Pulp extracts obtained with different buffers showed a strong tendency to gelify, and therefore, the supernatant (10,000 rpm, 30 min, 4°C) of a 1.8% NaCl (1 × 1 : 10 w/v, 90 min, 4°C) extract, dialyzed against H₂O and freeze-dried, was used only as a control in immunodetection experiments. Peels were defatted twice with cold acetone (1 : 5 w/v) for 1 h at 4°C, and, after drying, extracted with PBS buffer (100 mM sodium phosphate, pH 7.0, 150 mM NaCl; 1 : 5 w/v), for 1 h at 4°C, centrifuged (10,000 rpm, 30 min, 4°C) and the supernatant dialyzed against H₂O, freeze-dried and its protein content quantified according to Bradford [10].

The peel extract was fractionated by cation-exchange chromatography on an Acell Plus CM WatersTM SepPak^R cartridge (Waters Co, Milford, Mass., USA). Elution was performed with 20 mM formic acid, pH 4.0, followed by 0.75 M NaCl in the same buffer (1 ml/min). After dialysis against H₂O, fractions enriched in a 24-kDa IgE-binding protein were identified by immunodetection with a serum pool from orange-allergic patients. Such fractions were pooled, freeze-dried, and further fractionated by a second step of cation-exchange chromatography on a Mono-S HR 5/5 column (Pharmacia Biotech, Uppsala, Sweden), eluted with a linear gradient (0–100% in 120 min, 1 ml/min) up to 1 M NaCl in 10 mM sodium acetate, pH 5.4, buffer. Cit s 1-containing fractions, as tested by IgE immunodetection, were pooled, dialyzed against H₂O, quantified by the commercial bicinchoninic acid test (Pierce, Cheshire, UK), and freeze-dried.

N-terminal amino acid sequencing and matrix-assisted laser desorption/ionization mass spectrometry (MALDI) analysis of purified Cit s 1 were performed by standard methods with an Applied Biosystems 477A gas-phase sequencer (Foster City, Calif., USA) and a Biflex III spectrometer with delayed extraction (Brucker-Franzen Analytik, Bremen, Germany), respectively.

SDS-PAGE analysis was carried out on a Bio-Rad Miniprotein II System (Bio-Rad, Hercules, Calif., USA) gels (15% polyacrylamide), following the method of Laemmli [11].

Potential enzymatic activity of purified Cit s 1 was tested by methods previously described: oxalate oxidase by the solution assay of Requena and Bornemann [12], and superoxide dismutase activity using nondenaturing PAGE and riboflavin and nitro blue tetrazolium as substrates [13].

Chemical deglycosylation of Cit s 1 with trifluoromethylsulfonate acid (TFMS) was performed essentially as in Sojar and Bahl [14]. After being resuspended in anhydrous TFMS (15 µl/100 µg of protein), protein samples were kept at –20°C for 2 h under a nitrogen atmosphere, and then neutralized with ammonium bicarbonate.

Immunodetection Assays

Samples (20 µg of protein of crude orange extract and 3 µg of purified allergens) were separated by SDS-PAGE and then electrotransferred onto nitrocellulose membranes (Amersham Biosciences, Little Chalfont, UK; 0.45 µm). After blocking with 5% BSA in PBS buffer (1 h, 25°C), membranes were incubated overnight with a serum pool or individual sera from orange-allergic patients, or with control sera (1:7 dilution in PBS buffer, 0.5% BSA, 0.05% Tween-20), and then treated with mouse anti-human IgE mAb HE-2 ascitic fluid (1:3,000 dilution, 2 h, 25°C) [15]. After washing, a rabbit anti-mouse IgE peroxidase-conjugated antibody (DAKO A/S, Denmark; 1:5,000 dilution) was added (1 h, 25°C), and then IgE-binding components were revealed by enhanced chemiluminescence, following the manufacturer's instructions (Amersham Biosciences).

Alternatively, separated samples were electrotransferred onto polyvinylidene difluoride membranes, and, after blocking, immunodetected with anti-complex asparagine-linked glycan serum (kindly provided by Dr. M. Chrispeels, University of California, San Diego, Calif., USA; 1:20,000 dilution) [16]. Membranes were then treated with alkaline-phosphatase-conjugated anti-rabbit IgG (Sigma-Aldrich, St. Louis, Mo., USA; 1:500 dilution) and finally with a 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium solution.

Table 1. Demographic and clinical data of orange-allergic patients and specific IgE levels to purified Cit s 1 and SPT responses to Cit s 1, Cit s 2 and Cit s 3

Patient	Age	Sex	Symptoms ^a	Total IgE kU/l	Specific IgE to orange kU/l	SPT response to Cit s 1, mm ^b		Specific IgE to Cit s 1 OD units ^c	SPT responses ^d to	
						50 µg/ml	200 µg/ml		Cit s 2	Cit s 3
1	12	F	OAS	71	1.02	–	–	–	+	–
2	12	F	OAS	ND	0.47	–	–	–	+	–
3	19	F	OAS/EA	290	1.07	–	–	0.69 ± 0.08	+	–
4	16	M	OAS	164	1.88	–	ND	–	ND	–
5	13	M	OAS	99	2.59	–	ND	0.48 ± 0.02	ND	–
6	12	F	OAS	ND	6.65	–	ND	0.97 ± 0.05	+	–
7	18	M	OAS/S	120	3.39	–	–	–	–	+
8	15	M	OAS	1,309	49.90	–	ND	1.51 ± 0.01	+	–
9	16	F	OAS	150	1.10	–	–	–	+	–
10	10	F	OAS	ND	0.38	–	–	1.21 ± 0.01	+	–
11	15	M	OAS/G/V	285	6.10	ND	4	0.72 ± 0.01	+	–
12	13	M	OAS	263	0.87	–	–	0.34 ± 0.02	+	–
13	9	F	OAS/U	251	1.21	–	–	2.39 ± 0.14	+	+
14	14	M	OAS	729	11.90	–	–	1.79 ± 0.01	+	–
15	12	F	OAS/TT	598	6.18	–	–	–	+	+
16	15	F	G/V	4,963	4.91	–	ND	0.89 ± 0.01	+	–
17	12	F	OAS/AD	809	2.48	ND	–	0.30 ± 0.06	ND	ND
18	14	M	OAS	244	0.84	ND	–	–	+	–
19	29	F	OAS	30	0.35	ND	–	–	+	–
20	7	M	OAS	739	6.86	3	3	0.50 ± 0.01	+	+
21	9	M	OAS	2,103	20.60	–	–	1.27 ± 0.01	+	+
22	0.5	F	OAS/V	ND	0.37	–	3	0.26 ± 0.01	–	–
23	16	M	OAS	197	0.72	ND	–	–	–	+
24	11	M	OAS	699	3.67	–	–	2.59 ± 0.04	+	–
25	16	M	CT	1,708	25.70	ND	–	0.62 ± 0.02	+	+
26	12	M	OAS	1,204	2.07	ND	–	0.26 ± 0.01	+	–
27	19	F	OAS	399	5.49	–	–	–	–	–
28	19	F	OAS	66	0.39	ND	–	1.10 ± 0.17	+	–
29	10	F	OAS	394	2.49	–	–	–	+	–
Positive responses						1/21	3/24	18/29	22/26	7/28

^a 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.

^b Values are means (n = 2) of the mean diameter obtained in each patient. – = Mean wheal diameter < 3 mm; + = mean positive SPT; ND = not determined.

^c Values are means (n = 3) ± SE. – = OD < 0.20 units (5 × OD of the mean (n = 8) obtained for BSA used as negative control).

^d SPT responses to purified Cit s 2 (profilin) and Cit s 3 (LTP) according to Lopez-Torrejon et al. [3] and Ahrazem et al. [4], respectively.

Skin Prick Tests

SPT were carried out in duplicate with purified natural Cit s 1 (50 and 200 µg/ml), prepared in GMP-certified facilities according to standard procedures [17]. Histamine phosphate (10 mg/ml) and PBS buffer/glycerol solution were used as positive and negative controls, respectively. 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.

ELISA Assays

Specific IgE-binding to Cit s 1 was determined by using a method previously described [18]. The purified allergen (3 µg/ml) was used as solid phase, and 29 individual sera from patients allergic to oranges (1:2 dilution) were tested. BSA (1% in PBS buffer) was assayed as negative control, as well as a serum pool from control subjects (OD below 0.05 units when tested towards Cit s 1). All tests were performed in triplicate.

Cross-reactivity between Cit s 1 and horseradish peroxidase (Sigma-Aldrich) was tested by ELISA inhibition assays performed

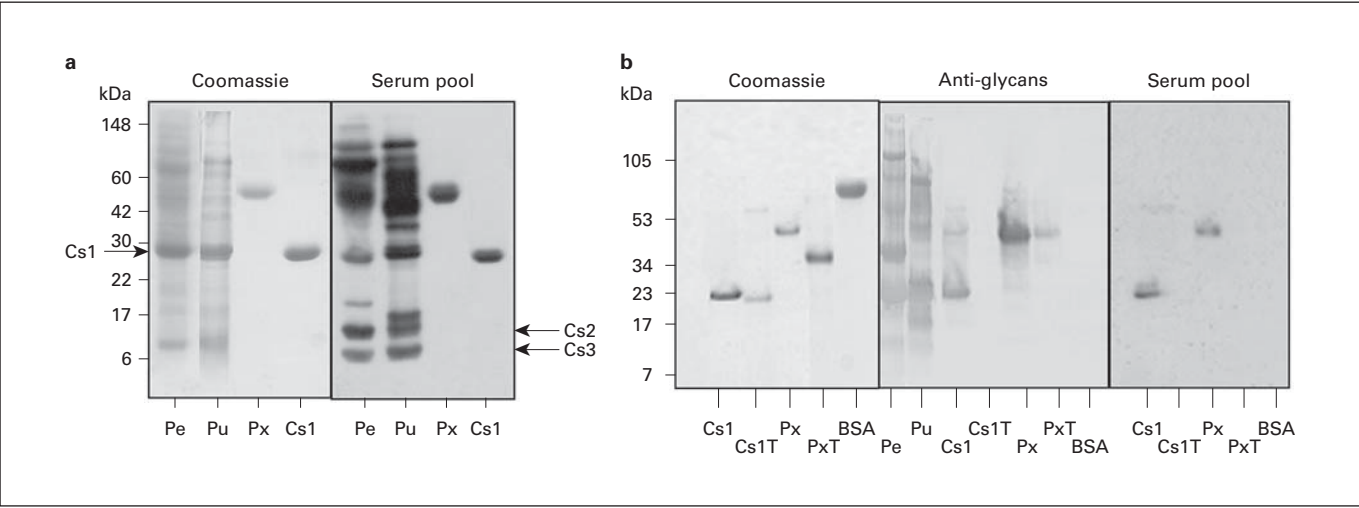


Fig. 1. a Protein staining (Coomassie) and IgE immunodetection with a serum pool from orange-allergic patients (serum pool) of a peel (Pe) and pulp (Pu) orange extract, and purified horseradish peroxidase (Px) and orange germin-like protein Cit s 1 (Cs1). Bands corresponding to Cit s 1, Cit s 2 (Cs2) and Cit s 3 (Cs3) are indicated. **b** Effect of TFMS treatment on purified horseradish peroxidase and Cit s 1. Untreated or treated (T) samples were protein stained (Coomassie), or immunodetected with rabbit IgG to complex N-linked glycans of plant glycoproteins (Anti-glycans) or human IgE from orange-allergic patients (Serum pool). BSA was included as negative control, as well as peel (Pe) and Pulp (Pu) orange extract in the Anti-glycans panel.

by the same method, with 2 individual sera (No. 8 and 14 of table 1) and each purified allergen as solid phase and as inhibitors (3 µg/ml). Additional tests were carried out to determine the IgE binding capacity of untreated and TFMS-treated Cit s 1 and horseradish peroxidase samples (9 µg/ml) against orange extracts in solid phase (20 µg/ml). All assays were performed in triplicate.

Results

IgE immunodetection using a serum pool from orange-allergic patients (fig. 1a) disclosed a main IgE-binding band of around 24 kDa, which corresponded to the major protein component of the Coomassie staining pattern of orange extracts, both from pulps or peels (fig. 1a).

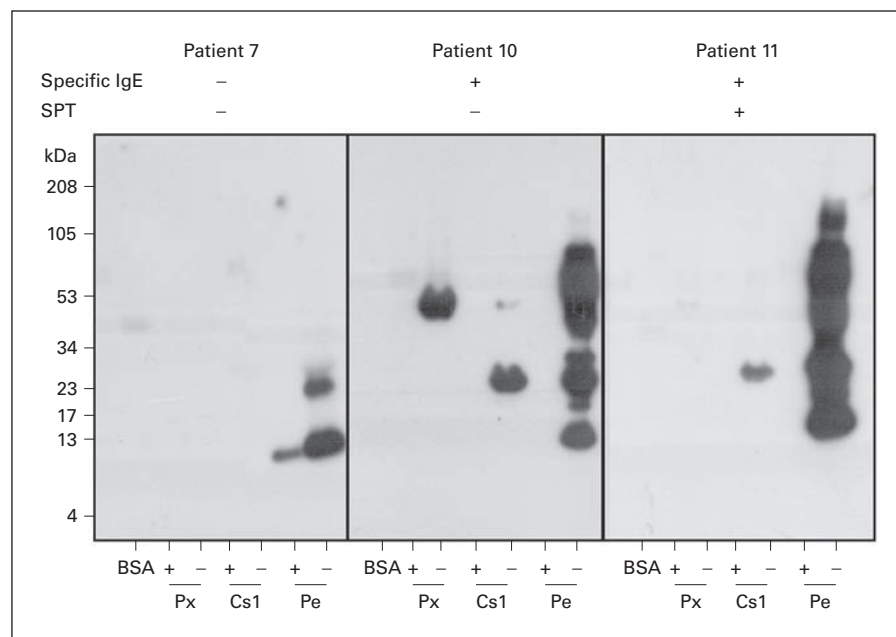
The 24-kDa IgE-binding protein was isolated from a PBS peel orange extract by means of a two-step cation-exchange chromatographic protocol (see Material and Methods). The purified allergen, named Cit s 1, showed a single band of the expected apparent molecular weight in SDS-PAGE, which strongly reacted with human IgE from orange-allergic patients (fig. 1a). N-terminal amino acid sequencing of Cit s 1 rendered the single sequence shown in figure 2. Homology of the orange allergen with allergenic members of the germin family previously reported [6, 7] was uncovered based on the significant iden-

Allergen	N-terminal amino acid sequence
Cit s1	TDPGHLQDVXVAINDPKXGVFVNRK
Pepper GLP	- DPSPQLQDFXVADL -- KSPIRVNGF
Wheat germin	TDPDPLQDFCVAIDL DGK - AVSVNGH
Arabidopsis GLP	YDPSPQLQDFCVAI DDPKNGVFVNGK

Fig. 2. Alignment of the determined N-terminal amino acid sequence for Cit s 1 with those for pepper GLP and wheat germin allergens and for Arabidopsis GLP (accession No. Q9FIC9). Residues identical to those of Cit s 1 are shown in bold letters. The amino acids that defined the conserved box A of the germin family [5] are boxed.

tity of their N-terminal region (40% with pepper GLP, 52% with wheat germin and 68% with Arabidopsis GLP). MALDI analysis of Cit s 1 disclosed three sharp peaks of 23,504, 23,298 and 23,095 Da, which were in the typical molecular size range of GLPs and, furthermore, suggested different degrees of modification of the orange allergen (see below). Its biochemical characteristics allowed to define Cit s 1 as germin-like protein. Some members of the

Fig. 3. IgE immunodetection with individual sera from orange-allergic patients of untreated (–) or TFMS-treated (+) samples of a peel orange extract (Pe) and purified Cit s 1 (Cs1) and horseradish peroxidase (Px). Specific IgE and SPT responses to Cit s 1 (table 1) of each patient are indicated.



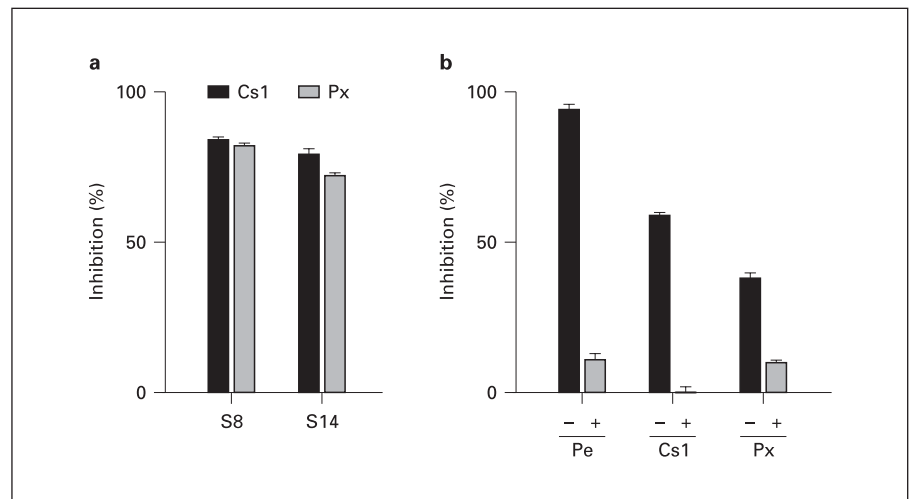
germin family act as oxalate oxidase and/or superoxide dismutase [5, 8], enzymatic activities that were not found for Cit s 1 (not shown).

The role of Cit s 1 as a novel orange allergen was explored both in vitro and in vivo (table 1). Specific IgE to the purified allergen was detected in 18 out of 29 (62%) individual sera from orange-allergic patients. In contrast, positive SPT responses were found in only 3 (10%) subjects, even after testing a high dose (200 µg/ml) of Cit s 1. No positive SPT response was obtained in any of the healthy control subjects. These results, together with the glycoprotein nature of most GLPs [5], led to suspect a potential role of complex N-linked glycans (usually designed as cross-reactive carbohydrate determinants) in the allergenic capacity of Cit s 1. This assumption was confirmed by three different experimental approaches. Firstly, the presence of complex N-linked glycans in Cit s 1 was ascertained by immunodetection with rabbit IgG against glycans of plant glycoproteins that contains β1→2 xylose and/or α1→3 fucose (fig. 1b). Besides Cit s 1, several other bands (mainly with high apparent size) of both the peel and pulp crude orange extracts were also recognized by the anti-glycans antibodies (fig. 1b, lines Pe and Pu). Interestingly, commercial horseradish peroxidase, which is not structurally related to the germin family, and is usually tested as model of glycoprotein harboring complex N-linked glycans, also clearly reacted with IgE of sera from orange-allergic patients (fig. 1a). Secondly, enzymat-

ic and chemical deglycosylation of purified Cit s 1 was attempted. The glycan could not be removed even after incubation with PNGase A for 24 h (not shown), whereas chemical deglycosylation with TFMS was fully effective, as indicated by the shift in electrophoretic mobility and the lack of recognition of the treated sample by anti-glycan antibodies (fig. 1b). Furthermore, deglycosylated Cit s 1 did not bind IgE from sera of orange-allergic patients (fig. 1b). Similar results were obtained analyzing horseradish peroxidase. r Pru p 3, the major allergen and nonglycosylated lipid transfer protein from peach fruit [18], was used as control to evaluate the effect of TFMS treatment on the IgE-binding properties. Treated r Pru p 3 retained its capacity to react with both rabbit antibodies and human IgE from peach-allergic patients (not shown).

Individual sera from 3 patients with different in vivo SPT and in vitro (specific IgE) responses to Cit s 1 were analyzed to further explore the effect of chemical deglycosylation (fig. 3). The absence of specific IgE led to recognition of neither Cit s 1 nor horseradish peroxidase, as expected. The 2 sera with specific IgE showed different patterns according to the SPT responses: both natural glycoproteins were revealed with serum from an SPT-negative patient, whereas only Cit s 1 reacted with serum from an SPT-positive patient. However, none of the TFMS-treated samples, including the crude peel orange extract, reacted with any of the 3 tested sera.

Fig. 4. ELISA inhibition assays. **a** Cit s 1 in the solid phase and Cit s 1 (Cs1) and horseradish peroxidase (Px) as inhibitors, testing 2 individual sera [No. 8 (S8) and 14 (S14); see table 1] from orange-allergic patients. **b** Orange peel crude extract as solid phase and untreated (–) or TFMS-treated (+) samples of the same extract (Pe), Cit s 1 (Cs1) and horseradish peroxidase (Px) as inhibitors. The assays were performed with a serum pool from orange-allergic patients. Means ($n = 3$) $\pm$ SD (bars) are represented.



Finally, two types of ELISA inhibition assays were performed (fig. 4). Cross-reactivity between Cit s 1 and horseradish peroxidase was confirmed by the similar inhibition values (74–84%) obtained using both proteins as inhibitors and Cit s 1 as solid phase. On the other hand, the relevance of the complex N-linked glycans was highlighted by the decrease in IgE binding to the crude orange extract using Cit s 1 and horseradish peroxidase as inhibitors (59 and 38% inhibition, respectively), in contrast to the lack of a substantial effect exerted by their deglycosylated forms.

Discussion

The information available on citrus fruit allergens is very scarce at present. Regarding oranges, only two allergenic proteins have been isolated and characterized from fruit extracts. Profilin Cit s 2 is a major allergen, showing high reactivity both in vitro (87% of sera from orange-allergic patients had IgE to the purified profilin) and in vivo (78% of positive SPT responses) [3]. Nonspecific lipid transfer protein Cit s 3 displayed lower in vitro (48%) and in vivo (27%) allergenic potency than Cit s 2 in the group of orange-allergic patients analyzed [4]. However, the potential best candidate to be a relevant orange allergen, the major 25-kDa protein component of fruit extracts (fig. 1), that strongly bound IgE from all 6 sera previously analyzed [2], remained to be identified.

This 25-kDa allergen has been isolated here, and named Cit s 1. Its biochemical characteristics (N-terminal amino acid sequence and molecular mass) clearly in-

dicated that Cit s 1 is a germin-like protein. Only two members of the germin family, which is extensively distributed in plant species [5], have been previously reported as allergens. The 28-kDa pepper (*Piper nigrum*) GLP [7] reacted in immunoblotting with 93% (15/16) of the sera from Central European patients with mugwort-birch-celery-spice syndrome. Unfortunately, the allergen was not isolated, and, therefore, could not be assayed in vivo. On the other hand, wheat germin (expressed in transgenic tobacco plants) [6] bound IgE from 50% (6/12) of patients sensitized to wheat, induced histamine release from blood of sensitized subjects, and elicited positive SPT reactions in 4 out of 5 cases. We have found specific IgE to Cit s 1 in 62% of the individual sera from orange-allergic patients, thus suggesting that it is a major allergen. However, its low in vivo reactivity (10% of positive SPT responses) makes it a minor allergen.

The sharp difference between the in vitro and in vivo reactivity of Cit s 1 could be partly explained by its glycoprotein nature. The purified allergen was recognized by anti-complex N-linked glycan antibodies, but the structure of the complex glycans linked to Cit s 1 is unknown. However, previous studies by Wilson et al. [19] showed that orange fruit is particularly rich in Lewis a-carrying N-glycans with core α 1,3-linked fucose and β 1,2-linked xylose and branches containing galactose and fucose residues. Besides, shorter structures of the ManManXylFuc, similar to that present in horseradish peroxidase, and GlcNAcGlcNAcXylFuc types are abundant as well. Cit s 1 lost its IgE-binding capacity, as shown by both IgE immunodetection and ELISA inhibition assays, when chem-

ically deglycosylated. Commercial horseradish peroxidase, a plant glycoprotein not structurally related to the germin family, was also recognized by sera from orange-allergic patients, and, even more, strongly inhibited the IgE-binding to Cit s 1 in ELISA inhibition tests. No reaction of TFMS-treated peroxidase was found, as was also the case for Cit s 1. Interestingly, chemical deglycosylation of wheat germin also resulted in full loss of its IgE-binding potency [6]. Together, all these data indicate that the complex glycan(s) harbored by Cit s 1 plays a pivotal role in its IgE-binding potency. However, a specific involvement of this allergen, but not of peroxidase, in true orange allergy was suggested by its recognition by serum from a patient with a positive in vivo response to Cit s 1. The differential reactivity of both glycoproteins could lie in the distinctive structure of their complex N-linked glycans [19], or in the sequence and/or conformation of both proteins. The lack of IgE binding by the TFMS-treated Cit s 1 sample points to the former possibility, or alternatively, to a crucial role of the glycans to reach a proper and reactive folding. Moreover, disruption of the Cit s 1 conformation by the chemical deglycosylation procedure cannot be discarded.

The potential role of complex N-linked glycans in the allergenicity of plant glycoproteins is well known [20]. However, the clinical significance of specific IgE for these glycans is still a matter of debate [21, 22]. The monovalency of glycan epitopes and/or a low affinity of interac-

tion between IgE bound to the receptors of mast cells and basophils and the allergen have been suggested to explain the low in vivo reactivity of some glycoproteins [23]. Nevertheless, although the positive SPT responses induced by Cit s 1 in some subjects suggest its potential clinical relevance in some orange-allergic patients, this matter needs further investigation. Whether or not additional IgE epitopes (sequential or conformational) are present in the protein structure of Cit s 1 and other allergenic GLPs besides the complex glycans remains to be studied as well. Future research along the same line will clarify not only potential cross-reactivity between GLPs of plant foods, but also their immunological relationship with other important allergens included in the cupin superfamily of plant proteins, such as vicilins and legumins. On the other hand, the characteristics of Cit s 1 discussed above could lead to false positives when diagnosis of orange allergy is mainly based on in vitro specific IgE determination.

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