

Comparison of IgE-Binding Capacity, Cross-Reactivity and Biological Potency of Allergenic Non-Specific Lipid Transfer Proteins from Peach, Cherry and Hazelnut

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Key Words

Cor a 8 • Food allergy • LTP • Non-specific lipid transfer protein • Pru av 3 • Pru p 3

Abstract

Background: Whether the observed clinical pattern of non-specific lipid transfer protein (nsLTP)-mediated food allergies is attributable to a primary sensitization by Pru p 3 from peach and subsequent cross-reactivity with Rosaceae- and non-Rosaceae-derived foods expressing homologous allergens is still unclear. **Objective:** To investigate the allergenic properties of nsLTPs from Rosaceae and non-Rosaceae foods. **Methods:** In peach-, cherry- or hazelnut-allergic patients, prevalence of sensitization, IgE-binding capacity, cross-reactivity and allergenic potency of Pru p 3 was compared with Pru av 3 (cherry) and Cor a 8 (hazelnut). **Results:** Frequency of sensitization to corresponding nsLTPs was 88, 85, and 77% in peach-, hazelnut- and cherry-allergic patients, respectively. Concomitant allergic reactions to cherry and hazelnut were reported in 51 and 44% of peach-allergic patients, respectively. In contrast to cherry allergy, hazelnut allergy was not strictly associated to peach allergy. Sensitization to Cor a 8 or Pru av 3 was strongly correlated with IgE

reactivity to Pru p 3, even when subjects tolerated peach. Specific IgE was highest for Rosaceae LTPs, and cross-inhibition experiments confirmed a stronger IgE-binding capacity of Pru p 3 than Cor a 8. The biological potency of Pru p 3 and Pru av 3 was similar but stronger for both nsLTPs than that of Cor a 8. **Conclusion:** Clinical cross-reactivity of food-allergic patients in the Mediterranean area is likely attributed to a primary sensitization to Pru p 3 and serological cross-reactivity with homologous food nsLTPs. In comparison to Cor a 8, Rosaceae nsLTPs showed a stronger IgE-binding capacity and allergenic potency indicating a different epitope pattern.

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Introduction

In the Mediterranean area, food allergy to fruits of the Rosaceae family mainly occurs independently from birch pollinosis [1]. In these patients, allergic reactions are caused by non-specific lipid transfer proteins (nsLTPs) from members of the Rosaceae family, e.g. Pru p 3 from peach [2] and Pru av 3 from cherry [3, 4]. nsLTPs have been identified as pan-allergens also in plant foods of

non-Rosaceae families (nuts, other fruits, vegetables, legumes and cereals), pollens and latex [for a review, see ref. 5–7]. Sensitization to nsLTPs contributes to the so-called nsLTP syndrome [8], which is characterized by severe and/or systemic reactions in a high proportion of patients. The compact structure and physicochemical properties of nsLTPs are due to a structural stability against digestive enzymes and thermal treatment, and are likely responsible for their strong allergenicity [for a review, see ref. 9].

Pru p 3, the major allergen from peach, has been described as the clinically most important allergen of the nsLTP family with respect to the frequency of sensitization (up to 100% in peach-allergic subjects) and its allergenicity [2]. Pru p 3 is considered as a true (class I) food allergen, capable of acting as primary sensitizing agent after ingestion of peach [2, 10, 11]. There is an ongoing debate whether reactivity to pollen nsLTPs is the consequence of a primary sensitization to Pru p 3, or nsLTPs from pollen itself are capable of inducing a sensitization via the respiratory route. Although several studies showed a frequent co-sensitization of peach-allergic patients to nsLTP from mugwort, Art v 3 [12], or a co-recognition of plane pollen nsLTP, Pla a 3 [13], displaying a partial IgE cross-reactivity with Pru p 3, the same studies also reported reactivity to pollen nsLTP without sensitization to Pru p 3 in a minority of patients. In contrast, other allergenic pollen nsLTPs from ragweed (Amb a 6), olive (Ole e 7) or *Parietaria* (Par j 1 and 2) are not cross-reactive with Pru p 3 and not involved in pollen associated plant food allergy [for a review, see ref. 5]. Taking into account the complex clinical picture of the LTP syndrome, and considering clinical reactivity, amino acid sequence identity and cross-reactivity between nsLTPs, Zuidmeer and van Ree [8] suggested different types of the nsLTP syndrome, characterized either by a primary sensitization to food nsLTPs with or without concomitant pollen allergy or by a primary sensitization to the pollen nsLTP.

Beside the patterns of cross-reactivity among nsLTPs from food and pollen, clinical association of allergies to different plant foods is a well-known phenomenon. Since peach allergy is strongly associated with a clinical reactivity to other plant food nsLTP of the Rosaceae or non-Rosaceae family, several studies investigated a potential correlation between clinical reaction and serological cross-reactivity [14, 15, for a review, see 16]. Initially, applying either crude extracts or purified nsLTPs, IgE cross-inhibition studies were performed for the identification and characterization of peach nsLTP homologous allergens in Rosaceae fruits, e.g. cherry [4], apricot [4, 17],

plum [4, 18], pear [1], apple [1, 19] and strawberry [1, 20], and non-Rosaceae foods, i.e. walnut [14, 21], peanut [14], hazelnut [22], orange [23], cabbage [24], wheat [25], rice [26] and grape [27, 28]. Pre-incubation of patient sera with Pru p 3 or peach extract was capable of abolishing IgE binding to almost all homologous allergens, an observation underlining the strong cross-reactivity of IgE directed against Pru p 3. Although the majority of IgE inhibition studies were performed by semi- or non-quantitative assays, without using purified nsLTPs for dose-dependent cross-inhibition experiments, a limited cross-reactivity between non-Rosaceae food nsLTPs and Pru p 3 and vice versa was found, i.e. for orange [23], walnut [21], grape [27] and wheat [25]. Species-specific epitopes among nsLTPs were confirmed in a recent study showing only partial cross-reactivity between purified Lac s 1, the nsLTP from lettuce, and Pru p 3 [29]. So far, there is strong evidence that all patients with reactivity to non-Rosaceae food nsLTPs are reactive to Pru p 3, suggesting Pru p 3 as the sensitizing agent [8, 30]. Although it is likely that in peach-allergic patients with reactivity to Pru p 3, the primary sensitization is mostly, even if not exclusively [31], caused by ingestion of food, it is not known whether other food nsLTPs, either from Rosaceae or non-Rosaceae, are able to elicit primary sensitization independent of peach allergy.

In order to investigate the allergenic properties of nsLTPs from different plant food species, Pru p 3 was compared with nsLTP from cherry (Pru av 3) [3], a member of the same taxonomical family, and with nsLTP from hazelnut (Cor a 8) [32], representing a non-Rosaceae family. Purified nsLTPs were subjected to quantitative analysis in regard to the sensitization pattern and IgE-binding capacity by ImmunoCAP™ testing, cross-reactivity by dose-dependent cross-inhibition assays and basophil activation capacity by in vitro mediator release assays.

Patients and Methods

Patients

Food-allergic patients (n = 50) with a positive case history of allergic reactions to peach (n = 43), cherry (n = 22) or hazelnut (n = 26) were included in this retrospective study. All patients were recruited at the Allergy Department of the Institute Universitari Dexeus in Barcelona, Spain. The majority of the selected patients reported concomitant allergies to numerous foods and plane pollen. All sera were tested by ImmunoCAP (Phadia, Uppsala, Sweden) for specific IgE to the respective food extracts from peach (f95), cherry (f242) and hazelnut (f17). Skin prick tests (SPTs) were performed with commercial extracts of peach, cherry and hazelnut (ALK-Abello Laboratories, Madrid, Spain) in most

patients. Histamine dihydrochloride and phosphate-buffered saline (PBS, 20 mM phosphate, 150 mM NaCl, pH 7.4) solutions were applied as positive and negative controls, respectively. SPTs were considered positive if the wheal diameter was at least 3 mm 15 min after application. The study was approved by the local ethical committee of the Institut Universitari Dexeus of Barcelona. Oral challenge tests were not performed due to the risk of severe reactions in patients with sensitization to nsLTPs.

Cloning and Overexpression of Hazelnut LTP (Cor a 8) and Cherry LTP (Pru av 3) in Pichia pastoris

Recombinant Cor a 8 and Pru av 3 were produced as described recently for Cor a 8 [33] with slight modifications. Briefly, Cor a 8 cDNA was amplified with the sense primer 5'-CCG CTC GAG AAA AGA GAG GCT GAA GCT TCC CTG ACA TGC CCA CAG ATA AAA-3' and the antisense primer 5'-GCT CTA GAA ACT TCA CGT TGT TGC AGT TGG TGG-3'. Pru av 3 was cloned using the sense primer 5'-CCG CTC GAG AAA AGA GAG GCT GAA GCT CTA ACA TGT GGC CAA GTG TCC AG-3' and the antisense primer 5'-GCT CTA GAA ACT TCA CGG TGG CGC AGT TGG TG-3'; *Xho*I and *Xba*I restriction sites are underlined. The target cDNA was inserted into pPICZ α A (Invitrogen, Groningen, The Netherlands) in frame with the sequence encoding for the pre-pro-sequence of the *P. pastoris* α -factor necessary for the secretory pathway. A stop codon was inserted by site-directed mutagenesis (QuickChange site-directed mutagenesis kit, Stratagene, La Jolla, Calif., USA) to obtain the nsLTPs as non-fusion proteins. The linearized constructs of pPICZ α A/Cor a 8 and pPICZ α A/Pru av 3 were used to transform X-33 *P. pastoris* cells (Invitrogen) by electroporation. Transformed cells were grown on yeast extract peptone dextrose sorbitol medium plates containing 100 μ g/ml Zeocin at 30°C for 5–6 days. Positive transformants were cultivated in buffered glycerol-complex medium for 16–18 h at 30°C in a shaking incubator until the culture reached an OD₆₀₀ of 2. Cells were collected by centrifugation (1,500 g, 5 min, 20°C) and expression of the recombinant protein was induced by resuspension in buffered methanol complex medium. Methanol was added every 24 h to reach a final concentration of 0.5%. After 4–5 days secreted proteins were purified from supernatant after centrifugation (1,500 g, 4°C).

Preparation of Peach Extract

Natural Pru p 3 was purified from freshly prepared extract of peach peel as described by Pastorello et al. [2] with some modifications. Peach peel of approximately 1.5 kg peaches purchased from a local market was homogenized and incubated for 2 h at 4°C in PBS buffer containing 2% suspended polyvinyl pyrrolidone, 2 mM ethylenediamine tetraacetic acid disodium salt and 10 mM sodium diethyl dithiocarbamate [34]. Insoluble material was removed by centrifugation at 1,500 g for 30 min at 4°C. The supernatant was dialyzed against PBS buffer for 12 h using a membrane cutoff of 3.5 kDa (Roth, Karlsruhe, Germany).

Purification of Recombinant and Natural LTPs

Recombinant (r) Cor a 8, rPru av 3 and natural Pru p 3 were purified by a two-step FPLC purification strategy using an Äkta FPLC™ system (GE Healthcare Life Sciences, Uppsala, Sweden). Samples were dialyzed for 24–48 h against starting buffer (sodium acetate, 50 mM, pH 5.0) with a membrane cutoff of 3.5 kDa (Roth) and filtered through a membrane with 0.22- μ m pore di-

ameter (Sartorius, Göttingen, Germany). First, a cation exchange column (Mono S HR 5/5; GE Healthcare Life Sciences) was equilibrated with 50 mM sodium acetate (pH 5), the filtered samples were loaded onto the column and proteins were eluted with a linear salt gradient of 50 mM Na/Ac, 1 M NaCl (0–1 M), pH 5.0, at a flow rate of 1 ml/min. Collected fractions were analyzed by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), silver staining and immunoblotting. The fractions containing nsLTPs were pooled, lyophilized and reconstituted in 1 ml H₂O. In a second purification step, the solution was subjected to size exclusion chromatography using a Superdex 75 HR 10/30 with a bed volume of 24 ml (GE Healthcare Life Sciences) after equilibration with MOPS buffer [20 mM; 3-(N-morpholino) propane sulfonic acid, 0.5 M NaCl, pH 7.4]. Collected fractions were analyzed as described above. The protein content was determined by a bicinchoninic acid method following the manufacturer's instructions (Pierce, Chester, UK).

SDS-PAGE and Immunoblotting

Purity of the allergens was verified by SDS-PAGE analysis (T:15%) under non-reducing conditions and Coomassie brilliant blue R250 or silver staining [35]. For immune detection, the purified allergens were transferred onto a 0.2- μ m nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) by electroblotting (150 mV, 45 min). The membrane was blocked with Tris-buffered saline {TBS, 0.05 M Tris [tris(hydroxymethyl)aminomethane], 0.15 M NaCl} containing 5% skimmed milk powder, followed by an overnight incubation of sera (1:10) from allergic patients. Immunostaining of bound IgE antibodies was performed with an alkaline phosphatase (AP)-conjugated rabbit anti-human IgE antibody (1:750; KPL, Gaithersburg, Md., USA). The colorimetric detection was performed by the AP substrate kit (Bio-Rad, Munich, Germany).

Analysis of Protein Structure by Circular Dichroism Spectroscopy

Secondary structure content of the purified allergens was verified by circular dichroism spectroscopy. The proteins (250 μ g each) were dialyzed against 10 mM KH₂PO₄ buffer (pH 7.5) and analyzed by a Jasco J-810S spectropolarimeter (Jasco, Gross-Umstadt, Germany) with a step width of 0.2 nm and a band width of 1 nm. Ten scans with a spectral range of 185–255 nm, recorded with a scanning speed of 50 nm/min, were accumulated for each protein.

Determination of nsLTP-Specific IgE and IgE Inhibition

Specific IgE to purified nsLTPs (nPru p 3, rCor a 8 and rPru av 3) was determined using experimental ImmunoCAP as antigen solid phase. Approximately 5 μ g of purified protein were coupled to the solid phase, as described elsewhere [36]. Cross-reactivity between nsLTPs was determined by IgE ELISA inhibition assays according to Hartz et al. [29]. To compete IgE binding to nsLTPs, patient sera were preincubated either with nPru p 3, rPru av 3 or rCor a 8 (0.001–10 μ g/ml, respectively).

Basophil Histamine Release Assay

The in vitro histamine release test was performed as described by Foetisch et al. [37]. Briefly, peripheral blood mononuclear cells were isolated from a non-allergic donor and IgE antibodies were stripped off by lactic acid treatment, followed by re-sensitization

with sera of the allergic patients containing IgE against nsLTPs. Passively sensitized cells were stimulated in a dose-dependent manner (0.1–10 µg/ml) with purified allergens, and BSA served as negative control. The total amount of histamine was determined after lysis of basophils by twofold freezing and thawing. A histamine release >10% was classified as a positive reaction. All experiments were performed in duplicate.

Results

Allergy to Peach, Cherry and Hazelnut in Spanish Patients Is Frequently Linked to Systemic Reactions

Fifty patients with a clear case history of food allergy against peach (n = 43), cherry (n = 22) and/or hazelnut (n = 26) were included in this study (table 1). In 19/43 patients, peach allergy was linked to hazelnut allergy. Cherry allergy without peach allergy was not observed. In contrast, clinical tolerance to Rosaceae fruits (peach and cherry) was reported by 7/26 hazelnut-allergic patients; 74% (32/43) of peach-allergic patients had experienced severe or systemic symptoms such as anaphylactic reactions (n = 4), urticaria (n = 22) and/or angioedema (n = 9). The remaining 11 patients reported only mild oral allergy. Fifty-five percent (12/22) of cherry-allergic patients had a case history of systemic reactions with anaphylactic reactions (n = 5), urticaria (n = 4) and/or angioedema (n = 8). Systemic reactions had also occurred in 62% (16/26) of hazelnut-allergic patients, including anaphylactic reactions (n = 8), urticaria (n = 5) and/or angioedema (n = 3). Sensitization to the offending foods was confirmed by positive SPT and/or detection of specific IgE antibodies (≥ 0.35 kU_A/l; ImmunoCAP, Phadia) in 42/43 peach-, 20/22 cherry- and 24/26 hazelnut-allergic patients. In subjects tested with both in vivo and in vitro methods, an overall good correlation was observed between positive SPT and detection of specific IgE to extracts from peach (32/36), cherry (18/23) and hazelnut (28/30). Discrepancies between specific IgE and SPT were found for 11 sera and were due to negative ImmunoCAP (n = 8) or negative SPT results (n = 3), respectively. Moreover, all patients reported clinical symptoms either to plant pollen (80%, 40/50) or to other plant-derived foods, e.g. lettuce (57%, 28/50), peanut (52%, 26/50), walnut (38%, 19/50) and apple (26%, 13/50).

Purification of Biologically Active Recombinant and Natural Food nsLTPs

Recombinant Cor a 8 and rPru av 3 were produced in *P. pastoris* strains showing the Mut^s genotype. Natural Pru p 3 was purified from extract of peach peel. The yield

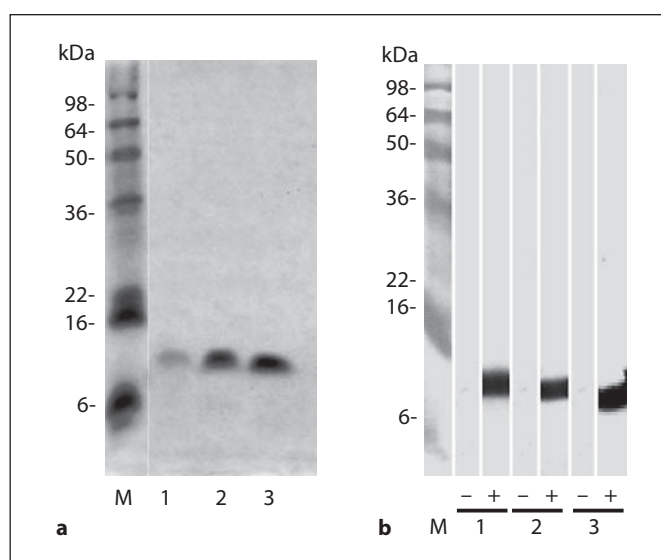


Fig. 1. SDS-PAGE and Coomassie staining (a) and immunoblotting (b) of the purified nsLTPs: rPru av 3 (lane 1), rCor a 8 (lane 2) and nPru p 3 (lane 3), and buffer control (–). IgE immune detection was performed using serum (+) from a food-allergic patient multi-sensitized to Pru av 3, Cor a 8 and Pru p 3.

of purified rPru av 3 and rCor a 8 obtained from 1 liter yeast culture was 6.0 and 4.3 mg, respectively, and 12.5 mg nPru p 3 was isolated from 1.5 kg of peaches. A purity of at least 95% of each protein preparation without any aggregates was verified by non-reducing SDS-PAGE and Coomassie brilliant blue staining (fig. 1a). The IgE reactivity was analyzed by immunoblotting using a patient serum (EDP, table 1) with cross-reactive IgE to all three food nsLTPs (fig. 1b). Secondary structure content was examined by circular dichroism spectroscopy (fig. 2). All purified LTPs showed similar α -helical structures with two minima at 208 and 222 nm, typical for members of the nsLTP family.

Sensitization to LTPs from Rosaceae and Non-Rosaceae Is Strongly Associated with a Sensitization to Pru p 3

Sensitization to nPru p 3, rPru av 3 and rCor a 8 was determined by experimental ImmunoCAP tests (table 1). The frequency of sensitization to nPru p 3, rPru av 3 and rCor a 8 was 88% (38/43), 77% (17/22) and 85% (22/26) in peach-, cherry- and hazelnut-allergic patients, respectively. The median concentration of nsLTP-specific IgE antibodies in the group allergic to the respective food was 6.83 kU_A/l (SD = 8.69 kU_A/l) for Pru p 3, 3.98 kU_A/l (SD = 5.54 kU_A/l) for Pru av 3 and 2.74 kU_A/l (SD = 3.31

Table 1. Clinical history and specific IgE of food-allergic patients

No.	Serum	Age/ sex	Peach				Cherry				Hazelnut			
			symp- toms	SPT	extract CAP class kU _A /l	nPru p 3 CAP class kU _A /l	symp- toms	SPT	extract CAP class kU _A /l	rPru av 3 CAP class kU _A /l	symp- toms	SPT	extract CAP class kU _A /l	rCor a 8 CAP class kU _A /l
Pru p 3, Pru av 3 and Cor a 8 reactive														
1	MLR 10560	33/F	A	+	3 (4.19)	3 (7.64)	–	–	ND	3 (12.3)	–	–	3 (8.46)	3 (10.8)
2	MAE 8608	34/F	A	+	3 (4.50)	3 (5.57)	–	ND	ND	3 (6.14)	–	–	ND	2 (2.06)
3	LCGH 9343	25/F	OAS	+	3 (16.4)	3 (11.2)	–	ND	ND	3 (11.0)	–	ND	3 (10.3)	3 (7.87)
4	YSF 8695	36/F	–	ND*	ND	2 (0.79)	–	ND*	ND	2 (0.79)	+	ND*	3 (6.89)	1 (0.69)
5	MNF 11539	28/F	OAS	+	4 (18.6)	3 (16.6)	OAS	+	3 (6.10)	3 (15.0)	OAS	+	2 (3.16)	3 (5.64)
6	AMA 7334	27/F	U	+	4 (19.0)	4 (27.2)	–	ND	ND	4 (24.0)	A	+	2 (3.50)	3 (4.09)
7	SGR 7268	33/F	U	+	3 (11.3)	3 (13.1)	–	–	0 (<0.35)	3 (13.6)	R	+	4 (22.2)	3 (8.78)
8	SSG 7799	28/F	OAS/U	+	4 (19.6)	3 (15.1)	ND	ND	ND	3 (14.4)	U	+	3 (5.68)	3 (8.70)
9	EDP 6491	25/F	AE	+	3 (5.00)	3 (6.68)	AE	+	3 (4.14)	3 (6.90)	–	–	ND	2 (3.16)
10	AFC 7241	34/M	OAS	+	2 (2.50)	2 (2.87)	OAS	+	2 (1.09)	2 (2.82)	OAS	+	2 (1.76)	2 (1.83)
11	MMG 4265	24/F	–	–	ND	2 (1.80)	–	ND	ND	2 (2.66)	A	+	0 (<0.35)	1 (0.51)
12	GGG 7306	37/F	–	–	ND	3 (6.08)	–	ND	ND	2 (1.77)	A	+	2 (0.88)	1 (0.42)
13	NSR 8059	31/F	OAS	+	3 (13.4)	3 (15.0)	ND	–	ND	3 (13.6)	–	–	0 (<0.35)	2 (0.91)
14	EBO 7957	30/F	U	+	3 (4.50)	2 (3.44)	–	ND	ND	2 (3.29)	OAS	+	2 (0.97)	2 (2.18)
15	EMB 8798	34/F	OAS	+	2 (2.32)	2 (1.63)	ND	ND	ND	2 (1.47)	–	–	ND	2 (0.77)
16	MSCH 7443	27/F	U	+	3 (10.5)	3 (15.3)	–	ND	ND	3 (15.8)	U	+	3 (4.98)	3 (8.39)
17	OSN 2932	22/F	U	+	3 (13.5)	4 (21.5)	OAS/AE/ A/D/U	–	2 (1.53)	4 (22.1)	OAS	+	3 (4.40)	3 (12.5)
18	MLM 7427	34/M	U	+	2 (1.10)	3 (5.38)	AE/D/R/A	+	1 (0.48)	3 (6.16)	–	–	ND	2 (2.73)
19	NMM 3986	32/F	U	+	2 (3.20)	2 (3.17)	–	ND	ND	2 (3.20)	A	+	1 (0.40)	1 (0.52)
20	FGT 7322	26/M	U	+	2 (1.95)	3 (8.05)	–	ND	ND	3 (8.22)	A	+	2 (1.37)	2 (1.28)
21	EMC 4359	8/M	–	–	ND	3 (6.07)	–	ND	ND	3 (5.48)	OAS	ND	2 (1.85)	2 (3.02)
22	PAL 7944	6/F	AE	+	3 (16.0)	3 (10.8)	ND	ND	ND	3 (10.4)	ND	ND	0 (<0.35)	3 (6.37)
23	PGR 10656	24/F	U/R	+	2 (1.37)	2 (1.56)	OAS	+	2 (0.79)	2 (1.52)	U/AE	–	0 (<0.35)	1 (0.43)
24	EMG 10949	30/F	U	+	3 (12.1)	3 (12.0)	A	+	3 (8.13)	3 (10.7)	U	+	2 (1.10)	2 (2.04)
25	ABR 11714	35/F	U	+	3 (3.70)	3 (4.83)	–	+	2 (1.47)	3 (4.91)	–	–	ND	2 (1.90)
26	RRM 8838	38/M	U	+	3 (6.77)	3 (5.28)	+	+	2 (2.06)	2 (3.17)	A	+	2 (1.23)	2 (1.17)
27	DPM 1252	26/F	AE	+	3 (5.82)	2 (3.19)	AE	+	2 (2.79)	2 (1.27)	AE	+	1 (0.56)	1 (0.64)
28	GVC 6391	23/F	–	–	ND	3 (11.4)	–	–	ND	3 (8.35)	A	+	2 (0.75)	1 (0.44)
29	MMM 1365	15/F	OAS	+	3 (4.00)	3 (6.02)	ND	ND	ND	3 (6.03)	OAS	+	2 (1.08)	2 (2.59)
30	MJBC 3549	24/F	AE	+	2 (1.12)	1 (0.64)	AE/R/A	–	1 (0.38)	1 (0.62)	ND	–	ND	1 (0.49)
31	OMS 8181	19/M	U/AE	+	3 (4.05)	3 (5.30)	OAS/U	+	2 (2.76)	3 (4.16)	AE	+	1 (0.60)	2 (2.08)
32	AGG 8066	7/F	A	+	3 (5.32)	3 (4.25)	AE	+	2 (1.21)	2 (1.95)	–	+	1 (0.40)	1 (0.62)
33	SCP 7535	26/F	AE	+	4 (37.5)	4 (44.8)	–	ND	3 (9.99)	4 (46.3)	–	+	3 (15.1)	3 (13.9)
34	RGP 8074	20/F	U	+	3 (3.90)	3 (5.70)	OAS	+	2 (0.84)	3 (5.40)	OAS	+	2 (0.71)	2 (1.86)
35	LGB 10005	14/F	U/OAS	+	2 (1.00)	2 (1.43)	–	ND	ND	2 (1.41)	–	+	1 (0.56)	1 (0.51)
Pru p 3- and Pru av 3 reactive														
36	RFJ 1360	42/F	OAS	+	2 (1.24)	2 (0.73)	ND	ND	ND	1 (0.70)	ND	ND	ND	0 (<0.35)
37	STV 1035	30/F	U	+	2 (0.89)	2 (0.80)	OAS	ND	2 (0.75)	2 (1.00)	ND	–	ND	0 (<0.35)
38	EMM 11369	43/F	U	+	2 (0.79)	2 (0.71)	OAS	+	1 (0.57)	1 (0.62)	OAS	–	ND	0 (<0.35)
39	LME 8673	31/F	OAS	+	0 (<0.35)	1 (0.47)	AE/R/A	+	1 (0.54)	1 (0.46)	–	–	ND	0 (<0.35)
40	MDC 2473	17/F	OAS/AE	+	2 (2.05)	2 (2.05)	OAS/U	+	2 (1.19)	2 (1.93)	–	–	ND	0 (<0.35)
41	RMJ 10440	10/F	U	+	2 (1.28)	2 (1.05)	–	–	ND	1 (0.38)	–	–	ND	0 (<0.35)
Pru p 3 reactive														
42	OPN 4203	24/M	U/AE	+	2 (1.39)	1 (0.61)	OAS/U	+	0 (<0.35)	0 (<0.35)	–	–	ND	0 (<0.35)
43	NPT 6212	28/F	–	–	ND	1 (0.66)	–	ND	ND	0 (<0.35)	A	+	1 (0.50)	0 (<0.35)
44	CMC 10585	34/M	U	+	2 (0.79)	1 (0.37)	–	ND	ND	0 (<0.35)	–	–	0 (<0.35)	0 (<0.35)
nsLTP non-responder														
45	MPC 9994	26/F	OAS	+	0 (<0.35)	0 (<0.35)	–	+	ND	0 (<0.35)	–	ND	ND	0 (<0.35)
46	MRO 7528	38/F	OAS	+	1 (0.38)	0 (<0.35)	OAS	+	0 (<0.35)	0 (<0.35)	OAS	+	2 (2.00)	0 (<0.35)
47	IGB 7881	30/F	U/AE	+	0 (<0.35)	0 (<0.35)	OAS/AE	–	0 (<0.35)	0 (<0.35)	–	–	0 (<0.35)	0 (<0.35)
48	ELC 9318	30/F	OAS	+	0 (<0.35)	0 (<0.35)	AE	–	0 (<0.35)	0 (<0.35)	–	–	ND	0 (<0.35)
49	JSM 8547	33/M	–	–	ND	0 (<0.35)	–	ND	ND	0 (<0.35)	U	+	2 (2.14)	0 (<0.35)
50	JGL 10571	12/M	A	–	0 (<0.35)	0 (<0.35)	OAS	+	0 (<0.35)	0 (<0.35)	–	–	ND	0 (<0.35)
Σ	50		43 (n)			38/43 (n)	22 (n)			17/22 (n)	26 (n)			22/26 (n)

OAS = Oral allergy syndrome; U = urticaria; A = anaphylaxis; AE = angioedema; R = rhinitis; D = dermatitis; AS = asthma; ND = not determined; ND* = not determined because of severe atopic dermatitis or aversion to the food; ImmunoCAP = class (U/ml); – = no symptoms; + = symptoms not specified.

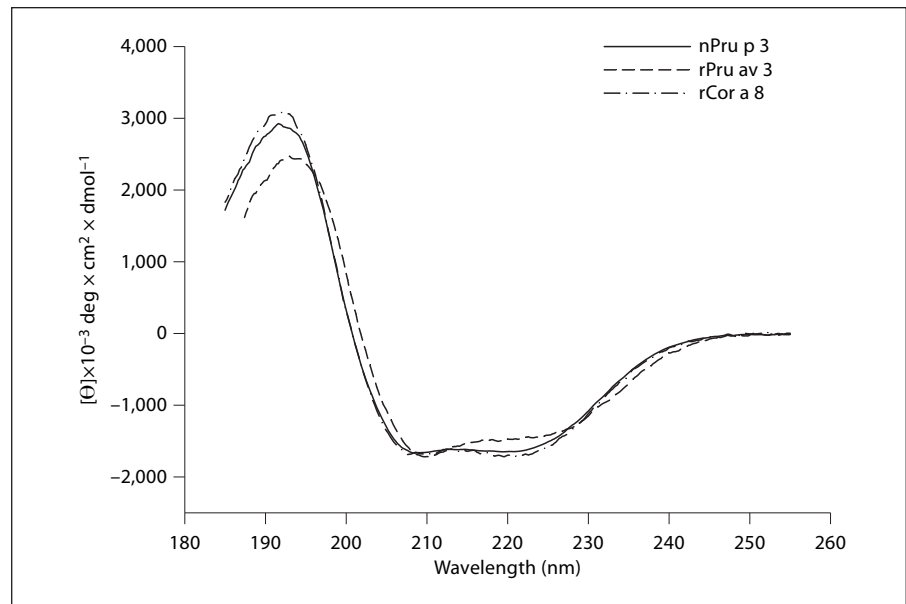


Fig. 2. Circular dichroism spectroscopy of nPru p 3, rCor a 8 and rPru av 3.

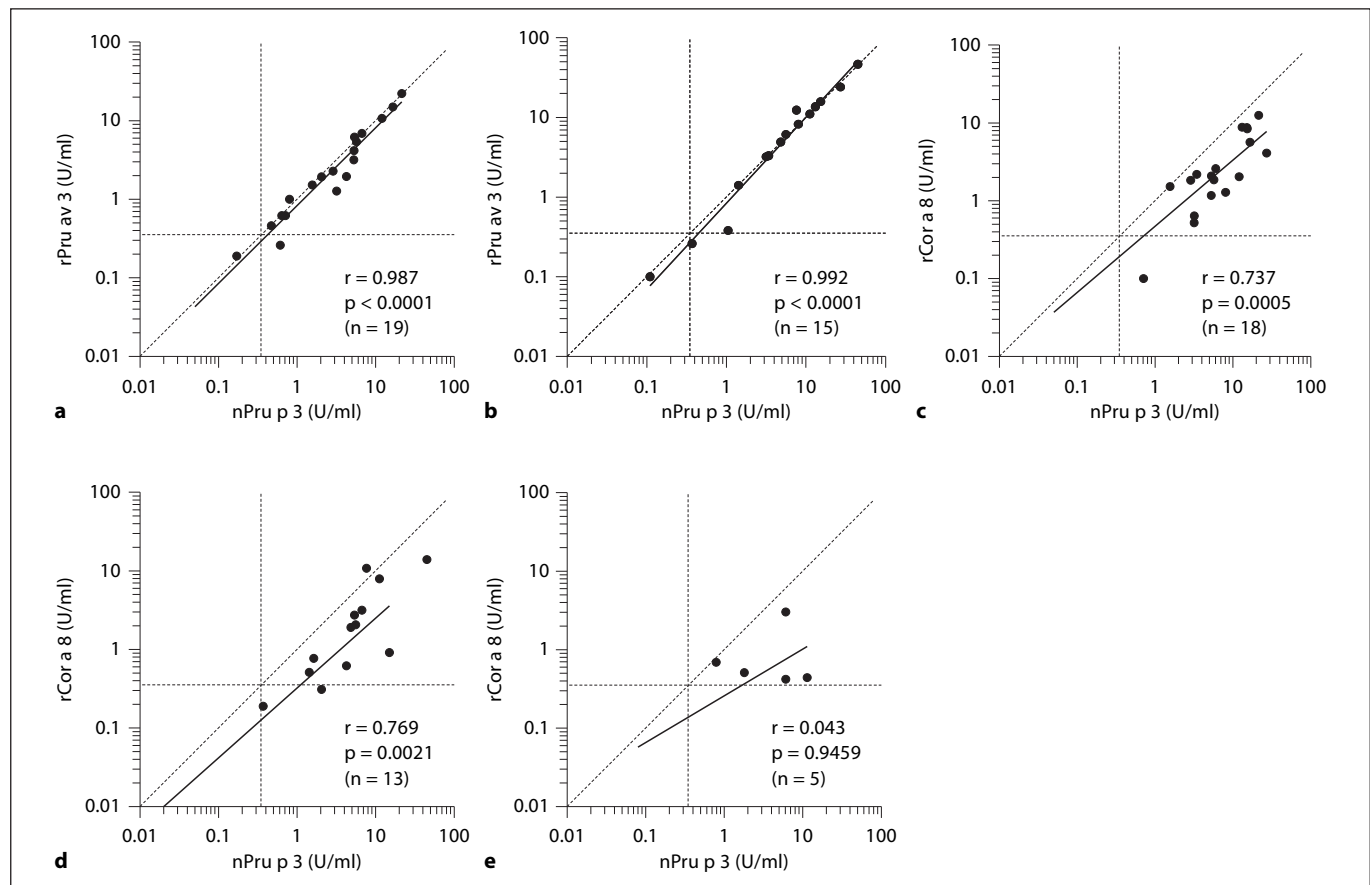


Fig. 3. Comparison of IgE-binding capacity of nsLTPs by ImmunoCAP™; Pru p 3 versus Pru av 3 (**a**, **b**) and Pru p 3 versus Cor a 8 (**c**–**e**). Results above the detection limit of 0.1 kU_A/l are expressed. Linear regression lines, correlation coefficient (*r*) and *p*

values (two-tailed) are given. **a**–**d** Peach-allergic patients with ImmunoCAP™; Pru p 3 versus Pru av 3 (**a**; *n* = 19) and without cherry allergy (**b**; *n* = 15), and with (**c**; *n* = 18) and without hazelnut allergy (**d**; *n* = 13). **e** Hazelnut-allergic patients without peach allergy (*n* = 5).

kU_A/l) for Cor a 8, respectively. All investigated LTPs were confirmed as major allergens in the respective food-allergic groups. In comparison to food extracts, the application of purified nsLTPs by ImmunoCAP testing showed similar sensitivity, except for 3 hazelnut-allergic patients who were negative for Cor a 8, but showed IgE binding to hazelnut extract. All 3 patients did not show IgE binding to Pru p 3 and Pru av 3 indicating sensitization to hazelnut allergens other than Cor a 8. The correlation of frequency and capacity of IgE binding to nsLTPs from Rosaceae fruits (peach and cherry) and non-Rosaceae (hazelnut) is shown in figure 3. Peach nsLTP (Pru p 3) and cherry nsLTP (Pru av 3) revealed almost the same IgE-binding capacity independently of a concomitant cherry allergy in peach-allergic patients ($r > 0.98$; $p < 0.0001$, fig. 3a, b). Sensitization to Pru p 3 in all peach-allergic patients was strongly associated with sensitization to Cor a 8, regardless of hazelnut allergy (fig. 3c, d). For all except 1 of these patients, the specific IgE binding was higher to Pru p 3 than to Cor a 8 ($r = 0.737$, $p = 0.0005$ and $r = 0.769$, $p < 0.0021$). In addition, all hazelnut-allergic patients with sensitization to Cor a 8 (22/26) showed IgE binding to Pru p 3, regardless of their allergy ($n = 17$, fig. 3c) or tolerance to peach ($n = 5$, fig. 3e). Noteworthy, sensitization to the investigated food nsLTPs without the manifestation of clinical symptoms was detected in each subgroup of patients. Moreover, we were not able to detect any serum with reactivity to cherry or hazelnut nsLTP without reactivity to Pru p 3.

nsLTPs from Rosaceae and Non-Rosaceae Are Partially IgE Cross-Reactive

IgE cross-reactivity of Cor a 8 (fig. 4) and Pru av 3 (fig. 5) with Pru p 3 was determined by ELISA inhibition experiments with purified nsLTPs on the solid phase. For each inhibition experiment, 3 selected sera from patients with IgE reactivity to Pru p 3, Cor a 8 and Pru av 3 were pre-incubated with a series of doses of each nsLTP. IgE binding to Pru p 3 was partially inhibited by Cor a 8 (fig. 4a), whereas Pru p 3 completely inhibited IgE binding to Cor a 8 (fig. 4b). For 2 patient sera (MSCH 7443 and SCP 7535), only 25–35% inhibition of IgE binding to Pru p 3 was obtained using the highest inhibitor concentration of Cor a 8 (10 μ g/ml, fig. 4a) whereas ‘self’-inhibition of approximately 90% was achieved at the highest concentration of Pru p 3. For both patients (MSCH 7443 and SCP 7535), half-maximal inhibition of IgE binding to Cor a 8 was achieved by 10- to 100-fold lower antigen concentration of Pru p 3 than Cor a 8 (fig. 4b) itself. These results suggested a different epitope recognition pattern between

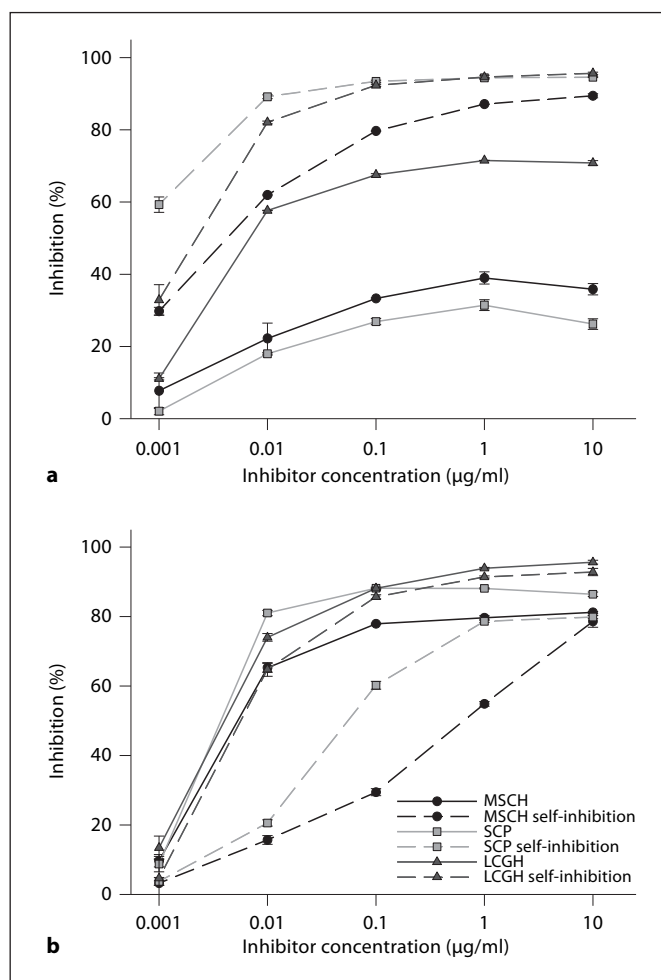


Fig. 4. ELISA inhibition with Pru p 3 (a) and Cor a 8 (b) on the solid phase. Three patients' sera (MSCH, SCP and LCGH) were pre-incubated with Pru p 3 and Cor a 8 in concentrations ranging from 0.001 to 10 μ g/ml. The broken line indicates the homologous ('self') inhibition of each nsLTP. Results are expressed in percent inhibition. Indicated values are mean values from a double determination $\pm$ SEM.

Cor a 8 and Pru p 3, with respect to both the number of epitopes and the affinity of antibody/allergen interaction. In contrast, Pru av 3 showed almost identical inhibition properties as Pru p 3, either using Pru av 3 or Pru p 3 on the solid phase, indicating that IgE epitopes of the two Rosaceae nsLTPs were very similar (fig. 5).

Cor a 8 Displays Low Biological Potency in Comparison to nsLTPs from Rosaceae

Biological potency was investigated by histamine release from human basophils passively sensitized with IgE of 4 different patient sera (MNF 11539, MSCH 7443, SGR

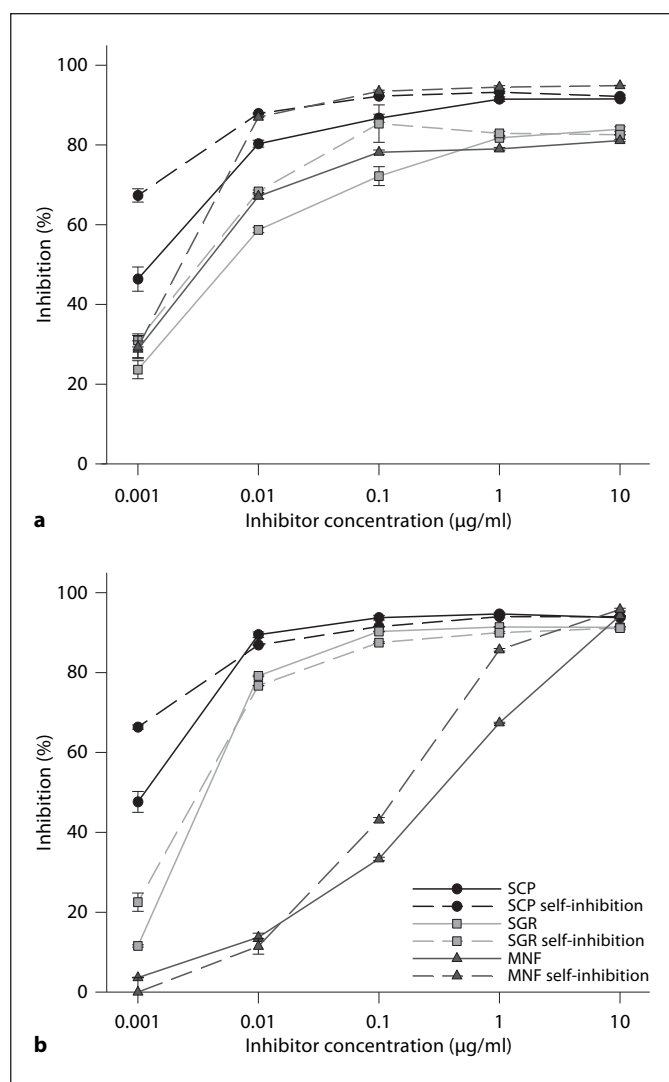


Fig. 5. IgE ELISA inhibition with Pru p 3 (**a**) and Pru av 3 (**b**) on the solid phase. Three patient sera (SCP, SGR and MNF) were pre-incubated with Pru p 3 and Pru av 3 in concentrations ranging from 0.001 to 10 µg/ml. The broken line indicates the homologous ('self') inhibition of each nsLTP. Indicated values are mean values from a double determination $\pm$ SEM.

7268 and SSG 7799). The sera were selected for multiple IgE reactivity to Pru p 3, Pru av 3 and Cor a 8 (table 1) and high specific IgE concentrations to the respective allergens. Sensitization to nsLTPs correlated with clinical symptoms to all three foods only in patient MNF 11539. Patients MSCH 7443 and SGR 7268 tolerated cherry. For patient SSG 7799 clinical reactivity to cherry was unknown, but a high specific IgE concentration to Pru av 3 was detected. Pru p 3 and Pru av 3 elicited a clear histamine

release in a low concentration range (0.1–1 ng/ml) and induced the strongest mediator release of up to 30–50% (fig. 6). Dose-dependent mediator release of both Rosaceae nsLTP was similar for basophils sensitized with at least 2 patients' sera. In contrast, Cor a 8 induced not more than 20% histamine release (SSG 7799), and release curves were shifted towards higher allergen concentrations despite the presence of significant levels of specific IgE antibodies in all patients' sera (table 1). No significant histamine release was observed in 2 of 4 patient sera using concentrations of up to 1 µg/ml of rCor a 8 (fig. 6a, b). In summary, in comparison to Rosaceae nsLTPs, the biological potency of Cor a 8 was substantially lower. No mediator release was induced by the negative control BSA.

Discussion

nsLTPs have been described as major allergens in numerous plant-derived foods and are clinically relevant particularly for patients from the Mediterranean area. Pru p 3, the nsLTP from peach, is considered to be a 'true' food allergen causing a primary sensitization and eliciting clinical symptoms via the gastrointestinal route. However, it is still unknown whether other food nsLTPs can also act as a primary sensitizing agent. In this study, we compared the allergenic properties of Pru p 3 with those of Pru av 3, another Rosaceae nsLTP, and Cor a 8, a non-Rosaceae nsLTP. The frequency of sensitization to selected nsLTPs, their IgE-binding capacity, cross-reactivity and allergenic potency are regarded as evidence for the relevance of nsLTPs in the manifestation of food allergies.

Food-allergic patients (n = 50), reporting either peach, cherry and/or hazelnut allergy, were recruited from Barcelona, Spain. According to the overall number of recruited patients, peach allergy seems to be clinically more important in this population than cherry and hazelnut allergy. Since patients were selectively recruited by reports of at least one the respective food allergies, data only indicate the frequency of peach, cherry or hazelnut allergy in patients with adverse reactions to food. Frequency of systemic reactions to the respective foods was high in all three patient groups, ranging from 55% for cherry, 62% for hazelnut to 74% for peach. Remarkably, for reasons we cannot explain, anaphylactic reactions after ingestion of hazelnut (31%) and cherry (28%) were reported to be more frequent than to peach (9%). Allergy diagnosis based on anamnestic data was in good concor-

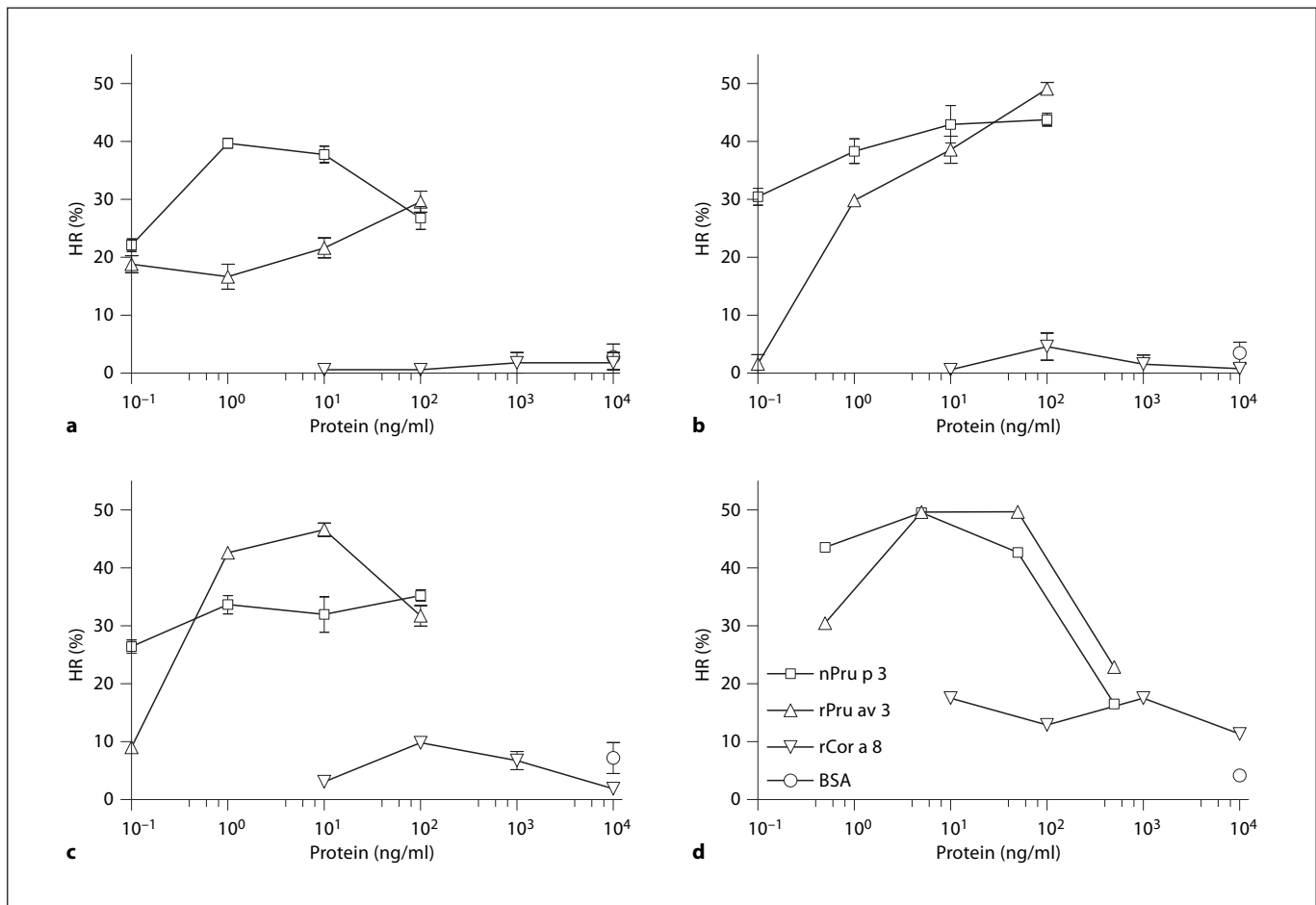


Fig. 6. Histamine release (%) from passively sensitized basophils of 4 different patients with IgE reactivity to Pru p 3, Pru av 3 and Cor a 8. Stimulation was done by the purified nsLTPs in different concentrations ranging 0.1 ng/ml to 10 µg/ml. Results are expressed in percent release.

dance with specific IgE detection (42/43 for peach, 20/22 for cherry and 24/26 for hazelnut), either by serological testing or skin testing using the respective food extracts. Interestingly, at least 70% of peach-allergic patients had a concomitant allergy to cherry and/or hazelnut. In contrast, all patients allergic to cherry and almost all patients allergic to hazelnut had a clinical manifestation of peach allergy; only 7 patients with allergic reaction to hazelnut tolerated peach. Therefore, in regard to the frequency of symptoms, peach allergy is clinically more important than cherry and hazelnut allergy in this patient group.

All recombinant nsLTPs used in the study had similar secondary structures, a strong indication of correct protein folding, and were biologically active. We described for the first time the heterologous overexpression of Pru

av 3 in *P. pastoris*, which was performed according to the method used for Cor a 8 [33]. In comparison to *Escherichia coli*, the eukaryotic yeast expression system was used to improve protein expression, correct disulfide bond formation and to avoid protein aggregation by oligomerization.

In our patient panel, the frequency of IgE-dependent sensitization confirmed all investigated food nsLTPs (rPru p 3: 88%; rPru av 3: 77%, and rCor a 8: 85%) as major allergens. Whereas reactivity to Pru p 3 without co-recognition to Pru av 3 and Cor a 8 was detected in a limited number of subjects, no Pru av 3- or Cor a 8-sensitized patients without simultaneous sensitization to Pru p 3 were found in our patient group. The results are in line with a previous study showing that almost all lettuce-allergic patients with sensitization to the nsLTP Lac s 1

were reactive to Pru p 3 [29] and none of the Pru p 3-negative sera demonstrated a positive RAST to carrot, broccoli and wheat LTPs [30]. The median IgE binding capacity in allergic patients was calculated highest for Pru p 3 (6.83 kU_A/l) and lowest for Cor a 8 (2.74 kU_A/l). Pairwise comparisons of nsLTP IgE reactivity in peach allergics with concomitant cherry and hazelnut allergy showed an almost identical IgE-binding capacity of Rosaceae nLTPs, but a weak correlation for Pru p 3 and Cor a 8. Even 5 hazelnut-allergic patients with clinical tolerance to peach were sensitized to both, Cor a 8 and Pru p 3. Noteworthy, we determined a high frequency of positive reactions to all purified nsLTPs in patients who tolerated the respective foods. A similar IgE reactivity of Pru p 3 and Pru av 3 was observed even if the patients tolerated cherry. This may be explained by the fact that both nsLTP are derived from plants of the same taxonomical family (Rosaceae) and show high amino acid sequence identity (87%) and a strong cross-reactivity. In addition, positive reactions to Cor a 8 were detected in peach-allergic patients who tolerated hazelnut. Except for 1 patient (MLR 10560), IgE binding was lower to Cor a 8, showing 59% amino acid identity with Pru p 3, than to Pru p 3. We conclude that sensitization to non-Rosaceae nsLTP from hazelnut does not occur independently from peach nsLTP sensitization. Considering the frequency of sensitization, the IgE-binding pattern and the specific IgE values, indirect evidence is provided that Pru p 3 (Rosaceae) acts as sensitizer.

Cross-reactivity between Pru p 3 and food nsLTPs from taxonomically distantly related plant species was assessed in previous studies either by using extracts containing unknown amounts of nsLTP and/or mainly in a semi-quantitative manner by immunoblotting inhibition experiments [20, 21, 24, 26–28]. In the present study, experimental conditions were clearly defined, as purified and structurally characterized allergen molecules were used in dose-dependent assays, allowing a quantitative evaluation of data. Our cross-inhibition results confirm the role of Pru p 3 as the predominant nsLTP allergen. Pru p 3 completely inhibited IgE binding to Cor a 8, whereas IgE binding to Pru p 3 was only partially inhibited by Cor a 8. Moreover, it is remarkable that heterologous inhibition with Pru p 3 for 2 sera is stronger than self-inhibition with Cor a 8. In comparison to Pru p 3, 10- to 100-fold higher concentrations of Cor a 8 were required for half-maximal inhibition values. Similar results displaying different IgE inhibition capacities of nsLTPs were shown previously for Pru p 3 and nsLTPs from lettuce and citrus fruits [23, 29]. From our study we conclude that Cor a 8 comprises a lower number of particular

IgE epitopes present also on Pru p 3, but vice versa Pru p 3 reflects all epitopes of Cor a 8. In addition, the avidity of IgE might be higher to Pru p 3 than to Cor a 8. Moreover, our results show almost identical IgE-binding properties of Pru p 3 and Pru av 3, which is in accordance with a previous study showing a complete cross-reactivity between Pru p 3 and Mal d 3 (nsLTP from apple) [12], both belonging to the Rosaceae family. Structural analyses of epitopes by peptide [38] and mimotope [39] mapping have shown conserved conformational IgE-binding epitopes on nsLTPs from members of the Rosaceae family which presumably account for IgE cross-reactivity. The question of whether the observed epitope disparity between Pru p 3 and Cor a 8 (non-Rosaceae) and the strong IgE reactivity of Pru p 3 are due to the sensitization process and the result of a primary sensitization to Pru p 3 or to inherent properties of non-Rosaceae and Rosaceae nsLTPs remains to be answered.

A strong biological potency of Pru p 3 and Pru av 3 was determined by in vitro histamine release assay using passively sensitized human basophils, whereas the biological activity of Cor a 8 was low. The results are in concordance with a stronger biological potency reported for Pru p 3 in comparison to nsLTP from strawberry [20] and plane pollen [13]. Although all tested patients reported adverse reaction to hazelnut and Cor a 8-specific IgE was at least 5.6 kU_A/l (CAP class 3), only 2 patients showed a moderate in vitro histamine release induced by Cor a 8. These results are in agreement with the lower IgE-binding capacity of Cor a 8 in comparison to Pru p 3. The correct folding and biological activity of *P. pastoris*-derived Cor a 8 has been confirmed in previous studies indicating that the lower biological potency of Cor a 8 is not attributable to inadequate quality of the recombinant protein preparation [33, 40].

Our results indicate that Pru p 3 is the most important nsLTP investigated, acting as primary sensitizing agent. Due to the strong IgE cross-reactivity of Pru p 3, and therefore the lack of tested patients without sensitization to Pru p 3, we finally cannot conclude to which extent other food nsLTPs are capable of acting as primary sensitizing agents. The possibility of performing longitudinal cohort studies showing the time-dependent development of an IgE response to particular nsLTPs and manifestation of food allergies is limited by unavoidable simultaneous exposure to several food allergens via the daily diet. Since no data on the consumption behavior of patients, in particular the amount of foods ingested, are available and the accumulation of nsLTPs varies in different foods, it is not possible to calculate the amount of

nsLTPs to which the patients are exposed. Therefore, the impact of antigen dose on sensitization remains unclear. Recently, in contradiction to our study, sensitization to Cor a 8 without IgE reactivity to Pru p 3 was reported in hazelnut-allergic children without any allergic reaction or even without exposure to peach [41]. From these findings we may speculate that Cor a 8 may act as primary sensitizing agent, but it should be considered that the natural Cor a 8 preparation used in that study appeared to contain some impurities which were IgE reactive. Since the induction of allergen-specific T cells is a prerequisite for the induction of a humoral immune response, the capability of individual nsLTPs to stimulate T cells is an important aspect of the mechanism of sensitization. Recently, two immunodominant T-cell epitopes were identified in allergic patients upon stimulation with Pru p 3 [42]. So far, studies addressing the T-cell cross-reactivity of food nsLTPs from different species, as well as the immunogenicity of individual LTPs, e.g. in mouse models, are lacking.

In summary, taking into account the prevalence of IgE sensitization, IgE cross-reactive capacity and allergenic potency, Pru p 3 is the most potent and clinically relevant nsLTP. Whilst Pru p 3 shows IgE reactivity equivalent to Pru av 3, species-specific IgE epitopes exist between nsLTPs from the Rosaceae (Pru p 3 and Pru av 3) and non-Rosaceae family (Cor a 8). Serological data provide indirect evidence that Pru p 3 is the primary sensitizing agent for subsequent reactivity to Cor a 8 and probably for the manifestation of the food nsLTP syndrome.

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