

Pru p 3, the nonspecific lipid transfer protein from peach, dominates the immune response to its homolog in hazelnut

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Abstract

Background: Nonspecific lipid transfer proteins (nsLTPs) are important food allergens. Often, patients allergic to the nsLTP in peach suffer from allergy to hazelnuts. We aimed to analyse the T-cell response to Cor a 8, the nsLTP in hazelnut and its immunological cross-reactivity with the nsLTP in peach, Pru p 3.

Methods: Cor a 8-reactive T-cell lines (TCL) established from patients allergic to hazelnut and peach were stimulated with 12-mer peptides representing the complete amino acid sequence of Cor a 8 to identify its T-cell-activating regions and with Pru p 3 to investigate cellular cross-reactivity. T-cell clones specific for different major T-cell-activating regions of Pru p 3 were stimulated with Cor a 8. Both nsLTPs were subjected to endolysosomal degradation assays. Immunoglobulin E (IgE) cross-reactivity between Cor a 8 and Pru p 3 was assessed in inhibition enzyme-linked immunosorbent assay.

Results: No major T-cell-activating region was found among 26 T-cell-reactive peptides identified in Cor a 8. Although generated with Cor a 8, 62% of the TCL responded more strongly to Pru p 3. This cross-reactivity was mediated by T cells specific for the immunodominant region Pru p 3_{61–75}. Peptide clusters encompassing this region were generated during lysosomal degradation of both nsLTPs. Cor a 8 was more rapidly degraded by lysosomal proteases than Pru p 3. Pre-incubation of sera with Pru p 3 completely abolished IgE binding to Cor a 8, which was not the case *vice versa*.

Conclusions: T-cell reactivity to Cor a 8 is predominantly based on cross-reactivity with Pru p 3, indicating that the latter initiates sensitisation to its homolog in hazelnut. The limited allergenic potential of Cor a 8 seems to be associated with rapid lysosomal degradation during allergen processing and the lack of major T-cell-activating regions.

Nonspecific lipid transfer proteins (nsLTPs) are a family of small proteins ubiquitously expressed in plants. In the Mediterranean area, nsLTPs are the predominant allergens in

Rosaceae fruits causing symptoms ranging from oral allergy syndrome (OAS) to more severe systemic reactions (1, 2). Only recently, nsLTPs were identified as the most frequent triggers of food-induced anaphylaxis in adult Italian individuals (3). The severity of nsLTP-induced symptoms has been linked to their compact structure consisting of four tightly packed alpha-helices, which are further stabilised by four disulphide bridges. This stable fold enables nsLTPs to withstand gastrointestinal digestion and food processing (4, 5).

Abbreviations

aa, amino acid; cpm, counts per minute; dpm, delta cpm; nsLTP, nonspecific lipid transfer protein; OAS, oral allergy syndrome; PBMC, peripheral blood mononuclear cells; TCC, T-cell clone; TCL, T-cell line; Th, T helper; SI, stimulation index.

Among *Rosaceae* fruits, peach is the most frequent cause for nsLTP-allergy (2, 6). Consequently, Pru p 3, the nsLTP from peach, has been extensively studied and its crystal structure (7), immunoglobulin E (IgE) (8) and T-cell-activating regions (9–11) have been identified. Other fruits of the *Rosaceae* family, e.g. apple and cherry, as well as non-*Rosaceae* foods, e.g. hazelnuts, peanuts and other plant-derived foods and certain pollen can also cause nsLTP-mediated allergic reactions upon exposure by either ingestion or inhalation, respectively (12, 13).

On the basis of the clinical observations and experiments on IgE reactivity *in vitro*, it has been suggested that Pru p 3 may play a precursor role in the sensitisation to other nsLTPs (14–18). To address this hypothesis further, we investigated the cellular and humoral cross-reactivity between nsLTP from peach and hazelnut because hazelnut is a frequently offending non-*Rosaceae* food in the Mediterranean area (12). Cor a 8, the nsLTP in hazelnut, has been cloned, sequenced and produced as recombinant protein (Acc. No. AAK28533) (19). Its amino acid (aa) sequence is 59% identical to the sequence of Pru p 3 (Acc. No. CAB96876). However, not much is known about the immunological characteristics of Cor a 8. Therefore, we first investigated the T-cell response to Cor a 8 and identified its T-cell-activating regions employing Cor a 8-specific T-cell lines (TCL) generated from peripheral blood from adult Italian patients allergic to hazelnut and peach. Cor a 8-generated TCL were stimulated with Pru p 3 to assess the cellular cross-reactivity between both nsLTPs. *Vice versa*, Pru p 3-specific T-cell clones (TCC) were stimulated with Cor a 8. In addition to T-cell activation experiments, both nsLTPs were subjected to endolysosomal degradation assays, and the peptides generated during antigen processing were characterised. The cellular investigations were complemented with the analysis of the IgE cross-reactivity between both nsLTPs.

Materials and methods

Allergens

Recombinant Cor a 8 was expressed and purified from *Pichia pastoris* as described (20). Pru p 3 was purified from peach skin (21) and the preparation contained only one Pru p 3-isof orm (Acc. No. CAB96876) as determined by mass spectrometry (data not shown). Single batches of Cor a 8 and Pru p 3, each containing <0.3 endotoxin units/mg protein as determined with the Limulus Amoebocyte Lysate assay (Bio Whittaker, Walkersville, MD, USA), were used throughout the study. Synthetic 12-mer peptides overlapping by nine aa residues and representing the aa sequence of mature Cor a 8 (acc no. AAK28533) including the cloning tag EAEA were purchased from Mimotopes/Biotrend, Köln, Germany.

Patients

Twenty nsLTP-sensitised patients who suffered from allergic reactions to both hazelnut and peach were included (Table 1). Cor a 8 and Pru p 3-specific IgE values were deter-

Table 1 Clinical data of patients

Patient	Sex	Age	IgE specific for		Symptoms to Hazelnut and Peach
			Cor a 8	Pru p 3	
1	M	32	2.01*	7.73*	ANA, AE, OAS, URT
2	F	38	1.74	1.57	AE, ECZ
3	F	60	0.96	9.34	ANA, AS, OAS
4	M	33	1.57	8.63	RHIN
5	F	47	0.57	1.00	AE, OAS, RHIN
6	M	17	2.64	13.47	AS, GT, URT
7	F	20	11.38	12.45	ECZ, OAS, URT
8	M	40	0.39	1.84	AE, RHIN, URT
9	F	19	2.73	2.90	OAS, RHIN
10	F	25	3.38	23.00	AE, OAS, RHIN, URT
11	F	27	0.86	5.46	AE, URT
12	F	30	0.78	20.98	AS, GT, RHIN
13	M	31	2.84	6.45	AS, OAS
14	M	27	0.05	0.71	AS, GT, RHIN
15	F	37	3.00	3.39	ANA, AE
16	F	35	1.29	2.55	AS, RHIN, URT
17	F	25	1.71	3.30	AE, ECZ
18	F	44	0.67	0.83	AE, AS, RHIN, URT
19	F	33	0.32	2.59	ANA, AS, OAS
20	F	42	3.97	16.44	OAS

ANA, anaphylaxis; AE, Angioedema; AS, asthma; ECZ, eczema; GT, gastritis; OAS, oral allergy syndrome; RHIN, rhinitis; URT, urticaria.

*Immunoglobulin E (IgE) values tested by immuno solid-phase allergen chip are expressed as kU_A/l.

mined by means of Immuno Solid-phase Allergen Chip (ISAC; VBC-Genomics, Vienna, Austria) assays. In this test system, IgE values >0.01 are considered positive. This study has been approved by the IDI-IRCCS ethics committee (26/CE/2008). Six nsLTP-negative birch pollen-allergic patients were included who all suffered from rhinoconjunctivitis in spring and showed positive skin prick tests to birch pollen and birch pollen-specific IgE >0.35 kU/l as determined by ImmunoCAP.

Primary responses

Peripheral blood mononuclear cells (PBMC) (2×10^5) were cultured in triplicate in 96-well plates (Nuncclone; Nunc, Roskilde, Denmark) in 200 µl of serum-free Ultra Culture Medium (BioWhittaker) supplemented with 2 mmol/l glutamine and 2×10^{-5} mol/l 2-βME in medium alone or in the presence of titrated amounts of Cor a 8 for 7 days. Proliferation was measured by uptake of tritiated thymidine during the last 16 h of culture. Stimulation indices (SI) were calculated as the ratio between counts per minute (cpm) obtained in cultures stimulated with allergen and cpm obtained from cultures incubated in medium alone.

Identification of T-cell-activating regions

Cor a 8-specific TCL were generated as described from PBMC (1.5×10^6) that had been incubated with Cor a 8

(20 µg/ml) (9). Control cultures were generated with medium alone. To identify T-cell-activating regions, TCL were stimulated with a panel of 28 peptides (5 µg/ml) in the presence of irradiated autologous PBMC. After 48 h, peptide-induced proliferation was assessed.

T-cell cross-reactivity studies

Cor a 8-specific TCL were stimulated with titrated concentrations (10, 5 and 2.5 µg/ml) of Cor a 8, and Pru p 3 in triplicate and highest proliferative responses to each nsLTP were compared. Pru p 3-specific TCC that had been generated in a previous study (9) were thawed, expanded and re-stimulated with Cor a 8 (10 µg/ml) and Pru p 3 (10 µg/ml) in triplicate. Proliferation was determined after 48 h.

Endolysosomal degradation assays

Endolysosomal degradation assays were performed as described (22). Briefly, monocyte-derived dendritic cells (mdDC) generated from CD14⁺ cells purified from PBMC of allergic patients were stimulated for 5 days with IL-4 (1000 U/ml; Strathmann Biotec, Hamburg, Germany) and GM-CSF (50 ng/ml; Peprotech, London, UK). The resulting mdDC were homogeneously CD14⁺HLA-DR⁺CD1a⁺ and were homogenised in TRIS acetate (10 mmol/l, pH 7) containing sucrose (250 mmol/l), and microsomes were isolated by ultracentrifugation. Cor a 8 and Pru p 3 (6 µg of each) were digested in separate reaction set-ups with microsomal enzymes (7 µg) in 100 mmol/l citrate buffer (pH 4.8) containing dithiothreitol (2 mmol/l) at 37°C. Reactions were stopped at the indicated time points by heat denaturation and analysed by SDS-PAGE (15%) and mass spectrometry using an ESI-QTOF mass spectrometer fitted with capillary reversed phase HPLC (Waters, Milford, MA, USA).

IgE-inhibition ELISA

Microtiter plates (Maxisorp; Nunc) were coated with Cor a 8 (1 µg/ml) or Pru p 3 (1 µg/ml) in carbonate buffer (pH 9.6) overnight at 4°C. Sera were incubated with fivefold diluted concentrations from 1 to 0.0016 µg/ml of either Cor a 8, Pru p 3 or Bovine Serum Albumin (BSA) overnight at 4°C. Allergen-coated plates were washed and saturated with 1% BSA in phosphate buffer saline/0.05% Tween 20 for 6 h at room temperature. Subsequently, the pre-absorbed sera were transferred onto the microtiter plates and incubated overnight at 4°C. After washing, bound IgE was detected using an alkaline-phosphatase-conjugated mouse anti-human IgE (BD Biosciences Pharmingen, San Diego, CA, USA). 4-nitrophenyl phosphate salt hexahydrate was used as substrate (Sigma-Aldrich, Steinheim, Germany).

Statistical analysis

Statistical significance of differences was determined using the Wilcoxon Signed Ranks Test using the software package

SPSS (SPSS Inc., Chicago, IL, USA). Differences of $P < 0.05$ were considered statistically significant.

Results

Primary responses to Cor a 8

The clinical data of the 20 adult Italian patients included in this study are summarised in Table 1. All patients displayed IgE antibodies specific for Cor a 8 and Pru p 3 and experienced allergic reactions after the consumption of hazelnut and peach. These foods induced systemic reactions in all but one patient who experienced OAS only. Peripheral blood mononuclear cells could be obtained from 14 patients and were incubated with titrated amounts of Cor a 8. Six birch pollen-allergic patients, who displayed no nsLTP-specific IgE antibodies, served as controls. All subjects showed dose-dependent T-cell responses to Cor a 8 (Fig. 1). Combining nsLTP-sensitised and nonsensitised individuals, the strongest proliferative responses were induced with 50 µg/ml of allergen when compared to lower concentrations. At this concentration proliferative responses in Cor a 8-allergic Italian patients ranged from 407 to 41535 cpm (median, 24128 cpm). Proliferative responses of control individuals at 50 µg/ml of Cor a 8 were significantly lower when compared to the Cor a 8-allergic patients ($P = 0.02$) and ranged from 179 to 34568 cpm (median, 8970 cpm). Background levels ranged from 190 to 21221 cpm (median, 7077 cpm).

T-cell-activating regions of Cor a 8

Twelve Cor a 8-specific TCL could be expanded from PBMC of 12 different nsLTP-allergic patients to cell numbers sufficient for the identification of T-cell-activating regions with 28 overlapping 12-mer peptides covering the entire aa sequence of Cor a 8. In general, very low proliferative responses to the peptides were found. Regarding a SI of ≥ 2 as positive, 26 T-cell-reactive peptides dispersed throughout the complete

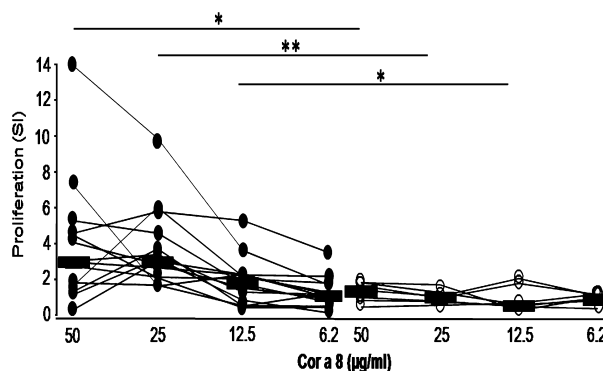


Figure 1 Proliferation of peripheral blood mononuclear cells derived from patients with (solid circles) and without (open circles) hazelnut allergy to titrated amounts of Cor a 8. Stimulation indexes are shown. Horizontal bars indicate median values. (Wilcoxon Signed Ranks Test, * $P < 0.05$; ** $P < 0.01$.)

		Patients											
aa	peptide	1	2	3	4	5	6	7	8	9	10	11	12
–4–7	<u>EAEAS</u> LTCPQIK												
1–12	LTCPQIKGNLTP												
4–15	PQIKGNLTPCVL												
7–18	KGNLTPCVLYLK												
10–21	LTPCVLYLKNGG												
13–24	CVLYLKNGGVLP												
16–27	YLKNGGVLPSPC												
19–30	NGGVLPSPSCCKG												
22–33	VLPPSCCKGVRA												
25–36	PSCCKGVRAVND												
28–39	CKGVRAVNDASR												
31–42	VRAVNDASRTTS												
34–45	VNDASRTTSDRQ												
37–48	ASRTTSDRQSAC												
40–51	TTSDRQSACNCL												
43–54	DRQSACNCLKDT												
46–57	SACNCLKDTAKG												
49–60	NCLKDTAKGIAG												
52–63	KDTAKGIAGLNP												
55–66	AKGIAGLNPILA												
58–69	IAGLNPILAAGL												
61–72	LNPILAAGLPGK												
64–75	NLAAGLPGKCGV												
67–78	AGLPGKCGVNIP												
70–81	PGKCGVNIPYKI												
73–84	CGVNIPYKISPS												
76–87	NIPYKISPSSTNC												
79–91	YKISPSSTNCNNVK												

Figure 2 T-cell-activating regions of Cor a 8. Cor a 8-specific T-cell lines from 12 hazelnut-allergic donors (1–12) were stimulated with 28 overlapping peptides representing the mature protein sequence

of Cor a 8 including the cloning tag EAEA (underlined). Peptides inducing stimulation index (SI) ≥ 2.0 are indicated by grey boxes, peptides inducing SI ≥ 5.0 are shown as black boxes.

hazelnut allergen were identified (Fig. 2). The peptide Cor a 8_{46–57} was recognised by four patients. Three patients harboured T cells specific for peptides Cor a 8_{4–7}, Cor a 8_{1–12} and Cor a 8_{52–63}. The peptides Cor a 8_{7–18}, Cor a 8_{16–27}, Cor a 8_{19–30}, Cor a 8_{28–39}, Cor a 8_{40–51}, Cor a 8_{49–60}, Cor a 8_{58–69}, Cor a 8_{61–72}, Cor a 8_{64–75}, Cor a 8_{67–78}, Cor a 8_{73–84} and Cor a 8_{76–87} were recognised by two patients each, and 10 peptides stimulated a proliferative response from T cells of one single patient (Cor a 8_{4–15}, Cor a 8_{10–21}, Cor a 8_{13–24}, Cor a 8_{25–36}, Cor a 8_{31–42}, Cor a 8_{34–45}, Cor a 8_{43–54}, Cor a 8_{55–66}, Cor a 8_{70–81} and Cor a 8_{79–91}). The peptides Cor a 8_{22–33} and Cor a 8_{37–48} did not induce proliferation in TCL from any patient. Except for patients 10 and 11, all individuals recognised more than one peptide and patient 12 recognised 12 peptides (Fig. 2).

Cor a 8 and Pru p 3 cross-react at the T-cell level

To evaluate the cellular cross-reactivity between Cor a 8 and Pru p 3, 13 TCL established with Cor a 8 were re-stimulated with either nsLTP (Fig. 3). Full-length allergens instead of synthetic peptides were used in these assays to account for the possibility that homologous allergens are differently processed by antigen-presenting cells and to avoid false positive results by synthetic peptides that do not correspond to naturally processed and presented peptides. Proliferative responses to Cor a 8 ranged from 74 to 12035 cpm (median, 2454 cpm), and to Pru p 3 from 200 to 11279 cpm (median, 2523 cpm). Background levels ranged from 99 to 4445 cpm (median, 1116 cpm), respectively. Three TCL proliferated only in response to Cor a 8 (Fig. 3). Six TCL reacted with both

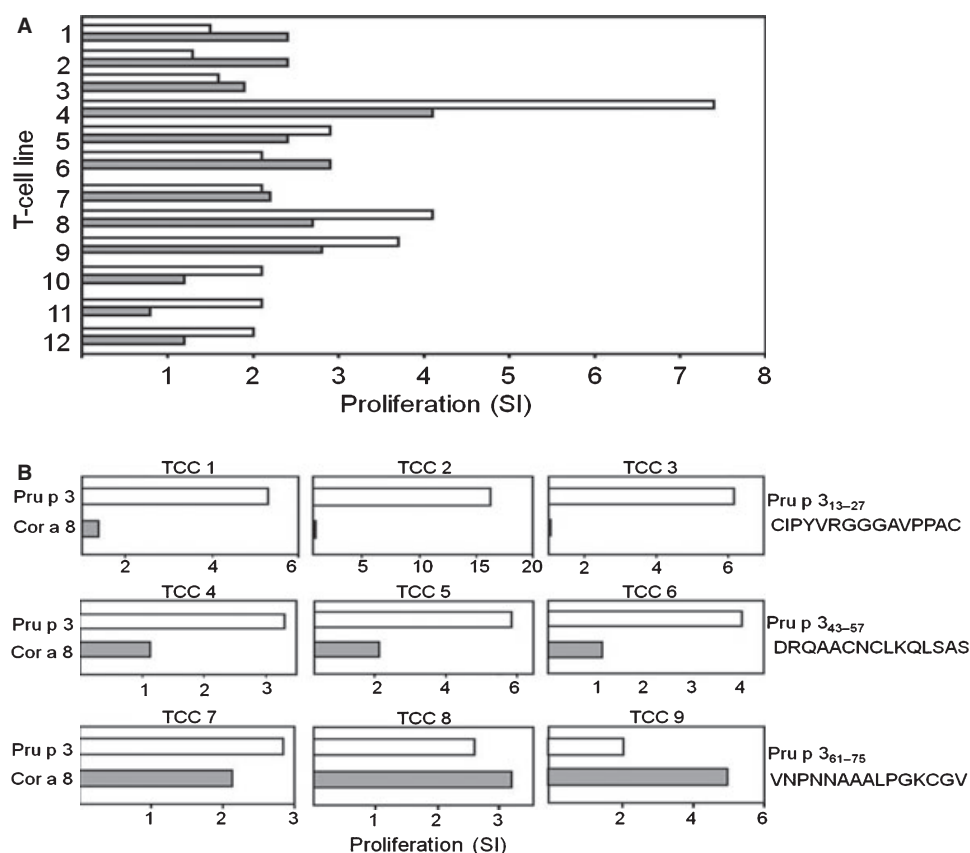


Figure 3 Cellular cross-reactivity of Cor a 8 and Pru p 3. (A) Proliferation of Cor a 8-generated T-cell lines stimulated with Pru p 3 (white bars) or Cor a 8 (grey bars). (B) Proliferation of Pru p 3-

specific T-cell clone (TCC) stimulated with Pru p 3 (white bars) or Cor a 8 (grey bars). Peptides recognised by the respective TCC are shown on the right side. SIs are shown.

allergens of which 4 (67%) responded stronger to Pru p 3 than to Cor a 8. Four TCL had lost lymphoproliferative responses to the original *in vitro* stimulus Cor a 8 but still reacted to Pru p 3. In summary, 10/13 TCL generated with Cor a 8 cross-reacted with Pru p 3. Eight of these 10 TCL proliferated stronger with Pru p 3 than with Cor a 8.

The immunodominant T-cell-activating region of Pru p 3 mediates cross-reactivity with Cor a 8

Cellular cross-reactivity studies indicated that stimulation of PBMC with Cor a 8 expanded Pru p 3-reactive T cells. Therefore, we sought to analyse whether T cells specific for major T-cell-activating regions of Pru p 3 were involved in cross-reactivity with Cor a 8 (9). Clones specific for the regions Pru p 3₁₃₋₂₇, Pru p 3₄₃₋₅₇ and Pru p 3₆₁₋₇₅ were stimulated with Cor a 8. None of the 3 TCC specific for Pru p 3₁₃₋₂₇ proliferated in response to Cor a 8 (Fig. 3). One of 3 TCC specific for Pru p 3₄₃₋₅₇ showed reactivity to Cor a 8. However, all TCC specific for Pru p 3₆₁₋₇₅, the immunodominant T-cell-activating region of Pru p 3, reacted with nsLTP from hazelnut indicating that it is important for cross-reactivity with Cor a 8.

Endolysosomal processing of Cor a 8 and Pru p 3

To complement T-cell activation studies with insights into antigen processing of Cor a 8 and Pru p 3, both nsLTPs were subjected to endolysosomal degradation assays (22). At defined time intervals, lysosomal degradation was monitored by SDS-PAGE and proteolytic fragments of either nsLTP were sequenced by tandem mass spectrometry. Full-length Cor a 8 remained detectable for up to 5 h whereas full-length Pru p 3 was detectable for up to 24 h (Fig. 4). After 0.5 h, two peptide clusters derived from the C-terminus Cor a 8₅₈₋₆₉ and Cor a 8₇₂₋₉₁ were identified (Fig. 4). After 5 h, a third peptide cluster located at Cor a 8₃₃₋₅₇ was detected. Peptides originating from these regions were detectable for up to 48 h of degradation. In the case of Pru p 3, a peptide cluster from the C-terminal region, Pru p 3₅₂₋₈₀, was detected first after 3 h of lysosomal degradation (Fig. 4). Additional peptides within Pru p 3₄₋₂₈ and Pru p 3₂₉₋₄₇ appeared after 3 and 12 h of incubation, respectively. Peptides within these regions also remained detectable for up to 48 h of incubation. Remarkably, these peptide clusters matched the major T-cell activating regions Pru p 3₁₃₋₂₇, Pru p 3₄₃₋₅₇ and Pru p 3₆₁₋₇₅ (9-11).

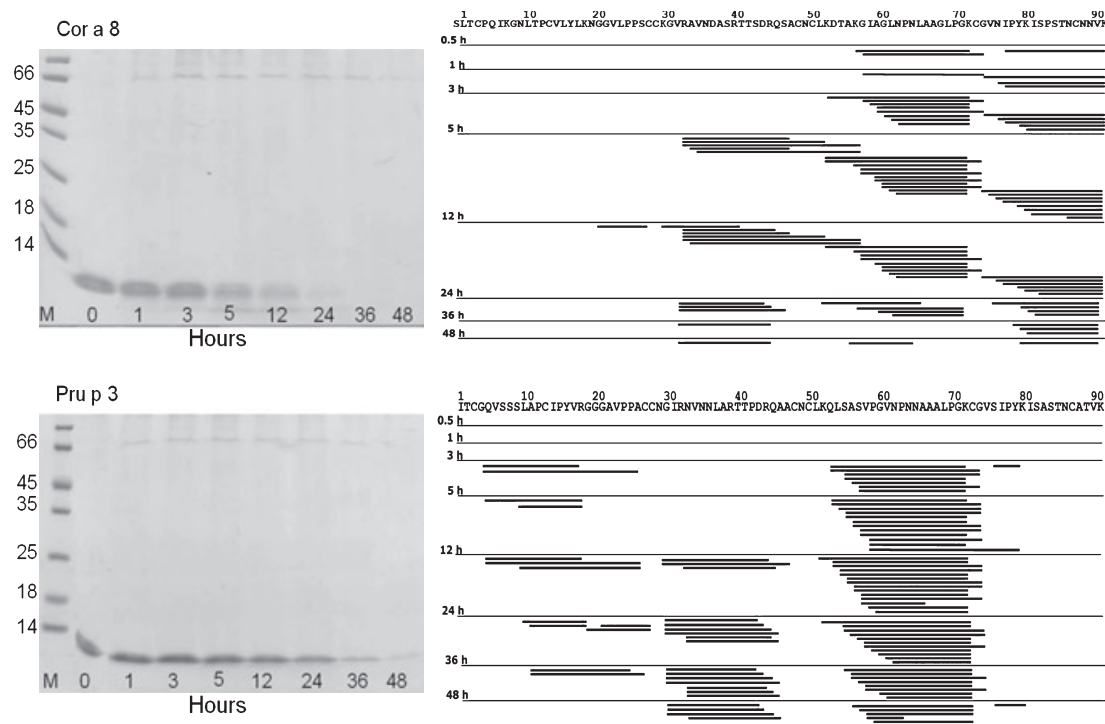


Figure 4 Time kinetics of endolysosomal processing of Cor a 8 and Pru p 3. After 0.5–48 h of incubation with endolysosomal proteases, 10 μ l of the digestion reactions containing 2.5 μ g of

allergen were analysed by SDS–PAGE (15%) and Coomassie staining. Generated peptides were sequenced by mass spectrometry. M, Molecular weight marker (kDa).

Cor a 8 and Pru p 3 cross-react at the humoral level

In addition to cellular cross-reactivity, the degree of IgE cross-reactivity between the nsLTPs from hazelnut and peach was analysed (Fig. 5). For this purpose, sera were pre-incubated with serial concentrations of either Cor a 8 or Pru p 3. Thereafter, the IgE reactivity to Cor a 8 or Pru p 3 was assessed in enzyme-linked immunosorbent assay. At all concentrations used, Pru p 3 inhibited IgE binding to Cor a 8 significantly stronger when compared to Cor a 8 (Fig. 5A).

In contrast, Cor a 8 only marginally reduced IgE binding to Pru p 3 (Fig. 5B). These results suggest that IgE reactivity to Cor a 8 is mainly because of cross-reactive Pru p 3-specific IgE antibodies.

Discussion

This study is the first detailed characterisation of the cellular and humoral cross-reactivity between two nsLTPs from botanically nonrelated plants and indicates that Pru p 3 is

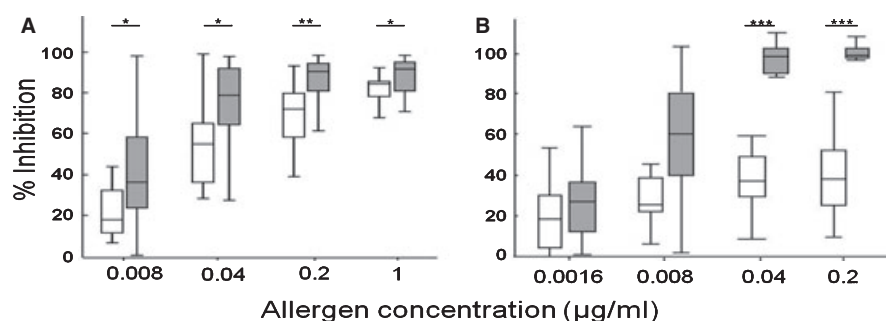


Figure 5 Immunoglobulin E-inhibition enzyme-linked immunosorbent assay. Immunoglobulin E binding to immobilised Cor a 8 (A) and Pru p 3 (B) was inhibited by pre-incubation of sera from 19 hazelnut and peach allergic patients with titrated amounts of Cor a 8 (white box plots) or Pru p 3 (grey box plots). Each box represents

the interquartile range containing the 50% values. The line across the box indicates the median. The whiskers extend from the box to the highest and lowest values. Wilcoxon Signed Ranks Test, * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

the leading cause for sensitisation to Cor a 8 in a selected Italian population suffering from hazelnut and peach allergy. The strong allergenic potential of Pru p 3 seems to result from its low susceptibility to lysosomal degradation which is characteristic for immunogenic proteins (22).

We performed proliferation assays using PBMC and identified T-cell-activating regions by employing Cor a 8-specific TCL to characterise the T-cell response to Cor a 8 prior to the analysis of its cellular cross-reactivity with Pru p 3 (Figs 1 and 2). Despite the weak proliferative responses to the individual 12-mer peptides, we could identify 26 T-cell-reactive regions in Cor a 8. Most T-cell-stimulating activity was located within aa 46–75 of Cor a 8 because peptides belonging to this region induced proliferation in 8/12 (67%) of Cor a 8-specific TCL (Fig. 2). However, none of the 26 T-cell-reactive peptides was recognised by more than 33% of the patients under investigation. In contrast, Pru p 3 contains several major T-cell-activating regions recognised by more than 50% of patients located within aa 12–27, aa 34–48 and aa 57–80 as previously shown by us and others (9–11).

T-cell cross-reactivity between both nsLTP was evaluated by stimulation of Cor a 8-reactive TCL with Pru p 3. Of note, Pru p 3 induced proliferative responses in 77% of Cor a 8-generated TCL. In 80% of cross-reacting TCL, Pru p 3 was a stronger stimulus than Cor a 8 indicating that cellular reactivity to hazelnut LTP was mainly based on cross-reactive Pru p 3-specific T cells. To identify cross-reacting regions of Pru p 3, three TCC specific for either Pru p 3_{13–27}, Pru p 3_{43–60} or Pru p 3_{61–75} were stimulated with Cor a 8. All TCC specific for the immunodominant T-cell-activating region Pru p 3_{61–75} reacted with Cor a 8 whereas very limited reactivity was found for clones specific for the more N-terminally located regions of Pru p 3 (Fig. 3). Thus, the most frequently recognised T-cell-activating region of Pru p 3 seems to be the key player for cross-reactivity with the nsLTP from hazelnut. The aa sequence identity of 80% between both allergens within the region Pru p 3_{61–75} may be one reason for this region-restricted cross-reactivity as less identity exists within Pru p 3_{13–27} (47%) and Pru p 3_{43–60} (60%). To address whether antigen processing of Cor a 8 contributed to the observed region-restricted cross-reactivity, we performed endolysosomal degradation assays of Cor a 8 and Pru p 3 employing mdDC. In previous work, we have demonstrated that this approach delivers sequences matching naturally processed and presented peptides of *in vivo* relevance (23). In endolysosomal degradation assays of Cor a 8, early and continuously generated peptide clusters appeared within its C-terminal regions aa 54–74 and aa 79–91 (Fig. 4). After 5 h, a cluster covering the region aa 31–54 appeared. However, no peptide clusters were detectable in the N-terminal region of Cor a 8. The prominent cluster Cor a 8_{54–74} encompasses the most relevant T-cell-activating region identified using Cor a 8-reactive TCL (Fig. 3) and shares 71.4% sequence similarity with the corresponding region of Pru p 3 which contains the immunodominant T-cell-activating region Pru p 3_{61–75}. Employing a consensus prediction

approach of Major Histocompatibility Complex (MHC) class II-peptide binding (24), no differences in the binding of Cor a 8- and Pru p 3-derived peptides to prevalent MHC class II molecules were found (data not shown). Thus, the continuous loading of Cor a 8-derived peptides highly identical to Pru p 3_{61–75} to MHC class II molecules during allergen processing may determine its prominent role in the cross-reactivity of both nsLTPs.

Clinical observations have suggested a precursor role for the nsLTP from peach in the sensitisation to other food nsLTPs (16). In our study population, allergic reactions to peach also preceded allergic reactions to hazelnut. This clinical evidence is supported by our immunological analyses demonstrating that Pru p 3 dominates the immune response to Cor a 8 in our study population. Similar to a previous study in Spanish patients allergic to both nsLTPs, which showed that Pru p 3 dominates the IgE reactivity to Cor a 8 (25), IgE-inhibition experiments in our laboratory revealed that Pru p 3 strongly inhibited IgE binding to Cor a 8 whereas this was not the case *vice versa* (Fig. 5). From these data, we conclude that Pru p 3 contains most relevant IgE epitopes of the hazelnut nsLTP.

Overall, Pru p 3 is highly reminiscent of the major birch pollen allergen, Bet v 1. Bet v 1 has also been demonstrated to dominate the cellular and IgE responses to other members of the Bet v 1-family (26–28). It contains one immunodominant T-cell-activating region important for cellular cross-reactivity with its homologs (29) and shows limited susceptibility to endolysosomal processing (22). As partial resistance to proteolysis for more than 5 h has been associated with the capacity to induce efficient T-cell priming and antibody responses (22), we have speculated that slow endolysosomal degradation may contribute to the high allergenic potential of the major birch pollen allergen (22). Pru p 3 is the second major allergen that was found to remain intact for more than 5 h when exposed to endolysosomal proteases and contains major T-cell-activating regions. In contrast, Cor a 8 was degraded more rapidly, did not contain major T-cell-activating regions and showed less sensitising capacity. Nevertheless, a small number of Dutch children have been reported to display IgE antibodies to Cor a 8 but not to Pru p 3, indicating that under certain circumstances, e.g. an immature gastrointestinal immune system or when not exposed to Pru p 3, also nsLTPs with less allergenic potential may possess sensitising capacity (30).

In summary, we compared antigen processing, T-cell and IgE reactivity of nsLTP from peach and hazelnut to study the basis of cross-reactivity of allergens within the same protein family. We found that Pru p 3 shares many immunological characteristics with Bet v 1, the primary sensitising allergen of allergic responses to members of the Bet v 1-like superfamily. Combining the information on the immunological behaviour of Bet v 1 and Pru p 3 may contribute towards a better understanding of what determines the strong allergenic potential of major allergens. Furthermore, these insights could have consequences for allergy treatment because specific immunotherapy with the primary sensitising allergen may concomitantly cure allergic reactions resulting

from cross-reactivity (31, 32). However, successful treatment with birch pollen does not always effectively reduce birch pollen-related food allergy even when Bet v 1 dominates the response to its dietary homologs in an individual (33, 34). Whether vaccines containing the nsLTP from peach can concomitantly cure Pru p 3-related cross-allergy needs to be further investigated.

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