

Peamaclein – A new peach allergenic protein: similarities, differences and misleading features compared to Pru p 3

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Summary

Background Among the peach-derived allergens which are already known, the lipid transfer protein (Pru p 3) seems to be the one to exert severe allergic reactions.

Objective To identify and characterize a new peach allergen causing a clinical picture similar to that of Pru p 3.

Methods Patients were selected on the basis of their severe clinical reactivity and negative results to a panel of peach allergens available on the ISAC103 microarray. Several in-house and commercial preparations were compared. Several methods were used to characterize the newly identified molecule. Specific IgE and inhibition assays were performed using the Allergen micro-Beads Array (ABA) assay.

Results Negative ISAC results to Pru p 3 were confirmed by additional testing in contrast with the positive results obtained by commercial Pru p 3-enriched peach peel extracts. The analyses of one of these preparations led to the identification of Peamaclein, a new allergenic protein. It is a small, basic, cysteine-rich, heat-stable, digestion-resistant protein, homologous to a potato antimicrobial peptide. Peamaclein was able to trigger positive skin test reactions and to bind IgE in the ABA assay. It displays an electrophoretic mobility and chromatographic behaviour similar to that of Pru p 3; therefore, it can be hidden in Pru p 3 preparations. In fact, Pru p 3-enriched peach peel extracts were found to contain both Pru p 3 and Peamaclein by means of comparative *in vivo* testing, and by biochemical and immunochemical assays. Commercially available anti-Pru p 3 polyclonal antibodies were found to have a double specificity for the two molecules.

Conclusions and Clinical Relevance A new allergen from peach belonging to a new family of allergenic proteins has been identified and characterized. This knowledge on Peamaclein will improve our understanding on the clinical aspects of the peach allergy and the quality of diagnostic reagents.

Keywords allergen, cysteine-rich protein, direct protein sequencing, inhibition, microarray, new allergenic protein family, peach, severe food allergy, stability

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Introduction

Food allergy prevalence is on the increase worldwide, and recent reports claim that it is going to be a greater problem than in the past [1]. A major contribution to a

better understanding of food allergy is coming from the characterization of the increasing number of allergenic molecules so far identified (www.allergome.org/script/statistic.php). However, after a sharp increase in the identification of new allergenic molecules during the nineties, not as many new allergen groups have been identified in the last decade. The Allergome database (www.allergome.org) currently lists four peach

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allergens (search performed using *Prunus persica*), namely a pathogenesis-related protein group 10, a thaumatin-like protein, a lipid transfer protein (LTP) and a profilin. These four allergens were named by the WHO-IUIS subcommittee for allergen nomenclature as Pru p 1–4 (www.allergen.org) respectively. To date, literature relating peach allergens to severe reactions mainly concerns Pru p 3 [2], the non-specific LTP1. This protein seems to be not homogeneously distributed in the fruit, being reported to be mainly present in the outer part [3–5], thus sometimes not causing symptoms when the peeled peach is eaten [6].

To correctly identify Pru p 3-allergic patients, several tools are used in *in vivo* and *in vitro* diagnosis, the former being based on the peach peel extract whose Pru p 3 content is known [7–9], and the latter based on the purified molecules, which are either natural or recombinant products [9–11]. The same peach peel extract was reported to be suitable for peach immunotherapy [12, 13], and the modified Pru p 3-based immunotherapy is one of the two branches of the FAST EU-funded study aimed at developing new preparations for food allergy immunotherapy [14].

We herein report the identification of a new peach allergen named Peamaclein, which was identified using a combined strategy already reported by Bernardi et al. [11] for the identification of kiwifruit LTP. The strategy is based on recording patients' clinical profiles and their overall diagnostic phenotypes, using some of the above-listed tools, mainly the multiplexing diagnostics [15]. In contrast to the strategy adopted in the study of Bernardi et al., we have mainly concentrated on characterizing discrepancies recorded by comparing several diagnostic tests. A comprehensive biochemical, immunological and clinical characterization of Peamaclein is reported in this study, highlighting the similarities and differences with Pru p 3. A finer characterization of the Pru p 3-enriched peach peel extracts available for *in vivo* diagnosis of the LTP allergy is also reported.

Methods

Preparation of protein extracts from peach peel and pulp

Peach fruits (*Prunus persica*, cultivars Percoca Ionia, Crimson Lady, Isabella d'Este) at the commercial ripening stage were provided by the Research Council for Experimentation in Agriculture, Research Unit on Fruit Trees (Caserta, Italy).

The peel and pulp were manually separated and homogenized in a blender after the addition of 1 M NaCl (1 : 1 w/v). After protein extraction, the peel and pulp samples were centrifuged and the supernatants, representing the protein extract, were collected.

Fresh peach preparations, peach extracts, Pru p 3-enriched peach peel extracts, Pru p 3 preparations

The peach pulp and peel of the Crimson Lady cultivar were manually separated and cut into small pieces, sufficient for a single patient prick–prick testing procedure (P–P). The preparations were immediately stored in plastic containers at -20°C , thawed immediately before the testing and discarded afterwards. A commercial whole peach fruit extract preparation for the skin prick test (ST) was purchased from Allergopharma (Reinbeck, Germany); two Pru p 3-enriched peach peel extracts for the ST were obtained from ALK-Abelló (ALK, Madrid, Spain) and BIAL Industrial Farmacéutica S.A. (BIAL, Bilbao, Spain). Different batches of the two commercial preparations were available. A purified natural (n)Pru p 3 preparation was purchased from BIAL. A second purified nPru p 3 was produced in-house as previously described [16].

Already described procedures were used for the estimation of protein concentration, protein extract fractionation by RP-HPLC, MALDI-TOF mass spectrometry, amino acid sequencing [11], circular dichroism (CD) experiments [17], preparation of Peamaclein and Pru p 3 solutions for skin testing and stability to the simulated gastric digestion (SGF) and intestinal digestion (SIF) [11, 18]. Details are reported in the online Supplementary Information.

Purification of Peamaclein from peach pulp

Peamaclein was purified from the pulp protein extract by ionic exchange and by size exclusion chromatographies as described in the Supplementary Information. The protein concentration was estimated on the basis of the molar extinction coefficient at 280 nm ($6710 \text{ M}^{-1} \text{ cm}^{-1}$). The purity of the protein preparations was checked by SDS–PAGE, Reverse phase-high performance liquid chromatography (RP-HPLC) and N-terminal amino acid sequencing as previously reported [19].

Structural modelling

The modeller v9 and QUARK methods [20, 21] were used to generate about 400 3D models of the Peamaclein structure. All the models were analysed for their secondary structure assignment with the DSSP program [22] and the results compared with the available data obtained by CD experiments. Moreover, the distances between all cysteine pairs were evaluated, to explore the possibility of disulphide bridge formation and to check if the results agreed with the experimental data. The selected model (Figure S3), generated with QUARK [21], was then subjected to very limited energy refinements (100 cycles with the insight II Discover Module, steepest descent algorithm).

Allergic subjects, skin test and double-blind placebo-controlled food challenge testing, sera collection, ISAC IgE routine testing

The study was approved by the Institutional Review Board of IDI-IRCCS, Rome, Italy (28/CE/2008). Patients or caregivers signed an informed consent when the patients were undergoing tests not in the routine work-up. Patients' demographic and clinical data, namely cutaneous, respiratory and ingestion-related symptoms on peach exposure, as well as all the *in vivo* and *in vitro* diagnostic data were recorded for all patients by an allergy specialist, or transferred real time from the laboratory into the InterAll software, a customized allergy electronic record in use at the Center for Molecular Allergology (CAM) at IDI-IRCCS (version 3.0; Allergy Data Laboratories, Latina, Italy). Peach-specific clinical information was collected adapting a previously reported standard questionnaire [19]. All enrolled subjects underwent specific IgE detection by the ISAC 103 microarray testing system (Phadia Multiplexing Diagnostics, PMD, Vienna, Austria). ST was performed and recorded as weal areas using a standard methodology as already reported [11]. The used preparations were – 20°C-stored fresh peach pulp and peel; a commercial peach extract (Allergopharma); the two commercial Pru p 3-enriched peach peel extracts (ALK and BIAL); and the in-house nPru p 3. A double-blind placebo-controlled food challenge (DBPCFC) was performed in enrolled subjects when not reporting a recent anaphylaxis episode [19]. The recipe used for blinding the peach preparation was the one adopted during the first phase of the FAST project with minor modifications [14]. In the case of patients reporting recent anaphylaxis after ingestion of peaches, this was taken as proof of clinical allergy in accordance with the most recent European guidelines [23]. Sera for *in vitro* testing were collected from patients during either the routine work-up, or during the further *in vivo* testing steps. The sera were stored at – 20°C until use.

IgE binding by immunoblotting after SDS–PAGE

IgE binding to Peamaclein preparation was first checked by immunoblotting (IB) following the already reported procedure [17]. Two different secondary antibodies were used; a goat polyclonal anti-IgE from Bioallergy (Fiumicino, Italy) and a mouse monoclonal anti-IgE from Becton Dickinson Biosciences (San Jose, CA, USA), both conjugated to alkaline phosphatase.

IgE detection by ImmunoCAP and dot blot singleplexing

Total and specific IgE using a singleplexed system were determined in patients' sera obtained at the time of

enrolment using the ImmunoCAP system (Thermo Fisher Scientific, Uppsala, Sweden). IgE detection included the peach extract and the recombinant (r)Pru p 3 preparation as available from the manufacturer. Values were considered positive when > 0.35 kU/L. IgE dot blotting technique is detailed in the Supplementary Information.

IgE detection by Allergen micro-Beads Array as a flexible multiplexing assay

A recently developed microbead-based immunoglobulin detection system called Allergen Beads Array (ABA) [24] was used for the detection of IgE recognizing in-house produced allergens and control molecules, to measure non-human antibody reactivity and for the IgE inhibition assay. The details are given in the Supplementary Information.

IgE detection by ISAC multiplexing

Specific IgE for allergenic molecules, including commercial rPru p 1, nPru p 3 and profilin preparations were detected by the ISAC 103 microarray test (PMD); the ISAC tests were performed as previously reported [25]. An experimental version of the ISAC microarray (ISAC Exp96, PMD) was developed to carry out the characterization of the IgE reactivity against allergens not available on the commercial ISAC 103 [11, 26], including our nPru p 3 in-house preparation. All proteins were immobilized on ISAC Exp96 following the same methodology as for the routine ISAC 103 [11]. Furthermore, for control purposes, the recently released ISAC 112 (Phadia Austria, Vienna, Austria) was used following the manufacturer instructions. This ISAC version bears the same rPru p 3 preparation produced in *Escherichia coli* available in the ImmunoCAP system (Christian Harwanegg, Phadia Austria, personal communication). For all ISAC microarray versions, values were considered positive when > 0.01 kU/L.

IgE single point highest inhibition achievable assay

IgE inhibition on ISAC: IgE inhibition experiments were performed using the single point highest inhibition achievable assay (SPHIAa) as previously reported [11, 19] (see the Supplementary Information).

IgE inhibition on ABA: the deglycerinated extract preparations (ALK and BIAL), the two nPru p 3 preparations, namely from BIAL and in-house ones, the highly purified Peamaclein preparation and the three pulp and peel peach cultivar preparations were used for the IgE inhibition experiments following the established ISAC SPHIAa protocol on the new ABA assay (see the Supplementary Information).

Results

Patients clinically allergic to peach but lacking any positive peach allergenic molecule-based IgE positive test

A periodical survey of patients' e-records led to the observation that some peach clinically allergic patients, reporting severe reactions, had no ISAC-detectable IgE to Pru p 3 or to any other available allergen. For that reason, two subjects, no. 1 and 2 in Table 1, underwent further Pru p 3 testing by using the in-house nPru p 3 preparation for ST and the rPru p 3 available on ImmunoCAP. The results of both tests were also negative. The same two patients were subjected to additional tests. In contrast with the above described negative results, both the subjects showed a positive ST when tested with the commercial whole peach extract (Allergopharma) and with two Pru p 3-enriched peach peel preparations (ALK and BIAL) (Table 1). On the basis of these conflicting observations, the presence of a new allergen in some peach extract preparations was hypothesized.

Identification of Peamaclein as a new protein component of peach extracts

To investigate the possible presence of a new allergen as a contaminant of Pru p 3-enriched peach peel extracts, the two commercial preparations, namely ALK and BIAL, giving positive ST results in enrolled patients, were analysed by electrophoretic and chromatographic separations: SDS-PAGE (Fig. 1, lanes A and B) and RP-HPLC, after glycerine removal as reported in the Supplementary Information for analytical purposes. SDS-PAGE showed a band at the Pru p 3 MW for each preparation. Chromatographic profiles displayed high amounts of Pru p 3 and small amounts of components eluted at retention times different from that of Pru p 3 (data not shown). The eluted peaks were manually collected and analysed. However, due to the low amount of the starting material, only Pru p 3 was clearly identified by N-terminal sequencing.

To avoid the step of glycerine removal and to collect contaminant components in amounts sufficient for biochemical identification of the molecules, a lyophilized preparation of nPru p 3 from one of the two Pru p 3-enriched peach peel extract manufacturers (BIAL) was subjected to chromatographic separation by RP-HPLC (Fig. 2, panel A). Eluted peaks were collected and analysed by N-terminal amino acid sequencing. Peaks eluted at retention times between 29 and 34 min were identified to be Pru p 3. The elution of Pru p 3 in different peaks can reasonably be assumed to be due to heterogeneities associated with the expression of different isoforms in the peach. The analysis of the other collected peaks indicated that some of them prob-

ably do not have a protein nature as they did not produce any identifiable sequence, other than the peak eluted at 26 min that produced the N-terminal sequence GSSFCDKCGVRCSKAG. Fig. 2, panel B, shows that the same peak is not observed in the chromatographic profile of the in-house Pru p 3 preparation. We have originally named this new protein component 'Peamaclein'.

Biochemical analysis of Peamaclein distribution in the peach pulp and peel

To investigate the Peamaclein distribution in the peach tissues, peel and pulp protein extracts were obtained from the cultivar Percoca Ionia and analysed by SDS-PAGE (Fig. 1, lanes C and D). The extracts were also fractionated by RP-HPLC (Fig. 2, panel C and D) and the peak eluted at the retention time of Peamaclein was collected and analysed by N-terminal sequencing. The results obtained indicated that Peamaclein was contained in almost comparable amounts in both the peach tissues. The analysis by N-terminal sequencing of other peaks allowed the identification of Pru p 3 as the major component of the peel extract. The fractions of the pulp extract eluted around the retention time of Pru p 3 were also collected and analysed by direct protein sequencing. However, Pru p 3 was not detected.

Purification and structural characterization

Peamaclein purified to homogeneity from the peach pulp was used for the structural and immunological characterization. It showed a single band with a migration rate very close to that of Pru p 3 (Fig. 1, lanes E and F). As shown in the 15% SDS-PAGE, the two different proteins cannot be seen separated either in our extracts (Fig. 1, lanes C and D) or in the two Pru p 3-enriched preparations (Fig. 1, lanes A and B). Peamaclein and Pru p 3 were also run, side-by-side and mixed together in the same lane, using a 17% SDS-PAGE, but again their electrophoretic mobility showed very similar apparent molecular masses (Supplementary Information, Figure S1). However, in line with the slightly different theoretical isoelectric point (pI) values estimated for Peamaclein (8.97) and Pru p 3 (9.25) using the ProtParam algorithm at www.expasy.org, the two allergens were separated by the strong cation exchanger Mono-S HR 10/10 column in a Fast Protein Liquid Chromatography system (Amersham-Pharmacia, Uppsala, Sweden) (Supplementary Information, Figure S2).

Primary structure and homology search

A homology search, performed in the Allergome database, as of May 19, 2012, using the N-terminal sequence of Peamaclein (GSSFCDKCGVRCSKAG) and the

Table 1. Peach allergic patients and controls – Demographic data, clinical and diagnostic profiles

Skin test [†]										IgE [‡]		ISAC 103				
Molecules					Extracts		Fresh fruit		ImmuCAP		ISAC 103					
Groups	N	Age	Gender	Symptoms*	Total IgE	Pru p 3 in-house	Pru p Peamaclein	Pru p		Pru p [Fruit] Allergopharma	Pru p [Peel]	Pru p [Pulp]	Pru p 3 in-house	Pru Peamaclein p 3	Peach	Pru p 3
								[Enriched Peel] Alk	[Enriched Peel] BIAL							
A	1	45	F	ANG, U	50	Neg	46.30	40.70	16.80	40.50	43.70	40.50	142	201	Neg	Neg
	2	37	F	ANA, ANG, AR, U	51	Neg	68.60	54.40	26.40	53.40	48.70	45.60	149	515	Neg	Neg
	3	16	F	ANG, AR, GI, U	154	Neg	82.00	98.70	43.20	Neg	47.60	63.00	130	6444	Neg	3.73 2.57
	4	25	F	ANG, U	99	Neg	21.30	30.50	Neg	Neg	7.90	7.20	173	7776	Neg	6.84 0.29
	5	23	F	ANG, GI, U	88	Neg	17.40	26.40	Neg	Neg	NT	NT	42	208	Neg	0.41 Neg
	6	20	F	ANG, GI, O	229	Neg	38.90	18.40	24.70	NT	NT	NT	41	4993	Neg	2.65 1.90
	7	41	F	ANG, U	492	Neg	29.68	NT	NT	NT	NT	NT	196	2165	Neg	0.76 Neg
	8	36	M	ANA, ANG, U	95	21.00	16.50	12.40	24.70	NT	NT	NT	323	2855	1.10 1.71 0.03	
	9	40	F	ANG, O	18	132.40	44.70	125.50	206.00	13.50	109.30	39.30	7509	55	1.40 1.17 5.74	
	10	36	F	ANG, AR, U	21	212.00	34.50	246.30	140.00	26.80	104.80	77.00	10 760	52	2.68 2.62 1.33	
B	11	27	M	ANG, U	133	53.70	126.70	66.70	45.60	Neg	NT	NT	120	875	1.45 1.64 0.75	
	12	7	M	ANG, GI, U	298	37.00	14.00	26.50	16.60	NT	NT	NT	132	129	1.62 2.68 Neg	
	13	5	M	ANG, U	NT	211.20	12.30	NT	NT	NT	NT	NT	2724	98	7.64 6.42 20.01	
	14	8	M	ANG, O, U	47	107.70	13.00	57.20	55.80	NT	NT	NT	3032	78	8.59 7.49 13.52	
	15	47	F	ANA, ANG, O, U	38	192.00	Neg	59.50	45.60	13.30	33.30	28.00	438	62	3.44 2.97 2.92	
	16	41	F	O	23	113.60	Neg	31.40	31.80	12.00	74.00	12.00	112	103	6.89 6.78 0.97	
	17	15	M	ANG, U	310	100.40	Neg	66.70	56.50	Neg	56.00	61.80	11 667	99	100.00 100.00 37.34	
	18	38	F	ANG, O, U	452	30.40	Neg	10.20	7.50	Neg	18.70	6.50	775	102	21.70 14.20 1.08	
	19	19	F	GI	250	18.40	Neg	25.70	20.30	Neg	35.80	Neg	302	109	5.83 5.56 7.46	
	20	47	F	ANG, AR, O	20	100.30	Neg	67.80	57.40	Neg	89.40	27.80	9132	132	4.16 3.60 4.78	
C	21	33	F	ANG, U	58	168.00	Neg	162.20	170.00	Neg	193.50	59.00	773	112	1.78 NT 0.51	
	22	37	M	ANG, GI, U	11	315.30	Neg	72.50	100.30	Neg	44.40	118.70	988	102	2.70 3.03 2.72	
	23	37	F	ANG, U	224	71.60	Neg	34.70	23.00	Neg	79.40	Neg	1017	88	NT 7.87 2.61	
	24	39	F	ANG, AR	29	28.60	Neg	26.60	12.00	Neg	12.40	14.30	97	94	Neg Neg Neg	
	25	37	M	ANG, U	33	49.20	Neg	74.50	59.40	Neg	48.80	Neg	141	88	Neg Neg 0.16	
	26	57	M	ANG, GI, U	16	62.70	Neg	50.30	53.30	Neg	56.00	31.20	280	113	Neg Neg Neg	
	27	14	M	ANG, U	138	52.60	Neg	40.80	31.20	Neg	78.30	71.40	548	122	10.70 12.40 3.75	
	28	30	M	ANA, AR, O, U	NT	74.80	Neg	40.70	16.70	Neg	56.50	Neg	3765	78	57.40 51.00 5.78	
	29	18	M	GI, O	102	83.50	Neg	38.20	14.50	Neg	118.50	73.50	163	87	0.93 1.58 Neg	
	30	27	F	ANG, U	12	215.90	Neg	167.40	217.40	6.70	48.00	87.40	1117	74	4.69 4.12 4.32	
D	31	13	F	ANA, ANG, O, U	115	97.00	Neg	49.00	38.70	1.70	48.80	8.80	1659	87	39.30 26.70 5.85	
	32	24	F	ANA, ANG, U	33	83.50	Neg	64.00	114.00	Neg	42.00	47.60	226	92	8.72 7.27 3.72	
	33	48	F	AR	112	9.70	Neg	8.50	7.60	10.20	11.60	9.80	436	104	1.54 Neg 0.64	
	34	47	F	–	93	Neg	Neg	Neg	Neg	Neg	Neg	Neg	143	66	NT NT Neg	
	35	54	M	–	350	Neg	Neg	Neg	Neg	Neg	Neg	Neg	141	71	NT NT Neg	
	36	32	F	–	178	Neg	Neg	Neg	Neg	Neg	Neg	Neg	145	74	NT NT Neg	

(continued)

Table 1 (continued)

Groups	N	Age	Gender	Symptoms*	Skin test†										IgE‡		
					Molecules					Extracts					Fresh fruit		
					Total IgE	Pru p 3 in-house	Pru p 3	Pru p Peamaclein	Pru p [Enriched Peel] Alk	Pru p [Enriched Peel] BIAL	Pru p [Fruit] Allergopharma	Pru p [Peel]	Pru p [Pulp]	Pru p 3 in-house	ABA	ImmunoCAP	ISAC 103
37	33	F	–	–	30	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	10	10	NT	Neg
38	59	F	–	–	NT	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	96	91	NT	Neg
39	51	F	–	–	20	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	49	75	Neg	Neg
40	30	M	–	–	29	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	91	35	Neg	Neg
41	28	F	–	–	3266	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	41	78	NT	Neg
42	6	M	–	–	98	Neg	Neg	Neg	Neg	Neg	Neg	Neg	Neg	160	111	Neg	Neg

*Symptoms are either as reported by patients or as recorded after double-blind placebo-controlled food challenge (DBPCFC): ANA, Anaphylaxis; ANG, Angioedema; AR, Asthma and Rhinitis; GI, Vomiting and Diarrhoea; O, Oral Allergy Syndrome; U, Urticaria; NT, Not tested.

†Skin tests are expressed as mean weal areas in mm²; ABA, Allergen micro-Beads Array; ISAC 103, Immuno Solid Phase Allergen Chip 103.

‡IgE values are reported as follows: ABA: Median Fluorescence Index (MFI; positive value > 200 MFI); ImmunoCAP and ISAC 103 kU/L (positive values > 0.35 and 0.01 kU/L respectively). Total IgE are expressed as IU/L.

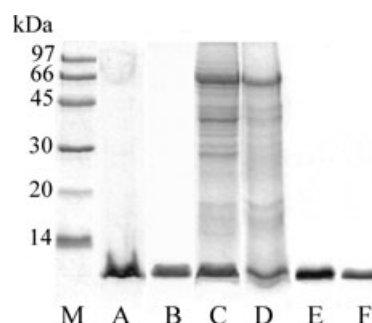


Fig. 1. SDS-PAGE of peach protein samples – Lane A: Pru p 3-enriched peach peel preparation (ALK; 6 µg); Lane B: Pru p 3-enriched peach peel preparation (BIAL; 6 µg); Lane C: Peach peel extract (cultivar Percoca Ionia; 30 µg); Lane D: Peach pulp extract (cultivar Percoca Ionia; 30 µg); Lane E: Peamaclein (4.5 µg); Lane F: in-house nPru p 3 (4 µg); Lane M: molecular weight markers.

AllergomeAligner tool (www.allergome.org/script/tools.php?tool=blaster), indicated that the list of known allergens does not contain any homologue of the protein. The search carried out in UniProtKB (www.expasy.org) revealed the absence of any peach homologues of Peamaclein in this database. However, a high sequence identity with a potato antimicrobial peptide (82%), snakina 1 [27], and with some other sequences from several plant organisms was observed. Even though the electrophoretic mobility of Peamaclein was similar to that of Pru p 3, the results obtained by the homology search in UniProtKB, using the BLAST algorithm, indicated the lack of sequence identity, and therefore the lack of homology, between Peamaclein and Pru p 3. A further homology search performed using the N-terminal sequence of Peamaclein to query the Expressed Sequence Tags (EST) database by the BLAST algorithm at NCBI (www.ncbi.nlm.nih.gov/Blast.cgi) showed the presence of some clones deriving from peach pulp (BU043773, BU044134, BU047758, BU046575) and peel (AM287841, AM290970, AM287653) coding for a single protein molecule having the N-terminal region identical to that of Peamaclein. Afterwards, the amino acid sequence of 18 residues located in the internal regions of Peamaclein (residues 25–42, Fig. 3) was elucidated by sequencing some of the peptides deriving from the trypsin digestion of the purified protein. These additional sequence data confirmed the identity between the primary structure of Peamaclein, and that of the above-mentioned EST clones. Accordingly, the molecular mass (6909.9 Da) of the protein coded by the EST clones was in agreement with that obtained for the purified Peamaclein by MALDI-TOF mass spectrometry (6910.84 ± 20 Da). This observation also suggests the absence of post-translational modifications, such as glycosylation.

The analysis of the primary structure of Peamaclein (Fig. 3) indicates that it is a small protein of 63 amino acid residues characterized by a high cysteine content,

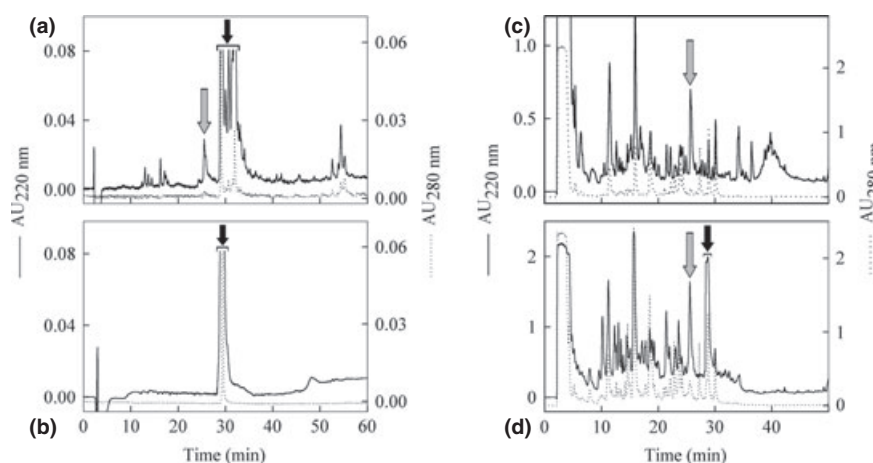


Fig. 2. RP-HPLC analysis of peach-derived preparations – Panel A: Pru p 3-enriched peel extract (BIAL; 0.25 mg); Panel B: in-house Pru p 3 (0.25 mg); Panel C: pulp extract (cultivar Percoca Ionia; 0.3 mg); Panel D: peel extract (cultivar Percoca Ionia; 0.7 mg). Grey and black arrows indicate Peamaclein and Pru p 3 peaks respectively.

	1	10	20	30	40	50	60	Identity (%)
PEAMACLEIN	G S S F C D S K C G V R C S K A G Y Q E R C L K Y C G I C C E K C H C V P S G T Y G N K D E C P C Y R D L K N S K G N P K C P							
B9MVY8_POPTR	A - G - - - - - S - - - - - I K D - - - - - K - - - - - H - - - - - M - - - - - K - - - - -							84
E0CP56_VITVI	- - G - - - - - A A - - - - - M K D - - - - - E - K - - - - - H - - - - - K - K - - - Q - - - - -							82
B9R733_RICCO	- - G - - - - - E K - E - - - - - V K D - - - - - H - - - - - M - - - - - K - - - - -							82
Q948Z4_SOLTU	- - - - - - - - K L - - - - - L A D - - - - - E - K - - - - - H - - - - - K - - - - - K S - - - -							82
C6SY10_SOYBN	- - P - - - - - A Q - A - V D - - - - - R F - - - - - N - - - - - M - - - - - K D - - - -							81

Fig. 3. Peamaclein amino acid sequence alignment with homologues from *Populus trichocarpa* (black cottonwood), *Vitis vinifera* (grape), *Ricinus communis* (castor bean), *Solanum tuberosum* (potato) and *Glycine max* (soybean), whose UniProt Knowledgebase accession numbers are P86888, B9MVY8, E0CP56, B9R733, Q948Z4, C6SY10 respectively. Peamaclein residues conserved in the homologues are indicated by dashes. Cysteine residues are on grey background. The amino acid regions identified by sequencing have been underlined. Percent sequence identity between Peamaclein and its homologues is indicated on the right.

representing 19% of the total residues, and a theoretical isoelectric point of 8.97, calculated using the ProtParam tool at www.expasy.org. A multiple alignment of the new peach protein with some of the most similar homologues found in the UniProt database is shown in Fig. 3. A high sequence identity between Peamaclein and the homologues from different botanical families was observed. The full sequence of Peamaclein was registered in the Uniprot Knowledgebase under the accession number P86888.

Analysis of structural features by circular dichroism and molecular modelling

Far-UV CD spectra recorded at 25°C showed that Peamaclein has a mixed alpha-helical/beta-sheet structure (Fig. 4, panel A). In fact, the curve is characterized by the two minima typical of the alpha-helix, around 222 and 206 nm, although the non-fully canonical shape reflects the contribution of other secondary structures, i.e. beta structures and/or random coils. Analysis by molecular modelling suggests that the helical structure characterizes the N-terminal region of the molecule, whereas there is a high probability that all the

beta-structure is in the C-terminal region (Figure S3). Although the connectivity of disulphide bridges could not be clearly assigned, the molecular modelling results suggest that the cysteine residues are located in the central part of the 3D structure and could stabilize, by disulphide bridges, the secondary structure elements of the N-terminal and C-terminal region (Figure S3).

Evaluation of the resistance to proteolysis and heating

The CD spectrum recorded at 90°C was similar to that obtained at 25°C, thus suggesting that Peamaclein is stable at this high temperature (Fig. 4, panel A). The analysis by SDS-PAGE and RP-HPLC of Peamaclein subjected to digestion in SGF and SIF showed that this molecule is insensitive to proteolysis by pepsin, trypsin and chymotrypsin (Fig. 4, panel B).

IgE immunoblotting and dot blotting analysis

Purified Peamaclein was used for IgE IB along with the peach pulp extract and the in-house nPru p 3 preparation. A pool of the two sera from patients with Peamaclein⁺/Pru p 3⁻ ST results were tested using the

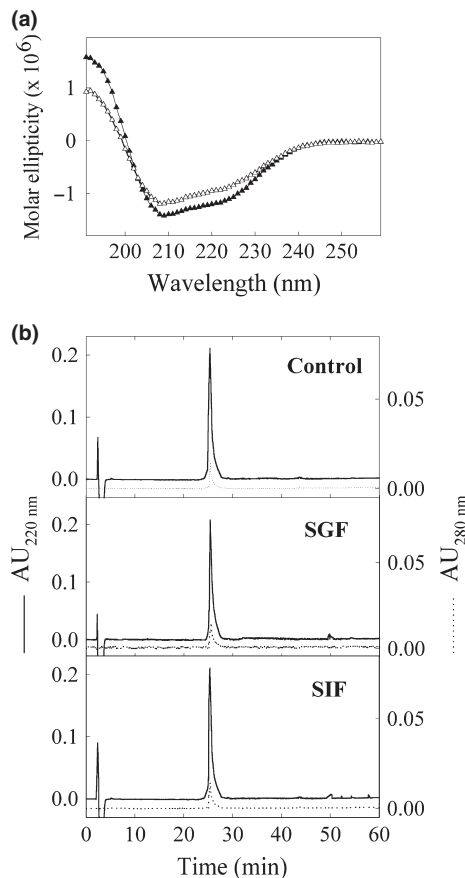


Fig. 4. Peamaclein heat and digestion stability assays. Panel A: Circular dichroism (CD) spectra of Peamaclein at 25°C (filled triangles) and 90°C (empty triangles). Panel B: RP-HPLC profiles of Peamaclein incubated for 2 h in simulated gastric fluid (SGF), or simulated intestinal fluid (SIF). The chromatographic profile labelled as 'control' shows the untreated protein.

BioAllergy anti-IgE secondary antibody. Although the pool was positive on Peamaclein, several control sera, namely a Peamaclein⁻/Pru p 3⁺ serum, two sera with total IgE equal to 350 IU/L (subject 35, Table 1) and to 3266 IU/L (subject 41, Table 1) and tested ST negative for both peach allergen in-house preparations and a myeloma IgE were positive as well (data not shown). Using a different secondary anti-IgE (the mouse monoclonal antibody, BD Biosciences), all primary antibody preparations turned out to be positive again. Instead, positive IgE results on Peamaclein and negative results on controls were obtained by dot blotting performed using the mouse monoclonal anti-IgE antibody (Figure S4).

Peamaclein and Pru p 3 skin tests in patients with documented peach allergy

As reported in Table 1, in total, 33 peach-allergic patients were recruited on the basis of a reliable clinical

history of severe reactions to peach or a positive DBPCFC. All underwent ST with the two in-house peach allergen preparations, and the majority of them with the other ST preparations as reported in Table 1. Seven subjects showed ST response to Peamaclein but not to Pru p 3 (Table 1; group A, $n=1-7$), all showing symptoms shortly after peach exposure, namely generalized urticaria and associated angioedema of the lips, tongue and larynx, and asthma in two cases. In one case, asthma and anaphylaxis were associated with cutaneous and mucosal symptoms. To control the generalized symptoms, all the subjects required assistance at a hospital emergency ward and the related records were made available to us afterwards. Seven additional peach-allergic subjects, all having almost the same clinical profile, showed a positive ST to both the allergen preparations, and thus they were labelled Peamaclein⁺/Pru p 3⁺ (Table 1; group B, $n=8-14$). Notably, all the above 14 patients experienced a single severe reaction, which forced them to stop eating peaches thereafter as a precaution. Nineteen additional patients, all being clinically allergic to peach were ST negative to Peamaclein and positive to Pru p 3 (Table 1; group C, $n=15-33$).

From the ST results, it turned out that the two Pru p 3-enriched peach peel extracts caused a positive ST in 31 tested subjects, regardless of their being single or double ST positive to Peamaclein and/or Pru p 3 (groups A–C), except for the BIAL preparation, which was negative in two of six Peamaclein ST-positive subjects. A similar outcome was recorded for the commercial whole peach extract (Allergopharma), which was recorded negative in three of five tested Peamaclein single-positive subjects (group A), and also in the majority of the Pru p 3 single-positive ones (group C). Furthermore, 4 Peamaclein⁺/Pru p 3⁻ subjects were tested by P–P using peel and pulp with no differences observed.

Nine peach-tolerant, but otherwise allergic patients, underwent the same testing as reported in Table 1 (group D), all showing ST negative results with all tested preparations.

Detection of IgE specific for Peamaclein and Pru p 3 by Allergen Beads Array, ImmunoCAP and three different ISAC microarrays

The new ABA assay was used for Peamaclein-specific IgE detection, along with the in-house nPru p 3 and other control allergens. All seven ST Peamaclein⁺/Pru p 3⁻ subjects (group A) turned out to have specific IgE to Peamaclein, and no detectable IgE to Pru p 3. Among the seven double-positive subjects (group B), just two and five had IgE levels above the cut-off limit for Peamaclein and Pru p 3 respectively. All 19 Peamaclein⁻/Pru p 3⁺ subjects (group C) were recorded negative to

Peamaclein and 15 (79%) were Pru p 3⁺ using ABA. All nine ST double-negative control subjects (group D) were negative for the IgE ABA detection.

Using the ImmunoCAP peach extract, IgE were detected in all subjects positive to one of the two allergens, except for six Peamaclein mono sensitized subjects (two in group A and four in group C). Comparing the IgE results obtained *in vitro* with different Pru p 3 preparations, negative fully matching results were recorded for the seven Peamaclein⁺/Pru p 3⁻ subjects using ABA and ImmunoCAP, whereas three of seven subjects of this group were IgE positive by ISAC 103, where commercial nPru p 3 is spotted (Table 1, group A, patients 3, 4 and 6). The positive results obtained with these three subjects by ISAC 103 were replicated three times for each subject, with minor differences between runs (replicates are not reported in Table 1). In contrast, the same three subjects showed negative results when tested by ISAC Exp96 and ISAC 112, where the in-house nPru p 3 and rPru p 3, respectively, are immobilized (data not reported in Table 1).

IgE inhibition experiments by ISAC 103

The SPHIAa was then used to find out whether the positive IgE results obtained by ISAC 103, where a natural purified Pru p 3 allergen was immobilized, were due to the presence of Peamaclein in the immobilized Pru p 3 preparation. The serum from subject 3 (Table 1) was used as a probe. The Pru p 3 IgE result equal to 2.57 kU/L remained almost unchanged when the IgE inhibition was searched with the in-house nPru p 3 preparation, whereas the Peamaclein preparation gave 100% IgE inhibition by ISAC 103 Pru p 3 (data not shown). The SPHIAa results were replicated three times with identical outcomes.

Allergen Beads Array IgE Inhibition using different allergenic preparations

The SPHIAa on ABA was used to run IgE inhibition experiments using the in-house Peamaclein and nPru p 3 preparations, the BIAL and ALK deglycerinated Pru p 3-enriched peach peel extracts prepared as reported in the Supplementary Information, the commercial nPru p 3 preparation (BIAL) and the pulp and peel peach extracts from the three different peach cultivars. The results are shown in Fig. 5. The main findings can be summarized as follows: homologous specific almost total IgE inhibitions using Peamaclein and in-house nPru p 3 were obtained lacking any inhibition on the heterologous molecule; the nPru p 3 commercial preparation from BIAL contains both Pru p 3 and Peamaclein in a sufficient amount to cause an almost complete inhibition on both allergens; the ALK deglycerinate Pru p 3-enriched peach peel extract contains both Peamac-

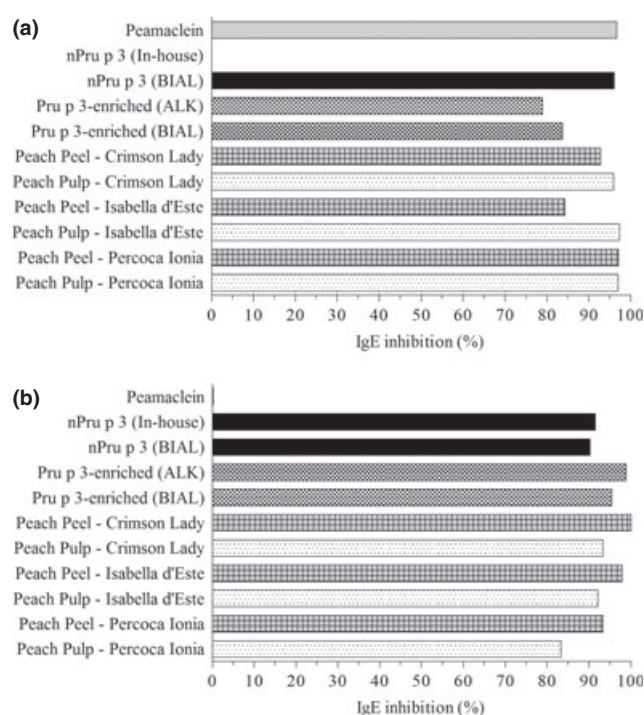


Fig. 5. Allergen micro-Beads Array (ABA) IgE Inhibition assays using the SPHIAa method on Peamaclein and in-house nPru p 3. Panel A: Peamaclein as antigen on the solid phase; inhibitors as listed on the Y-axis; Serum: Peamaclein IgE-positive sample from subject 4 as in Table 1; ABA Peamaclein IgE MFI value = 7263 (serum + buffer); Panel B: Pru p 3 in-house preparation as antigen on the solid phase; inhibitors as listed on the Y-axis; Serum: Pru p 3 IgE-positive sample from subject 17 as in Table 1; ABA Pru p 3 IgE MFI value = 25 997 (serum + buffer).

lein and Pru p 3, giving 79% and 99% IgE inhibition respectively; the BIAL deglycerinate Pru p 3-enriched peach peel extract contains Peamaclein sufficient to give 83% IgE inhibition and Pru p 3 to reach 95%; the two allergens seem to be equally distributed in the peel and pulp in the three different peach cultivars, although, lacking reference curves, we could not estimate the actual allergen concentrations.

Non-human Pru p 3-specific antibodies binding to Pru p 3 and Peamaclein on Allergen Beads Array

The results obtained by testing the three non-human antibody preparations on the in-house nPru p 3 and Peamaclein conjugated to the microbeads are described in the Supplementary Information and shown in Figure S5.

Discussion

We herein report for the first time the comprehensive characterization of a new peach allergen named Peamaclein. Its identification was made possible combining the systematic observation of patients' clinical

features, namely severe allergy symptoms on peach exposure, associated with the result discrepancies among several diagnostic tools applied to the early identified patients, followed by biochemical and immunological procedures.

Peamaclein was identified in peach pulp and peel and purified from the fruit pulp for the structural and immunological characterization. It is a small protein of about 7 kDa, with an electrophoretic mobility very similar to that of the 9-kDa Pru p 3. In fact, these two peach proteins produce overlapping bands in SDS–PAGE, and thus cannot be discriminated. Like Pru p 3, Peamaclein is a basic protein with a pI close to 9. Therefore, due to the similar molecular mass and pI, and depending on the chromatographic separations used, in some conditions Pru p 3 copurifies with Peamaclein. These observations can explain why the Pru p 3 preparations can easily be contaminated by Peamaclein, and how difficult it is to detect the contamination when appropriate controls are not carried out. Nevertheless, the slightly different theoretical pI values estimated for Peamaclein (8.97) and Pru p 3 (9.25), allow their separation into two distinct peaks by ion exchange chromatography. Peamaclein also shares with Pru p 3 a stability to heating and to simulated gastric digestion, but seems more resistant than Pru p 3 to simulated intestinal digestion. In fact, Pru p 3 is partially digested by trypsin [11, 28], whereas Peamaclein is totally resistant to this protease.

Although Peamaclein and Pru p 3 share the above-mentioned features, they have distinct primary structures and a different secondary structure pattern. Peamaclein has a quite unusual high content of cysteines (19% of total residues), supposed to form six disulphide bridges stabilizing the small molecule. Homology searches in data banks show a high sequence identity with homologues from different botanical families, thus suggesting a high spread of Peamaclein-like proteins and a high conservation of their molecular structure among plants. In peach, in line with the finding in the EST clone data bank deriving from both peach pulp and peel and coding for Peamaclein, this protein was detected and isolated from both fruit tissues. It probably has a pathogenesis-related function as an antimicrobial activity was reported for the homologue from potato [29], named snakin, with which Peamaclein shares 82% of amino acid sequence. As there are no reports in the literature concerning the 3D structure of Peamaclein homologues, we could not obtain a refined molecular model of this protein. However, the results from CD experiments provide evidence that unlike Pru p 3 which displays a four-helical-bundle structure, Peamaclein has a mixed alpha-helical/beta-sheet structure. At first glance, the helical segments could be located in the N-terminal region of Peamaclein, as suggested by our *in silico* molecular modelling analysis.

The immunological investigations started with the problematic and difficult to explain IgE IB results. In fact, unlike the native protein showing a specific IgE binding in the dot blot assay, the denatured Peamaclein had a non-specific binding of the two secondary antibodies in the IB. This observation suggests that denaturation could cause the exposure of hidden parts of the molecule responsible for non-specific binding of the immunoglobulins from different sources. It was hypothesized that this peculiar Peamaclein behaviour in the IB assay could have been misleading researchers in previously performed experiments where fully controlled positive/negative IB results are the basis for new allergen identifications. Combined with the above-discussed overlapping migration in SDS–PAGE, this could be the cause of Peamaclein being overlooked as an allergen up to now. For the time being, we have no explanation of the recorded IB-unspecific binding. However, this was not present when Peamaclein was tested in native conditions in the dot blot assay using the same reagents. Thus, for a better evaluation of the specific IgE reactivity of Peamaclein, we used the new ABA assay. The ABA IgE results largely matched the skin test positive results with both Peamaclein and the in-house Pru p 3 preparation; although in patients lacking a strong ST reactivity, we were unable to detect Peamaclein IgE by ABA. This would certainly require an improvement of the overall quality of ABA as an IgE detection system for future use, as also discussed in our feasibility study [24].

A further element which directed our attention and guided our approach to Peamaclein identification was the conflicting results obtained with the available natural Pru p 3 preparations compared with our highly purified natural one and the recombinant form available on the ImmunoCAP. Regardless of their being natural or recombinant forms, the availability of highly purified and perfectly characterized allergenic molecule preparations seems to be mandatory [30, 31]. We speculated that even molecular preparations could require special attention when reporting data in our recent studies [11, 24]. In these two studies, a certain discrepancy between the results obtained with the two different Pru p 3 preparations was reported when both were tested either using the same ISAC microarray technology [11] or comparing the two microtechnology systems, namely the ISAC and the ABA [24]. The explanation of this discrepancy at the time those studies were performed was elusive, whereas now we could argue that the nPru p 3 preparation immobilized on the ISAC 103 contained Peamaclein as well, thus causing the IgE detection of two different compounds at once. The same manufacturer has recently launched a new ISAC microarray with 112 different proteins, where the immobilized Pru p 3 preparation is a recombinant form produced in *E. coli*, like the one available on the

ImmunoCAP system. As reported in this study, this new ISAC version does not cause the false positive IgE results in Peamaclein mono-sensitized subjects like those obtained with the previous ISAC 103 microarray.

The same problematic Peamaclein contamination of the so-called Pru p 3-enriched peach peel commercial preparations is reported in this study. Several authors have been describing the usefulness of ST with such preparations, claiming that it is possible to perform diagnosis and epidemiology of LTP or other allergen sensitizations by using just one representative molecule for each group, and Pru p 3-enriched peach peel commercial preparation is currently the one most frequently adopted [8, 9, 32–34]. As shown in our previous studies and in those of other authors on allergen groups, namely LTP, hevein-like, Bet v 1-like, tropomyosin, profilin [11, 25, 35–37] this claimed approach is an oversimplification of the complexity of homologous molecule IgE recognition. Indeed, the biochemical, immunochemical and diagnostic findings on Pru p 3-enriched peach peel preparations reported in this study suggest the need for an in-depth quality control of allergenic molecule preparations complying with the already established International regulations on molecular compounds [38, 39]. For the integrity of the diagnostic and therapeutic molecular approach to allergic diseases, available products should be clearly labelled as non-molecular compounds depending on their characterization level, and a grading system should be adopted similar to that of any chemical preparation. Notably, an important difference was recorded using anti-Pru p 3 MoAb and PoAbs. Regardless of the immunogen, the MoAb retains its Pru p 3 specificity, whereas we would argue that rabbits immunized with the Peamaclein/Pru p 3 preparations do produce IgG against both antigens. Without an affinity purification step against a highly purified antigen, the non-human primary PoAb preparations are shown to carry both specificities.

On the basis of these very early observations, the Peamaclein-related clinical pattern of symptoms is indistinguishable from that of the reported Pru p 3. Generalized reactions were recorded in all our Peamaclein-positive subjects, but that could be caused by a selection bias due to our recruitment approach. As reviewed by Mari, discussing a comprehensive combined bio-, micro-, information technology approach to define proteins as allergens [40], weighting the allergenicity of a molecule and evaluating its risk should include the broadest testing at least within allergic populations, recording the presence or not of an IgE reactivity first and then a clinical reactivity. That is certainly feasible as suggested by several of our recent studies [10, 11, 19, 25, 26, 35, 36, 41–44] by adopting molecule microarray testing within a bioinformatics infrastructure as the tool for routine diagnosis [15]. New

allergens, such as the herein reported Peamaclein, could definitely be evaluated for their real impact after such broad testing, better still if performed in geographically distinct populations.

Differing from Pru p 3, reported to be almost exclusively present in peach peel [5], Peamaclein seems to be present in both peach pulp and peel. Although a different degree of Pru p 3 distribution between peel and pulp has been reported by several authors [3, 4, 45], not all of them had the same findings. For instance, Carnes and collaborators, using a direct Pru p 3 detection based on a rabbit PoAb, showed Pru p 3 to be present also in the pulp, though sevenfold less compared to the peel. Although we could argue that even in this study the rabbit PoAb could have detected two allergens instead of just Pru p 3, the use of a non-human PoAb makes that assay very similar to our ABA IgE inhibitions due to the polyclonality of the probe. We may thus deduce that the tissue distribution could depend on the detection method used in each study, revealing the advantages and limitations of each of them. We have already faced such differences between detection methods when evaluating the presence of Act d 10 and Act c 10 in kiwifruit pulp or seeds; in that case, as in the present report, the immunochemical assay detecting the LTP molecule in the kiwi/peach pulp was demonstrated to be superior to the biochemical method that failed in this detection [11]. However, further evaluation with our SPHIAa on ABA should be carried out to try to define its sensitivity compared to a standard reference to avoid an overestimation of the Pru p 3 concentration in peach pulp. Due to the low number of observations and the severity of the reactions reported by patients who were afraid to eat peaches again, we could not investigate whether a different Peamaclein distribution in the peach leads to a different 'real life' reactivity. For the time being, we may just hypothesize that Pru p 3⁺ patients reporting a tolerance to peeled peaches could be Peamaclein⁻/Pru p 3⁺ or be classified as weak reactors to the lower amounts of Pru p 3 in the pulp, whereas those reporting a clinical reactivity also to peach pulp ingestion should be tested to find out whether they are also Peamaclein⁺ or not. Further studies with a larger number of enrolled patients, and based on fully characterized peach cultivars, are indeed needed to reach a reliable conclusion on this issue.

In summary, we have identified and fully characterized a new peach allergen whose original biochemical, immunological and clinical features share at the same time some similarities and some differences with Pru p 3. Further epidemiological and clinical studies are required to improve the diagnostic approach to and the therapeutic decisions for peach allergic patients.

Note: The Peamaclein full amino acid sequence, along with the biochemical, clinical and immunological data have been submitted to the WHO-IUIS subcommittee for

allergen nomenclature, which assigned the designation of Pru p 7 to the new peach allergen.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Electrophoretic mobility of the two purified peach proteins on 17% SDS-PAGE.

Figure S2. Separation of Peamaclein and Pru p 3, based on their different pI, by FPLC ion exchange chromatography using a Mono-S HR 10/10 column.

Figure S3. Peamaclein molecular model, showing two parallel helices in the N-terminal region, is displayed as a white ribbon and the side-chains of the cysteines are in yellow.

Figure S4. IgE Dot Blot Assay.

Figure S5. Non-human antibody direct binding to Peamaclein and Pru p 3 using the Allergen micro-Beads Array (ABA).

Appendix S1. Methods and Results.

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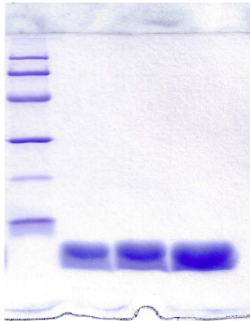
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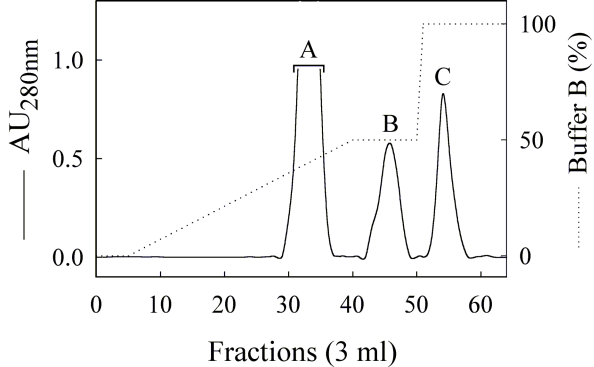
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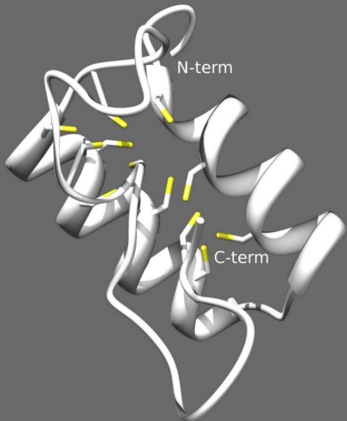
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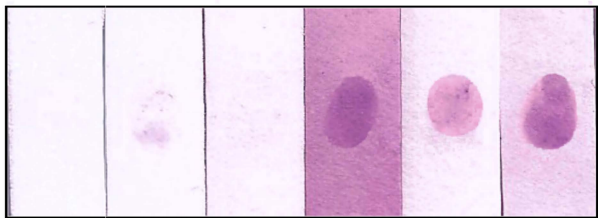
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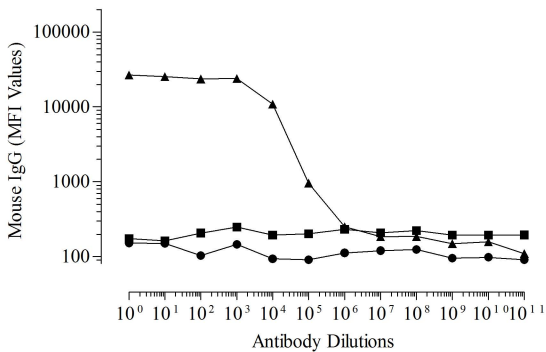
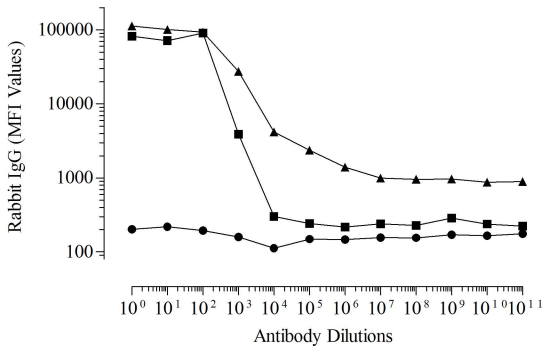
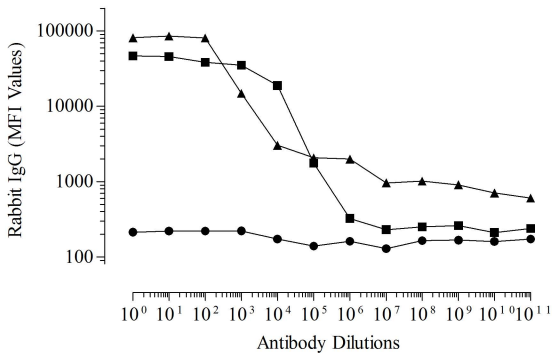
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Supplementary Information

Peamaclein – A new peach allergenic protein: similarities, differences and misleading features compared to Pru p 3

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These authors equally contributed to the study

Short title: Peamaclein – A new risky self-hiding peach allergen

Keywords: Severe food allergy, allergen, microarray, inhibition, stability, cysteine-rich protein, peach, direct protein sequencing, new allergenic protein family

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Supplementary Information

Methods

Estimation of the protein concentration

The protein concentration in the extracts was estimated by the BIO-RAD Protein Assay (Bio-Rad, Milan, Italy), using calibration curves made with bovine serum albumin.

Protein Extract Fractionation by RP-HPLC

Reverse Phase-High Performance Liquid Chromatography (RP- HPLC) of peach extracts was performed on a Vydac (Deerfield, IL, USA) C8 column (0.21 x 25 cm), using a Beckman System Gold apparatus (Fullerton, CA, USA). Elution was accomplished by a multistep linear gradient of eluant B (0.08% TFA in acetonitrile) in eluant A (0.1% TFA) at a flow rate of 1 ml/min. The eluate was monitored at 220 and 280 nm. The separated fractions were manually collected and analysed.

MALDI-TOF Mass Spectrometry

The molecular mass of purified proteins was estimated by MALDI-TOF mass spectrometry measurements carried out on a PerSeptive Biosystems Voyager-DE Biospectrometry Workstation (Framingham, MA, USA). Analyses were performed on pre-mixed solutions prepared by diluting samples (final concentration 5 pmol/ml) in 4 volumes of matrix, namely 10 mg/ml α -ciano-4-hydroxycinnamic acid in 60% acetonitrile containing 0.3% TFA.

Amino Acid Sequencing

The N-terminal sequence was obtained by direct sequencing of the purified protein using a Procise 492 automatic sequencer (Applied Biosystems, Foster City, CA, USA). Moreover, Peamaclein was denatured and its sulfhydryl groups were alkylated with 4-vinylpyridine as already described [1].

The denatured and alkylated protein was subjected to proteolytic cleavage by trypsin (Roche Diagnostics, Mannheim, Germany), following manufacturer's instructions. The recovered peptides were separated by RP-HPLC and some of them were analysed by direct sequencing.

Purification of Peamaclein from Peach Pulp

The pulp protein extract was dialysed against 10 mM Tris-HCl, pH 7.2 and loaded on a DE52 (Whatman, Brentford, UK) column, equilibrated in the same buffer. Peamaclein was eluted in the column flow-through, and then loaded on a SP-Sepharose column (Amersham Biosciences, Uppsala, Sweden), equilibrated in 10 mM sodium acetate, pH 5.0 (buffer A). Elution was carried out by increasing the concentration of buffer B (50 mM sodium acetate, pH 5.0, containing 0.5 M NaCl). The fractions containing Peamaclein were identified by RP-HPLC analysis. The sample was dialysed against buffer A (see above) and concentrated by ultrafiltration on Ultracel 3K Amicon Ultra filters (Millipore, Carrigtwohill, Ireland). Further purification was achieved by Fast Protein Liquid Chromatography (FPLC) on a cation exchanger Mono-S HR 10/10 column (Amersham-Pharmacia, Uppsala, Sweden), eluting with a linear gradient from 0% to 100% of buffer B (see above). The fractions containing the protein were concentrated by ultrafiltration as above, and loaded on a Superdex 75 HR 10/30 column (Amersham-Pharmacia, Uppsala, Sweden) equilibrated in 10 mM phosphate buffer pH 7.4, containing 0.25 M NaCl. Elution from gel filtration provided Peamaclein purified to homogeneity.

Separation of Peamaclein and Pru p 3 by Ion-exchange Chromatography

The peel protein extract was dialysed against 10 mM Tris-HCl, pH 7.2 and loaded on a DE52 column (Whatman), equilibrated in the same buffer. Peamaclein and Pru p 3 were eluted in the column flow-through, and then loaded on a SP-Sepharose column (Amersham Biosciences), equilibrated in 10 mM sodium acetate, pH 5.0 (buffer A). Elution was carried out by increasing the

concentration of buffer B (50 mM sodium acetate, pH 5.0, containing 0.5 M NaCl). The fractions containing Peamaclein were identified by RP-HPLC analysis. The sample was dialysed against buffer A and concentrated by ultrafiltration on Ultracel 3K Amicon Ultra filters (Amicon). Further purification was achieved by FPLC on a Mono-S HR 10/10 column (Amersham-Pharmacia), eluting with a linear gradient from 0% to 100% of buffer B (Figure S2). The fractions of each peak were pooled, analysed by SDS-PAGE and identified by N-terminal amino acid sequencing as Pru p 3 (peak A), pectin methylesterase (peak B) and Peamaclein (peak C).

Circular Dichroism Experiments

Circular dichroism (CD) spectra were recorded on a JASCO J-810 spectropolarimeter (Easton, MD, USA) as already reported [2]. A quartz cell of 0.1 cm path length was used to record the spectra over the wavelength range 260-190 nm with a bandwidth of 1.0 nm. Measurements were performed in 5 mM sodium phosphate pH 7.4, at 25°C and 90°C, using a protein concentration of 0.10 mg/ml. Each spectrum was baseline corrected for the contribution of the solvent.

Stability to the Simulated Gastric and Intestinal Digestion

In vitro simulated gastric fluid (SGF) digestion of the purified Peamaclein was performed as already described [3]. For intestinal digestion porcine trypsin and bovine chymotrypsin (Roche Diagnostics) were used. Simulated intestinal fluid (SIF) was prepared as described in the United States Pharmacopeia [4], and consists of a mixture of trypsin and chymotrypsin in 0.05 M potassium phosphate buffer pH 6.8. Digestions were performed at 37°C, at an enzyme to protein ratio of 1:50 (w/w), both for trypsin and chymotrypsin. Aliquots of the digest were withdrawn at 0 and 120 minutes and analysed by SDS-PAGE and RP-HPLC.

Preparation of Peamaclein and Pru p 3 solutions for Skin Testing

Peamaclein and nPru p 3 samples were solubilised in deionised water, mixed with sterile glycerin in a 1:1 (v/v) ratio and sterilised by membrane filtration through a 0.22- μ m filter (Millex, Millipore, Bedford, MA, USA), in a sterile horizontal laminar flow hood. The final protein concentration was 0.25 mg/ml.

Removing Glycerin from Pru p 3- enriched Peach Peel Extract Commercial Preparations for Skin Test

For biochemical and immunochemical assays, 50% glycerin was removed from commercially glycerinated Pru p 3-enriched peach peel preparations from ALK-Abelló (ALK; Madrid, Spain) and BIAL Industrial Farmacéutica S.A. (BIAL; Bilbao, Spain) using two different methods. For analytical purposes, samples were diluted five times by the addition of bidistilled water then the volume was reduced five times by ultrafiltration using Ultracel 3K Amicon Ultra centrifugal filters (Millipore). To obtain a negligible concentration of glycerin in the samples, the procedure was applied twice again. The samples were then analysed by RP-HPLC. For immunochemical purposes, glycerin was removed using PD Spin Trap G-25 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). PD Spin Trap G-25 was prepared following the procedure recommended by the manufacturer. Then, 100 μ l of each preparation was pipetted from the commercial vials and placed in the middle of the packed bed and eluted by centrifugation (800 x g for 2 minutes). The de-glycerinated preparations were available in the collection tube without further adjustment of their protein concentrations and used as inhibitors in SPHIAa (see below).

IgE detection by Allergen micro-Beads Array as a Flexible Multiplexing Assay

In-house nPru p 3 and Peamaclein preparations were set at 1 mg/ml concentration before conjugation. The color-coded polystyrene micro-beads (Becton Dickinson, New Jersey, USA) of

distinct and non-overlapping fluorescence emission intensities were used and covalently coupled with allergenic molecules using the sulfosuccinimidyl 4-N-maleimidomethyl cyclohexane 1-carboxylate (sulfo-SMCC) chemistry. The conjugation procedure consisted of four steps: micro-bead preparation, protein modification, buffer exchange, and protein conjugation, all detailed in the paper by Pomponi *et al.* [5]. One μl of conjugated micro-beads was used for each test for each specific allergen, re-suspended in capture micro-bead diluent for serum and plasma to a final volume of 50 μl /test. Fifty μl of micro-bead mix was combined with 50 μl of the biological sample to be tested, being either human sera or non-human antibodies. The PoAbs, the MoAb, and the human serum samples were used in each given experiment as reported in the Results section. Tested samples were then washed, the supernatant was aspirated and the pellet re-suspended in 100 μl capture micro-bead diluent for serum and plasma (Becton Dickinson); 50 μl of each secondary antibody were then added to each tube, followed by incubation for 1 hour at room temperature in the dark. Human IgE were detected using 20 μl of phycoerythrin-conjugated anti-human IgE (PE; Becton Dickinson), whereas non-human antibodies were detected by a fluorescein isothiocyanate-conjugated mouse monoclonal anti-rabbit IgG (FITC; GenWay Biotech Inc., San Diego, USA) or 5 μl of PE-conjugated goat anti-mouse IgG (Dako Cytomation, Milan, Italy). Samples were then washed once as described above and finally re-suspended in 300 μl of washing buffer. Specimens were analysed using the FACS Aria flow cytometer (Becton Dickinson). FACSDiva software (Becton Dickinson) was used for data capturing and a first analysis. From raw data analysis, the median fluorescence intensity (MFI) of each micro-bead cluster was quantified. No reference curves have been used to normalise the antibody quantification from each experiment. Measurements were performed counting at least 500 events for each analysis. ABA-measured antibody values were expressed as MFI as given by the final readout of the cytometer. A blank reading for each run, made with conjugated micro-beads and micro-beads plus serum without

secondary antibody to measure background fluorescence, was performed always with comparable very low range fluorescence results. As a totally negative MFI was seldom recorded for allergic sera when the PE-anti-IgE was added, we set an ABA negative cut-off point as previously reported [5].

IgE Single Point Highest Inhibition Achievable assay (SPHIAa) by ISAC

25 µl of the patient's serum have been incubated overnight with 25 µl of a solution containing the highly purified allergen preparation of Peamaclein or in-house nPru p 3, at a concentration of 100 µg/ml. The above preparations acted as inhibitors of the IgE binding. After o.n. incubation, the IgE binding inhibition was evaluated by running the ISAC 103 microarray (PMD) where the IgE reactivity was evaluated on immobilised commercial nPru p 3. A control sample, where only the buffer solution was added, was used as reference value for no IgE inhibition. For control purposes of the specific IgE inhibition several other allergens which were recognised by the IgE of the same patient were used. A no-inhibition value was required to record the experimental results as valuable. All IgE inhibition data were stored in the InterAll, and percent inhibition values were calculated real time by a specific procedure developed for the InterAll application.

SPHIAa IgE inhibition by ABA

Briefly, 20 µl of all preparations, either as obtained after removing glycerin or at 100 µg protein/ml concentration, were incubated o.n. with 20 µl of human sera or non-human antibodies as detailed in the Results section. For control purposes a buffer/serum preparation was used. The o.n. incubated preparations were then tested for antibody binding on in-house nPru p 3- and Peamaclein-conjugated micro-beads as reported above, using the species specific secondary antibody. Additional micro-beads either conjugated with allergens recognised by the tested

human serum, for no-inhibition control, or conjugated with irrelevant allergens, for non-specific secondary antibody binding control, were used. Results were exported from the FACSDiva software, uploaded by a semi-automatic procedure in InterAll, and processed as for the above ISAC IgE SPHIAa results

Dot blotting

To assess the IgE binding of Peamaclein in native condition 4 µl of a 1 mg/ml protein solution of the in-house purified preparation have been dotted on a nitrocellulose membrane and let to dry. The probing with the patient's serum and secondary antibody were performed as already described for the immunoblotting (IB) procedure [2], using the mouse monoclonal anti-IgE conjugated to alkaline phosphatase (Becton Dickinson Biosciences) as secondary antibody. Three sera from Peamaclein allergic subjects, as detected by skin test (ST), were tested; a control serum from a peach tolerant, Peamaclein ST negative allergic subject having very high total IgE, and an intravenous human IgG preparation were used as controls.

Non-human anti-Pru p 3 antibodies

Anti-Pru p 3 non-human antibodies were obtained as follows: a mouse monoclonal antibody (MoAb) and a rabbit polyclonal (PoAb) from ALK [6], and a second PoAb from BIAL. The same non-human antibodies have been successfully used in a previous study from us under similar experimental conditions [5].

Supplementary Information

Results

Non-human Pru p 3 specific antibodies binding to Pru p 3 and Peamaclein on ABA

The three non-human antibody preparations were tested on the in-house nPru p 3 and Peamaclein conjugated to the micro-beads, using an irrelevant plant-derived allergen (Act d 1) for control purposes. As shown in Figure 6 (Supplementary Information), no unspecific binding was recorded for the latter allergen with all three Ab preparations, whereas as expected the undiluted Pru p 3-specific MoAb gave a net signal on Pru p 3 which started to decrease after the forth dilution showing no binding at the 10^6 dilution (Figure S5, panel A). Negative results were recorded on Peamaclein with all Pru p 3-specific MoAb concentrations (Figure S5, panel A). Higher MFI values were recorded for the two undiluted anti-Pru p 3 specific PoAbs, but both, nPru p 3 and Peamaclein, were recognised with almost the same MFI values. The two PoAbs gave two different dilution curves comparing either allergens or PoAbs each other (Figure S5, panel B and C). A higher background has been recorded when testing the highest dilutions of the two PoAbs on nPru p 3.

Supplementary Information

References

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Supplementary Information

Figure S1. Electrophoretic mobility of the two purified peach proteins on 17% SDS-PAGE. Lane A: nPru p 3 (5 μ g); Lane B: Peamaclein (5 μ g); Lane C: nPru p 3 (5 μ g) *plus* Peamaclein (5 μ g); Lane M: molecular weight markers.

Figure S2. Separation of Peamaclein and Pru p 3, based on their different pI, by FPLC ion exchange chromatography using a Mono-S HR 10/10 column. The molecules contained in the fraction obtained from the SP-Sepharose column were separated into three peaks. The protein components of each peak were identified by N-terminal amino acid sequencing as (A) Pru p 3, (B) pectin methylesterase (not discussed in the present study), and (C) Peamaclein.

Figure S3. Peamaclein molecular model, showing two parallel helices in the N-terminal region, is displayed as a white ribbon and the side-chains of the cysteines are in yellow.

Figure S4. IgE Dot Blot Assay. Peamaclein has been probed with the following preparations: (A) PBS; (B) human polyclonal intravenous IgG; (C) serum from a peach tolerant, Peamaclein skin test negative subject having 20,000 IU/L total IgE (not listed in Table 1); (D, E, F) sera from peach allergic, Peamaclein skin test positive subjects, #1 #2 #3 in Table 1, respectively.

Figure S5. Non-human antibody direct binding to Peamaclein and Pru p 3 using the Allergen micro-Beads Array (ABA). Panel A: Mouse monoclonal anti-Pru p 3 antibody - ALK; Panel B: Rabbit polyclonal anti-Pru p 3 antibody - ALK; Panel C: Rabbit polyclonal anti-Pru p 3 antibody - BIAL. MFI: Median Fluorescence Index. Squares: Peamaclein; Triangles: Pru p 3; Circles: Act d 1 (control).

Editor's Choice

The Editor takes a closer look at some of this month's articles

Obesity and asthma: does weight loss help?

In this issue, two original papers, an excellent review by Dr Boulet (pp. 8-21) and an insightful editorial by Chapman and Salome (pp. 2-4), address various aspects of the link between obesity and asthma. The association between obesity and asthma is well established, but whether this is due to obesity causing symptoms through deconditioning and dysfunctional breathing or is due to a more direct and causal effect is not clear [1-9]. The extent to which weight loss helps disease control is also not clear. Scott and colleagues (pp. 36-49) have shed light on this issue in a study in which dietary and exercise interventions were correlated with changes in airway inflammation. They demonstrated that a modest degree of weight loss over ten weeks in overweight and obese adults with asthma improved measures of both asthma quality of life and symptoms as well as the numbers of neutrophils and eosinophils in sputum. Further encouragement to focus on this aspect of management in our asthma clinics.



Hayley Scott



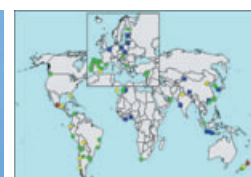
Bathroom scales, courtesy of Elinor D, Wikimedia Commons

Obesity and asthma: what can epidemiology tell us?

The obesity–asthma link is present in children as well as adults. Mitchell and colleagues (pp. 73-84) have taken advantage of the ISAAC database to further confirm this association as well as determine if it extends to other manifestations of allergic disease. Their study of over 76 000 children aged 6–7 years and over 200 000 adolescents, as well as showing a worrying global rate of obesity in children, confirmed the link between increased BMI and asthma and demonstrated that this extended to eczema, but not rhinitis. Excess television viewing (> 5 h) was also associated with asthma in both adults and children. Although this points to the importance of exercise in children, the authors also noted a link between allergic disease and vigorous exercise in adolescents but not the 6–7-year olds. This was also seen for eczema and rhinitis so this observation cannot be simply explained by exercise provoking exercise induced asthma. A case of epidemiology begging as many questions as it answers.



Ed Mitchell



Prevalence of overweight and obesity (combined) in children 13–14-year-old children in ISAAC centres.

Peach allergy: a new kid on the block

Severe food allergic reactions to peach are a common problem, particularly in Mediterranean. Pru p 3, a lipid transfer protein found mainly in the skin of the peach, is thought to be the major allergen involved. Tuppo and colleagues (pp. 128-140) explored the possibility that there are other important anaphylaxis causing allergens in peach, prompted by observations that some patients with allergic reactions to this fruit were negative to Pru p 3 in the ISAC 103 microarray. After much work, they have identified a new peach allergen which they have called Peamaclein which appears to be a member of a new family of proteins related to a potato antimicrobial peptide. The clinical importance of this allergen now needs to be fully characterized.



Lisa Tuppo



Peamaclein (Pru p 7) – A new risky, self-hiding, very stable peach allergen. Image by Adriano Mari

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Caption to cover illustration: Prevalence of overweight and obesity (combined) in children 6–7-year-old children in the International Study of Asthma and Allergies in Childhood (ISAAC) centres. The symbols indicate prevalence values of < 10% (blue square), 10 to < 20% (green circle), 20 to < 30% (yellow diamond) and ≥ 30% (red star) [see figure 1 in E. A. Mitchell *et al.* (pp. 73–84)].

EC This logo highlights the Editor's Choice articles on the cover and the first page of each of the articles.