

Extraction and mass spectrometry identification of a major peach allergen Pru p 1

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Abstract

BACKGROUND: Peach allergy can be caused by the allergen Pru p 1. This occurs by cross-reactivity with the homologous birch pollen allergen Bet v 1. However, the direct identification of Pru p 1 as an immunoglobulin E (IgE)-binding protein extracted from peach fruit has never been reported.

RESULTS: Phosphate-buffered saline (PBS) and phenol extractions were applied to solubilise the proteins from peach peel and pulp, and IgE immunoblotting with sera of individual peach-allergic patients was used to detect the potential allergens. Most of the patients showed binding to an 18 kDa band in IgE immunoblotting performed with the phenolic extracts of peach peel and pulp, but not when the PBS extracts were used. Mass spectrometry of the 18 kDa spot excised from a two-dimensional electrophoretic gel showed this protein to correspond to the peach allergen Pru p 1.

CONCLUSION: Phenol extraction was necessary to detect by IgE immunoblotting a major peach allergen, which showed very low extractability with PBS, indicating the appropriateness of adopting different extraction procedures to identify plant allergens. The 18 kDa peach protein was definitively identified as the Bet v 1-homologous peach allergen Pru p 1.

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Keywords: peach allergy; Pru p 1; Pru p 3; mass spectrometry; electrophoresis

INTRODUCTION

Rosaceae fruits are probably those most frequently involved in food allergy in adult patients in the European area. Patients allergic to peach show two different patterns of clinical response according to their geographical area of residence. In southern Europe the clinical manifestations are often severe with a high probability of systemic symptoms,^{1,2} whereas in central and northern Europe the oral allergy syndrome (OAS) is the most frequent allergic manifestation to peach.^{3,4}

For patients living in birch-free areas of Mediterranean countries, peach allergy seems to be the result of primary sensitisation to the lipid transfer protein (LTP)¹ named Pru p 3, which is homologous to the apple allergen Mal d 3 (Allergen Nomenclature Sub-Committee, www.allergen.org). This LTP is a 9 kDa protein belonging to the pathogenesis-related (PR) protein family 14, is mainly concentrated in the fruit pericarp,^{5,6} is readily extractable in water solutions and shows high pepsin and heat resistance.⁷ Different immunoglobulin E (IgE)-binding intensities to LTP can be found in peach cultivars, indicating the presence of different levels of this allergen.⁸ Similar LTPs have been identified as allergens in several plant foods, latex and pollens, and substantial clinical evidence has allowed defining LTPs as relevant plant panallergens.⁷

In contrast, the peach allergen mainly involved in OAS is named Pru p 1. In this case, peach allergy seems to be a consequence of primary sensitisation to birch pollen causing cross-reactivity between the birch allergen Bet v 1 and the homologous peach allergen Pru p 1, and also with a large number of other plant

proteins, all belonging to the Bet v 1 family, including the apple allergen Mal d 1.⁹ Pru p 1 is an 18 kDa protein belonging to the PR-10 protein family,¹⁰ is unstable to denaturation,¹¹ accumulates in both peach peel and pulp and varies in quantity among different peach and nectarine cultivars.¹²

The involvement of Pru p 1 in peach allergy has been deduced only indirectly from data obtained using homologous proteins from other plant sources.¹² More recently, because of its low abundance in the fruit, Pru p 1 has been expressed in *Escherichia coli*, purified and characterised in terms of its structural properties.¹¹ Also, the identification of Pru p 1 by a proteomic approach has recently been reported.¹³ However, the direct identification of the peach 18 kDa protein bound by the IgE of peach-allergic patients¹⁴ as the Pru p 1 allergen directly extracted from the fruit has never been reported.

In the present study the potential allergens from peach peel and pulp were detected by IgE immunoblotting with the

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sera of peach-allergic patients living in northern Italy using extracts obtained with two different protein extraction methods. Moreover, the IgE-binding protein involved in peach allergy of birch pollen-sensitised patients was identified by two-dimensional electrophoresis and mass spectrometry.

MATERIALS AND METHODS

Patients

A case-series of 12 consecutive patients (eight women and four men aged between 25 and 45 years, all living in the Lombardy region of northern Italy) who reported allergic manifestations (OAS and/or rhinitis/rhinoconjunctivitis and asthma) after eating peach were selected. The clinical data of these patients are given in Table 1. All patients but one (patient N) reported mild or moderate symptoms of OAS after eating peach, and reaction to this fruit was confirmed, after informed consent, by open challenge. All these 11 patients suffered from seasonal rhinoconjunctivitis with or without secondary symptoms of asthma and resulted positive in the clinical tests for pollinosis. In contrast, patient N reported more or less important occasional episodes of asthma and rhinitis after ingestion of peach and peach juice, but never showed OAS symptoms.

After written informed consent the selected patients were subjected to skin prick tests (SPTs) using the standard method. Reading and scoring were done according to EAACI guidelines.¹⁵ All patients were prick tested with a complete series of commercially available pollen extracts (Lofarma, Milan, Italy). The prick-by-prick technique was also used with the fruits and vegetables reported by the patients as responsible for their allergic manifestations. In particular, prick-by-prick tests were performed with both peach peel and pulp.

SPTs with phosphate-buffered saline (PBS) extracts of both peach peel and pulp and with purified LTP solution, prepared as previously reported,⁸ were also performed.

Specific IgE to the main recombinant birch (Bet v 1, Bet v 2, Bet v 4) and peach (Pru p 1, Pru p 3, Pru p 4) allergens was determined with a Uni-CAP system (Phadia, Uppsala, Sweden) according to the manufacturer's instructions.

Peach protein extraction

Fruits of the peach variety 'Royal Gem' (yellow flesh) grown at the Experimental Farm of Verona Province were harvested at commercial ripeness.

Peaches were peeled and the peel was mechanically made free of pulp residues. Peel and pulp were ground in liquid nitrogen and extracted using two different methods, PBS extraction and phenol extraction, as follows.

PBS extraction

Proteins were extracted from ground peach peel and pulp using PBS (0.01 mol L⁻¹ Na₂HPO₄/NaH₂PO₄, 0.015 mol L⁻¹ NaCl, pH 7.2) (3 : 1 w/v) containing 20 g kg⁻¹ insoluble polyvinylpyrrolidone (Sigma, Milan, Italy) by stirring for 1 h in an ice bath. The samples were then centrifuged (10 000 × *g*, 30 min, 4 °C) and the resulting supernatants were stored at -20 °C or used for SPTs.

Phenol extraction

Samples (5 g) of ground peel and pulp were washed with 10 mL of cold trichloroacetic acid in acetone (100 g L⁻¹) and centrifuged. This washing was repeated four times on the pellet. After the last

centrifugation the pellet was resuspended in 15 mL of 300 g L⁻¹ sucrose, 20 g L⁻¹ sodium dodecyl sulfate (SDS), 0.1 mol L⁻¹ Tris-HCl (pH 7.5) and 20 mL L⁻¹ 2-mercaptoethanol (2-ME) in water, following the modified method described by Vincent *et al.*¹⁶ Briefly, samples were stirred for 30 min in an ice bath, then 15 mL of phenol solution (Fluka, Milan, Italy) was added and mixed. The mixtures were placed at -20 °C for 30 min with vortexing every 10 min. The phenol phase was separated from the water phase by centrifugation (5000 × *g*, 30 min, 4 °C), collected, mixed with 5 volumes of cold ammonium acetate in methanol (0.1 mol L⁻¹) and stored for 3 h at -20 °C. The mixtures were then centrifuged (10 000 × *g*, 20 min, 10 °C) and the protein pellet was recovered.

Protein determination

The protein content of all samples was estimated by the method of Smith *et al.*¹⁷ using a bicinchoninic acid reagent kit (Pierce, Rockford, IL, USA) according to the manufacturer's instructions and with bovine serum albumin (BSA) as the standard. For protein determination the protein pellet derived from phenol extraction was solubilised in 1 mL of PBS.

One-dimensional SDS polyacrylamide gel electrophoresis (SDS-PAGE)

All protein extracts obtained as described above were analysed by SDS-PAGE according to Laemmli¹⁸ in a Mini Protean II unit (Bio-Rad, Milan, Italy). Gels were prepared with a total polyacrylamide concentration of 140 g L⁻¹. Before electrophoresis the samples were processed as follows. The PBS extracts were added (3 : 1 v/v) to a concentrated loading buffer (1.33 mol L⁻¹ Tris-HCl, pH 7.4, 400 g L⁻¹ glycerol, 80 g L⁻¹ SDS, 100 mL L⁻¹ 2-ME), while the protein pellet obtained by phenol extraction was resuspended in 1 mL of the same loading buffer diluted threefold with water. All samples were heated at 100 °C for 8 min and a volume corresponding to 50 µg of protein was loaded on the gel. Electrophoresis was carried out at 50 mA until the tracking dye bromophenol blue ran off the gel. Gels were stained with Coomassie brilliant blue (CBB) or used for blotting. Molecular weight standard proteins (Bio-Rad) were phosphorylase B (97.4 kDa), BSA (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa), soy trypsin inhibitor (21.5 kDa) and lysozyme (14.4 kDa). Gels were scanned using a Biorad ChemiDoc station, and gel images were subjected to densitometric analysis using Image Lab software (Bio-Rad).

Two-dimensional polyacrylamide gel electrophoresis (2D PAGE)

Following the phenol extraction of peach peel, the protein pellet was solubilised in urea (7 mol L⁻¹), thiourea (2 mol L⁻¹), dithiothreitol (DTT, 0.060 mol L⁻¹) and 3-[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonic acid (40 g L⁻¹). Protein concentration was determined with a Bio-Rad protein quantisation kit according to the manufacturer's instructions.

Isoelectric focusing (IEF) was performed with immobilised pH gradient (IPG) strips (11 cm, pH range 4–7) using a Multiphor II system (GE Healthcare Biosciences, Uppsala, Sweden) at 20 °C with a maximum voltage of 8000 V. IPG strips were loaded with 150 µg of protein, 10 mL L⁻¹ ampholytes and traces of bromophenol blue in a final volume of 210 µL, and rehydration was done overnight.

Following IEF, the strips were equilibrated for 15 min in a buffer solution (0.05 mol L⁻¹ Tris-HCl, pH 8.8, 6 mol L⁻¹ urea, 300 g L⁻¹ glycerol, 20 g L⁻¹ SDS) containing 10 g L⁻¹ DTT for protein

Table 1. Clinical characteristics of patients and specific IgE to recombinant allergens of birch (rBet v 1, rBet v 2, rBet v 4) and peach (rPru p 1, rPru p 3, rPru p 4)

Patient	Symptoms	Pollen allergy		Specific IgE (kU L ⁻¹)					
		Birch	Others	rBet v 1	rBet v 2	rBet v 4	rPru p 1	rPru p 3	rPru p 4
A (f)	OAS/R	+	+	15.7	<0.1	<0.1	5.8	<0.1	<0.1
B (f)	OAS/R	+	—	11.4	<0.1	<0.1	4.3	<0.1	<0.1
C (f)	OAS	+	+	31.1	<0.1	<0.1	20.4	<0.1	<0.1
D (f)	OAS	+	—	>100	<0.1	<0.1	69.9	<0.1	<0.1
E (m)	OAS/R	+	+	35.2	<0.1	<0.1	11.2	<0.1	<0.1
F (f)	OAS	+	+	<0.1	1.4	<0.1	<0.1	<0.1	1.1
G (m)	OAS	+	—	6.6	<0.1	<0.1	4.0	<0.1	<0.1
H (f)	OAS	+	—	14.9	<0.1	<0.1	17.2	<0.1	<0.1
I (f)	OAS/R	+	+	7.0	<0.1	<0.1	4.5	4.2	<0.1
L (m)	OAS	+	+	NT	NT	NT	NT	NT	NT
M (m)	OAS	+	+	NT	NT	NT	NT	NT	NT
N (f)	A/R	—	—	<0.1	<0.1	<0.1	<0.1	12.2	<0.1

OAS, oral allergy syndrome; R, rhinitis/rhinoconjunctivitis; A, asthma; +, positivity to both skin prick test and specific IgE; others, pollens of Fagales, grass, pellitory, mugwort; NT, not tested; (m), male, (f), female.

reduction, and then for 15 min in the same buffer containing 25 g L⁻¹ iodoacetamide for alkylation.

Strips were subsequently placed on top of precast polyacrylamide gels (8–16% T, Bio-Rad) and sealed with melted agarose. Separation on the second dimension was performed according to Laemmli¹⁸ at 20 mA. Gels were stained with CBB or used for blotting.

IgE immunoblotting

After electrophoretic separation, proteins were transferred onto a nitrocellulose membrane (Opitran BA-S 83, Whatman, Maidstone, UK) using a semi-dry transfer cell (Bio-Rad). Membranes were blocked with Tris-buffered saline (TBS) containing 0.5 mL L⁻¹ Tween 20 (TBS-T) and 50 g L⁻¹ skim milk powder (M-TBS-T) for 4 h. Membranes were then incubated overnight with individual or pooled sera from peach-allergic patients, previously diluted with 0.5 volumes of M-TBS-T. After five washings, blots were incubated for 1 h with an anti-human IgE peroxidase-conjugate antibody (Sigma) diluted 1:6000 (v/v) in M-TBS-T. After a further wash with TBS, peroxidase activity was revealed by chemiluminescence using a Super Signal detection kit (Pierce) according to the manufacturer's instructions.

Mass spectrometry (MS) analysis and protein identification

The protein spot corresponding to that recognised by the IgE of the patients was excised from the 2D gel, washed with a solution containing acetonitrile (ACN) and 0.1 mol L⁻¹ NH₄HCO₃ (1:1 v/v) and vacuum dried. The gel fragments were incubated for 45 min at 55 °C in NH₄HCO₃ (0.1 mol L⁻¹) containing DTT (0.01 mol L⁻¹) to reduce disulfide bonds. After cooling, the DTT solution was replaced with 0.055 mol L⁻¹ iodoacetamide in NH₄HCO₃ (0.1 mol L⁻¹). The gel pieces were then washed with a solution containing ACN and 0.1 mol L⁻¹ NH₄HCO₃ (1:1 v/v), dried and subsequently swollen in 10 µL of digestion buffer containing 12.5 ng L⁻¹ trypsin (Promega, Madison, WI, USA) in NH₄HCO₃ (0.025 mol L⁻¹) and incubated overnight at 37 °C. Tryptic peptides were extracted according to the protocol of Kim *et al.*¹⁹ Liquid chromatography/tandem mass spectrometry

(LC/MS/MS) analyses were performed using a Micromass CapLC unit (Waters, Milford, MA, USA) interfaced with a Micromass Q-TOF mass spectrometer (Waters) equipped with a nanospray source. A 6.4 µL aliquot of tryptic digest was injected at a flow rate of 20 µL min⁻¹ into an Atlantis dC18 trap column (Waters). After valve switching, the sample was separated in an Atlantis dC18 NanoEase column (0.075 mm × 150 mm, 3 µm particle size; Waters) at a flow rate of 0.20 µL min⁻¹ with a gradient from 100 to 550 mL L⁻¹ of solvent B in solvent A over 35 min (solvent A: 949 mL L⁻¹ H₂O, 50 mL L⁻¹ ACN, 1 mL L⁻¹ formic acid; solvent B: 49 mL L⁻¹ H₂O, 950 mL L⁻¹ ACN, 1 mL L⁻¹ formic acid). Instrument control, data acquisition and processing were performed with MassLynx Version 4.0 software (Waters). Database searching was done with the online version of the Mascot program (www.matrixscience.com) using the following parameters: one missed cleavage; 0.8 and 0.6 Da mass accuracy allowed for parent and fragment ions respectively; carbamidomethyl as fixed modification; oxidised methionine as variable modification; 100 hits allowed.

All searches were performed in both the National Centre for Biotechnology Information (NCBI) complete database with no species specified and the NCBI Viridiplantae-specified database.

RESULTS

Allergological characterisation of patients

Specific IgE measurements showed that all patients with OAS (11/12) were sensitised to birch pollens (Table 1). Detection of IgE to recombinant birch allergens indicated that all OAS patients tested except one (patient F) were sensitised to the major birch pollen allergen Bet v 1. In contrast, patient F showed IgE to the birch allergen Bet v 2, whereas no one had IgE against the allergen Bet v 4. All patients with IgE against Bet v 1 were sensitised also to Pru p 1 from peach, while patient F showed IgE to peach profilin Pru p 4 (Table 1). Specific IgE against Pru p 3 allergen, which corresponds to peach LTP,⁷ was found in only two of the patients tested: one (patient I) showed IgE to Pru p 1 and Bet v 1, while the other (patient N) did not. This latter patient showed asthma and rhinitis after eating peach, but neither OAS symptoms nor pollen sensitisation.

Table 2. Skin test and IgE-immunoblotting results for peach-allergic patients

Patient	Peach peel		Peach pulp		Purified LPT	Immunoblotting	
	SPT (PBS)	PbP	SPT (PBS)	PbP	SPT	9 kDa	18 kDa
A	+	++	—	+	—	—	+
B	—	++	—	++	—	—	+
C	+	+++	—	++	—	—	+
D	—	+++	—	++	—	—	+
E	—	+++	—	++	—	—	+
F	+	+	—	—	—	—	—
G	++	+++	—	++	—	—	+
H	—	++++	—	+++	—	—	+
I	++++	++++	++	+++	+++	+	+
L	—	++	—	++	—	—	+
M	—	+++	—	++	—	—	+
N	++	+++	+	+	+++	+	—

SPT (PBS), skin prick test with PBS extracts; PbP, prick-by-prick; +, positive response; —, negative response. For skin tests the number of + is related to the intensity of the cutaneous response.

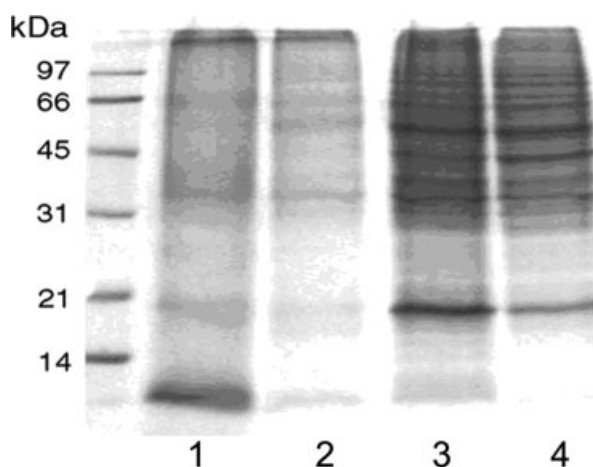


Figure 1. SDS-PAGE analysis of peach peel (lanes 1 and 3) and pulp (lanes 2 and 4) proteins obtained by PBS (lanes 1 and 2) and phenol (lanes 3 and 4) extractions.

Prick-by-prick tests were performed on all selected patients with both the peel and pulp of the peach fruit. SPTs were also carried out with the PBS extract of the same peach samples and with a purified peach LTP solution⁸ (Table 2). The results obtained in a given patient with peach peel and pulp showed only minimal differences between the fruit tissues, indicating that the allergens were present in both of them. In general, stronger skin responses were elicited by the prick-by-prick technique compared with SPTs with the PBS extracts, indicating low solubility of the allergens in saline solution. Finally, SPTs with the purified peach LTP preparation were positive only for the two patients sensitised to Pru p 3 (patients I and N) (Tables 1 and 2).

SDS-PAGE

Peach peel and pulp were treated with both PBS and the phenol method (PM) in order to evaluate the best conditions for extracting peach allergens from the two types of tissue. Extraction with PBS gave 0.73 g protein kg⁻¹ peel and 0.6 g protein kg⁻¹ pulp (fresh

weight), while extraction with the PM gave 1.1 g protein kg⁻¹ peel and 0.9 g protein kg⁻¹ pulp (fresh weight).

SDS-PAGE analysis of the samples prepared by PBS extraction resulted in the detection of few bands of poor quality for both peach peel and pulp (Fig. 1, lanes 1 and 2). Only a band with an M_r corresponding to 9 kDa, which can be identified as the peach LTP,⁷ was clearly visible in the peel sample (Fig. 1, lane 1), while this band was barely detectable in the pulp (Fig. 1, lane 2), confirming the results obtained by other authors.^{5,20} Moreover, some CCB-stained material could be detected at the top of the gel, indicating that a fraction of the protein extracted by PBS was present in the solution as aggregates of very high molecular weight, likely comprising protein–polyphenol complexes.²¹

Better electrophoretic results were obtained with the PM extracts (Fig. 1, lanes 3 and 4). The electrophoretic patterns showed a clear separation of a large number of protein bands in the 20–100 kDa range. Apparently, there were no major differences between the PM extracts of peel and pulp, and, in particular, a strong protein band with an M_r around 18 kDa, barely detectable in the PBS samples, was evident in both PM extracts (Fig. 1). Moreover, in these latter, no high-molecular-weight aggregates appeared at the top of the gel, indicating that extraction with the PM minimised their formation.²² Densitometric analysis performed on gel images indicated that the band at 18 kDa from PM extracts was 5.1–5.5 times more intense than the corresponding band from PBS samples (Fig. 1). However, the 9 kDa band detectable in the peach peel extracted by PBS was very faint in the PM extract, indicating a different behaviour of the peach LTP in the two extraction systems.

IgE immunoblotting

The peach IgE-binding proteins present in the samples obtained with the two extraction systems were identified by IgE immunoblotting with the sera of individual patients (Fig. 2). Owing to the limited quantity of sera, this experiment was done only with the samples derived from the peach peel, which seemed to contain a larger number of protein bands compared with the pulp (Fig. 1).

Different results were shown for the samples obtained by the two extraction methods. When the PBS extract was analysed, only

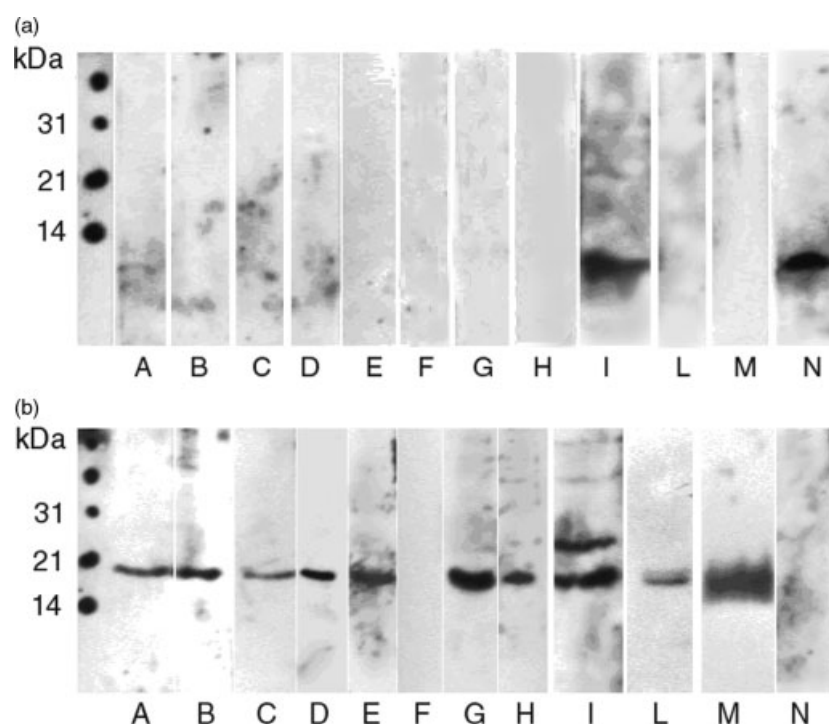


Figure 2. Immunoblotting with individual sera on peach peel proteins obtained by PBS (a) and phenol (b) extractions.

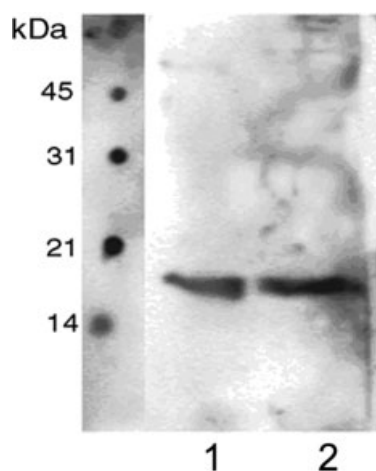


Figure 3. Immunoblotting with pooled sera on peach peel (lane 1) and pulp (lane 2) proteins obtained by phenol extraction.

two patients (I and N) showed IgE binding, whereas the sera of the other patients did not bind any band. For both patients I and N only, a band was recognised showing a molecular weight corresponding to that of the peach LTP (Fig. 2a). These results agreed with those obtained by both SPT with the purified LTP preparation (Table 2) and IgE detection of the recombinant allergen Pru p 3 (Table 1), which corresponds to the peach LTP.

In contrast, a similar experiment performed on the proteins extracted with the PM showed that 10/12 patients recognised a band of 18 kDa, and only one of them (patient I) had IgE binding to another band of lower mobility (Fig. 2b). The remaining patients (F and N) did not show any binding for the PM extract of the peach peel. These latter patients were those resulting negative in specific IgE detection of Bet v 1 and Pru p 1 allergens (Table 1).

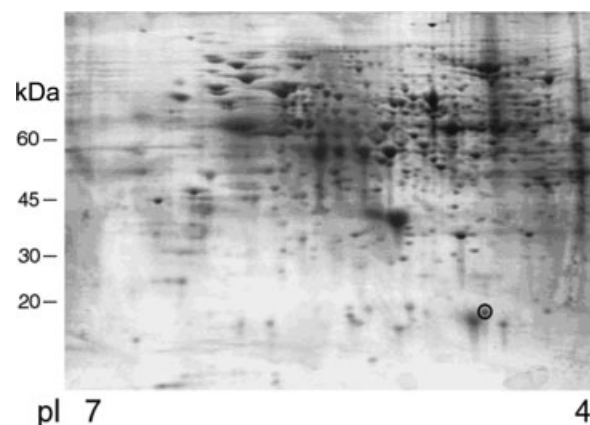


Figure 4. Two-dimensional electrophoretic analysis of peach peel proteins obtained by phenol extraction, stained with CBB. The circle indicates the IgE-reactive spot.

The pool of sera of all patients showing IgE binding to the peel 18 kDa protein recognised the same protein band also in the PM extract of the peach pulp (Fig. 3), confirming that this allergen was present in both tissues.¹²

2D PAGE and protein identification by MS

The protein profile of peach peel extracted with the PM and subjected to high-resolution 2D PAGE yielded hundreds of clearly defined spots (Fig. 4). Two electrophoretic separations were carried out in parallel in two different gels, one of which was used for IgE immunoblotting with the pool of sera recognising the 18 kDa band in one-dimensional SDS-PAGE (Fig. 2b). This allowed the recognition of only one IgE-reactive spot (data not shown) located in a position corresponding to an isoelectric point of around 6 and an M_r of 18 kDa (circle in Fig. 4). To unequivocally identify

this protein, the spot was excised from the CBB-stained 2D gel, submitted to digestion with trypsin and analysed by LC/MS/MS. The sequences of the identified peptides were KAFVLDADNLVPK, ITFGEQSQYGYVK and LVASPSGGSIK. Data analysis using the Mascot software and NCBI protein database identified the protein spot as a major allergen Pru p 1 from *Prunus persica* with 23% coverage of its amino acid sequence and a protein score of 149.

DISCUSSION

PBS extraction of proteins is the normal procedure used for the preparation of extracts to be used in experiments aimed at the identification of potential allergens in fruits. However, it has been recently pointed out that other buffers with acidic or basic pH can be more effective than PBS, at least for the extraction of some allergens from peach. In particular, one of the main peach allergens, Pru p 1, could be extracted from both the pulp and the peel by using Na₂CO₃ solution at pH 9.4, but was unextractable in PBS. However, the presence of this allergen was detectable only by immunoblotting and not by CBB staining of the gel, indicating a very low quantity of the corresponding protein in the extracts. This very low content in peach extracts obtained by aqueous solutions seems to be the reason for the lack of information about this protein.¹² As a matter of fact, to overcome this problem, recombinant Pru p 1 was expressed in *E. coli* in order to obtain a protein amount sufficient for biochemical characterisation.¹¹

Phenol extraction has been shown to be a suitable method for the extraction of proteins present in low amounts in plant tissues, which can show low solubility in aqueous buffers.²³ The application of this extraction method (PM) to the peach peel and pulp allowed us to obtain a great improvement in the SDS-PAGE pattern in comparison with that obtained after extraction of the peach tissue with PBS, especially for bands located in the upper part of the gel (Fig. 1). In particular, a strong band with the same mobility as the Pru p 1 allergen (about 18 kDa) appeared, which was only barely detectable in the PBS extract. On the contrary, the PM allowed the detection of only a very low quantity of the 9 kDa band, the peach LTP, which could be extracted in a larger amount by PBS (Fig. 1). This result indicates the suitability of the PM as a preliminary step for the detection of IgE binding to Pru p 1 and possibly also to other peach proteins different from LTP. Similar good results following phenol extraction were obtained for Pru p 1 in peach¹³ and for the Bet v 1-homologous allergen Mal d 1 in apple.²³ Therefore the use of different complementary solvents such as PBS and phenol must be recommended in order to avoid the loss of a part of the proteins from plant tissues, which are often recalcitrant to be extracted and analysed, and also owing to the presence of compounds interfering with the electrophoretic analysis.²⁴

The results were confirmed by the IgE-immunoblotting experiments, where the sera of our patients were used in order to detect peach allergens. Despite the clinical reactivity to peach, no bands were detectable in the PBS extracts, with the exception of the two patients showing IgE against the recombinant allergen Pru p 3 (Table 1) and skin test positivity to purified LTP (Table 2). These sera in fact recognised the 9 kDa LTP (Fig. 2a), confirming this protein to be potentially responsible for their peach allergy. In contrast, to identify the allergens potentially responsible for the OAS shown by the majority of the peach-allergic patients, it was necessary to use the extract obtained with the PM in IgE-immunoblotting experiments, which revealed clear IgE binding to the 18 kDa band (Fig. 2b). Therefore this protein can be identified

as the potential peach allergen responsible for the symptoms of most of the peach-allergic patients considered in this study. The *M_r* of this IgE-binding band corresponds to that of Pru p 1, the peach allergen homologous to Mal d 1 from apple, Pru av 1 from sweet cherry and other Bet v 1 homologues.¹¹ However, the nature of this IgE-binding protein has never been determined directly in peach fruit, most of the available information deriving from characterisation of the recombinant Pru p 1 expressed in *E. coli*¹¹ or indirectly from RAST inhibition experiments with the homologous allergens Mal d 1²⁵ and Bet v 1.²⁶ The reason for the difficulty in characterising the 18 kDa allergen of peach fruit was claimed to be the very low quantity of Pru p 1 in peach tissues.^{11,12} Based on the results presented here, this low quantity, however, seems to be due to the inappropriateness of the extraction methods; indeed, PM extraction results in a fivefold increase in intensity of the band at 18 kDa compared with PBS extraction.

The possibility of extracting a sufficient amount of the 18 kDa protein by the PM allowed us to separate the peach extracts by high-resolution 2D PAGE (Fig. 4) and to analyse by MS the protein spot bound by the IgE of patients suffering from the pollen/fruit syndrome. This analysis showed homology of the peach 18 kDa band to the putative Pru p 1 sequence as determined by nucleotide sequencing, two of the identified peptides (KAFVLDADNLVPK and LVASPSGGSIK) being indicated as potential IgE epitopes.²⁷ Therefore the close similarity of the peach protein indicated as Pru p 1 to the allergens belonging to the Bet v 1 family can be definitely established.

In patients living in Mediterranean areas free of birch trees, the allergic manifestations are a consequence of primary sensitisation to a major peach allergen Pru p 3, corresponding to the LTP. This LTP is responsible for the cross-reactivity between peach and other related and unrelated species of fruits and vegetables. As confirmed here, this allergen is present mainly in the peach peel,¹² which is the reason for the proposal of industrial peeling of peach fruit as a means to reduce its allergenicity.²⁸ In contrast, in areas in which trees of the order Fagales are present, peach allergy seems to be a consequence of primary sensitisation to birch pollens.⁴ Both Pru p 3 and Pru p 1 allergens have been reported to be involved in peach allergy in patients living in northern Italy.^{29,30} However, among the 12 peach-allergic patients considered in this study, only two proved to be allergic to peach LTP (Tables 1 and 2), whereas the great majority suffered from the pollen/fruit syndrome with OAS, which in fact is the most frequent allergic problem in Italy.²⁹ In these cases the peach allergen, present in both the peel and pulp, cannot be eliminated by peeling the fruit as suggested for removal of the peach LTP, which is almost exclusively located in the peel.²⁸

Moreover, variations in the expression of allergen-encoding genes, including those controlling Pru p 1 accumulation, were recently reported in peaches at different ripening stages and grown under different conditions,^{10,27} suggesting the possibility of variations in the allergenic potential of peach fruit. The phenol extraction of the Pru p 1 allergen described here will allow the development of a method for Pru p 1 quantification useful to verify if the variations detected at the gene expression level actually result in a different allergen content in the fruit.

In conclusion, it has been demonstrated that peach allergy for patients suffering from the birch/fruit syndrome is caused by a protein present in both the peach peel and pulp, which was definitively identified by MS as the allergen Pru p 1, a member of the Bet v 1 family. However, this was possible only after extraction of the peach fruit with the PM, suggesting that, to detect potential

allergens in peach and reasonably in other fruits and vegetables, it is necessary to consider more than one suitable extraction procedure to avoid loss of allergenic proteins.

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