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Characterization of the major allergen of plum as a lipid transfer protein

Elide A. Pastorello[a,*], Laura Farioli[b], Valerio Pravettoni[a], Maria Gabriella Giuffrida[c], Claudio Ortolani[d], Donatella Fortunato[c], Chiara Trambaioli[a], Elisabetta Scibola[a], Ambra M. Calamari[a], Anna M. Robino[a], Amedeo Conti[c]

[a]*Allergy Centre, Third Division of General Medicine, Ospedale Maggiore IRCCS, Milan, Italy*
[b]*Unità Operativa Ospedaliera di Medicina del Lavoro (UMOOL), Milan, Italy*
[c]*National Research Council, Turin, Italy*
[d]*Bizzozzero Division, Niguarda Ca' Granda Hospital, Milan, Italy*

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Abstract

Background: Allergy to *Prunoideae* fruit (plum, peach, cherry and apricot) is one of the most frequent food allergies in southern Europe. All these fruits cross-react in vivo and in vitro, as they share their major allergen, a 9 kD lipid transfer protein (LTP). *Objective:* The aim of the study was the identification and molecular characterization of the major allergen of plum. *Methods:* The IgE pattern of reactivity to plums was investigated by SDS–PAGE and immunoblotting with the sera of 23 patients. The identified major allergen was purified by HPLC, using a cationic-exchange column followed by gel-filtration. Further characterization was achieved by periodic-Schiff stain, isoelectrofocusing and N-terminal amino acid sequencing. *Results and conclusions:* The major allergen of plum is a 9 kD lipid transfer protein, not glycosylated and with a basic character (p*I*>9), highly homologous to the major allergen of peach.

Keywords: Food allergy; Skin prick test; *Prunoideae* fruit; Lipid transfer proteins

1. Introduction

The origins of plum tree cultivation date back to ancient times; this tree probably came from the Caucasian area, but was introduced to Europe some 2000 years ago. Until recently, the most important production area was Europe, while nowadays China and the USA are the main producers. Many varieties of plum are cultivated throughout the world, but the most widespread are *Prunus domestica* (European plum) and *Prunus salicina* (Japanese plum). They are mainly consumed as fresh fruit, but part of the crop is dried into prunes. Uses of plums include jams, jellies and sauces. Prunes are widely used in baked goods.

Even if plum production in Europe has decreased over the last decade, plum consumption is still prominent in our continent, especially in southern countries such as Italy, France and Spain. Thus, it is not surprising that allergic reactions to plums have

*Corresponding author. Fax: +39-2-5519-2842.
E-mail address: elidepa@box1.tin.it (E.A. Pastorello).

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been described in these countries, as we reported in previous studies [1,2].

Plum allergy usually appears as local oropharyngeal symptoms within a few minutes after ingestion, sometimes followed by systemic symptoms. This clinical picture is characteristic of the so-called oral allergy syndrome (OAS). Life-threatening reactions to plums are rare, but anaphylactic shock has been reported [3].

Patients allergic to plums frequently show allergic reactions also to other fruit belonging to the same botanical family *Rosaceae*, subfamily *Prunoideae*, namely peaches, cherries and apricots. We found that all *Prunoideae* fruit share a cross-reacting low molecular mass protein acting as a major allergen [2]. This allergen, characterized in the peach, corresponds to a basic protein with an isoelectric point of 9.25 and a molecular mass of 9 kD; sequence analysis shows it to be a lipid transfer protein (LTP) [4]. In a further study we isolated the major allergen from the apricot, which is also an LTP and has 95% sequence homology with peach 9 kD protein [5].

The aim of this study was to achieve the identification and the molecular characterization of this major allergen also in plums.

2. Material and methods

2.1. Patients

For this study we selected patients allergic to plums referred to the Allergy Center of the Third Division of General Medicine of the University of Milan, or to the Bizzozzero Division of the Niguarda Ca' Granda Hospital of Milan. Patients reporting symptoms after ingestion of plums were recruited and studied by the following protocol: (1) clinical history of allergic reactions to plums and to other foods, especially fruit belonging to the *Prunoideae* subfamily (peach, apricot and cherry); (2) skin prick tests (SPT) with fresh fruit by the prick+prick technique [6]; (3) measurement of plum specific IgE antibodies by the CAP System (Pharmacia & Upjohn Diagnostics AB, Uppsala, Sweden); (4) an open oral challenge with fresh plums, made by chewing increasing amounts of the fruit, ranging from 4 to 64 g, as reported elsewhere [2].

Patients were also studied for pollinosis by recording a clinical history of seasonal respiratory symptoms and by performing SPT and CAP System with commercial pollen extracts. Blood was drawn from all patients and sera were stored at −80°C until used for the in vitro tests to identify plum allergens.

2.2. In vitro methods

All the in vitro methods have been described in detail elsewhere [4].

2.2.1. Plum extract

The plum extract was prepared by the method of Björksten et al. [7], using about 250 g of plum peels diluted in 100 ml of 10 mmol/l PBS (pH 7) with 2% polyvinylpolypyrrolidone, 2 mmol/l EDTA disodium salt and 10 mmol/l sodium diethyldithiocarbamate. After homogenizing and centrifuging at 16000 rpm at 4°C for 30 min, the supernatant was dialyzed against 10 mmol/l PBS (pH 7) for 48 h at 4°C, with dialysis tubing Spectra/Por Membrane (M_r cut off at 3500 D). We thus obtained about 200 ml of the final extract with a protein content of 10.5 mg/ml, as assessed by the Lowry's method [8]. This extract was used for SDS–PAGE analysis, but was too dilute for allergen purification, so we further concentrated it by centrifugation at 3000 rpm at 4°C with a Centripep 3 concentrator (Amicon, Inc., Beverly, USA) with a M_r cut-off at 3000 D. The extract was concentrated to achieve a final concentration of about 130 mg/ml.

2.2.2. SSD–PAGE/immunoblotting

The plum extract was separated in a discontinuous buffer system in an SDS–PAGE gel with a 6% stacking gel and a 7.5–20% separation gradient, essentially as described by Neville [9]. The sample was diluted 1:2 in sample buffer containing Tris–SO_4 (pH 6.1) (hydroxymethyl-aminomethane), 10% SDS, 2-mercaptoethanol, 50% glycerol and 1% Bromophenol blue. The samples were denatured at 100°C for 5 min. In the same gel reference markers at known M_r (i.e., 94, 67, 43, 30, 20.1 and 14.4 kD, Pharmacia-Upjohn) were run. Plum extract (0.63

mg/cm gel) and reference markers were run at 6 mA for 16 h in a BIO-RAD Protein IIxi vertical electrophoresis slab cell (Bio-Rad Labs, Richmond, USA).

After separation, a portion of gel was fixed and stained with Coomassie Brilliant Blue R-250 (Pharmacia-Upjohn) and another part was used for the immunoblotting studies.

The separated proteins were transferred to a nitrocellulose membrane (pore size=0.45 μm, Amersham, UK) using a trans-blot cell from Bio-Rad at 0.45 A, 100 V, for 4 h at 4°C. After transfer, the unoccupied protein-binding sites in the nitrocellulose membrane were blocked by incubation with a phosphate buffer, pH 7.4, containing 0.5% Tween 20, for 30 min at 37°C. The nitrocellulose was then cut into strips, which were incubated overnight at room temperature with the serum of each patient. Specific IgE-binding was detected by incubation with ^{125}I-labeled anti-human IgE diluted 1:4 in blocking solution, for 6 h at room temperature, and exposure to X-ray film at −70°C for 4 days. Sera from ten healthy subjects were used as negative controls.

A 9 kD protein was identified as the major allergen and purified.

2.2.3. Purification of plum 9 kD allergen by high-performance liquid chromatography

2.2.3.1. Cationic exchange. The 9 kD protein from plum was isolated and purified by analytical cationic-exchange chromatography with an high-performance liquid chromatographic (HPLC) system (AKTA Purifier Pharmacia Biotech, Uppsala, Sweden) injecting 1 ml of the raw extract, after dilution 1:3 in buffer A to reach the correct pH and molarity, in a 1-ml Resource-S column (column dimensions=6.4×30 mm, particle size=15 μm, pore size=200–10 000 Å, Pharmacia-Biotech) at room temperature. The mobile phase was buffer A — 30 m*M* sodium citrate dihydrate, pH 2.2 — and buffer B — 30 m*M* sodium citrate dihydrate plus 1 *M* NaCl, pH 2.2. The gradient length was 15 CV (column volume) with a flow-rate of 3 ml/min. Absorbance was monitored at 280 nm. After the peak corresponding to the unbound material, only one further peak was detected. This fraction, analyzed by SDS–PAGE, contained the 9-kD protein with other higher molecular mass impurities, so further resolution was achieved by gel-filtration.

2.2.3.2. Gel-filtration. Cationic-exchange concentrated fractions were separated on a Superdex™ 75 HR 10/30 column (bed dimension=10×300–310 mm, bead diameter=13 μm) equilibrated and eluted with 30 m*M* sodium citrate dihydrate at a flow-rate of 0.7 ml/min. Before the first run, a calibration curve was prepared by measuring the elution volumes of various standard substances of known molecular mass: ribonuclease, 13.7 kD; chymotrypsinogen A, 25 kD; ovalbumin, 43 kD; bovine serum albumin, 67 kD (Pharmacia Biotech). The injection volume was 500 μl and absorbance was monitored at 280 nm. The chromatogram showed several peaks. The fractions corresponding to the 9 kD peak were concentrated, analyzed by SDS–PAGE and stored at −20°C, after measuring their protein content by the method of Warburg and Christian [10] and reading by spectrophotometer at 260 and 280 nm.

2.2.4. Purity of the 9-kD protein and its IgE-binding capacity

To assess the purity of the 9-kD protein, we used SDS–PAGE/immunoblotting following the method described above, using pooled sera from all the patients in the study.

2.2.5. Blotting inhibition of plum 9-kD protein by peach 9-kD allergen

An immunoblotting–inhibition experiment was done to demonstrate the homology between the 9-kD purified plum protein (loaded at 0.021 mg/cm gel) and the 9-kD purified peach protein (loaded at 0.050 mg/cm gel). Briefly, 500 μl of pooled sera were inhibited with 500 μl of the peach 9-kD purified protein. After incubation, the inhibited serum was matched with a nitrocellulose strip of the blotted SDS–PAGE of the plum 9-kD purified protein. Amounts of 0.31, 0.031, 0.0031, 0.00031, 0.000031 and 0.0000031 mg of peach 9-kD protein were used as inhibitor. The experiment then followed the steps described above.

2.2.6. Amino acid sequencing

N-terminal sequence analysis was done on an Applied Biosystems 492 pulse-liquid sequencer (Per-

kin-Elmer Applied Biosystems, Foster City, CA, USA).

2.2.7. Isoelectrofocusing

The 9-kD purified protein was focused by a Pharmacia-LKB Phast System, using Phast Gel pH gradient 3–9. The process was run according to the manufacturer's instructions and then stained with Coomassie Brilliant Blue R-250.

2.2.8. Periodic acid-Schiff stain

Periodic acid-Schiff (PAS) staining was done to detect glycosylation of proteins. Purified 9-kD protein was run into minigel and blotted onto Problot membrane (Perkin-Elmer, Applied Biosystem) at a constant 16 V for 60 min, as described by Towbin and Gordon [11], except that methanol and SDS were omitted from the buffer. Two identical membranes were blotted at the same time, one stained with Coomassie R-250 and the other used for detection of glycoproteins by PAS staining. The latter was fixed in 12% trichloroacetic acid for 1 h. The membrane was then immersed in Schiff's reagent and kept in the dark at 4°C overnight. The background was destained in methanol, and the glycoproteins appeared as purple bands. Milk whey proteins were used as the control.

3. Results

3.1. Patients

Twenty-three patients, 15 women and 8 men, aged from 18 to 45 years (mean age=29.8 years), were recruited for the in vitro study. All reported OAS to plums. Table 1 gives their age and sex, the plum specific IgE values (CAP-system), the pollens causing respiratory symptoms, and sensitizations to other foods as assessed from history and from skin prick test and CAP-system findings. All patients also had OAS to peaches, and 15 and 12 were also allergic to cherries and apricots, respectively. Patients 1–7 were allergic to plums and/or other *Prunoideae* fruit but

Table 1
Characteristics of patients

Patient no.	Sex	Age	Plum CAP-system (kU/l)	Pollens	Other foods causing symptoms
1	F	30	2.88	–	peach, apple
2	M	33	6.74	–	peach, cherry
3	M	18	1.53	–	peach, cherry
4	M	24	16.15	–	peach, cherry
5	F	34	3.47	–	peach, cherry, apricot
6	F	25	3.23	–	peach, apricot
7	F	35	7.33	–	peach, cherry
8	F	32	4.31	grasses	peach, cherry, apricot
9	F	29	17.52	grasses	peach, cherry, apricot, apple
10	F	39	3.35	grasses, birch	peach, cherry
11	M	22	3.80	grasses	peach, cherry, apricot
12	F	25	2.85	grasses, birch	peach, cherry, apricot
13	M	20	1.66	grasses	peach, apple
14	F	42	1.48	grasses, birch	peach, cherry, apricot, apple
15	F	23	14.08	grasses, birch, mugwort	peach, cherry
16	F	26	11.59	grasses, birch	peach, cherry, apricot
17	M	45	18.54	grasses, birch	peach, cherry, apricot
18	F	39	15.91	grasses, birch	peach
19	M	23	13.44	grasses, birch	peach
20	F	32	0.61	birch	peach, apricot
21	F	31	13.90	grasses	peach, cherry, apricot
22	F	41	2.37	grasses, birch	peach, apricot
23	M	19	15.50	grasses, birch	peach

not to birch pollen, whereas patients 8–23 had positive IgE levels for birch and/or grass pollen.

3.2. SDS–PAGE/immunoblotting

Coomassie Brilliant Blue R-250 stained profiles of plum proteins showed different components with apparent molecular masss ranging from 9 to 94 kD. Fig. 1 depicts the IgE immunoblotting with sera from the 23 patients, showing an IgE-binding to proteins with M_r 9 kD (19/23 patients, 83%), 19 kD (9/23 patients, 39%), 30 kD (3/23 patients, 13%), 43 kD (7/23 patients, 30%), 67 kD (9/23 patients, 39%), 80 kD (6/23 patients, 26%).

3.3. Purification of plum 9-kD allergen by HPLC

Fig. 2A shows the chromatographic profile obtained from the cationic-exchange column. The first fraction contained the unbound material. SDS–PAGE analysis of the second fraction showed the presence of a 9-kD protein, which was not pure due to the presence of higher molecular mass proteins (data not shown). This fraction, having a volume of 5 ml, was then collected by repeated runs, thus obtaining 80 ml, which were concentrated to reach a final volume of 4 ml. A 500-μl aliquot of this concentrated fraction was injected into a gel-filtration column; the peaks are shown in Fig. 2B. After comparison with the chromatographic profile of the markers, we collected the fraction corresponding to the third peak, which had an appropriate molecular mass. About 2 ml of the 9-kD purified protein were collected at each run, obtaining a final volume of 12 ml, with a protein concentration of 0.03 mg/ml. This was concentrated again to a final volume of 1.5 ml, with a protein concentration of 0.18 mg/ml.

3.4. Purity of the 9-kD protein and its IgE-binding capacity

The 9-kD protein, analyzed by SDS–PAGE/immunoblotting with the pooled sera, was pure, as demonstrated by the absence of other allergenic components both in SDS–PAGE and in IgE immunoblotting, which also demonstrated the IgE binding capacity of this fraction (Fig. 3).

3.5. Blotting inhibition of plum 9-kD protein by peach 9-kD allergen

Fig. 4 depicts the IgE immunoblotting of the pooled sera for 9-kD purified plum and peach proteins and the inhibition of the 9-kD purified plum

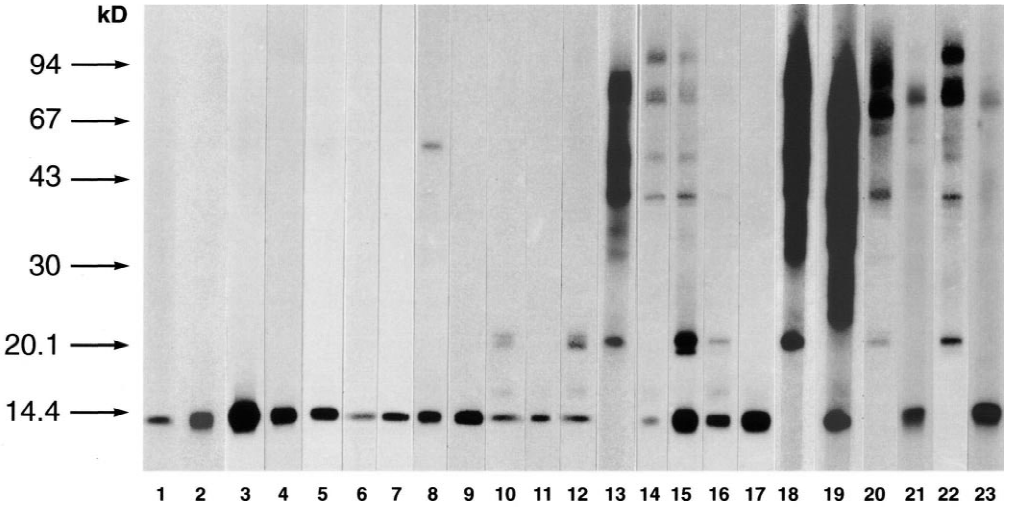

Fig. 1. IgE immunoblots of plum crude extract with sera from 23 plum-sensitive patients (patients 1–7 without specific IgE to pollens and patients 8–23 with specific IgE to grasses and/or birch pollens). The 9-kD protein is the major allergen, recognized by 19 of the 23 sera (83%).

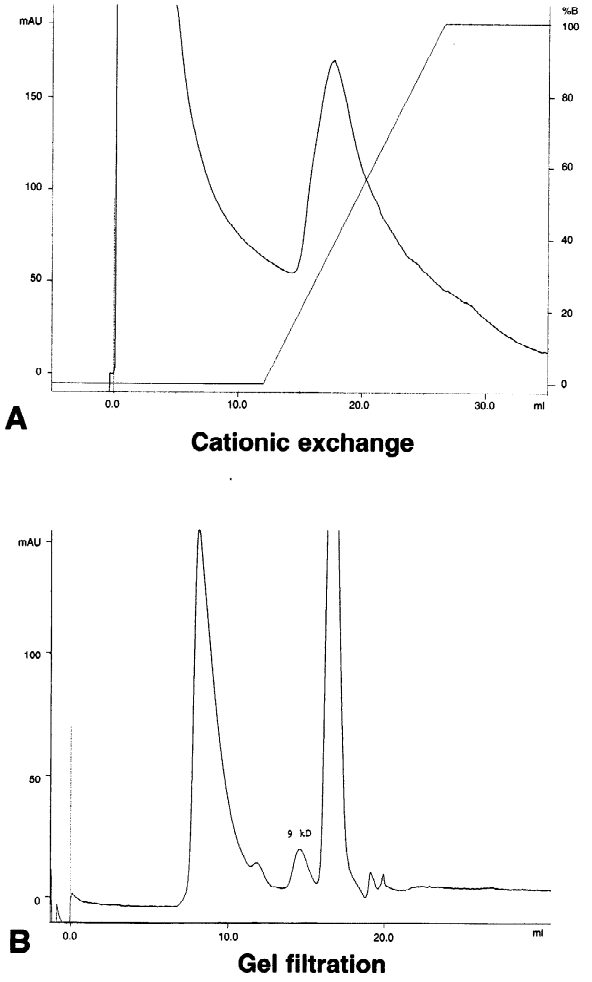

Fig. 2. Cationic-exchange of plum crude extract fractionated over the HPLC Resource-S column (A). Gel-filtration of 9-kD peak obtained in cationic-exchange chromatography (B).

protein from peach 9-kD protein at different concentrations. Inhibition was complete until 0.0031 mg of peach inhibitor.

3.6. Amino acid sequencing

The N-terminal sequence of the 9-kD allergen was determined for the protein in solution. The result was: Ile-Thr-x-Gly-Gln-Val-Ser-Ser-Asn-Leu-Ala-Pro-x-Ile-Asn-Tyr-Val-Lys-Gly-Gly-Gly-Ala-Val-Pro-Pro. A data base search revealed a very high degree of homology with several proteins belonging to the LTP family, such as peach LTP (94%).

3.7. Isoelectrofocusing

Isoelettrofocusing gave a p*I* value of >9.

3.8. Glycostaining

Glycostaining was negative for glycosylation of the purified 9-kD protein from plum.

4. Discussion

The present study demonstrates that the major allergen of plums is a 9-kD protein belonging to the lipid transfer protein (LTP) family. Sequence comparison made on the basis of N-terminal sequences shows more than 90% sequence homology with LTPs from peaches and apricots, identified in our previous studies as the major allergens of these fruits [4,5]. The high homology between these proteins acting as major allergens accounts for the frequent clinical cross-reactivity between *Prunoideae* fruits observed in patients' histories. This cross-reactivity found in vivo is confirmed by in vitro evidence, such as blotting inhibition of plum 9-kD by peach 9-kD protein.

One of the relevant allergens of apples, a fruit belonging to the botanical family of *Rosaceae* but not to the *Prunoideae* subfamily, is also a lipid transfer protein [12]. This evidence, combined to the observation that the major allergens from *Parietaria judaica*, Par j 1 and Par j 2, have the LTP characteristics [13], suggests that LTPs can be considered as panallergens, since they are shared by non-related plants.

LTPs are a class of ubiquitous plant proteins, whose main characteristics are low molecular mass (about 9–10 kD), basic character (isoelectric point >9), abundance in plant source and high stability [14]. As for their function, in the past LTPs have been investigated mainly for their lipid transfer activity, but more recent studies have also demonstrated a role in defense against pathogens or environmental stress [15].

It is interesting to note that other important allergens are also proteins playing a defending role in plants, for example pathogenesis related proteins (PRPs) of group 5 (thaumatin) and group 10 (ribonu-

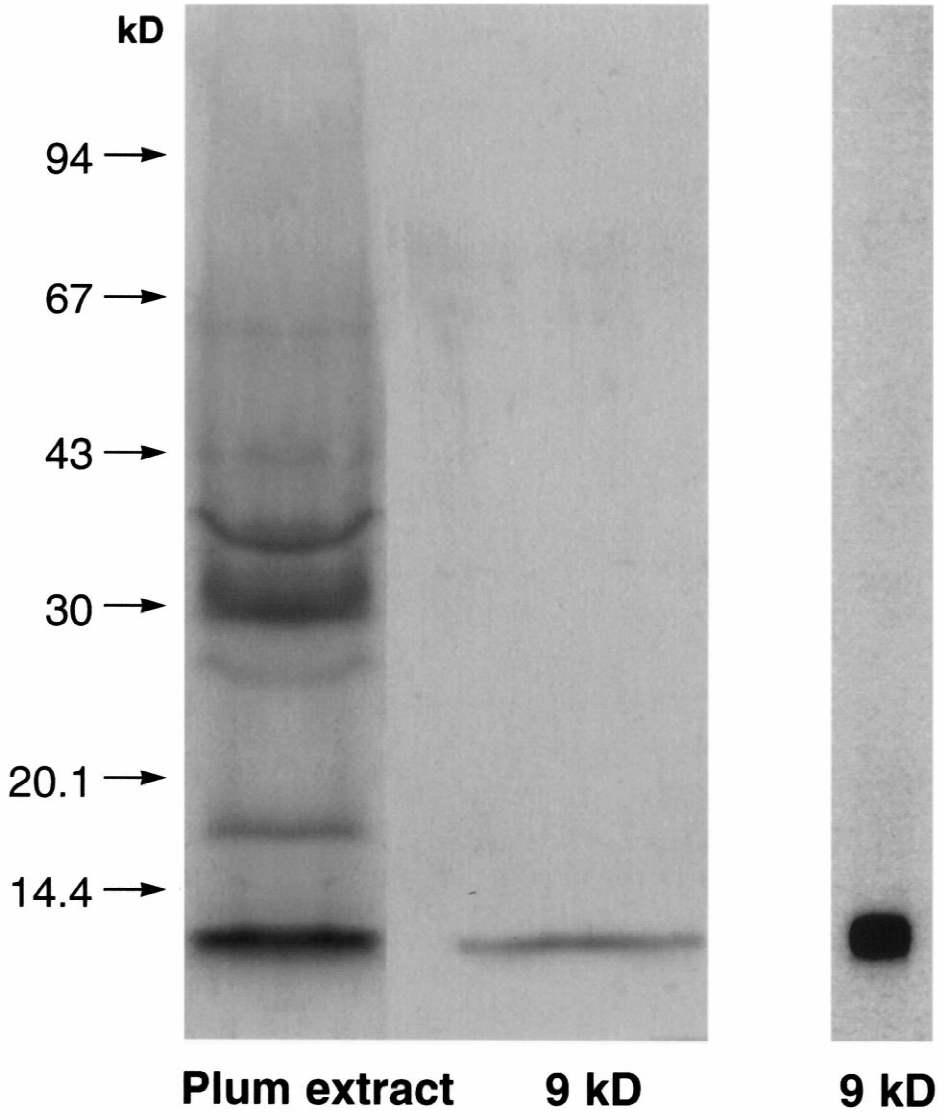

Fig. 3. SDS–PAGE of plum crude extract and 9-kD purified protein stained with Coomassie Brilliant Blue and IgE imunoblotting of 9-kD purified protein.

cleases). PRPs of group 5 include Pru a 2, a major allergen from the cherry [16], while PRPs of group 10 include several food allergens related to the major birch pollen allergen Bet v 1, such as Mal d 1 from apple [17], Api g 1 from celery [18], Dau c 1 from carrot [19] and Pru av 1 from cherry [20]. We presume that the defensive role of these proteins requires them to be highly resistant and stable, so that they can be produced even under stressful conditions; this characteristic of stability makes them ideal food allergens.

After our previous studies, in which we purified the major allergens from peach, apricot and apple, with this last study we have definitively established the purification procedure suitable for lipid transfer proteins from vegetable foods. First of all, their localization on the surface, especially in the fruit peel, allows easy extraction with a phosphate buffer.

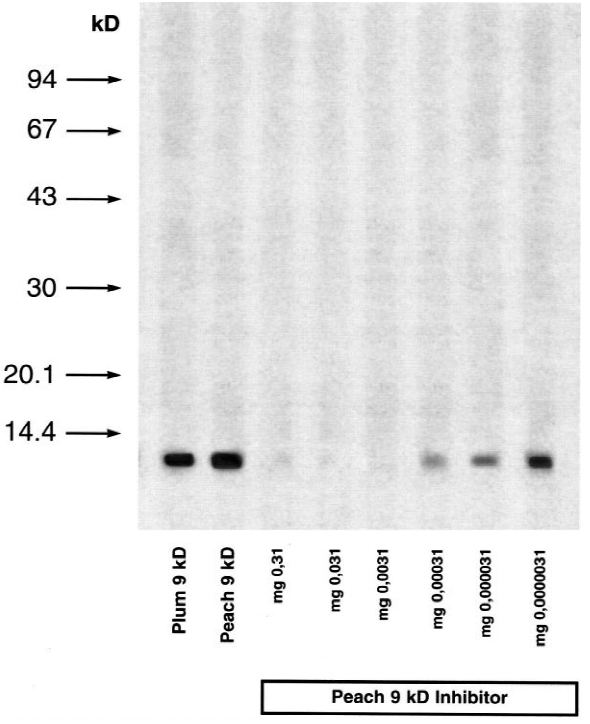

Fig. 4. IgE immunoblotting of 9-kD purified plum and peach protein and inhibition of the plum 9-kD purified protein from peach 9-kD protein at different concentrations by using pooled sera from patients allergic to plum.

Then, their basic character allows the use of a cationic-exchange column for the first step of purification, thus obtaining a good amount of material for the second step on a gel-filtration column, in order to obtain the pure protein. We think that this technique could be applied with small modifications of the buffer (pH and type of salt) for the purification of all vegetable LTPs.

It is worth observing that allergen purification is an essential step for the study of an allergenic protein, mainly because it allows the confirmation of its in vivo IgE-binding activity, and provides a reference material against which to compare recombinant molecules, the biological activity of which always needs to be compared with their natural counterparts. In the case of LTPs, which seem, at least in the Mediterranean area, to act as the most important allergens of fruit such as peaches, apricots and apples, the introduction of recombinant allergens for diagnostic, and possibly therapeutic, purposes will allow diagnostic accuracy to be greatly improved. The relevance of a diagnostic procedure for allergies to *Prunoideae* fruit that entails using extracts, standardized on the basis of the 9-kD allergen, appears from the observation that also in plums, as in peaches, apricots and apples, this allergen was the only one without cross-reactivity to pollen. In fact we found [4,5,12] that patients allergic to fruits but not to pollens recognized only the 9 kD allergen, while patients also allergic to pollens, and especially to birch pollen, reacted against higher molecular mass allergens. This pattern of IgE reactivity was detected also in patients allergic to plums. Almost all the plum/birch allergic patients recognized a 19-kD protein, which is likely a Bet v 1 homologue, and other allergens of higher molecular mass. These proteins thus appear to sensitize through an inhalative route, while LTP seems to be the only allergen capable of sensitizing through the gastrointestinal route.

The protein we have described can be considered the only major allergen of plums, and the name we suggest on the basis of the IUIS nomenclature is Pru d 3.

5. Nomenclature

OAS	Oral allergy syndrome
M_r	Molecular weight
kD	Kilodalton
SDS–PAGE	Sodium-dodecylsulphate–polyacrylamide-gel electrophoresis
HPLC	High-performance liquid chromatography
SPT	Skin prick test
LTP	Lipid transfer protein

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References

[1] C. Ortolani, M. Ispano, E.A. Pastorello et al., Ann. Allergy 61 (1988) 47.

[2] E.A. Pastorello, C. Ortolani, L. Farioli et al., J. Allergy Clin. Immunol. 94 (1994) 699.
[3] C. Ortolani, E.A. Pastorello, L. Farioli et al., Ann. Allergy 71 (1993) 470.
[4] E.A. Pastorello, L. Farioli, V. Pravettoni et al., J. Allergy Clin. Immunol. 103 (1999) 520.
[5] E.A. Pastorello, F. Purello D'Ambrosio, V. Pravettoni et al., Evidence for a lipid transfer protein as the major allergen of apricot, J. Allergy Clin. Immunol. (in press).
[6] S. Dreborg, T. Foucard, Allergy 38 (1983) 167.
[7] F. Björksten, L. Halmepuro, M. Hannuksela, A. Lahti, Allergy 35 (1980) 671.
[8] O.H. Lowry, N.S. Rosebrough, A.L. Fair, R.J. Randall, J. Biol. Chem. 93 (1951) 265.
[9] D.M. Neville, J. Biol. Chem. 246 (1971) 6328.
[10] H. Warburg, D. Christian, Biochem. Z. 310 (1941) 384.
[11] H. Towbin, J. Gordon, J. Immunol. Methods 72 (1984) 313.
[12] E.A. Pastorello, V. Pravettoni, L. Farioli et al., J. Allergy Clin. Immunol. 104 (1999) 1099.
[13] P. Colombo, D. Kennedy, T. Ramsdale et al., J. Immunol. 160 (1998) 2780.
[14] J.C. Kader, Annu. Rev. Plant Physiol. Plant Mol. Biol. 47 (1996) 627.
[15] W.F. Broekaert, B.P.A. Cammue, M.F.C. De Bolle et al., Crit. Rev. Plant Sci. 16 (1997) 297.
[16] C. Inschlag, K. Hoffmann-Sommergruber, G. O'Riordain et al., Int. Arch. Allergy Immunol. 116 (1998) 22.
[17] S. Vieths, K. Jamek, H. Aulepp, A. Petersen, Allergy 50 (1995) 421.
[18] H. Breiteneder, K. Hoffmann-Sommergruber, G. O'Riordain et al., Eur. J. Biochem. 233 (1995) 484.
[19] K. Hoffmann-Sommergruber, G. O'Riordain, H. Ahorn et al., Clin. Exp. Allergy 29 (1999) 840.
[20] S. Scheurer et al., Mol. Immunol. 34 (1997) 619.