

Pomegranate (*Punica granatum* L.) Expresses Several nsLTP Isoforms Characterized by Different Immunoglobulin E-Binding Properties

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Key Words

Pomegranate allergy · Two-dimensional gel electrophoresis · Mass spectrometry · Non-specific lipid transfer protein

Abstract

Background: Pomegranate allergy is associated with sensitization to non-specific lipid transfer proteins (nsLTPs). Our aim was to identify and characterize the non-specific nsLTPs expressed in pomegranate at the molecular level and to study their allergenic properties in terms of immunoglobulin E (IgE)-binding and cross-reactivity with peach nsLTP (Pru p 3). **Methods:** A non-equilibrium two-dimensional (2-D) electrophoretic approach based on acid-urea PAGE and sodium dodecyl sulfate PAGE was set up to separate pomegranate nsLTPs. Their immunoreactivity was tested by immunoblotting carried out with anti-Pru p 3 polyclonal antibodies and sera from pomegranate-allergic patients. For final identification, pomegranate nsLTPs were purified by chromatography and subjected to trypsin digestion and mass spectrometry (MS) analysis. For this purpose, the sequences obtained by cDNA cloning of three pomegranate

nsLTPs were integrated in the database that was subsequently searched for MS data interpretation. **Results:** Four nsLTPs were identified by 2-D immunoblotting. The detected proteins showed different IgE-binding capacity and partial cross-reactivity with Pru p 3. cDNA cloning and MS analyses led to the identification of three nsLTP isoforms with 66–68% amino acid sequence identity named Pun g 1.0101, Pun g 1.0201 and Pun g 1.0301. **Conclusions:** By 2-D electrophoresis, we could separate different nsLTP isoforms possessing different IgE-binding properties, which might reflect peculiar allergenic potencies. The contribution of Pru p 3 to prime sensitization is not central as in other plant nsLTPs.

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Introduction

Pomegranate (*Punica granatum* L.), a plant of the Lythraceae family, endemic to the Middle East and India, has been used for centuries in traditional medicine. Pomegranate fruit is commonly consumed in the Mediterranean areas in its raw and processed forms. Recently,

it has been spreading among northern countries because of its elevated phenolic content which correlates with a strong antioxidant activity and several health-promoting properties.

Besides these valuable characteristics, pomegranate ingestion has been associated with immediate hypersensitivity reactions. Recently, it has been demonstrated that non-specific lipid transfer proteins (nsLTPs) are involved in the development of type I allergic reactions towards this fruit [1, 2]. nsLTPs are small, basic proteins initially studied for their capacity to transport fatty acids and phospholipids across cell membranes [3] and their role as pathogenesis-related (PR) proteins in plant defense against biotic and abiotic stresses [4, 5]. They are subdivided into two main families according to their molecular mass, i.e. LTP1 (9–10 kDa) and LTP2 (6–7 kDa), although LTP2s have so far rarely been associated with allergic disease [6]. In the last 15 years, nsLTPs have been indicated as potent allergens involved in the elicitation of severe reactions [6, 7], especially in the Mediterranean population [8, 9]. Pru p 3, the peach nsLTP, has been described as the most potent sensitizer capable of priming subjects who can thus suffer from the ingestion of foods containing cross-reacting nsLTPs [8–11]. However, data regarding the role of Pru p 3 in mediating the elicitation of symptoms after ingestion of pomegranate are limited [1, 2].

In pomegranate juice, two nsLTPs belonging to the LTP1 group have been identified, but only one is recognized by the allergic patient's immunoglobulin E (IgE) [1]. It is known that an allergenic source may express different isoforms of the same allergen, but this has rarely been described for nsLTPs at the protein level. In some cases, nsLTPs or nsLTP isoforms showed the same allergenic potency [12, 13], whereas in others, e.g. green beans [14], both immunoreactivity and biological activity displayed different profiles.

So far, no data are available on the primary structure of Pun g nsLTPs, especially about their possible involvement in allergic disease. The results of this study will enable us to elucidate the presence of different nsLTPs and their contribution to the overall allergenic potency of pomegranate, and to evaluate the potential involvement of Pru p 3 in the development of food allergy to this fruit.

Materials and Methods

The source material for all the analyses carried out in this work consisted of the red fleshy part (i.e. the edible pulp) surrounding the seeds of commercial pomegranates.

cDNA Cloning of nsLTPs from Pomegranate

The pomegranate pulp tissue (400 mg) was finely ground in liquid nitrogen. Total RNA was extracted by the Spectrum™ Plant Total RNA kit (Sigma Aldrich, St. Louis, Mo., USA) and subjected to reverse transcription with the SuperScript III First-Strand Synthesis System (Life Technology, Paisley, UK) using the modified *Poly-T* primer previously employed [14]. The cDNA was amplified by PCR using different forward primers, designed on conserved sequence regions identified by the alignment of different nsLTPs sequences, among which Pru p 3 (Acc. No. AJ277163) and other homologous nsLTP cDNAs belonging to Rosaceae and other taxonomically distantly-related families (Fra a 3: DQ066727; Mal d 3: AJ277164; Pha v 3: GQ434008; Dau c 3: M64746; Pla a 3: AM286249; Art v 3: EU564847; Lac s 1: EF101532). In some cases, the primer carried degenerations (for a complete list of the primers employed, see online suppl. table S1; for all online suppl. material, see www.karger.com/doi/10.1159/000362761). *PNotI* was used as a reverse primer. On the basis of obtained 3'-cDNA region sequences, specific reverse primers were designed and used in combination with a hexamer primers mix (SMARTer™ RACE Kit; Clontech Laboratories, Mountain View, Calif., USA), in order to obtain the 5'-cDNA region of LTP genes. The mix assembly and amplification conditions were set following the manufacturer's instructions. By overlapping the partial sequences of the 3'-ends and the 5'-ends, the full cDNA sequences of three LTPs named LTP1A, LTP1B and LTP1C were deduced. For each sequence, specific terminal primers were designed to isolate the complete coding region (online suppl. table S1). The N-terminus of the mature protein was inferred by comparison with known nsLTP sequences using the Signal P 4.0 program (www.cbs.dtu.dk/services/SignalP-4.0/). All PCR products obtained were cloned in pGEM®-T Easy vector (Promega, Madison, Wisc., USA), and sequenced (BMR Genomics, Padova, Italy).

Extraction of Natural Pomegranate nsLTPs

Pomegranate pulp was squeezed and 1% (w/w) polyvinylpyrrolidone (Sigma) was added to the juice collected (final pH: 3.0). After 30 min of mixing, the juice (180 ml) was centrifuged at 4°C (9,000 g for 15 min) and filtered through Whatman paper. The obtained clarified juice was equilibrated in 20 mM sodium acetate buffer (pH 4.2) by chromatography onto PD10 columns (GE Healthcare Biosciences, Uppsala, Sweden) following the manufacturer's procedures. The eluate was fractionated by cation exchange chromatography (CEC) on an AKTA FPLC apparatus (GE Healthcare Biosciences) equipped with a Mono S 5/50 GL column (GE Healthcare Biosciences). The elution was performed in 20 mM sodium acetate buffer (pH 4.2) with a 0–0.25 M NaCl gradient (30 min) at a constant flow of 1 ml/min. The absorbance was monitored at 280 nm.

Protein Quantification

Protein concentration was measured by bicinchoninic acid assay (BCA assay, Pierce, Cheshire, UK) using bovine serum albumin as standard. The assay was performed on 96-well microplate (Sarstedt, Numbrecht, Germany) using a Powerwave reader (Bio-Tek, Winooski, Vt., USA). The absorbance was monitored at 562 nm.

Sodium Dodecyl Sulfate PAGE

Samples of pomegranate juice preparation (100 µl precipitated with cold acetone) were mixed with a 2× loading buffer [62.5 mM

Table 1. Clinical data of patients with type I food allergy to pomegranate

No.	Sex, M/F	Age, years	Symptoms to pomegranate	CAP Pru p 3 class	Food allergens ¹	SPT wheal diameter, mm		Aero-allergens ²
						SPT pomegranate	SPT peach	
1	F	31	A	4	a, l, n, pg, p	12 × 6	pos ³	Pl
2	F	24	OAS	2	c, f, gb, pg	12 × 8	n.d.	M, Ph
3	F	45	OAS	1	h, pg	5 × 5	n.d.	B, C, Pa
4	F	28	OAS	3	al, h, n, pg, pn	10 × 5	15 × 5	–
5	F	26	U, UE	2	P ⁴ , pg, h, n	6 × 5	5 × 4	–
6	F	43	OAS	2	a, ch, p, pg, s	6 × 8	14 × 7	D

CAP classes were defined as follows: 0: <0.35, 1: 0.35–0.7, 2: 0.7–3.5, 3: 3.5–17.5, 4: 17.50–50, 5: 50–100 and 6: >100 kU_A/l.

A = Anaphylaxis; n.d. = not determined; OAS = oral allergy syndrome; U = urticaria; UE = uvular edema.

¹ a = Apple; al = almond; c = carrot; ch = cherry; f = fig; gb = green bean; h = hazelnut; l = lettuce; n = nut; p = peach; pg = pomegranate; pn = peanut; s = shrimp.

² B = Birch pollen; C = hazelnut pollen; D = house-dust mites; M = mugwort pollen; Pa = pellitory pollen; Ph = timothy grass pollen; Pl = plane pollen.

³ SPT was positive, but the wheal diameter is not available.

⁴ For patient No. 5, symptoms to peach were limited to contact urticaria. She tolerated peach ingestion.

Tris-HCl pH 6.8, 1.3% (w/v) SDS and 10% (w/v) glycerol] and heated at 100°C for 5 min. Proteins were separated in 16% SDS-PAGE at 20 mA of current by means of a Mini Protean III apparatus (Bio-Rad, Hercules, Calif., USA) in 25 mM Tris-192 mM glycine, 0.1% SDS (pH 8.4) under non-reducing conditions.

Two-Dimensional Gel Electrophoresis

First Dimension

Acid-urea-PAGE (AU-PAGE) was performed by Mini Protean III (Bio-Rad) according to previously reported protocols [15–17] with some modifications. A 12% polyacrylamide running gel (acrylamide:bis acrylamide 29:1) was prepared incorporating 8 M urea and 5% acetic acid. For the polymerization of a minigel (10 ml) 50 µl TEMED (N,N,N',N'-tetramethylethylenediamine) and 37 µl ammonium persulfate were added. The stacking gel (5 ml), containing 8 M urea, was prepared at 8% polyacrylamide concentration by adding 50 µl TEMED and 22 µl ammonium persulfate (270-mg/ml stock). An overnight pre-run was carried out at 110 V in 5% acetic acid as running buffer. The buffer was discarded and substituted with a fresh one.

Samples of pomegranate juice preparation or CEC fractions (25 µg/cm slot) were diluted with 2 volumes of 8 M urea solution containing methyl green as tracking dye and loaded. The run was carried out at 130 V until the dye ran out of the gel.

Second Dimension

The lane of interest was cut with a razor blade and incubated in a Falcon tube for 10 min with 10 ml equilibrating buffer containing 0.2 M Tris-HCl, pH 6.8, 10% glycerol, 3% SDS and bromophenol blue. The solution was substituted with a fresh one and the lane was incubated for a further 5 min. The lane was finally loaded onto a 16% polyacrylamide SDS-PAGE gel and run at 20 mA of constant current as described above.

The gels were stained in Coomassie brilliant blue R250 (0.1% dye in 40% ethanol and 5% acetic acid) for 1 h and immediately

destained with a solution containing 40% methanol and 5% acetic acid. Alternatively, the gels were subjected to electroblotting as described below.

Patients

Six patients reporting adverse reactions after eating pomegranate were recruited at the University Hospital Dexeus of Barcelona (patient No. 1), the University Hospital of Verona (No. 2), the Hospital Carlo Poma of Asola (No. 3), the Clinical University of Navarra (Nos. 4 and 5) and the Hospital of Sagunto (No. 6) (table 1). Written informed consent was obtained from all patients. For patients Nos. 1, 2, 4 and 5, a formal approval from the local ethic committee was obtained. In the case of patients Nos. 3 and 6, the local ethical committee considered the written informed consent as sufficient since the interventions were based on routine clinical procedures, were not founded on the use of drugs and were without any additional cost for the hospitals. The patients underwent clinical investigation by recording a detailed case history and skin prick testing with a panel of commercial inhalant and plant food allergens (ALK-Abello, Madrid, Spain or Lofarma, Milano, Italy) performed following standard procedures [18] and by prick-to-prick with fresh pomegranate and peach fruits. Histamine dihydrochloride (10 mg/ml) and PBS/glycerol solution served as positive and negative controls, respectively. A mean wheal diameter of ≥3 mm (15 min after puncture) compared with that of the negative control was considered a positive response. Specific IgE for Pru p 3 (f420) was measured by ImmunoCAP™ (Thermo Fisher Scientific, Uppsala, Sweden). Values >0.35 kU_A/l were considered as positive. The serum from a healthy donor served as a negative control.

Immunoblotting

Proteins were electroblotted onto 0.45 µm PVDF membrane (Millipore, Billerica, Mass., USA) at 50 V for 150 min. The membrane was blocked with 3% dry defatted milk, 0.05% Tween-20 in

PBS and probed with a rabbit anti-Pru p 3 polyclonal antibody [1] at 1:5,000 dilution for 2 h or overnight with patients' sera at 1:10 dilution (for IgE-immunoblotting inhibition, 10 µg of pooled Pun g nsLTPs or Pru p 3 was incubated with patients' sera). Rabbit IgG was detected with alkaline phosphatase-conjugated anti-rabbit IgG antibody (1:5,000, Sigma). Enzymatic staining was achieved by BCIP/NBT solution (Bio-Rad). Human IgE was detected by monoclonal HRP-conjugated anti-human IgE antibody (Southern Biotech, Birmingham, Ala., USA) at 1:1,500 dilution, followed by a chemiluminescence reaction with Immobilon™ Western HRP substrate (Millipore). In this case, a ChemiDoc XRS apparatus was used for image acquisition (Bio-Rad).

Tryptic Digestion of Purified Pomegranate LTPs

The proteins from the CEC fractions (25 µg each) were lyophilized and subsequently dissolved in 25 µl of 0.1 M Tris-HCl (pH 8.0). They were then treated following a reversed approach with respect to the more common protocols, i.e. digesting the proteins before reduction and alkylation. In our hands, this gave the best results in terms of sequence coverage. Tryptic digestion was performed by adding 0.4 µl of trypsin gold (Promega) from a 200-ng/µl stock. The mixture was kept at 37°C for 15 h. A volume of 1.4 µl dithiothreitol (Sigma) from a 0.2-M solution was added and incubated for 1 h at room temperature. Subsequently, 5.6 µl iodoacetamide (Sigma) from a 0.2-M solution was added. After 1 h of incubation in the dark, residual free iodoacetamide molecules were blocked by the addition of an equimolar amount of dithiothreitol. Reduced and alkylated peptides were subjected to Ziptip cleaning (Millipore) following the manufacturer's protocol, in order to remove salts, and were analyzed by mass spectrometry (MS) as described below.

Peptide Sequencing by Nano-High-Performance Liquid Chromatography Chip Tandem MS

Peptides from 5 µl of each sample treated with trypsin were separated by reversed-phase nano-high-performance liquid chromatography (HPLC) chip technology (Agilent Technologies, Palo Alto, Calif., USA) online-coupled with a 3-D ion trap mass spectrometer (model Esquire 6000, Bruker Daltonics, Bremen, Germany). The chip was composed of a Zorbax 300SB-C18 (150 mm × 75 µm, with a 5-µm particle size) analytical column and a Zorbax 300SB-C18 (40 nl, 5 µm) enrichment column. The complete system was fully controlled by ChemStation (Agilent Technologies, Santa Clara, Calif., USA) and Esquire Control (Bruker Daltonics, Billerica, Mass., USA) softwares. The scan range used was 300–1,800 m/z. For tandem MS (MS/MS) experiments, the system was operated with automatic switching between MS and MS/MS modes. The three most abundant peptides of each m/z were selected to be further isolated and fragmented. The MS/MS scanning was performed in the normal resolution mode at a scan rate of 13,000 m/z per second. A total of five scans was averaged to obtain an MS/MS spectrum.

Database Searching

An ad hoc database was created by generating a FASTA file containing all sequences present in the entire SwissProt database integrated with the sequences of the cloned cDNAs. The resulting database contained 518,836 protein sequences. The FASTA file was parsed into the software Mascot 2.3.0 (Matrix Science Ltd., London, UK). The searches were carried out with Mascot version

2.3.0 allowing a tolerance of 100 ppm for mass data and 0.6 Da for the MS/MS data. The modifications considered were cysteine carbamido methylation, asparagine and glutamine deamidation and methionine oxidation. Since the proteins were digested before the reduction and alkylation steps, a condition in which the activity of trypsin is hindered by the incomplete protein denaturation, we considered results obtained by up to 3 missed cleavages. We considered as statistically significant those identifications with Mascot scores [defined as $-10 \times \log(P)$, where P is the probability that the observed match is a random event] over a threshold level ($p < 0.05$).

Results

The Main Symptom in Patients Allergic to Pomegranate Is Oral Allergy Syndrome

Six female pomegranate allergic patients were recruited (table 1). The most frequently referred symptom (4/6 patients) upon ingestion of pomegranate fruits was oral allergy syndrome. In case of patient No. 1, severe systemic reactions were reported. Sensitization to pomegranate was confirmed by skin prick testing. Although all 6 patients displayed positive CAP value to Pru p 3, 4 of them tolerated peach ingestion (Nos. 2–5), which suggests that peach and pomegranate food allergy are not strictly clinically associated. In one case (No. 5), manipulation of peach peel induced contact urticaria.

Identification of Three nsLTP cDNAs Expressed in Pomegranate Pulp

PCR reactions were carried out on cDNA obtained from pomegranate pulp, using 20 combinations of forward primers (online suppl. table S1) and *Poly-T* primer to isolate the 3'-region of LTP transcripts. Only Prupha1, Artpla1, Prufra2 and GB1 primers gave amplification products of a length and sequence consistent with LTP1-like genes. The 3'-ends obtained were used for 5'-RACE PCR primer design. By overlapping the 3'- and 5'-sequences, it was possible to identify 3 cDNAs homologous to nsLTP family members (fig. 1a). LTP1A (GenBank ID: KC550307) and LTP1B cDNA (GenBank ID: KC550306) are 363 bp, coding for mature 93 amino acid (aa) proteins with predicted leader sequences of 27 amino acids. LTP1C cDNA (GenBank ID: KC550305) comprises 363 bp, coding for a mature 94 aa protein and a predicted leader sequence of 26 aa (fig. 1a). The amino acid sequence identity degree among the mature pomegranate LTPs (Pun g-LTP1) is 66–68% (fig. 1b). The degree of identity of the three Pun g-LTP1s with Pru p 3 ranges from 62 to 65%. The sequence data were used to imple-

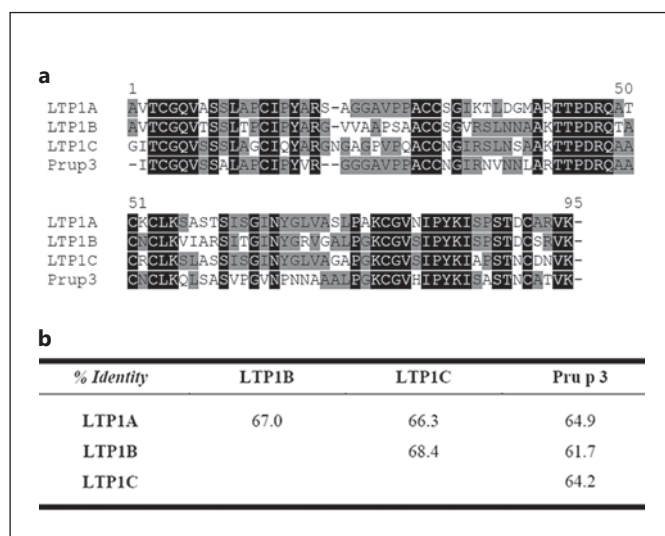


Fig. 1. a Alignment of the deduced mature protein sequences encoded by LTP1A, LTP1B and LTP1C cDNAs in comparison with Pru p 3. Identical amino acids are shown in black and highly conserved amino acids are shown in gray. **b** Degree of amino acid sequence identity of the cloned pomegranate LTP1 sequences between isoforms and in comparison with Pru p 3.

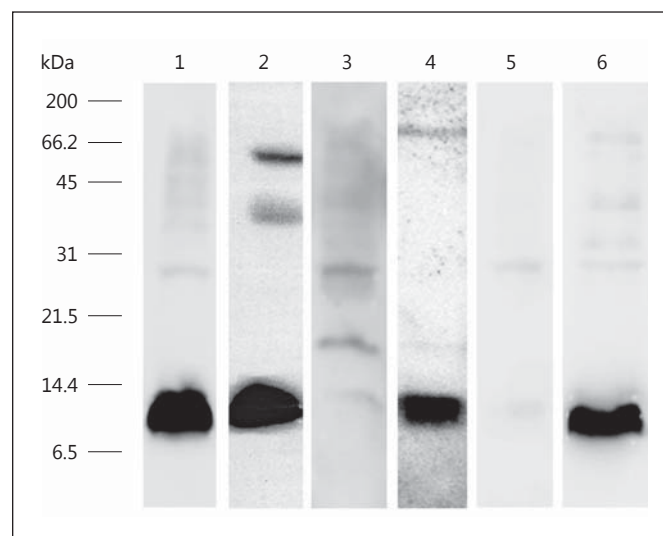


Fig. 2. SDS-PAGE and IgE-immunoblotting of pomegranate proteins. Pellets obtained by acetone precipitation of pomegranate juice preparation were separated by SDS-PAGE under non-reducing conditions and assayed by immunoblotting separately with the sera of 6 patients (lanes 1–6).

ment a SwissProt database (containing more than 500,000 sequences) to be employed for MS analysis (see below).

The molecular mass of the protein sequences was calculated and compared to the mass of two intact proteins purified in a previous work [1]. Despite a difference of 7–8 Da, the results obtained for LTP1A (9,349 Da) and LTP1B (9,475 Da) were very close to the values obtained previously (9,342 Da for LTP1a and 9,467 Da for LTP1b). The difference observed might be due to post-translational modifications, already described for nsLTPs, which can slightly modify the molecular mass of the proteins. For instance, very recently, Larocca et al. [19] demonstrated that Pru p 3 is naturally methylated.

Pomegranate Fruit Contains Multiple nsLTPs

The juice obtained by squeezing pomegranate pulp was analyzed first by SDS-PAGE (one-dimensional, 1-D) and IgE-immunoblotting and subsequently by a two-dimensional (2-D) approach more suitable for small and basic proteins. Figure 2 shows the IgE-immunoblotting after the 1-D separation of pomegranate proteins carried out with the patients' sera. Four sera (i.e. Nos. 1, 2, 4 and 6) strongly reacted with a 10–12-kDa band, and were thus compatible with nsLTPs, while serum No. 3 reacted with marked less intensity. Further protein bands were immunodetected at higher molecular masses

but with less frequency and intensity; the 29-kDa band has been already reported [1, 20]. Figure 3a shows that different proteins of approximately 8–14 kDa were separated by 2-D gel electrophoresis (2-DE). To verify whether these proteins belong to the nsLTP family, immunoblotting using anti-Pru p 3 polyclonal antibodies was carried out. The obtained immunodetection (fig. 3b) showed at least 3 proteins (spot Nos. 1, 3 and 4) sharing common epitopes with Pru p 3 (no signal was detected using the preimmune rabbit serum, not shown). The same analysis performed with the sera of patients (Nos. 1–6) allergic to pomegranate produced different IgE-binding patterns (fig. 3d–i). Five out of 6 patients' sera reacted to at least 1 spot representing the LTPs, but differences in immunodetection were observed. In general, 3 patients were apparently monosensitized to only one low-molecular mass protein (fig. 3e, f, i) while 2 were polysensitized to 4 spots potentially representing nsLTPs (fig. 3d, g). In addition to the detection with anti-Pru p 3, a further spot, i.e. No. 2, was recognized by 4/6 patients' sera. Specificity of immunodetection was shown by using serum from a healthy donor (fig. 3c). In order to gain more information on the identity of the immunoreactive proteins and to evaluate whether Pru p 3 cross-reacts with pomegranate LTPs, a second series of 2-D immunoblots with Pru p 3 as an inhibitor was car-

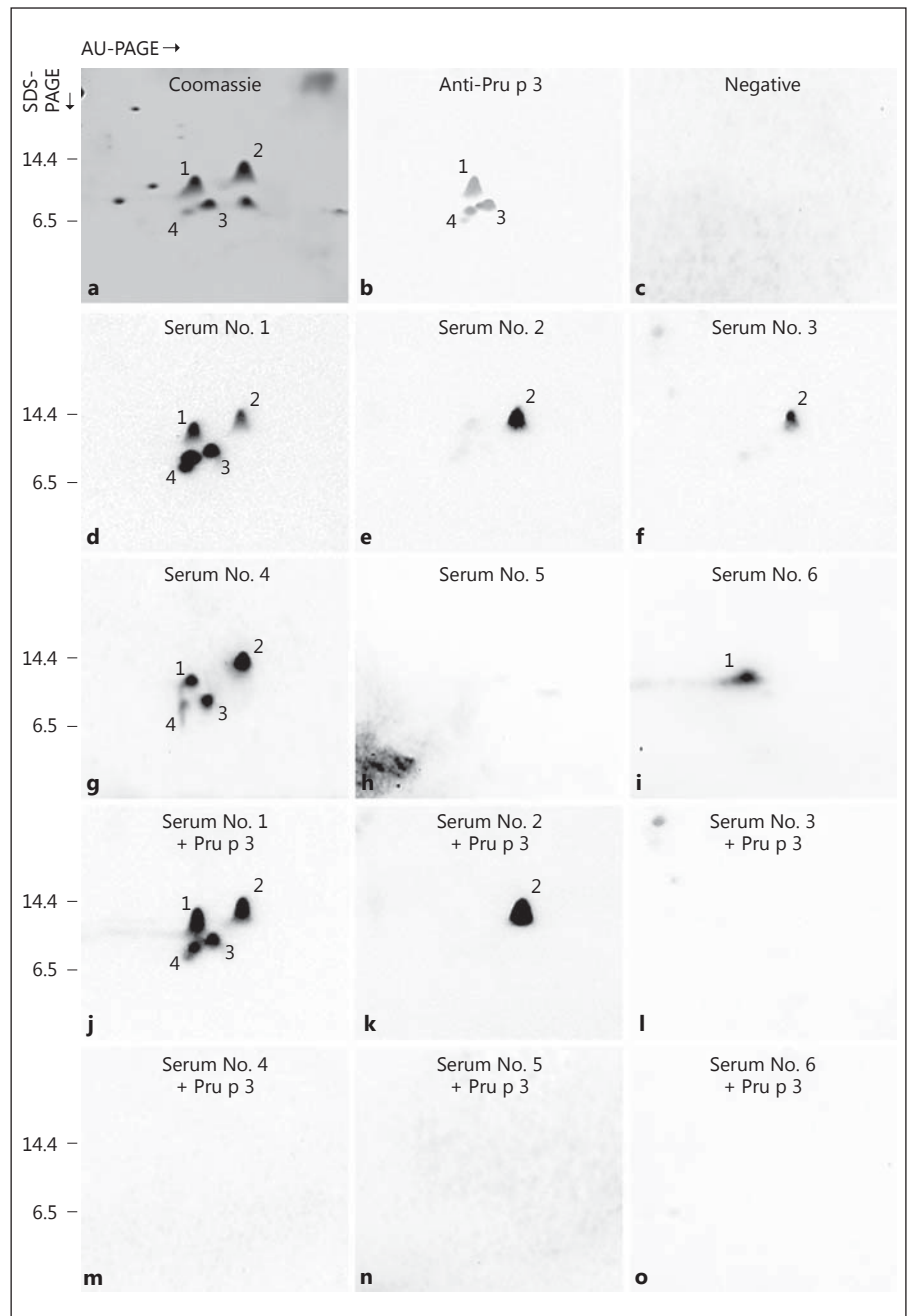


Fig. 3. Analysis of pomegranate juice proteins by 2-DE. Coomassie staining (**a**), immunoblotting developed with anti-Pru p 3 polyclonal antibody (**b**) and human sera (**c–o**). **c** Immunoblotting developed with the serum of the healthy donor. **j–o** Immunoblotting was developed with the same sera as for **d–i**, coincubated with Pru p 3 as an inhibitor.

ried out. The results (fig. 3j–o) showed that for the sera from 2 patients, the IgE-binding was not inhibited by the coincubation with Pru p 3 (fig. 3j, k), demonstrating that different epitopes are involved, while for other patients (fig. 3l, m, o), Pru p 3 totally inhibited the antibody binding. As in figure 2, the serum of patient No. 5 (fig. 3h, n) did not show any reactivity towards potential pomegranate nsLTPs despite a CAP class of 2 to Pru p 3. We suggest that this patient probably expressed very low

concentrations of pomegranate-specific IgE, even if these seemed to be sufficient to trigger allergic reactions. To verify the specificity of the IgE-binding to pomegranate proteins for patient No. 1, we performed a Pun g nsLTP self-inhibition experiment by coincubating a cocktail of the purified pomegranate nsLTPs with the serum. The addition of Pun g LTPs totally abolished IgE-binding (fig. 4), confirming that the epitopes involved were substantially different from those of Pru p 3.

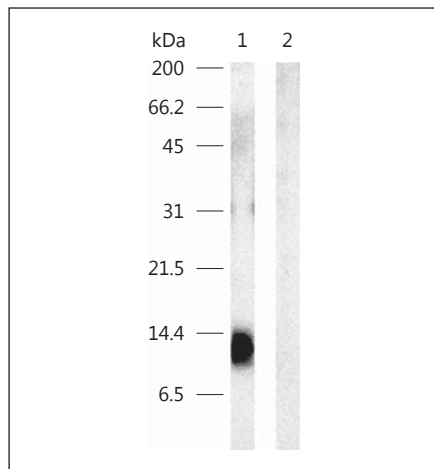


Fig. 4. SDS-PAGE under non-reducing conditions and IgE-immunoblotting of pomegranate proteins (lane 1) carried out with serum No. 1. The inhibition of IgE-binding was performed coincubating the serum with pooled Pun g nsLTPs (lane 2).

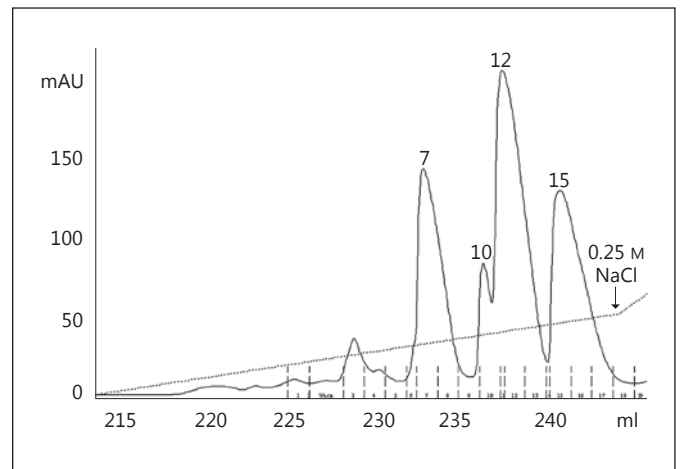


Fig. 5. CEC chromatogram of pomegranate juice proteins. Absorbance was read at 280 nm (solid curve). The dotted line represents the buffer B concentration.

Table 2. Mass spectrometry results (MS/MS) of the purified Pun g nsLTPs

Fraction	Name	Mascot score	Coverage	Sequence
7	LTP1C	240	87%	GITCGQVSSSLAGCIQYARGNGAGPVPQACCNGIRSLNSAAK TPDRQAACRCLKS-LASSISGINYGLVAGAPGKCGVSIPYKIAPSTNCDNVK
10	LTP1A	154	60%	AVTCGQVASSLAPCIPYARSAGGAVPPACCSGIKTLDGMA RTTPDRQATCKCLK-SASTSISGINYGLVASLPAKCGVNIPYKISPSTD CARVK
10	LTP1C	245	58%	GITCGQVSSSLAGCIQYARGNGAGPVPQACCNGIRSLNSAAK TPDRQAACRCLK-SLASSISGINYGLVAGAPGKCGVSIPYKIAPSTNCDNVK
12	LTP1A	150	49%	AVTCGQVASSLAPCIPYARSAGGAVPPACCSGIKTLDGMA RTTPDRQATCKCLK-SASTSISGINYGLVASLPAKCGVNIPYKISPSTD CARVK
15	LTP1B	124	39%	AVTCGQV TSSLT PCIPYARGVVAAPSAACCSGVRSLN NAK TPDRQTACNCLKVI-ARSITGINYGRVGALPGKCGVSIPYKISP STD CSRVK

The amino acid sequences covered by the identified peptides are in bold type.

Taken together, these results highlight the diversity of the sensitization profile of the patients.

nsLTPs from Pomegranate Consist of Different IgE-Binding Proteins

To confirm the identity of the immunodetected spots as pomegranate nsLTPs, the fruit proteins were purified by CEC (fig. 5). The fractions corresponding to the major peaks (Nos. 7, 10, 12 and 15) were separated by 2-DE in order to check their purity. Figure 6 shows the results of

the 2-D analysis of the fractions. Three (Nos. 1–3) out of four proteins were successfully isolated in fractions 12, 15 and 7, respectively, while the latter (No. 4 in fraction 10) still contained other proteins. Further attempts to purify the unresolved proteins by reverse-phase HPLC did not lead to better results (data not shown). A lane representing a pomegranate juice SDS-PAGE profile (1-D) is also included in figure 6 in order to show the strong resolution of low-molecular-mass proteins obtained by 2-DE in comparison to a monodimensional SDS-PAGE separation.

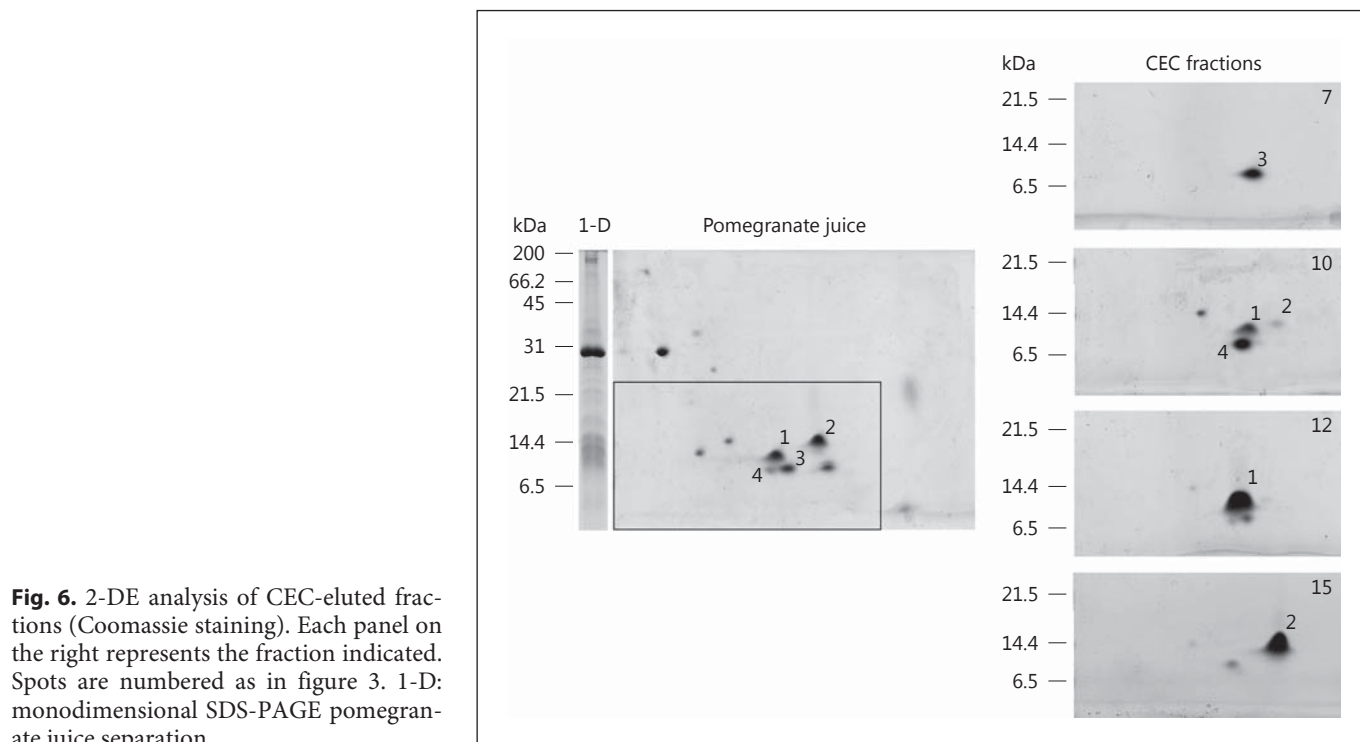


Fig. 6. 2-DE analysis of CEC-eluted fractions (Coomassie staining). Each panel on the right represents the fraction indicated. Spots are numbered as in figure 3. 1-D: monodimensional SDS-PAGE pomegranate juice separation.

Fractionated proteins underwent tryptic digestion. The peptides obtained were analyzed by HPLC-MS/MS. Mascot scores resulting from the interpretation of the spectra were well above the statistical significance (table 2), allowing the identification of the purified proteins as nsLTPs with a good sequence coverage (39–87%). In particular, fraction 7 contained peptide belonging to Pun g-LTP1C, and due to the high sequence coverage (87%), we reasonably suggest that the identified protein was actually corresponding to the cloned sequence. Fractions 12 and 15 contained specific peptides derived from Pun g-LTP1A and Pun g-LTP1B, respectively, but with a lower degree of sequence coverage. Interestingly, peptides homologous to the N-terminus of the cloned sequences were detected. This indicates that the start of the mature protein was correctly predicted. Fraction 10, as expected, contained peptides deriving from different Pun g-LTPs, making the identification of IgE-binding protein(s) represented by spot No. 4 impossible.

Discussion

The involvement of nsLTPs in pomegranate allergy has already been proven [1, 2]. In order to analyze the expression of nsLTPs and, in particular, of possible iso-

forms characterized by different IgE-binding properties possibly contributing to different allergenic potency, a new proteomic approach was necessary. The small molecular mass (<10 kDa) and the extremely basic isoelectric point (pI >9) of these proteins make their proteomic analysis particularly difficult due to their possible comigration or scarce focalization during isoelectrofocusing [21]. The use of a non-equilibrium electrophoretic approach, such as the AU-PAGE, helped to overcome this problem. This technique has been successfully applied to histones, which share the characteristics of small mass and high isoelectric points with nsLTPs [15–17]. The results obtained by combining AU-PAGE with SDS-PAGE showed that the general scenario of pomegranate nsLTPs is even more complex than expected. In fact, from the immunoblotting performed after 2-DE, several proteins were recognized by polyclonal antibodies and the patients' sera. In order to confirm that the spots were truly nsLTPs, we employed MS. However, to obtain statistically significant results ($p < 0.05$), a further step was necessary, i.e. the cloning of nsLTP sequences in order to gain their deduced amino acid sequence for incorporation in the database. Indeed, performing MS analyses without the knowledge of the sequence would have been extremely cumbersome, leading to inconsistent results. Hence, the rationale of our approach was to exploit the sequence in-

formation deriving from the cloned Pun g-LTP cDNAs in order to identify the nsLTPs expressed at the protein level.

In a first set of experiments (data not shown) the spots obtained by 2-DE separation giving a positive signal on immunoblotting were processed and analyzed by HPLC-MS/MS. Although some peptides were recognized as belonging to nsLTPs, the overall Mascot score was below the statistical significance even when the MS spectra were analyzed with the updated database. Since the reason for this missing identification is still unknown (possibly the low recovery of peptides from the gel or the occurrence of unpredictable modifications of some amino acid residues), we tried to overcome the problem by purifying the proteins recognized by immunoblotting and analyzing their 'in-solution' digestion products by MS. The results demonstrated that the target proteins (table 2) are actually nsLTP1s. Since the sequence identity was 66–68% (fig. 1b) (i.e. very close to 67%, the threshold set by Chapman et al. [22] to define allergen isoforms), the three pomegranate nsLTPs here identified are included in the official database of the International Union of Immunological Societies as isoallergens named Pun g 1.0101, Pun g 1.0201 and Pun g 1.0301. Interestingly, the LTP1 corresponding to spot No. 2, highly homologous to Pun g-LTP1B (Pun g 1.0201), was bound by the majority of patients' IgE (4/5 patients displaying Pun g LTP-specific IgE), thus representing a potential target for a future component-resolved diagnosis. The other Pun g-LTP1A (Pun g 1.0101) and Pun g-LTP1C (Pun g 1.0301) represented by spot Nos. 1 and 3, were recognized by 3/5 and 2/5 patients' sera respectively, while spot No. 4, still not assigned to any cloned Pun g-LTP1, was detected by 2/5 patients' sera.

It is generally accepted that Pru p 3 acts as the main sensitizer in the development of the clinical picture known as LTP syndrome [9]. In most of the cases, patients allergic to nsLTPs are sensitized to Pru p 3 and, due to the high degree of cross-reactivity, the IgE specific to peach nsLTP may mediate the onset of symptoms upon ingestion of other plant nsLTPs, even when taxonomically distant [23, 24]. In this study, 2-D spots were immunodetected by anti-Pru p 3 antibodies, and for 3 patients (Nos. 3, 4 and 6) Pru p 3 cross-reacted with pomegranate LTPs (as demonstrated by inhibition showed in fig. 3l, m, o), thus indicating epitope homology between Pru p 3 and Pun g-LTPs. Nevertheless, the sensitization to peach nsLTP seems to not be a strict prerequisite for the elicitation of symptoms to pomegranate. In fact, in spite of the positive ImmunoCAP to Pru p 3 for all patients' sera, food allergy upon ingestion of peach was referred only by pa-

tient Nos. 1 and 6 (No. 5 suffered from urticaria upon peach manipulation, but no symptoms were aroused after peach ingestion). Furthermore, the immunoblotting inhibition of sera from patient Nos. 1 and 2 performed with Pru p 3 did not show evident modifications of the 2-D immunodetection pattern, demonstrating that the epitopes involved did not cross-react. Interestingly, the spot recognized by the serum No. 2 was not detected by anti-Pru p 3 polyclonal antibodies.

However, patient No. 1 is the most remarkable. In fact, despite a CAP class of 4 to Pru p 3 and a clinical history characterized by anaphylactic reactions to peach, Pru p 3 was found to not cross-react with Pun g LTP. In order to study the specificity of the IgE-binding to pomegranate, we decided to perform a further experiment, i.e. a Pun g nsLTP self-inhibition by coincubating a cocktail of the purified pomegranate nsLTPs with the serum. The addition of Pun g LTPs totally abolished IgE-binding (fig. 4), confirming that the epitopes involved differ from those harbored on Pru p 3.

It remains unknown whether the elicitation of the allergic symptoms to pomegranate is caused by a direct priming of the fruit nsLTPs or by cross-reaction with other food or pollen nsLTPs that might be involved in the primary sensitization. However, because the number of patients recruited for this study was limited, it is impossible to draw general conclusions.

In summary, we demonstrated that the use of AU-PAGE combined with SDS-PAGE can be considered a valid approach for the separation of nsLTPs. We showed that pomegranate expresses different nsLTP isoforms characterized by different immunological properties related to specific epitopes.

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Supplementary material - Table S1. Primers used for Pun g LTP-cDNA cloning. Primer 1 was used for RT-PCR, primers 2-22 for 3'end cloning, primers 23 and 24 for 5'RACE, and primers 25-30 for cloning of the full-length cDNAs.

	Name	Sequence	T _m (C°)
1	Poly-T	5'- AACTGGAAGAATTCGCGGCCGCAGGAATTTTTTTTTTTTTTTTTT -3'	
2	PNot1	5'- AACTGGAAGAATTCGCGGCCG -3'	66
3	Prufra1	5'- GCTTGCTGCAAMGGMRTTAG -3'	61
4	Prufra2	5'- GACCACCSCTGACCGCCAG -3'	65
5	Prufra3	5'- CTTCCCGGCAAGTGTGG -3'	56
6	Prufra4	5'- CAAGATYAGCRCCTCCACCAACTGC	78
7	Prumal1	5'- CCAGCAGCCTTGACCATGC -3'	66
8	Prumal2	5'- CCTCCAGCTTGCTGCAAYGG -3'	65
9	Prumal3	5'- CTGACCGCCAGRCYGCTTGCAACTGC -3'	86
10	Prumal4	5'- GGWGTYAACCCTAACAATGCMGCAG -3'	77
11	Prumal5	5'- CCTCCACCAACTGCGCCACCG -3'	72
12	Prupha1	5'- ACCCCWGACCGCCAGGC -3'	61
13	Prurice1	5'- ACCACCSCYGACCGCCAGGC -3'	70
14	Dauc1	5'- TGTGGTGTCAACATTCC -3'	50
15	Prup1	5'- TGTGGAGTTAGTATTCCTTAC -3'	58

16	Artpla1	5'- AAACSACCCCWGACCGYC -3'	59
17	Artpla2	5'- TGAYTGCWMCAAGGTGAAGT -3'	60
18	Artlac1	5'- ACRACYCCTGAYCGYCAGAC -3'	64
19	Artlac2	5'- TGATTGCWMCAAGGTGMAGT -3'	59
20	Plalac1	5'- AACMACYCCWGAYCGTCAG -3'	57
21	Plalac2	5'- AAYMTCCCTTACAAGATCAGYCC -3'	67
22	GB1	5'- GACCACCCCWGACCGC -3'	57
23	GSP1	5'- GARGGGCTGATCTTGTATGG -3'	61
24	GSP4	5'- AGAAGGGGCGATCTTATAAGGG -3'	66
25	PGLTP1AFL_ fow	5'- CCCATTGCCACCGAAGCCGC -3'	68
26	PGLTP1AFL_ rev	5'- GCGGATCCTCATCACTTGACTCTAGCGCAGTCAGTG -3'	74
27	PGLTP1BFL_ for	5'- GGAATTCCATATGGCCGTCACGTGCGGCCAGGT -3'	70
28	PGLTP1BFL_ rev	5'- GCGGATCCTCATCACTTTACTCTGCTGCAGTCAGT -3'	78
29	PGLTP1CFL_ for	5'-GGAATTCCATATGGGCATAACGTGCGGCCAGGTG -3'	70
30	PGLTP1CFL_ rev	5'- GCGGATCCTCATCACTTCACGTTGTCACAGTTGGT -3'	70

Tm: melting temperature.