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Isolation and Identification of Two Lipid Transfer Proteins in Pomegranate (*Punica granatum*)

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Lipid transfer proteins (LTPs) are a family of low molecular mass (7–9 kDa) polypeptides, the members of which share 35–95% sequence homology. These proteins are widely distributed throughout the plant kingdom and are receiving attention for their biochemical characteristics and biological activity. LTPs are indeed studied in different research fields varying from allergy to food technology, and numerous molecules belonging to this class are progressively being identified and investigated. Proteins from pomegranate juice were fractioned by cation exchange chromatography and analyzed by SDS-PAGE. Two proteins were identified as putative LTPs on the basis of their molecular weights and their electrophoretic behaviors under reducing and nonreducing conditions. Finally, proteins were purified and characterized by mass spectrometry. This analysis confirmed that the two polypeptides are LTPs on the basis of an amino acid sequence common to LTPs from other plant sources and cysteine content. The two proteins, named LTP1a and LTP1b, showed similar molecular masses but different immunological profiles when immunodetected with rabbit antibodies specific for Pru p 3 and human IgE from a patient suffering from pomegranate allergy. The demonstration of the existence of two immunologically unrelated LTPs in pomegranate confirms the variability and the complexity of the plant LTP family. This should be taken into account when the role of these proteins as elicitors of allergies to fruits is investigated and could help to explain the contradictory literature data on pomegranate allergy.

KEYWORDS: Pomegranate (*Punica granatum*); LTP; immunoblotting; allergy; mass spectrometry

INTRODUCTION

Lipid transfer proteins (LTPs) are polypeptides ubiquitously expressed in the plant kingdom (*1*). They are subdivided into two main groups, LTP1 (≈9 kDa) and LTP2 (7 kDa). For their biochemical properties and biological activities LTP1s have attracted the interest of scientists devoted to various research areas such as plant physiology/pathology, food technology, and finally health science. For example, LTPs from barley malt, thanks to a marked amphiphilicity, are involved in the formation of beer foam (*2, 3*). As LTPs seem to possess antimicrobial activity against bacteria and fungi, and their expression is modulated by biotic and abiotic stimuli, they are now considered to be plant pathogenesis related proteins (PR-14) (*4, 5*). The binding properties of LTPs have been explored in pharmacology as a means to deliver drugs (*6, 7*). Many active agents used in pharmaceutics and cosmetics need a protective shield when they reach aggressive environments, and LTPs appear to provide it, being resistant to enteric proteolysis (*8–10*). However, resistance to enteric digestion is often associated with protein allergenicity. Such a characteristic, together with the observation that allergy to *Rosaceae* fruits may be acquired without any pollen sensitization, especially in the Mediterranean area, suggests that sensitization to LTPs, particularly abundant in these fruits, may occur by the oral route (*8, 10, 11*), reviewed in refs *12–15*.

LTPs are now considered to be panallergens causing severe systemic reactions to ingested fruits as many LTPs from evolutionarily related and unrelated plant species appear to share common allergenic epitopes (*8, 16*). For all of these reasons

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LTPs are increasingly studied and their characterization and identification in plants and fruits are the subject of numerous studies.

Pomegranate (*Punica granatum*), a plant of the *Punicaceae* family, endemic to the Middle East and India, has been used for centuries in popular medicine. Pomegranate fruits are commonly consumed in the Mediterranean areas in raw and processed forms such as juice, wines, flavors, and extracts, but they have rarely been reported to cause immediate hypersensitivity after ingestion (*17–22*). The limited number of cases obscures any conclusion on the possible presence and identification of common allergen(s) based on a solid statistical ground. Recently, however, a case of anaphylactic reaction to this fruit has been described (*22*). The presence of serum IgE specific to pomegranate was investigated with an immunoblotting technique that detected two proteins reacting with the patient's serum and that banded at 29 and 9 kDa. The latter molecular mass is highly suggestive that LTP family members could be involved also in this case.

Here, we show that two 9 kDa proteins can be isolated from pomegranate, and we demonstrate that they both belong to the LTP1 family. Notably, the two LTP isoforms have different immunological profiles, as shown by immunodetection with rabbit IgGs and IgEs from a pomegranate allergic patient.

MATERIALS AND METHODS

Protein Extraction and Purification. *Pomegranate.* The red gelatinous flesh from several pomegranate fruits was squeezed in the presence of 1% (w/w) polyvinylpolypyrrolidone (PVPP; Sigma, Milano, Italy). The juice was clarified by centrifugation at 4 °C (9000*g* for 15 min) and filtered through Whatman paper.

The obtained clarified juice (50 mL) 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 colorless PD10 eluate (corresponding to approximately 15 mg of protein) was then fractionated by cation exchange chromatography (CEC) with an AKTA FPLC apparatus (GE Healthcare Biosciences) on 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 0–0.35 M NaCl gradient (35 min) at a constant flow of 1 mL/min. The absorbance was monitored at 280 nm.

Peach. Peel from nectarine peaches was frozen in liquid nitrogen, finely crushed with a mortar and a pestle, and extracted in PBS (10 mM phosphate buffer saline, pH 7.2, 150 mM NaCl) 1:3 w/v, with the addition of 1% PVPP for 2 h at 4 °C. The obtained extract was clarified by centrifugation at 4 °C (9000*g* for 15 min) and filtered through Whatman paper.

Protein Determination. Protein concentration was determined in microtiter plates (Sarstedt, Numbrecht, Germany) by bicinchoninic acid assay (Pierce, Milano, Italy) following the manufacturer's instructions, with bovine serum albumin as standard protein. Absorbance was monitored by means of a PowerWave microtiter plate reader (Bio-Tek Instruments, Winooski, VT).

SDS-PAGE. Samples from pomegranate juice before the P10 equilibration (obtained by the precipitation of 100 μL of extract with 4 volumes of cold acetone, as in ref *23*), peach extract, and CEC fractions were dissolved in loading buffer [62.5 mM Tris-HCl pH 6.8, 1.3% (w/v) SDS, and 10% (w/v) glycerol)] and proteins separated in 16% polyacrylamide tricine SDS-PAGE (*24*) by means of a Mini Protean 3 apparatus (Bio-Rad, Milano, Italy) under reducing (with 4% 2-mercaptoethanol added) or nonreducing conditions. The gels were stained with Coomassie R250.

IgG Immunoblotting. Proteins separated by SDS-PAGE were electroblotted onto PVDF membranes (Millipore, Milano, Italy) at constant voltage (50 V for 150 min) and immunodetected with anti-peach LTP1 polyclonal antibodies (*25*), diluted 1:5000 in blocking solution (3% w/v of skimmed dry milk and 0.05% Tween-20 in PBS) for 2 h. After washings, an alkaline phosphatase-conjugated anti-rabbit IgG (clone RG-96, Sigma) was added at a dilution of 1:5000. Sigma-Fast NBT/BCIP (Sigma) substrate was used to detect bound IgGs.

IgE Immunoblotting. A serum sample was collected from a patient presenting oral allergic syndrome after eating pomegranate and highly positive to pomegranate juice by prick-by-prick test (*26*).

The IgE immunoblotting was performed on proteins transferred onto PVDF membranes as described above. The membranes were incubated with the patient serum diluted 1:5 in blocking solution overnight. Bound IgE were detected using a mouse monoclonal horseradish peroxidase-conjugated anti-human IgE antibody (Southern Biotech) at a dilution of 1:2000. Supersignal Ultra chemiluminescent substrate (Pierce) and a Chemi-Doc XRS acquiring apparatus (Bio-Rad) were utilized to detect bound antibodies.

Mass Spectrometry on Intact Proteins. Electrospray ionization mass spectrometry (ESI/MS) was performed by a Micromass ZMD single-quadrupole mass spectrometer. The solutions containing the separated proteins were further purified by means of a Ziptip pipet (Millipore), dissolved in a water/acetonitrile 1:1 mixture (added of 0.2% of formic acid), at a final protein concentration of 200 μg/mL, and then directly perfused into the ESI source at 10 μL/min rate. ESI/MS conditions were as follows: positive ion mode,; capillary voltage, 3.3 kV; cone voltage, 30 V; source temperature, 100 °C; desolvation temperature, 150 °C; cone gas (N_2), 100 L/h; desolvation gas (N_2), 400 L/h; scan range, *m*/*z* 200–2000; scan time, 4 s.

Mass Spectrometry on Reduced and Alkylated Proteins. One hundred micrograms of proteins for each CEC fraction was lyophilized and subsequently dissolved in a buffer containing 8 M urea and 0.1 M Tris-HCl (pH 8). Dithiothreitol (DTT, Sigma), was added from a 0.5 M stock to a 10 mM final concentration with incubation for 1 h at room temperature. Then iodoacetamide (Sigma, from a 0.5 M stock) was added to a 50 mM final concentration. After 1 h of incubation, residual free iodoacetamide molecules were blocked by the addition of an amount of DTT equal to that of alkylant previously added.

Alkylated proteins were purified by Ziptip pipet (Millipore) and analyzed by mass spectrometry as described above.

HPLC-MS of the Tryptic Digest. Tryptic digest was performed by mixing 10 μL of protein solution (5 μg/μL) with 100 μL of a 50 mM ammonium bicarbonate buffer (pH 7.8) and 2 μL of a 1 μg/μL solution of Trypsin Gold (Promega Corp., Milano, Italy) dissolved in 50 mM aqueous acetic acid. The mixture was kept at 37 °C for 24 h, and then the digestion was stopped by freezing. The protein digests were subjected to reduction of disulfide bonds and alkylation as described in the previous paragraph. The tryptic mixtures were finally analyzed by reversed phase HPLC interfaced with an ESI mass spectrometer by using a C18 column (250 × 4.6 mm, 5 μm, injection of 40 μL). Eluent A was water (0.2% acetonitrile and 0.1% formic acid), and eluent B was acetonitrile (0.2% water and 0.1% formic acid). The eluent program was as follows: 0–5 min, isocratic 100% A; 5–35 min, linear gradient from 100% A to 60% A; 35–45 min, isocratic 60% A. The flow rate was 1 mL/min. By using a T valve, only 10% of the incoming flow was directed into the ESI source. ESI/MS conditions were as follows: positive ion mode; capillary voltage, 3.3 kV; cone voltage, 30 V; source temperature, 100 °C; desolvation temperature, 150 °C; cone gas (N_2), 100 L/h; desolvation gas (N_2), 400 L/h; scan range, *m*/*z* 150–1900; scan time, 3 s.

Database Searching. Peptide sequence searching was performed in the Swiss-Prot database. Computation was performed by using the BLAST network service maintained by the Swiss Institute of Bioinformatics. Search parameters included taxonomic group Viridiplantae, expectation value (*E*) threshold 1000, gapped alignment OFF, identity BLAST ON. The other options were set as default values.

RESULTS

Electrophoretic Analyses and IgG Immunoblotting. The electrophoretic profile of pomegranate juice is shown in **Figure 1**. To identify LTPs we have used rabbit anti-peach LTP1 polyclonal antibodies. The rationale for this experiment is based on the described antibody cross-reactivity between *Rosaceae* LTPs and those from different plant sources (*8, 16*).

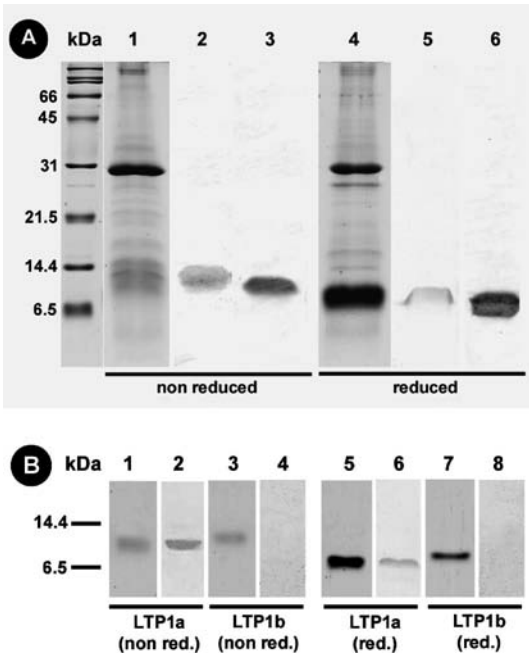

Figure 1. (**A**) SDS-PAGE (under reducing and nonreducing conditions) and immunoblotting analyses of pomegranate juice proteins (lanes 1, 2, 4, and 5) and peach peel proteins (lanes 3 and 6): Coomassie blue stain, lanes 1 and 4; immunodetection with antipeach LTP1 polyclonal antibodies, lanes 2, 3, 5, and 6. (**B**) SDS-PAGE (under reducing and nonreducing conditions) and immunoblotting analyses of purified pomegranate LTP1a and LTP1b: Coomassie blue staining, odd lane numbers; immunodetection with antipeach LTP polyclonal antibodies, even lane numbers.

Immunoblots carried out using the rabbit immune serum demonstrated that pomegranate expresses a protein cross-reacting with the peach homologue. These analyses, if performed under nonreducing conditions, showed a major protein band in pomegranate extract, at an approximate molecular mass of 12 kDa (**Figure 1A**, lane 2). As a control of the serum specificity, a lane containing peach peel extracts was also run (**Figure 1A**, lanes 3 and 6). The signal related to pomegranate proteins decreased when electrophoresis was performed under reducing conditions, leaving a faint 9 kDa band labeled by the rabbit serum (**Figure 1A**, lane 5). This band derived from the 12 kDa one as demonstrated by electrophoresis performed on the purified proteins (**Figure 1B**, compare lanes 1 and 5 and lanes 3 and 7). This electrophoretic mobility shift has already been shown for maize LTP1 (*27*). As hypothesized by Grosbois et al. (*27*), the nonreduced protein is probably more compact and less accessible to SDS than the extended reduced form, determining a lower electric charge on the molecule and then a slower electrophoretic mobility resulting in an apparent molecular weight increase. Accordingly, Coomassie dye seems to bind poorly to nonreduced LTPs (**Figure 1B**, compare lanes 1 and 3 to lanes 5 and 7).

Purification of LTP from Pomegranate Protein Extracts. To purify the immunodetected polypeptide, pomegranate juice was fractionated by CEC and analyzed by SDS-PAGE. Within a narrow range of salt concentrations (approximately at 21 and 24 mS/cm; see the chromatogram, **Figure 2**) two comigrating proteins with an apparent molecular mass of approximately 12 kDa (nonreducing conditions) were identified and considered as putative LTP1a and LTP1b (**Figure 1B**, lanes 1 and 3). The redox-induced mobility shift described above was the same for both proteins (**Figure 1B**, lanes 1 and 5 and lanes 3 and 7 for LTP1a and LTP1b, respectively).

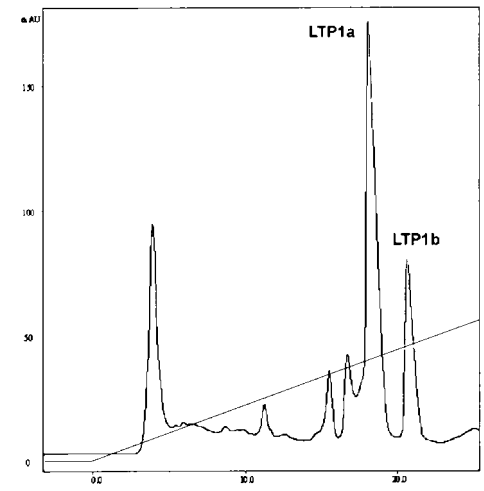

Figure 2. CEC chromatogram of the pomegranate juice proteins. Absorbance was measured at 280 nm wavelength. Peaks related to LTP1a and LTP1b are indicated.

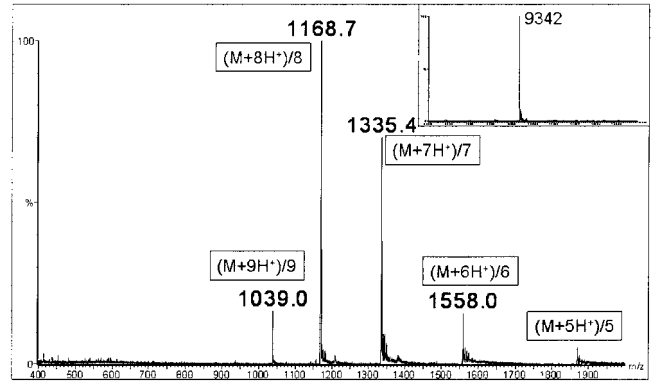

Figure 3. Annotated ESI/MS spectrum of the LTP1a protein showing the multicharged mass peaks together with the reconstructed spectrum indicating its molecular mass of 9342 ± 1 Da (inset).

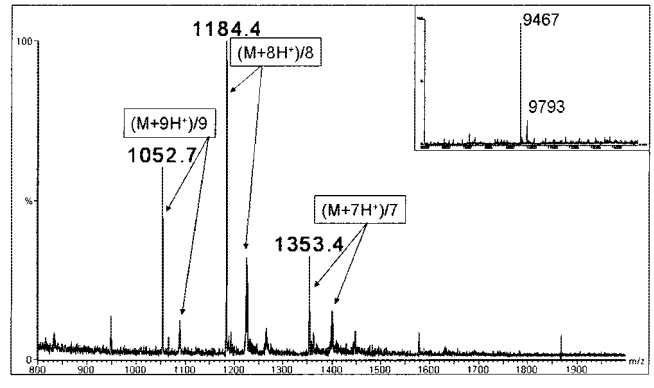

Figure 4. Annotated ESI/MS spectrum of the LTP1b protein showing the multicharged mass peaks together with the reconstructed spectrum indicating its molecular mass of 9467 ± 1 Da. A minor isoform having a molecular mass of 9793 ± 1 Da is also evident.

By immunoblots, rabbit antibodies recognized LTP1a but not LTP1b, especially when electrophoresis was performed under nonreducing conditions (see **Figure 1B**, lanes 2 and 4). To further characterize the isolated proteins, mass spectrometry experiments were carried out.

ESI/MS Analysis of Putative LTP1a and LTP1b. ESI/MS analysis was performed by directly perfusing both proteins into the ESI source (**Figures 3** and **4**) and their molecular masses turned out to be 9342 ± 1 Da for the LTP1a and 9467 ± 1 Da

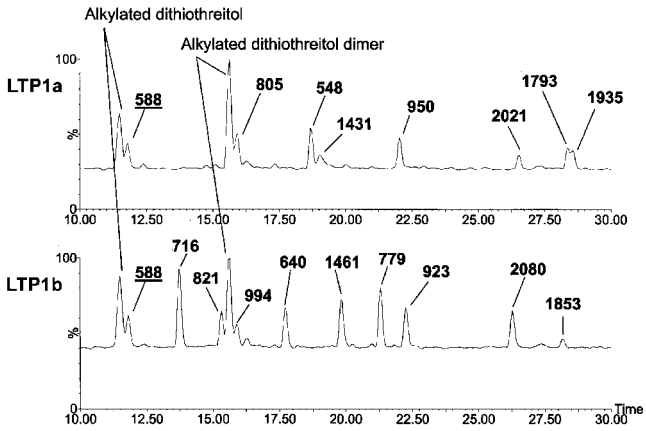

Figure 5. HPLC-ESI/MS chromatograms of the tryptic mixtures derived from LTP1a and LTP1b. The peaks related to the monomeric and dimeric forms of alkylated DTT are indicated. The only amino acid stretch common to both mixtures is underlined.

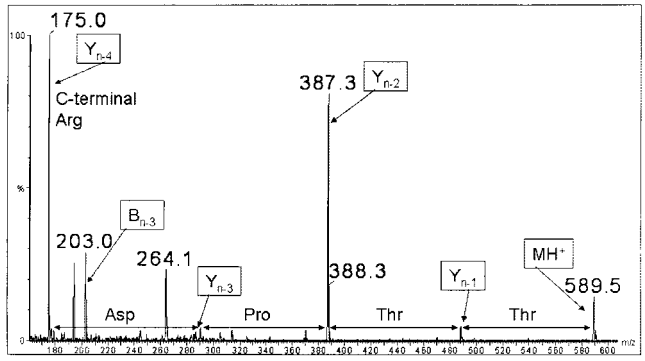

Figure 6. Annotated ESI/MS spectrum of the peptide with a molecular mass of 588.5 Da, common to both protein digestion mixtures.

for the LTP1b. A minor protein contaminating the LTP1b preparation was also evidenced, with a molecular mass of 9793 ± 1 Da. These molecular weights are perfectly consistent with those of all known LTP1s (*1*).

To gain more information on the molecular identity of both LTP1a and LTP1b, both proteins were digested with trypsin and subsequently analyzed by HPLC-ESI/MS (**Figure 5**). The molecular weights of the peptides formed upon digestion were searched in the Swiss-Prot database using the Aldente algorithm for protein identification from peptide mass fingerprinting data. The search was carried out without any hit, indicating that the proteins have never been previously identified. Quite interestingly, only one peptide with a molecular mass of 588.5 Da was found in both of the two digestion mixtures. From the fragments due to the in-source collisionally induced dissociation present in the ESI/MS spectrum of the common peptide with a molecular mass of 588.5 Da, it was possible to infer its complete sequence (**Figure 6**). As can be seen by the annotated spectrum, we were able to assign the following sequence: Thr-Thr-Pro-Asp-Arg (TTPDR). This sequence was searched in the Swiss-Prot Database by using the BLAST program, restricting the taxonomic groups to *Viridiplantae* only: among the first 25 matches containing this exact sequence, 23 proteins belong to the LTP family, supporting the identification of the putative LTP1a and LTP1b as members of the LTP family and indicating at the same time that this sequence is highly conserved in the LTP family.

As nearly all LTPs share a common eight-cysteine pattern (*1*, *16*), to further investigate whether the two purified proteins were truly members of the LTP family, the number of

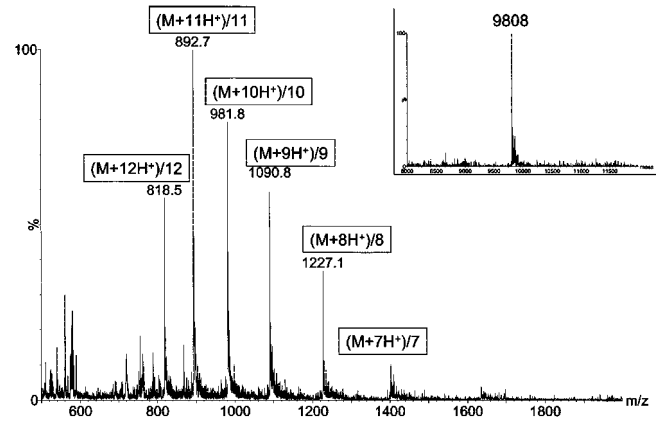

Figure 7. Annotated ESI/MS spectrum of the alkylated LTP1a protein showing the multicharged mass peaks together with the reconstructed spectrum indicating its molecular mass of 9808 Da (inset).

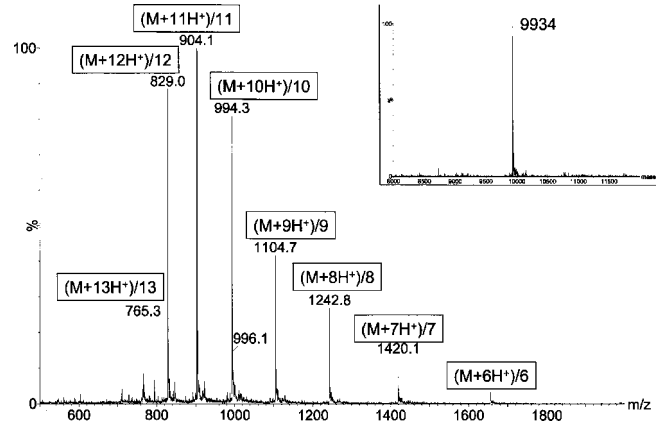

Figure 8. Annotated ESI/MS spectrum of the alkylated LTP1b protein showing the multicharged mass peaks together with the reconstructed spectrum indicating its molecular mass of 9934 Da (inset).

cysteines of both proteins was determined by reduction of disulfide bonds and subsequent alkylation of thiol groups by iodoacetamide, followed by mass spectrometric analysis.

The mass spectral analysis (**Figures 7** and **8**) clearly indicated that both proteins actually contain eight cysteines, because the molecular mass of the alkylated molecules showed an increase of about 466–467 Da (9808 Da for LTP1a and 9934 Da for LTP1b). Within the experimental error of mass measurements, these data are consistent with the addition of exactly eight acetamido groups (58 Da) to both proteins.

Thus, mass spectrometric analyses showed that the two isolated polypeptides are indeed members of the LTP family.

IgE Immunoblotting. The IgE immunoblotting experiments were performed on proteins separated under nonreducing conditions to preserve their conformational epitopes.

The results are shown in **Figure 9**. The IgEs from the serum of the pomegranate allergic patient recognized LTP1b but not LTP1a or Pru p 3. Two faint bands with higher molecular mass (38 and 60 kDa) were also detected.

DISCUSSION

It is known that different isoforms of LTPs can coexist in a given plant (*28*–*33*). Furthermore, LTPs are known to exhibit post-translational modifications such as phosphorylation as in the case of LTPs from wheat and barley (*34*). In addition, Jegou et al. (*3*) reported that an LTP isoform from barley seeds and derivatives has an excess mass of 294 Da with respect to the

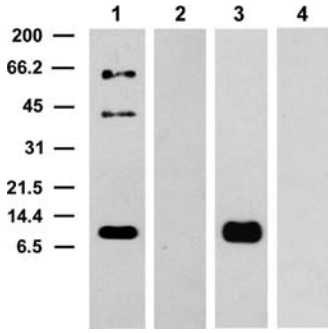

Figure 9. SDS-PAGE (under nonreducing conditions) and IgE immunoblotting analysis of pomegranate juice proteins (lane 1), pomegranate LTP1a (lane 2), LTP1b (lane 3), and peach peel proteins (lane 4).

known LTP. This LTP isoform displays the same N-terminal amino acid sequence and the low mass difference cannot be detected by SDS-PAGE. The 294 Da adduct was finally identified as a C_{17} lipid covalently bound to barley LTP (*35*).

In general post-translational modifications are well-known to affect the function and biological properties of proteins that may also include their immunogenicity and allergenic potential. LTPs have been identified as major allergens in fruits belonging to the *Rosaceae* family. The wide distribution and the highly conserved structure of these proteins in various plant species confer to these polypeptides a role as plant panallergens, and LTPs are in turn recognized as being responsible for immunological cross-reactivity among fruits, nuts, and/or pollens (*8, 10–16*).

Pomegranate is a plant of the *Punicaceae* family having fruits that have rarely been reported to cause immediate hypersensitivity after ingestion (*17–22*). Moreover, there is not a general consensus on which is the major pomegranate allergen.

In this paper we demonstrate that pomegranate fruits express at least two different LTP isoforms that share the common sequence "TTPDR". This sequence is also present in Pru p 3 (in position 40–44), Pru ar 3, Pru d 3 [LTPs from peach, apricot, and plum, respectively (*16*)]. Intriguingly, some amino acids present in this sequence, common to almost all plant LTPs, are reported to be critical for both lipid- and IgE-binding capacities (*14, 36*). To confirm the nature of the purified proteins and avoid the possibility of a false-positive assignment, we performed a further mass spectrometric analysis of the same proteins after reduction and alkylation of thiol groups. The results undoubtedly showed that the polypeptides contain eight cysteine residues, a structure common to all LTP members (*1, 16*).

The two pomegranate LTPs do not appear to be the result of post-translational modifications: the different patterns of the tryptic mixtures (**Figure 4**) suggest that they are probably encoded by different genes rather than the result of different splicings of the same gene as, for example, in maize (*37*). This behavior has been previously reported for other plant species (*4, 25, 38*). Here we further showed that LTP1a differs from LTP1b for at least one epitope because the antibodies raised in rabbits against Pru p 3 recognized LTP1a only. This hypothesis, on the other hand, is confirmed by the IgE immunoblotting with the serum of a pomegranate allergic patient, because only pomegranate LTP1b, but not Pru p 3 or LTP1a, was recognized. These preliminary observations suggest that LTP is involved in pomegranate allergy as reported for many other fruits (*8, 10–16*). To our knowledge, however, this is the first time that two LTP isoforms with different IgE binding capacities are described in a given plant (organ). Further studies involving a large group of patients allergic to pomegranate will contribute to the evaluation of the allergenicity of the different LTP isoforms.

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