



Isoform identification, recombinant production and characterization of the allergen lipid transfer protein 1 from pear (Pyr c 3)

Ileana Ramazzina ^a, Stefano Amato ^b, Elisabetta Passera ^a, Stefano Sforza ^c, Gianni Mistrello ^b, Rodolfo Berni ^a, Claudia Folli ^{a,*}

^a Department of Biochemistry and Molecular Biology, University of Parma, Parco Area delle Scienze 23/A, 43124 Parma, Italy

^b Lofarma SpA, Viale Cassala 40, 20143 Milan, Italy

^c Department of Organic and Industrial Chemistry, University of Parma, Parco Area delle Scienze 17/A, 43124 Parma, Italy

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ABSTRACT

Non-specific lipid transfer proteins belonging to LTP1 family represent the most important allergens for non pollen-related allergies to *Rosaceae* fruits in the Mediterranean area. Peach LTP1 (Pru p 3) is a major allergen and is considered the prototypic allergenic LTP. On the contrary, pear allergy without pollinosis seems to be under-reported when compared to other *Rosaceae* fruits suggesting that the as-yet-uncharacterized pear LTP1 (Pyr c 3) has in vivo a low allergenicity. We report here on the identification of four cDNAs encoding for LTP1 in pear fruits. The two isoforms exhibiting amino acid sequences most similar to those of peach and apple homologues were obtained as recombinant proteins. Such isoforms exhibited CD spectra and lipid binding ability typical of LTP1 family. Moreover, pear LTP1 mRNA was mainly found in the peel, as previously shown for other *Rosaceae* fruits. By means of IgE ELISA assays a considerable immunoreactivity of these proteins to LTP-sensitive patient sera was detected, even though allergic reactions after ingestion of pear were not reported in the clinical history of the patients. Finally, the abundance of LTP1 in protein extracts from pear peel, in which LTP1 from *Rosaceae* fruits is mainly confined, was estimated to be much lower as compared to peach peel. Our data suggest that the two isoforms of pear LTP1 characterized in this study possess biochemical features and IgE-binding ability similar to allergenic LTPs. Their low concentrations in pear might be the cause of the low frequency of LTP-mediated pear allergy.

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1. Introduction

The most relevant allergies to *Rosaceae* fruits in the Mediterranean area are caused by non-specific lipid transfer proteins belonging to

LTP1 family (Egger et al., 2010; Salcedo et al., 2007a; van Ree, 2002). The wide distribution of LTP1 in plant-derived foods, the recent evidence for food allergies induced by LTP1 from a number of botanically unrelated species distinct from *Rosaceae* (Ciardiello et al., 2010; Gadermaier et al., 2011; Krause et al., 2009; Pastorello et al., 2000; Pastorello et al., 2004; San Miguel-Moncin et al., 2003), and the demonstration of immunological cross-reactivity between LTP1 from such species (Asero et al., 2002, 2004; Tordesillas et al., 2009) indicate that these proteins represent plant pan-allergens. Allergenic LTP1 described so far are small (about 9 kDa) extracellular lipid-binding proteins that are ubiquitous in plants and whose biological function has not yet been precisely clarified. Initial in vitro experiments have shown that LTP1 are able to transfer lipid molecules between membranes, and their involvement in the intracellular trafficking of membrane lipids was hypothesized (Kader, 1996). However, the extracellular localization of LTP1 is not consistent with such a role, so that other functions, such as cell wall extension (Nieuwland et al., 2005) and cuticular wax deposition (Cameron et al., 2006), have been proposed for LTP1. Moreover, inhibitory activities of LTP1 against bacterial and fungal phytopathogens have been reported and, therefore, their possible roles in plant-defense mechanisms have been postulated (Salcedo et al., 2007b). The

Abbreviations: A, absorbance; ATP, adenosine 5'-triphosphate; bp, base pair(s); CD, circular dichroism; cDNA, DNA complementary to RNA; C-terminal, carboxyl-terminal; CTP, cytidine 5'-triphosphate; d, deoxyribo; Da, dalton(s); DEPC, diethyl pyrocarbonate; DIECA, diethyldithiocarbamate; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ESI-MS, electrospray mass spectrometry; GTP, guanosine 5'-triphosphate; ID, identification; Ig, immunoglobulin(s); IPTG, isopropyl β-D-thiogalactopyranoside; kDa, kilodalton(s); LDS, lithium dodecyl sulfate; LTP, lipid transfer protein(s); Lyso C₁₆, 1-palmitoyl-2-lysophosphatidylcholine; Mal d, *Malus domestica*; M-MLV, Moloney Murine Leukemia Virus; MOPS, morpholinopropanesulfonic acid; mRNA, messenger RNA; nt, nucleotide(s); N-terminal, amino-terminal; OD, optical density units; OAS, oral allergy syndrome; PAGE, polyacrylamide-gel electrophoresis; PBS, phosphate buffered saline; PCR, polymerase chain reaction; PDB, protein data bank; Pro, proline; Pru ar, *Prunus armeniaca*; Pru av, *Prunus avium*; Pru d, *Prunus domestica*; Pru p, *Prunus persica*; PVPP, polyvinylpyrrolidone; Pyr c, *Pyrus communis*; RAST, radioallergosorbent test; SDS, sodium dodecyl sulfate; SSC, 0.15 M NaCl/0.015 M sodium citrate pH 7; SYS, systemic symptoms; Tm, melting temperature; Tris, tris(hydroxymethyl)aminomethane; TTP, thymidine 5'-triphosphate; UTP, uridine 5'-triphosphate; UV, ultraviolet.

* Corresponding author. Tel.: +39 0521 905174; fax: +39 0521 905151.

E-mail address: claudia.folli@unipr.it (C. Folli).

recent determination of the 3D-structure of peach LTP1 (Pru p 3), the prototypic allergenic LTP, has revealed molecular details of the surface of this protein (Pasquato et al., 2006). The protein structure, stabilized by four disulfide bridges, basically consists of four α -helices packed against a C-terminal region, forming a tunnel-like hydrophobic cavity, able to lodge in vitro amphiphilic molecules. This compact fold confers a high resistance to heat denaturation (Gaier et al., 2008; Sancho et al., 2005; Scheurer et al., 2004) and proteolytic digestion to LTP1 (Asero et al., 2001; Cavatorta et al., 2010), which retain most of their immunogenic and allergenic capacity after passing through the gastrointestinal tract. This particular stability of LTP1 is thus believed to be associated with the primary sensitization mediated by the protein interaction with the gastrointestinal epithelial immune system, and to represent an important factor contributing to the severe systemic reactions often observed in allergic patients.

To date, little is known about non pollen-related allergies to pear fruits. In the Mediterranean area, only few cases of allergic reactions after ingestion of pear in LTP-sensitive patients have been reported. On the other hand, a clinical study including patients sensitive to *Rosaceae* fruits without related pollinosis showed an important cross-allergenicity between apple, pear and peach, even if RAST inhibition experiments suggested a lower allergen concentration in pear in comparison with apple and peach (Fernandez-Rivas et al., 1997). We report here on the identification, recombinant production, biochemical characterization and IgE-binding properties of different isoforms of LTP1 from pear to gain insight into the basis of the low frequency of LTP-mediated pear allergy.

2. Materials and methods

2.1. Isolation of total RNA and cDNA synthesis

Total RNA was extracted from fresh pear fruits belonging to the cultivar William's Bon Chretien. Pulp or peel were ground to obtain a fine powder in liquid nitrogen with a prechilled mortar and pestle. 250 mg of powder from pulp or peel were transferred to new tubes containing 1.5 ml of lysis buffer (100 mM Tris-HCl, pH 8, 600 mM NaCl, 4% SDS, 10 mM EDTA). The solution was twice phenol-extracted, ethanol precipitated and the pellet was resuspended in DEPC-treated water. An equal volume of 9 M LiCl was added, and the sample was incubated at 4 °C overnight. After centrifugation, the RNA pellet was dissolved in DEPC-treated water and extracted by using a 1:1 phenol:chloroform solution. The RNA was ethanol precipitated and finally dissolved in DEPC-treated water. Total RNA was analyzed by denaturing agarose gel-electrophoresis in 1× MOPS (20 mM morpholinopropanesulfonic acid, 2 mM sodium acetate, 1 mM EDTA, pH 7) containing 7% formaldehyde and quantified by spectrophotometric measurement. 1 µg of RNA from peel was then used to obtain cDNA by employing a M-MLV Reverse Transcriptase (Promega, Madison WI, USA) and an oligo (dT) following the manufacturer instructions.

2.2. Cloning, expression and purification of pear LTP1 isoforms

The region corresponding to the mature LTP1 coding sequence (276 bp) was PCR-amplified using the total cDNA from pear peel as template, a thermostable high fidelity DNA polymerase (*PfuTurbo*, Stratagene, La Jolla, CA, USA) and two specific primers, (LTP_{plus} 5'-GCTCATGCCA-TAACATGTAGCC-3' and LTP_{minus} 5'-TTACTTCACGGTGGCGCAGTTG-3'), designed on the basis of the sequence deposited in GenBank database with the accession number AF221503. The amplified fragment, after an incubation for 30 min at 72 °C in the presence of MasterTaq DNA polymerase (Eppendorf, Hamburg Germany) to add 3' terminal adenosine residues, was inserted into the vector pGEM T-easy (Promega) to generate the intermediate vector pGEM-LTP1. After transforming *E. coli* XL1

Blue cells, 8 recombinant plasmids were recovered from single colonies and sequenced on both strands. Two selected sequences, named LTPa and LTPb, were then amplified by means of *PfuTurbo* DNA polymerase (Stratagene), using a *Nde*I-tailed upstream primer (5'-CATATGATAA-CATGTAGCCAGGTG-3') and a *Bam*HI-tailed downstream primer (5'-GGATCCTTACTTCACGGTGGCG-3'). The restriction fragments obtained from *Nde*I/*Bam*HI digestion were then ligated into the *Nde*I and *Bam*HI sites of the expression vector pET11b (Novagen, Darmstadt, Germany) and the resulting plasmids (pET-LTPa and pET-LTPb) were sequenced on both strands. The expression of LTPa and LTPb in *E. coli* Origami (DE3) cells in 1 L of culture medium was induced by adding 1 mM isopropyl-1-thio- β -D-galactopyranoside and, after an overnight incubation at 28 °C, cells were lysed by twenty 30 second bursts of sonication. Pear LTPs were purified to homogeneity using a single-step procedure; after concentration in an Amicon Cell (Millipore, Billerica, MA, USA), the bacterial cell lysate was subjected to ion exchange chromatography on a *SP Sepharose fast flow* (Pharmacia AB, Helsingborg, Sweden) column. Protein concentrations were determined using absorption coefficients (A 1 mg/ml, 1 cm) at 280 nm of 0.376 for both LTPa and LTPb, as calculated according to Pace et al., (1995).

2.3. Mass spectrometry and fluorescence binding assays for pear LTPa and b

The identity of pear LTPa and b was verified by measuring their mass by electrospray mass spectrometry (ESI-MS). Aqueous solutions of both proteins were directly perfused into a Quattro Micro mass spectrometer (Waters, Milford, MA, USA) and mass spectra were recorded on the intact molecules setting the following instrumental parameters: positive ion mode, capillary voltage 3.3 kV, cone voltage 30 V, source temperature 100 °C, desolvation temperature 180 °C, cone gas (N_2) flow 90 l/h, and desolvation gas (N_2) flow 450 l/h. To avoid fragmentation, entrance energy was set up at 50, collision energy at 2, and exit energy at 50. Acquisition was performed in full scan mode from 500 to 2000 m/z, scan duration 1.5 s, interscan delay 0.01 s.

To verify that recombinant LTPa and LTPb were functionally active, lipid binding assays were performed, by using 1-palmitoyl-2-lysophosphatidylcholine (Lyso C₁₆, SIGMA) as a probe (Gomar et al., 1996; Lullien-Pellerin et al., 1999). Pear LTPa and b (10 µM) in 50 mM sodium phosphate (pH 7), 150 mM NaCl, were supplemented with equimolar Lyso C₁₆ dissolved in ultrapure water and stirred gently in the spectrofluorometer cuvette. Emission spectra were then recorded in the 290–340 nm range (excitation at 275 nm), to monitor changes in the intrinsic protein fluorescence caused by the binding of Lyso C₁₆, by means of a Fluoromax-3 (Jobin Yvon Horiba, Edison, NJ USA) spectrofluorometer.

2.4. Circular dichroism measurements

The secondary structures of the purified pear LTPa and b and of recombinant Pru p 3 (UniProt: Q5RZZ3, PDB: 2ALG, obtained and purified as described in Pasquato et al., (2006)), were analyzed by circular dichroism (CD) spectroscopy. Far UV CD spectra (200–250 nm) were recorded by means of a Jasco J-715 spectropolarimeter (Jasco, Easton, USA), using a 0.1 cm path length cuvette and a protein concentration of 0.5 mg/ml. Spectra were recorded during heating (2 °C/min) from 25 to 105 °C at intervals of 10 °C (5 min incubation), followed by the cooling to 25 °C. Thermal denaturation was revealed by the increase in molar ellipticity at the 220 nm minimum. This change was used to calculate the denaturation percentage according to the equation: Denaturation (%) = $(CD_t - CD_{25}) / (CD_{105} - CD_{25}) \times 100$, where CD_t is the ellipticity value obtained at different temperatures and CD_{25} and CD_{105} are the ellipticity values recorded at 25 °C and 105 °C, respectively. CD spectra were also recorded for all LTPs after an incubation at 85 or 95 °C (5 min incubation), followed by

the cooling to 25 °C. Data from three spectra were accumulated to obtain mean spectra.

2.5. Patient sera and immunoglobulin binding assays

A panel of 10 patients was recruited based on a reported history of allergic reaction to peach not related to pollinosis. Patients showed a good tolerance to pear as no allergic reaction after pear ingestion has been reported in their clinical history. ELISA assays were performed for each serum to establish the presence of IgE able to recognize recombinant Pru p 3 (Pasquato et al., 2006). IgE levels were expressed as optical density units (OD). Based on the IgE levels found in normal control sera, values <0.3 OD were considered as negative. All tested sera led to positive results and were collected for ELISA assays. Written informed consent was obtained from all patients and the ethics committees of the corresponding hospitals approved the study. The evaluation of IgE reactivity to pear and peach LTP1 was accomplished by means of IgE ELISA assays. Plate wells were coated with either recombinant Pru p 3 or pear LTPa and b in 50 mM sodium bicarbonate buffer (pH 9.6), saturated with 2% bovine serum albumin in PBS buffer (0.1 M phosphate-buffered saline, pH 7.4) for 2 h at room temperature, and then incubated with the serum pool from Pru p 3-sensitive patients (in serial dilution from 1:3 to 1:729). In the inhibition assays, the same serum pool from Pru p 3-sensitive patients (dilution 1:5) was preincubated with increasing quantities of pear LTPa or b for 2 h at room temperature and then added to the plates coated with recombinant Pru p 3. Specific IgE binding was detected by adding a peroxidase-conjugated anti-human IgE from goat (BiosPacific, Emeryville, CA) (dilution 1:1500), and tetramethylbenzidine/H₂O₂ as reaction substrates. The enzymatic reaction was stopped after 20 min by acidification, and absorbance values were determined at 450 nm.

The interaction of polyclonal murine anti-pear LTPa antibodies with recombinant Pru p 3 was also evaluated. To obtain polyclonal anti-pear LTPa antibodies, three BALB/c mice were immunized subcutaneously with 20 µl of pear LTPa (1 mg/ml) emulsified in Freud's complete adjuvant (SIGMA, Buchs, Switzerland). The same amount of antigen in incomplete Freud's adjuvant was injected every 3 weeks for further three times. Mice sera were collected 4 days after the last boost and checked by ELISA for the presence of IgG antibodies specific for pear LTPa. IgG ELISA assays were performed as described above using pear LTPa or Pru p 3 in the coating phase, and incubating for 1 h with serum pools from immunized or non-immunized mice in serial dilution (from 1:5 × 10² to 36.45 × 10⁴). Specific murine IgG binding was detected by adding a peroxidase-conjugated goat anti-murine IgG (SIGMA) (dilution 1:1500).

2.6. Northern blot analysis

10 and 20 µg of total RNA from pear peel or pulp were heated for 25 min at 65 °C and separated on denaturing (formaldehyde/MOPS) agarose gel. After electrophoresis, the gel was washed four times in 300 ml of water for 7 min, once in 300 ml of 50 mM NaOH for 20 min and quickly in 300 ml of 150 mM sodium citrate, 1.5 M NaCl (pH 7) (SSC 10×). RNA was vacuum blotted on a positively charged nylon membrane (Biodyne B Nylon Membranes, Pall Gelman Laboratory, MI USA) and fixed by UV-crosslinking. The blot was hybridized with a probe corresponding to the entire LTP1 coding sequence amplified from total cDNA from pear peel. The probe labeling was performed by standard PCR using GoTaq DNA polymerase (Promega), 0.1 mM biotin-labeled dUTP (Biotium, CA USA), 0.25 mM dATP, dCTP, dGTP, 0.15 mM dTTP, 25 pmoles of primers LTP_{plus} and LTP_{minus} and 10 ng of cDNA from pear peel as template. PCR products were purified using the QiaQuick PCR Purification Kit (Qiagen, Hilden Germany). Prehybridization was performed at 68 °C for 40 min in the ExpressHyb Hybridization Solution (Clontech

Laboratory, Palo Alto, CA USA) containing 0.1 mg/ml of salmon sperm DNA, and hybridization was carried out for 2 h at 68 °C in the same solution, in the presence of 3.6 µg of biotin-labeled cDNA probe. After hybridization, the blot was washed twice in 20 ml of SSC 2×, 0.1% SDS (25 °C, 30 min) and twice in 20 ml of SSC 0.1×, 0.1% SDS (50 °C, 30 min). The blot was then incubated with Odyssey Blocking Buffer (LI-COR Biosciences, Lincoln, NE USA) containing 1% SDS at 25 °C for 30 min and hybridized with streptavidin IRDye-680 (LI-COR) 0.2 µg/ml in the same solution at 25 °C for 30 min in the dark. Finally, the blot was washed three times in 20 ml of PBS 1×, 0.1% Tween 20 (25 °C, 5 min) and once in 20 ml of PBS 1× (25 °C, 5 min); the signal was visualized using a Odyssey scanner (LI-COR Biosciences).

2.7. Western blotting analysis

Total protein extracts from peach and from two different cultivars of pear were obtained as described in Bjorksten et al. (1980). Briefly 100 g of fresh pear or peach peel were homogenized in 200 ml of 0.1 M phosphate buffer pH 7, containing 2% polyvinylpyrrolidone (PVPP), 10 mM diethyldithiocarbamate (DIECA), 2 mM EDTA, 20 mM ascorbic acid and 3 mM sodium azide. PVPP, EDTA and DIECA were added to inhibit reactions between proteins and phenolic compounds. After 1 h mixing at room temperature, the homogenate was centrifuged for 40 min at 13,000 rpm at 4 °C and the supernatant was dialyzed against 5 mM ammonium bicarbonate, pH 8.4. The protein content was determined by Bradford method (Bradford, 1976). For western blot experiments were employed 30 µg for each extract and murine polyclonal anti-pear LTPa antibodies (for more details see Section 2.5.).

Extracts were mixed with LDS sample buffer Nupage Bis-Tris Novex (Invitrogen Ltd., Paisley, UK) containing 5% β-mercaptoethanol and then denaturated by heating at 100 °C for 10 min. Electrophoresis was carried out in a 10% polyacrylamide precast gel (Nupage Bis-Tris, Novex, Invitrogen) and the resolved proteins were transferred for 1 h onto a nitrocellulose membrane according to Towbin et al. (1979). The membrane was incubated with 0.1 M Tris-buffered saline solution containing 5% fat-free milk powder and the hybridization was performed for 16 h at 4 °C with murine polyclonal anti-pear LTPa antibodies or with serum of non-immunized mice (dilution 1:65). Detection was carried out by employing peroxidase-conjugated anti-murine antibodies from goat (dilution 1:8000) (SIGMA).

3. Results and discussion

3.1. Sequence analyses

After the retrotranscription and amplification from total RNA, the pear LTP1 cDNA was cloned and the recombinant plasmids extracted from 8 single colonies were sequenced. This analysis allowed us to identify 4 different sequences, named LTPa, LTPb, LTPc and LTPd, which were found 3, 2, 1 and 2 times, respectively. This result is consistent with LTP1 being encoded by multiple genes, as previously reported (Botton et al., 2002; Gao et al., 2005; Liu et al., 2010; Vignols et al., 1997). In Fig. 1, the amino acid sequences of the four pear LTP1 isoforms are compared with the unique sequence of pear LTP1 (Pyr c 3, accession number Q9M5X6) and with that of Pru p 3 (accession number Q5RZZ3) present in the UniProt database. Multiple sequence alignment was constructed by CLUSTALW (Thompson et al., 1994) and rendered with ESPrpt program (Gouet et al., 1999). Pear LTPb is identical to Pyr c 3 sequence and shares 97 and 95% identities with LTPa and LTPc, respectively. LTPd is the most different among isoforms, sharing identities of 76% with LTPb and LTPc and 78% with LTPa. As revealed by the comparison of these amino acid sequences with those of their homologues present in other *Rosaceae* fruits, all pear LTP1 share the highest identities with LTP from apple (Mal

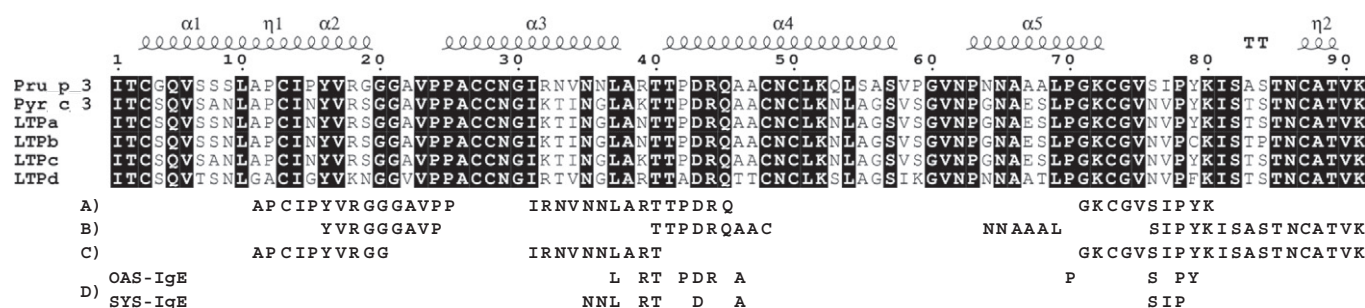


Fig. 1. Multiple amino acid sequence alignment for LTP1. The four pear sequences identified in this work are compared with Pyr c 3 and Pru p 3 sequences deposited with UniProt accession number Q9M5X6 and Q5RZZ3, respectively. Residues that are identical in all the sequences are boxed in black; numbering and secondary structure elements are based on the structure of Pru p 3 (PDB: 2ALG). Bottom lines: A, linear epitopes identified in Garcia-Casado et al. (2003); B, linear epitopes identified in Borges et al. (2008); C, linear epitopes identified in Tordesillas et al. (2009); D, conformational epitopes proposed in Pacios et al. (2008) for patients affected by oral allergy syndrome (OAS-IgE) or for patient showing systemic symptoms (SYS-IgE). Sequence alignment was constructed by CLUSTALW (Thompson et al., 1994) and rendered with ESPrpt program (Gouet et al., 1999).

d 3) and lower identities, ranging from 72 to 80%, with LTP1 proteins from other *Rosaceae* (Table 1).

In previous studies, by using different methodologies evidence was obtained to indicate the presence in Pru p 3 of sequential and conformational IgE binding epitopes (Borges et al., 2008; Garcia-Casado et al., 2003; Pacios et al., 2008; Tordesillas et al., 2009) (Fig. 1). In a study on the cross-allergenicity between *Rosaceae* LTP1 (Borges et al., 2008), four conserved sequences were found to partially overlap with those previously proposed for the peach protein (Garcia-Casado et al., 2003). It is worth noting that by comparing the amino acid sequences of peach and pear LTP1 only few significant amino acid substitutions are present in the regions potentially involved in IgE recognition (Fig. 1), in keeping with the in vitro immunological reactivity we have determined for pear LTPa and LTPb with IgE from Pru p 3-sensitive patients (see below, Fig. 6). In particular, most significant appear to be the P15N, G19S, N36G, R39N, N64G, A68S and A83T amino acid replacements in pear LTP1 isoforms as compared to Pru p 3 (similarity groups considered: HKR, DE, STNQ, AVLIM, FYW, PG, C) (Fig. 1). Evaluating the amino acids located in the conformational epitopes suggested by Pacios et al. (2008), the only significant replacement is N36G in all pear LTP1 isoforms. It should be noted that, with the only exception of the R39N amino acid replacement in pear LTPa and c, these substitutions do not involve positively charged residues

located on the protein surface of peach LTP (Pasquato et al., 2006), for which the direct participation in the interaction with IgE has been proposed (Garcia-Casado et al., 2003).

3.2. Heterologous expression and purification of functionally active pear LTPa and LTPb

LTPa and LTPb, whose amino acid sequences are the closest, among the pear LTP1 we have identified, to the allergenic LTP1 from both peach and apple, were obtained as recombinant proteins. By applying optimized induction conditions, recombinant proteins were found in the soluble fraction of the *E. coli* lysate (Fig. 2). Both proteins were purified by means of a single step procedure obtaining a purity higher than 95%, as revealed by SDS–PAGE (Fig. 2). The final yields of purified LTPa and LTPb were estimated to be about 3 mg and 2 mg for liter of cell culture, respectively.

The identity of pear LTPa and b was verified by determining their average molecular mass by electrospray mass spectrometry (ESI-MS). As reported in Figs. 3A and B, the measured average masses were perfectly consistent with the sequences of LTPa and LTPb reported in Fig. 1, with the addition of an N-terminal methionine, derived from the recombinant expression process, and by considering the eight cysteine residues all bound in four disulfide bridges. This feature is very important since it is consistent with a proper fold for both pear LTP1 isoforms. It is important to underline that, albeit the very small difference in the theoretical average mass between the two pear LTP1 isoforms (only 2 Da deriving from serine to alanine and

Table 1

Amino acid sequence identities (%) of mature pear LTP1 and related allergenic LTP1 from different *Rosaceae* species. UniProt accession numbers are reported. Q5RZZ3 is identical to Q9LED1 and to E7CLP9; Q5J009 is identical to Q5J000; Q2V6D8 is identical to Q5J026, to Q5GLH0 and to Q9M5X7; Q9M5X8 is identical to E7CLQ1; and P81651 is identical to E7CLQ6. Sequence alignment was constructed by CLUSTALW (Thompson et al., 1994).

		LTPa	LTPb	LTPc	LTPd
Pyr c 3	Q9M5X6	97	100	95	76
Pru p 3	Q5RZZ3	79	78	76	73
	Q8H2B2	79	78	76	73
	P81402	79	78	76	73
	Q4VUZ0	78	76	75	72
Mal d 3	Q5J009	91	91	89	78
	Q5JZZ6	90	90	87	76
	Q5JZZ5	90	90	87	76
	Q2V6D8	86	85	84	85
	Q5J011	84	83	82	85
Pru av 3	Q9M5X8	79	79	76	74
	E7CLQ2	79	79	76	74
Pru ar 3	E7CLQ4	80	80	78	78
	P81651	79	78	76	78
	E7CLQ7	78	76	75	76
	E7CLQ5	74	75	72	73
Pru d 3	P82534	80	79	78	80

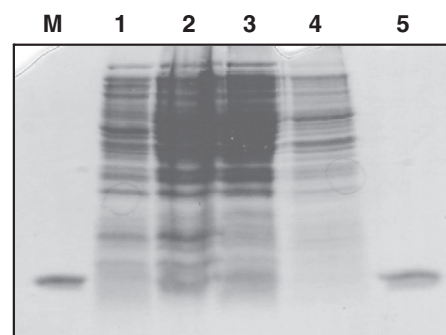


Fig. 2. Expression and purification of recombinant pear LTPa. Coomassie blue-stained SDS–PAGE of fractions containing LTPa. M: molecular weight marker (recombinant Pru p 3, 9 kDa), lane 1: whole uninduced cell extract, lane 2: whole IPTG induced cell extract, lane 3: supernatant after cell lysis, lane 4: insoluble proteins after cell lysis, and lane 5: pure protein fraction obtained at the end of the purification protocol (for details see Section 2.2.). Similar results were also obtained for LTPb expression and purification.

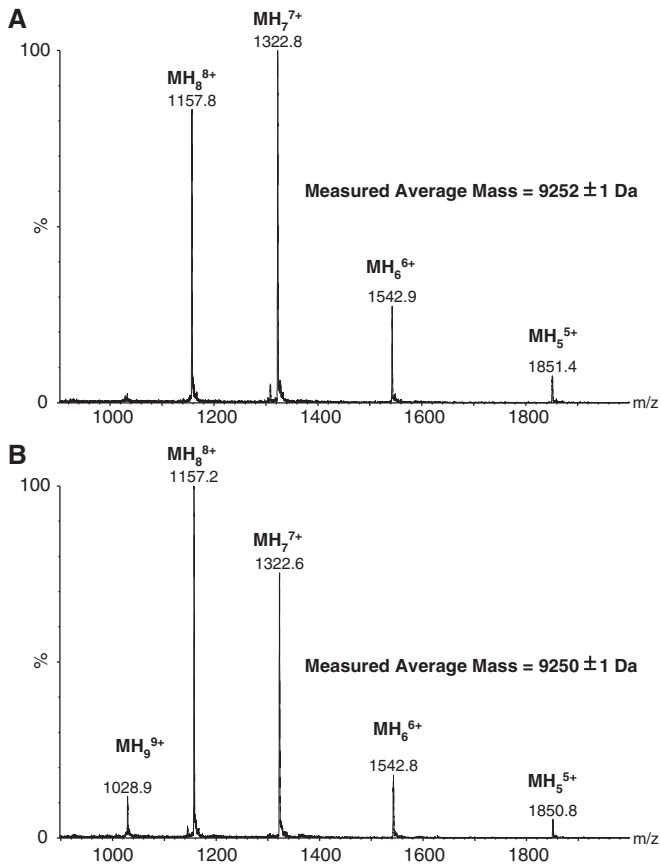


Fig. 3. Mass spectrometry measurements. A: Annotated mass spectrum of pear LTPa, indicating an average mass of 9252 ± 1 Da. The theoretical average mass for LTPa, including an N-terminal methionine and four disulfide bridges, is 9251 Da. B: Annotated mass spectrum of LTPb, indicating an average mass of 9250 ± 1 Da. The theoretical average mass for LTPb, including an N-terminal methionine and four disulfide bridges, is 9249 Da.

lysine to asparagine substitution), ESI-MS was perfectly able to significantly discriminate between them.

It is known that the binding of Lyso C_{16} to LTP1 causes a considerable enhancement of its intrinsic fluorescence, due to a protein conformational change involving the conserved residue Y79, upon formation of the LTP–Lyso C_{16} complex (Gomar et al., 1996; Lullien-Pellerin et al., 1999). Binding assays conducted with recombinant pear LTPa and LTPb, as well as with recombinant Pru p 3 used as a control, revealed a global increase in the intrinsic protein fluorescence of about 50% after the addition of equimolar Lyso C_{16} (Fig. 4), in keeping with the functionally active state of the recombinant proteins we have obtained.

3.3. Thermal stability of pear LTPa and LTPb

The CD spectra at 25 °C of pear LTPa and LTPb, showing two characteristic negative minima at about 208 and 220 nm (Fig. 5A), are typical of proteins, such as LTP1, containing a significant extent of α -helical structure. By increasing the temperature from 25 to 105 °C, the proteins underwent denaturation, as revealed by the extent of spectral change at 220 nm (Fig. 5B). For both pear proteins small changes in the native spectra were measured up to 75 °C, while the denaturation process proceeded quickly in the range 75–95 °C (Fig. 5B). Pru p 3 exhibited a higher stability if compared to pear LTPa and b as shown in Fig. 5B. In fact, in the case of Pru p 3 small changes in the native spectrum were measured up to 85 °C, while the denaturation process proceeded quickly in the range 85–105 °C (Fig. 5B). Taking into account these results, it was possible

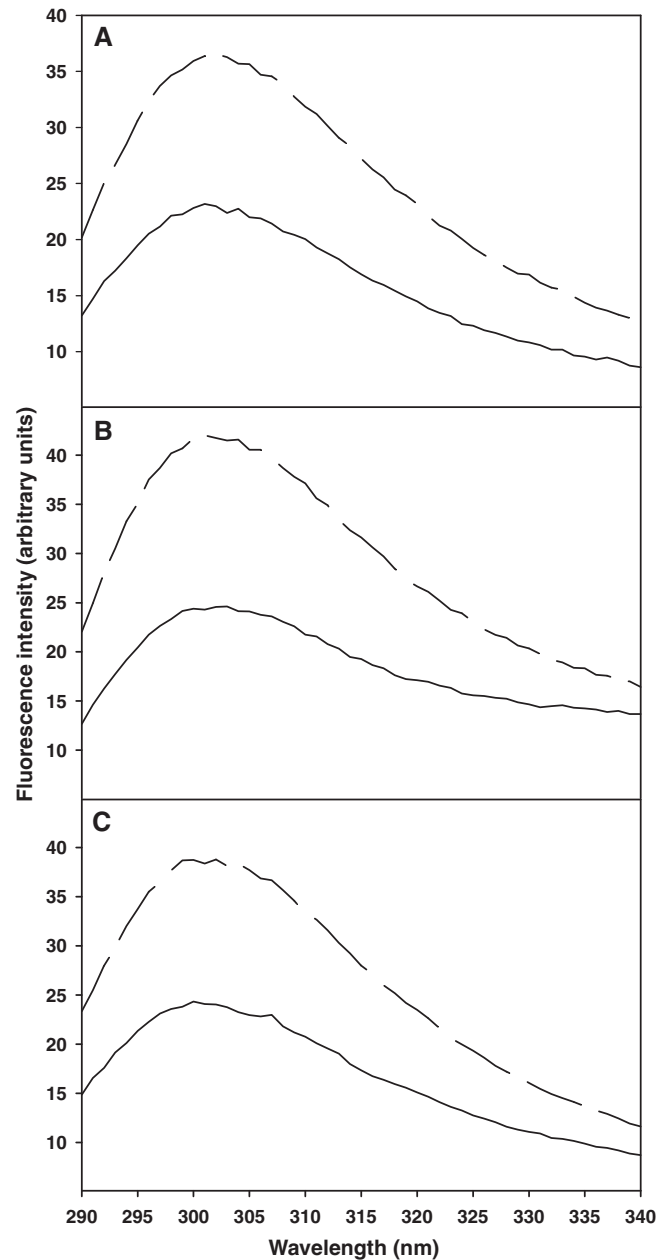


Fig. 4. Fluorescence spectra of LTP1 in complex with Lyso C_{16} . Fluorescence emission spectra (excitation at 275 nm) of LTP1 (continuous lines) and of LTP1–Lyso C_{16} complexes (dashed lines) are shown. Panel A: Pru p 3; panel B: pear LTPa; panel C: pear LTPb.

to calculate melting temperatures (T_m) of about 85 °C and >93 °C for the two pear proteins and for Pru p 3, respectively (Fig. 5B).

After heating up to 95 °C, the denaturation was irreversible for both pear LTPs, such that after the subsequent temperature decrease to 25 °C, the initial spectrum could not be recovered (Fig. 5A). On the other hand, the two pear proteins heated at 85 °C for 5 min recovered nearly completely the correct fold (Fig. 5A). Pru p 3 completely recovered its native spectrum after heating to 85 °C, and nearly completely after heating to 95 °C (Fig. 5A), while the heating to 105 °C led to an irreversible denaturation (not shown). These results indicate that depending on the particular source from which *Rosaceae* LTP1 are obtained, the temperatures to which allergenic proteins are exposed may differently affect their structure and, consequently, their allergenicity.

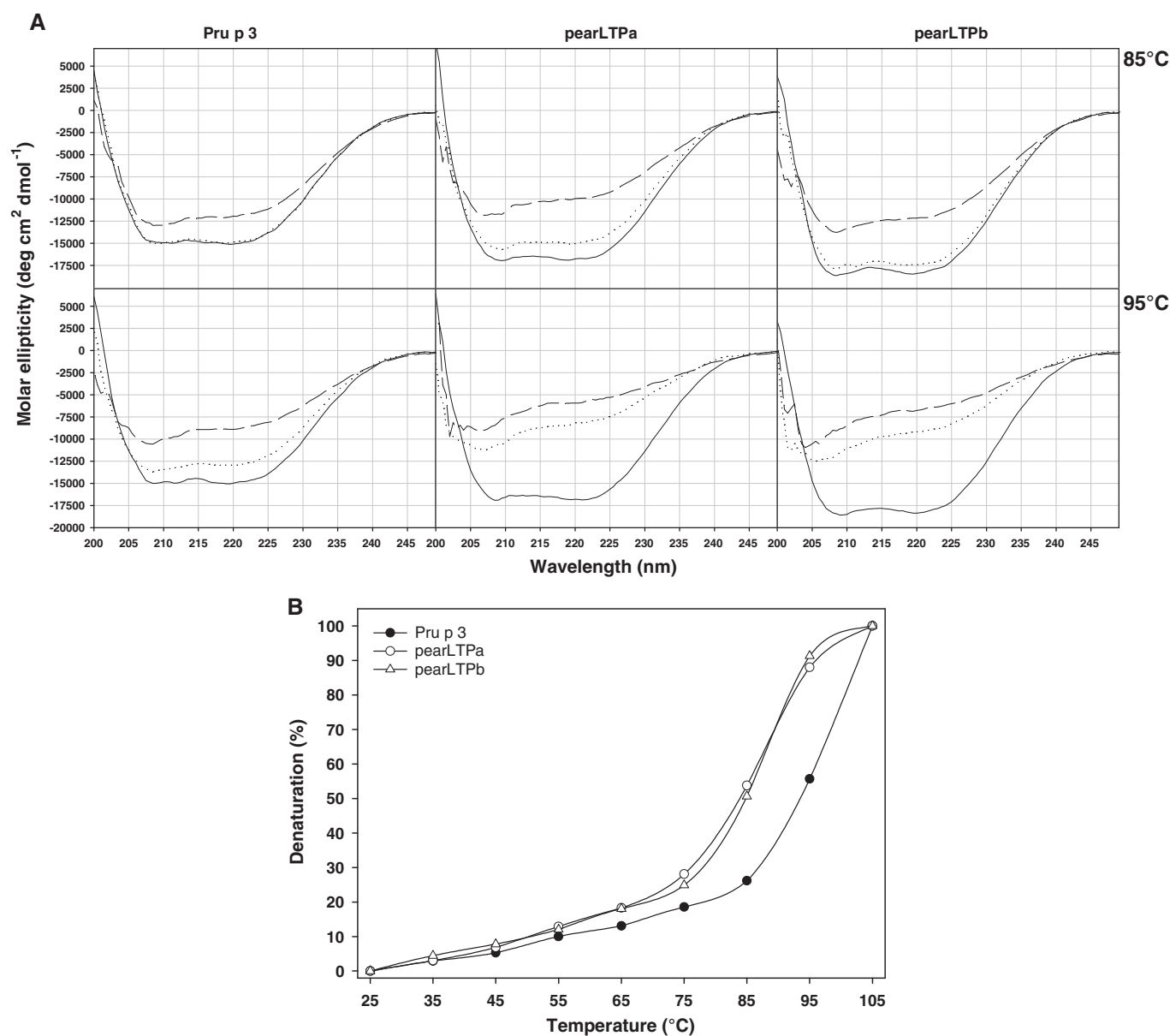


Fig. 5. Thermal stability of pear LTP1 isoforms. A: Effect of heating and cooling on LTP1 structure. CD spectra for Pru p 3 and pear LTP1 isoforms were recorded at 25 °C (continuous line), after heating to 85 °C (dashed line, upper panel) or 95 °C (dashed line, lower panel) and after cooling back to 25 °C (dotted line). B: Thermal denaturation of Pru p 3 and pear LTP1 isoforms. Changes in molar ellipticity at 220 nm were used to calculate denaturation percentages (see [Materials and methods](#) section for details), which were plotted as a function of the applied temperature.

3.4. Immunological reactivity of pear LTPa and LTPb

The IgE-binding ability of pear LTPa and LTPb has been analyzed *in vitro* by direct and inhibition IgE ELISA assays (Figs. 6A and B). Initially, the reactivity was determined by coating pear LTPa or LTPb in plate wells and incubating with sera from patients sensitive to Pru p 3. The immunoreactivity of both proteins to patient IgE immunoglobulins was significant, albeit weaker than that exhibited by the control Pru p 3 (Fig. 6A). Next, when IgE inhibition ELISA assays were performed, by coating Pru p 3 in plate wells and using pear LTPa or LTPb as inhibitors, an approximately 60% inhibition was observed for both pear LTP1 isoforms (Fig. 6B), while an approximately 90% inhibition was observed for the control Pru p 3. To verify whether antibodies raised against pear LTP1 also detected Pru p 3, anti-pear LTPa polyclonal IgG immunoglobulins were produced and used in ELISA assays. A considerable reactivity of murine anti-pear LTPa IgG to Pru p 3 was

observed (Fig. 6C), suggesting at least a partial sharing of antigenic epitopes.

3.5. Northern blot analysis

The expression of LTP1 mRNA in pear fruits was analyzed by Northern Blotting. Total RNA was extracted from pulp and peel, transferred onto nylon membrane and hybridized with a biotin-labeled LTP1 cDNA probe. A marked difference between peel and pulp in the expression of pear LTP1 was observed. In fact, in pulp no specific transcript could be detected, while a single band corresponding to RNA of about 700 nt, in agreement with the reported length of the complete Pru p 3 mRNA (Botton et al., 2006), was determined for peel samples (Fig. 7). On the other hand, a PCR experiment conducted by employing as template cDNA from pear pulp and LTP_{plus} and LTP_{minus} primers led to the amplification of LTP1 coding sequences

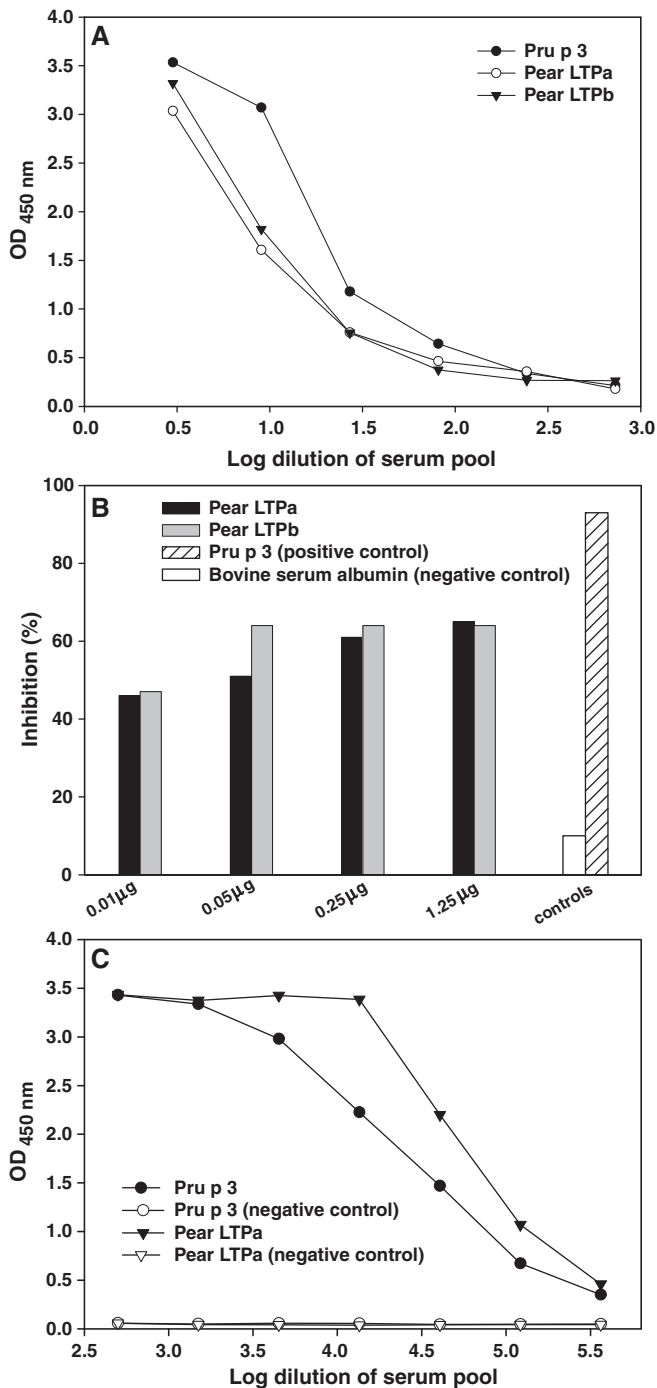


Fig. 6. Immunoglobulin-binding of pear LTP1 isoforms. A: IgE direct ELISA assays for the two pear LTP1 isoforms. Plate wells were coated with 0.05 µg of either Pru p 3 or one of the two isoforms of pear LTP1 and incubated with 100 µl of serum pool from Pru p 3-sensitive patients in serial dilution. The OD_{450 nm} values obtained from direct ELISA assays by employing serum pool from normal subjects (dilution 1:3) were 0.184, 0.142, and 0.181, for Pru p 3, pear LTPa and LTPb, respectively. B: IgE inhibition ELISA assays for pear LTPa and b. 100 µl of serum pool from Pru p 3-sensitive patients (dilution 1:5), preincubated for 2 h at room temperature with increasing quantities of pear LTPa or pear LTPb, were added to plate wells coated with 0.05 µg of Pru p 3. Inhibition assays were also performed using 0.25 µg of Pru p 3 and 1.25 µg of bovine serum albumin as positive and negative controls, respectively. C: Murine IgG cross-immunoreactivity between pear LTPa and Pru p 3. ELISA plate wells were coated with 0.05 µg of either pear LTPa or Pru p 3 and incubated with 100 µl of a serum pool from immunized (with pear LTPa) or non-immunized mice (negative control) in serial dilution (from 1:5 × 10² to 36.45 × 10⁴).

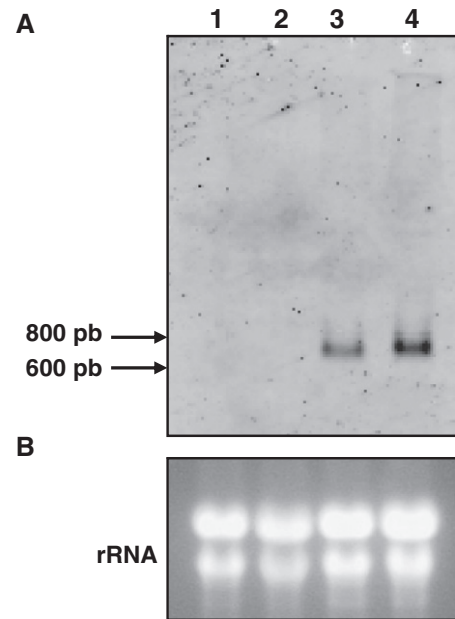


Fig. 7. Expression of LTP1 mRNA in pear fruit. A: Northern blot of RNA extracted from pear pulp and peel. 10 and 20 µg of total RNA extracted from pear pulp (lanes 1 and 2, respectively) and 10 and 20 µg of total RNA extracted from pear peel (lanes 3 and 4, respectively) were hybridized with a biotin-labeled cDNA probe specific for LTP1 coding regions. B: Denaturing (formaldehyde/MOPS) agarose gel electrophoresis of total RNA before blotting. The bands corresponding to ribosomal RNA were visualized by employing ethidium bromide.

(not shown). These results are in good agreement with a predominant presence of LTP1 in peel in comparison with pulp, which was previously found for the peach fruit by analysing the specific mRNA distribution or by the direct quantification of the protein using specific antibodies (Botton et al., 2002; Botton et al., 2009; Brenna et al., 2004; Carnes et al., 2002). In the case of peach, a high concentration of Pru p 3 in fuzz was also determined (Asero et al., 2006); this specific localization of the allergenic protein in peach is likely to be responsible for a recently described respiratory allergy caused by LTP1 (Borghesan et al., 2008; Palacin et al., 2007). All these data are in good agreement with the results of clinical studies, demonstrating a higher allergenicity of peel in comparison with pulp for *Rosaceae* fruits (Fernandez-Rivas and Cuevas, 1999; Fernandez-Rivas et al., 1997).

3.6. Western blot analysis

Western blot analyses have shown that the quantities of LTP1 in pear and peach peel are markedly different (Fig. 8). By using murine polyclonal anti-pear LTPa antibodies, whose ability to identify pear and peach LTP1 is demonstrated in Fig. 6C, a band, albeit weak, corresponding to LTP1 was detected for peach extract, while such a band was not revealed for pear extract from two different cultivars. Taking into account the higher specificity of the employed antibodies in the recognition of pear LTP1 (Fig. 6C), this result is consistent with a markedly lower concentration of LTP1 in pear peel as compared to peach peel.

4. Conclusions

Our screening of the cDNAs from pear fruit has led to the identification of four LTP1 isoforms, whose mRNA was essentially confined to the fruit peel. This result is in agreement with the multigenic origin of LTP1 within a single botanical species (Botton et al., 2002; Vignols et al., 1997), and with the predominant location of LTP1 in the peel in *Rosaceae* fruits (Botton et al., 2002; Brenna et al., 2004; Cavatorta et

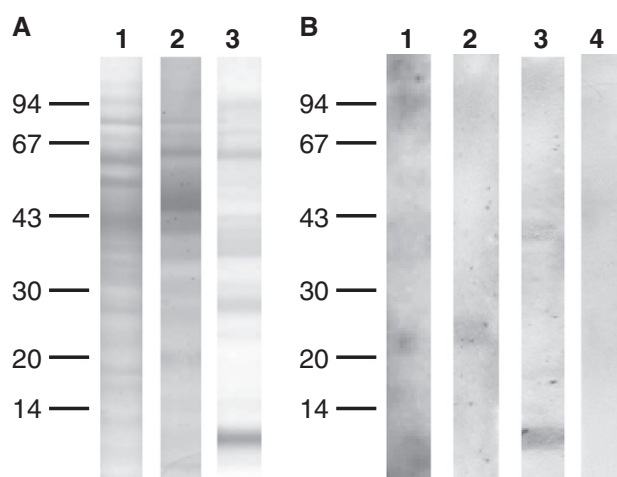


Fig. 8. SDS–PAGE and Western blot analyses of protein extracts from pear and peach peel. A: SDS–PAGE. Protein extract from pear peel, Beurre Bosc cultivar and William's Bon Chretien cultivar (lanes 1 and 2); protein extract from peach peel (lane 3). B: Western blot performed with murine polyclonal anti-pear LTPa antibodies. Protein extract from pear peel, Beurre Bosc cultivar and William's Bon Chretien cultivar (lanes 1 and 2, respectively); protein extracts from peach peel (lane 3); peach extract incubated with serum of non-immunized mice as a negative control (lane 4).

al., 2009). Two pear LTP1 isoforms (LTPa and LTPb), which were the most similar to the allergenic LTPs from apple and peach, were produced as pure recombinant proteins and were biochemically and immunologically characterized. LTPa and LTPb from pear exhibited CD spectra typical of LTP1 and the ability to bind Lyso C₁₆, in keeping with their correct fold. A high thermal stability, albeit lower than that of Pru p 3, was determined for both pear LTPa and LTPb. The IgE present in the sera of Pru p 3-sensitive patients were reactive to both LTPa and LTPb in ELISA assays, despite the lack of a clinical pear allergy in the patients. Moreover, pear and peach LTP1 exhibited immunological cross-reactivity to murine IgG immunoglobulins. Thus, the in vitro biochemical and IgE-binding properties of LTPa and LTPb from pear appear to be very similar to those reported for typical allergenic LTPs from other *Rosaceae*, even though the frequency of pear LTP-mediated allergy is quite low. Relatively different consumption of pear compared to other fruits and different eating habits (i.e. peeling or not peeling the fruits) could play a role in the limited diffusion of pear LTP-mediated allergy. However, western blotting analysis suggests that a quite low concentration of lipid transfer protein might also be the cause of the low frequency of LTP-mediated allergy from pear.

Data deposition

Pear LTP coding sequences have been submitted to GenBank with the following ID: HQ698354 for LTPa, HQ698355 for LTPb, HQ698356 for LTPc and HQ698357 for LTPd.

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