



Peel LTP (Pru p 3) – the major allergen of peach – is methylated. A proteomic study



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ABSTRACT

Lipid transfer protein (LTP, Pru p 3) is the major allergen of peach (*Prunus persica*), and is in a greater abundance in the peel than in the pulp of the fruit. Peel LTP is more allergenic than pulp LTP, but it is not clear whether this is due to its specific allergenic properties or to its higher concentration.

In this study, we have used a new one-step, rapid procedure for the purification of LTP from peel and pulp of four peach varieties [Gladys (white flesh), California (nectarine yellow flesh), Plusplus (yellow flesh), Red Fair (nectarine yellow flesh)] harvested in a field grown in Southern Italy.

Purification was based on miniature reversed-phase chromatography, a procedure suitable for proteomic study. Proteomic analysis of purified LTPs revealed that the amino acid sequence of LTP was identical in all peach genotypes but, for the first time, peel LTP was found to be methylated.

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1. Introduction

Lipid transfer protein (LTP) plays an important role in plant defense against fungi and bacteria. LTP has been identified as a major fruit allergen (Pru p 3) in individuals resident in Italy or in other Mediterranean countries (Fernández-Rivas et al., 2003). Peach (*Prunus persica*) is the fruit most frequently involved in cases of LTP allergy, and peel LTP appears to be more allergenic than pulp LTP in *Rosaceae* fruits (Fernández-Rivas & Cuevas, 1999). This may be ascribed to the more abundant presence of the allergenic protein in the epidermal tissues of the fruit (Carnés, Fernández-Caldas, Gallego, Ferrer, & Cuesta-Herranz, 2002), or to until now, unknown structural differences between peel and pulp LTP.

Several LTP-sensitized patients presented clinical allergy also to other plant-related foods including *Rosaceae*, nuts, peanut, cereals and other fruits and vegetables (Asero et al., 2002; Díaz Perales et al., 2002; Sanchez-Monge, Lombardero, Garcia-Selles, Barber, & Salcedo, 1999).

LTPs are small proteins of 9 kDa consisting of 90–95 amino acids, with pI >8. The typical structure of LTPs is characterised by four disulphide bridges, which contribute to the overall stability of these proteins against thermal denaturation or enzymatic digestion (Asero et al., 2000; Asero et al., 2001; Cavatorta et al., 2010).

To date, diverse methods have been reported for LTP purification. All are based on different steps, as follows: cation-exchange chromatography and gel filtration (Pastorello et al., 1999); gel filtration and hydrophobic interaction chromatography (Carnés et al., 2002); or by two steps hydrophobic interaction chromatography (semipreparative and analytical) (Cavatorta et al., 2009).

The aim of our study was the purification of LTP and its characterization by proteomics, in order to find differences that could justify the higher allergenic power of peel LTP, when compared with pulp LTP. To this end, we have analysed four varieties of peaches: Gladys (white flesh), California (nectarine yellow flesh), Plusplus (yellow flesh), Red Fair (nectarine yellow flesh) harvested from a field grown in Southern Italy.

The protocol includes: ethanol wash of dry powder tissue, acid extraction in the presence of acetonitrile and one-step purification of LTP by miniature reversed-phase chromatography. Proteomic study revealed that, differently than in the pulp, peel LTP is methylated in all genotypes.

2. Materials and methods

2.1. Chemicals

All reagents used were of the highest grade and were purchased from Sigma–Aldrich (St. Louis, MO, USA), Carlo Erba (Milan, Italy), Bio-Rad Laboratories (Segrate, Italy) and GE Healthcare (Uppsala, Sweden).

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2.2. LTP extraction from peach fruits

Four peach varieties: Gladys (white flesh), California (nectarine yellow flesh), Plusplus (yellow flesh) and Red Fair (nectarine yellow flesh) were harvested at the end of August 2011, from a field grown in Southern Italy (Azienda Sperimentale, Pantanello, Polignano, Basilicata, Italy). After peel separation, peel and pulps were cut in small pieces and ground into a fine powder using liquid nitrogen, and then stored at -70°C .

Two hundred and fifty milligram of peel or pulp powder were washed twice, in two cycles: the 1st cycle with 1.5 ml ice-cold ethanol (100%), the 2nd cycle with 1.5 ml deionised water. After 2 min incubation, the resuspended samples were centrifuged at $12,000\times g$ for 4 min at room temperature. The final sediments were dried in a speed-vac and dissolved in 150 μl 100% formic acid, with vortexing for 2 min. Then 150 μl of acetonitrile was added and thorough vortexed for 8 min. For each sample, two independent extractions were performed in duplicate. Protein content of the extracts was determined according to the method of Bradford (1976), using the Bio-Rad reagent and bovine serum albumin as standard protein.

2.3. One-step purification of LTP

LTP was purified from either peel or pulp peach extracts by miniature reversed-phase chromatography using ZipTip_{C18} micro pipette tips (Millipore, Bedford, MA, USA) containing 0.6 μl of a standard resin bed volume. The ZipTip was first equilibrated in 100% acetonitrile and then with 0.1% trifluoroacetic acid (TFA) in Milli-Q grade water. For protein binding to ZipTip pipette tips, samples (10 μl) were aspirated and dispensed for 8 cycles. After washing with 0.1% TFA in Milli-Q grade water, LTP was eluted with 4 μl 0.1% (v/v) TFA, 50% (v/v) acetonitrile.

2.4. SDS–polyacrylamide gel electrophoresis

SDS–PAGE was carried out using a 4% stacking gel and 16% running gel. Aliquots of freeze-dried peel and pulp extracts containing 20 μg of proteins were dissolved in the sample buffer of Laemmli (1970) and incubated at room temperature for 30 min. After centrifugation at $13,000\times g$ for 5 min, the supernatants were loaded onto the gel. Electrophoresis runs were performed on the Hoefer SE 600 vertical electrophoresis unit at 4°C first for 30 min at 200 V and then for 5 h at 280 V. The molecular mass of the proteins was estimated by comparison with the low range standard proteins kit of Bio-Rad. The protein bands were visualised by staining with 0.1% Coomassie Brilliant Blue G-250, and gels were analysed using the ImageMaster 1D program (Pharmacia Biotech, Uppsala, Sweden).

2.5. 2-D polyacrylamide gel electrophoresis (2-DE)

Aliquots (25 μg) of freeze-dried LTP purified from peach peel were resuspended with 250 μl rehydration solution containing 7 M urea, 2 M thiourea, 2% (w/v) CHAPS, 60 mM DTT, 0.5% (v/v) IPG buffer, plus a trace of bromophenol blue. IEF (isoelectric focusing) steps were performed on IPG Dry-Strips of 13 cm in linear pH gradient of 3–10 (GE-Healthcare). IPG Dry-Strips were rehydrated with a sample-containing rehydration solution for 10 h at 20°C . IEF was run using an IPGphor unit (Amersham Biosciences) at 20°C for a total of 32,450 V h. After IEF, the IPG-strip equilibration step was carried out for 20 min in 1% (w/v) DTT containing equilibration buffer (6 M urea, 30% glycerol, 2% SDS, 50 mM Tris–HCl, pH 8.8) and then for 20 min in the same solution with 4% (w/v) iodoacetamide plus a trace of bromophenol blue. Proteins were separated in the 2nd dimension (SDS–PAGE) in a 16% (w/v) polyacrylamide gel. Runs were carried out on the Hoefer SE 600 vertical

electrophoresis unit at 4°C first for 30 min at 200 V and then for 5 h at 280 V. Low range standard proteins (Bio-Rad) were used as molecular weight markers. Gels were silver stained, scanned using a ScanMaker 9800 XL-Microtek and imported to the ImageMaster 2D Elite software (Amersham Biosciences).

2.6. Immunoblot

After mono-dimensional SDS–PAGE and 2-DE separations, the bands (or, respectively, the spots) corresponding to purified peel LTP, were transferred for 1 h onto nitrocellulose membrane by semi-dry transfer cell (Bio-Rad), saturated with 0.1 M Tris Buffer Saline (TBS) containing 0.05% Tween 20 and 5% skim milk powder for 2 h, and then incubated overnight with a pool of four sera from peach allergic-patients, positive to skin prick tests (all patients had a clinical history of immediate allergic reactions after peach ingestion). After three washings, blots were incubated for 1 h with goat anti-human IgE peroxidase-conjugated (Sigma) (diluted 1:5000 in saturation buffer) and developed by ECL reagent (GE Healthcare).

2.7. Protein identification by MALDI-TOF mass spectrometry (MS)

Protein bands (or spots) were excised from the gels, destained and digested with trypsin (Shevchenko, Wilm, Vorm, & Mann, 1996). The extracted tryptic fragments were mixed with the matrix solution: 1% (w/v) α -cyano-4-hydroxy-cinnamic acid solution (CHCA), 50% (v/v) acetonitrile, 0.5% (v/v) TFA and analysed by MALDI-TOF MS. Mass spectra were acquired in positive reflectron mode at 20 kV using an Ettan MALDI-TOF Pro mass spectrometer (Amersham Biosciences) equipped with an UV nitrogen laser (337 nm) with delayed extraction mode and low mass rejection. For each spectrum 256 single shots were accumulated. Peptide spectra were internally calibrated using two trypsin peptides ($\text{M}+\text{H}^{+}$ 842.509 (monoisotopic) and $\text{M}+\text{H}^{+}$ 2211.104 (monoisotopic)). Protein identification was performed by the MASCOT search engine (<http://www.matrixscience.com>), against the NCBI nr protein and Swiss-Prot/TrEMBL databases using peptide mass fingerprinting (PMF). The following parameters were used for database search: (1) taxonomy group: *Viridae* plant (green plant); (2) mass tolerance of 0.2 Da; (3) zero missed tryptic cleavage allowed; (4) carbamidomethylation of cysteine (as a fixed modification) and (5) oxidation of methionine (as a variable modification).

2.8. Molecular mass of purified LTP

For the determination of the molecular mass of purified LTP, samples were prepared by mixing 0.5 μl of purified LTP with 0.5 μl CHCA matrix solution. Samples were analysed in duplicate. Mass spectra were acquired in positive linear mode and externally calibrated using the signals corresponding to cytochrome c from bovine heart ($\text{M}+\text{H}^{+}$ 12,231) and hACTH 18–39 ($\text{M}+\text{H}^{+}$ 2,465.191) (Sigma).

3. Results

3.1. Protein extraction

In all four peach varieties analysed, the extracts obtained from peel showed a higher protein content (range: 53–65 μg protein/mg of freeze dried material) than the corresponding pulp extracts (range: 22–32 μg protein/mg of freeze dried material) (Table 1). Both Red Fair extracts showed the highest protein content when compared to the other varieties. Data, corresponding to the mean of two independent extractions ($n=4$), were analysed by one way analysis of variance (ANOVA), and significant differences at

Table 1

Protein and LTP content in peach extracts.

Peach samples		Protein content ($\mu\text{g prot/mg freeze dried material}$)	LTP content ($\mu\text{g/mg freeze dried material}$)
Gladys	Peel	59.1 ± 2.6	16.9 ± 0.8 (28.6%)
	Pulp	26.4 ± 1.3	1.7 ± 0.1 (6.4%)
	Peel/pulp	2.2	9.9
California	Peel	57.1 ± 1.6	15.8 ± 1.1 (27.7%)
	Pulp	25.4 ± 1.2	1.6 ± 0.1 (6.3%)
	Peel/pulp	2.2	9.9
Plusplus	Peel	58.3 ± 3.3	16.3 ± 0.9 (28.0%)
	Pulp	27.1 ± 1.3	2.2 ± 0.2 (8.1%)*
	Peel/pulp	2.2	7.4*
Red Fair	Peel	$65.6 \pm 2.8^*$	19.3 ± 1.3 (29.4%)*
	pulp	$32.6 \pm 1.5^*$	1.8 ± 0.1 (5.5%)
	Peel/pulp	2.0	10.7

Protein and LTP content were determined using the Bradford method. Values are reported as mean $\pm$ SD of two independent experiments performed in duplicate ($n = 4$).* One-way ANOVA was carried out to test for significant differences and results were considered to be statistically significant at $p < 0.05$ (Student's t test).

$p < 0.05$ between the samples were determined by the Student's t -test.

3.2. SDS-PAGE of peach extracts

SDS-PAGE analysis of the peach extracts is shown in Fig. 1. The protein patterns of peel extracts (lanes 1, 3, 5 and 7) were similar to those obtained from the pulp (lanes 2, 4, 6 and 8). In particular, peel extracts showed an electrophoretic profile characterised by the presence of eleven protein bands in the range of 8.5–9 kDa. All lanes (1, 3, 5 and 7) showed a major band at 9 kDa that was identified by MALDI TOF MS analysis as Lipid Transfer Protein (LTP), the major allergen of peach fruit (Pru p 3). For pulp extracts (lanes 2, 4, 6 and 8), the analysis revealed the presence of 14 bands with apparent molecular mass between 8.5 and 9 kDa, with a major band of 3.1 kDa. Densitometric analysis of the gel showed that the LTP band in the peel extracts represented more of the 50% (54–59%) of the total bands intensity with respect to the 5–8% measured in the LTP band of pulp extracts.

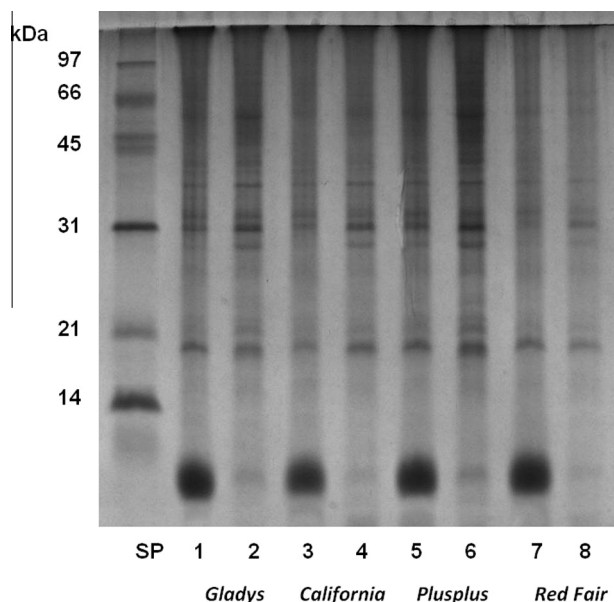


Fig. 1. SDS polyacrylamide gel electrophoresis of peach extracts. SDS-PAGE (16% polyacrylamide) of the extracts obtained from four different peach varieties (Gladys, California, Plusplus, Red Fair). SP: low range standard proteins Bio-Rad, lanes 1, 3, 5 and 7: peel extracts; lanes 2, 4, 6 and 8: pulp extracts. 20 μg of proteins were applied to each lane.

3.3. LTP purification

LTP was purified from peel and pulp peach extracts by miniaturized reversed-phase chromatography using ZipTip_{C18} micro pipette tips (Fig. 2). LTP content in the purified fractions was determined by using the method of Bradford (1976) and expressed as μg of LTP/mg of freeze dried material. As shown in Table 1, LTP content in the eight extracts was as follows: Gladys: peach peel 16.9 and peach pulp 1.7; California: nectarine peel 15.8 and nectarine pulp 1.6; Plusplus: peach peel 16.3 and peach pulp 2.2; Red Fair: nectarine peel 19.3 and nectarine pulp 1.8.

LTP content in peel and pulp extracts were significantly higher in Red Fair and Plusplus extracts, respectively, compared to other peach varieties. LTP percentages were between 27% and 30% of the total protein in peel and between 5% and 8% in pulp, respectively. Overall, LTP was approximately 9.5 times greater in peel extracts than in pulp extracts.

3.4. LTP identification by MALDI-TOF MS and immunoblot analysis

The identification of LTP in the purified fractions obtained from peel and pulp extracts was performed both by MALDI-TOF and IgE immunoblotting analysis. Purified fractions in both lanes 2: gel A for peel and gel B for pulp (Fig. 2) showed only a band of 9 kDa.

For protein identification, bands were excised from the gel and, after reduction and alkylation, were digested with trypsin and applied to MALDI-TOF mass spectrometry. By applying the MASCOT search engine, both bands were found to correspond to peach LTP (Swiss-prot/TrEMBL accession number: Q9LED1). In the mass spectrum obtained from the digested peel-LTP band (Fig. 3A) six peaks were identified as matching peptides covering the 82% of the entire sequence, whereas two peaks (Fig. 3B) covering 29% of the sequence were identified as pulp-LTP band, providing a reliable indication on protein identity.

In the mass spectra obtained from the digested peel-LTP band (A), two mass signals at m/z 1399.57 and 2021.76 were attributed to the methylated peptides 19–32 (GGGAVPPACCNGIR) and 1–18 (ITCGQVSSSLAPCIPYVR), respectively (predicted methylation sites are reported in bold) (<http://web.expasy.org/cgi-bin/findmod/find-mod0.p1>). In fact, peaks at m/z 1399.57 and 2021.76 had indeed masses of 14 Da greater than the calculated masses for peptides 19–32 and 1–18, respectively, indicating the peptides were methylated. The signals were absent in the mass spectra (B) corresponding to the digested pulp-LTP band.

The immunoblotting experiments showed the capability of both purified LTP (Fig. 2, lanes WB) to bind to IgE from pooled sera of peach allergic-patients.

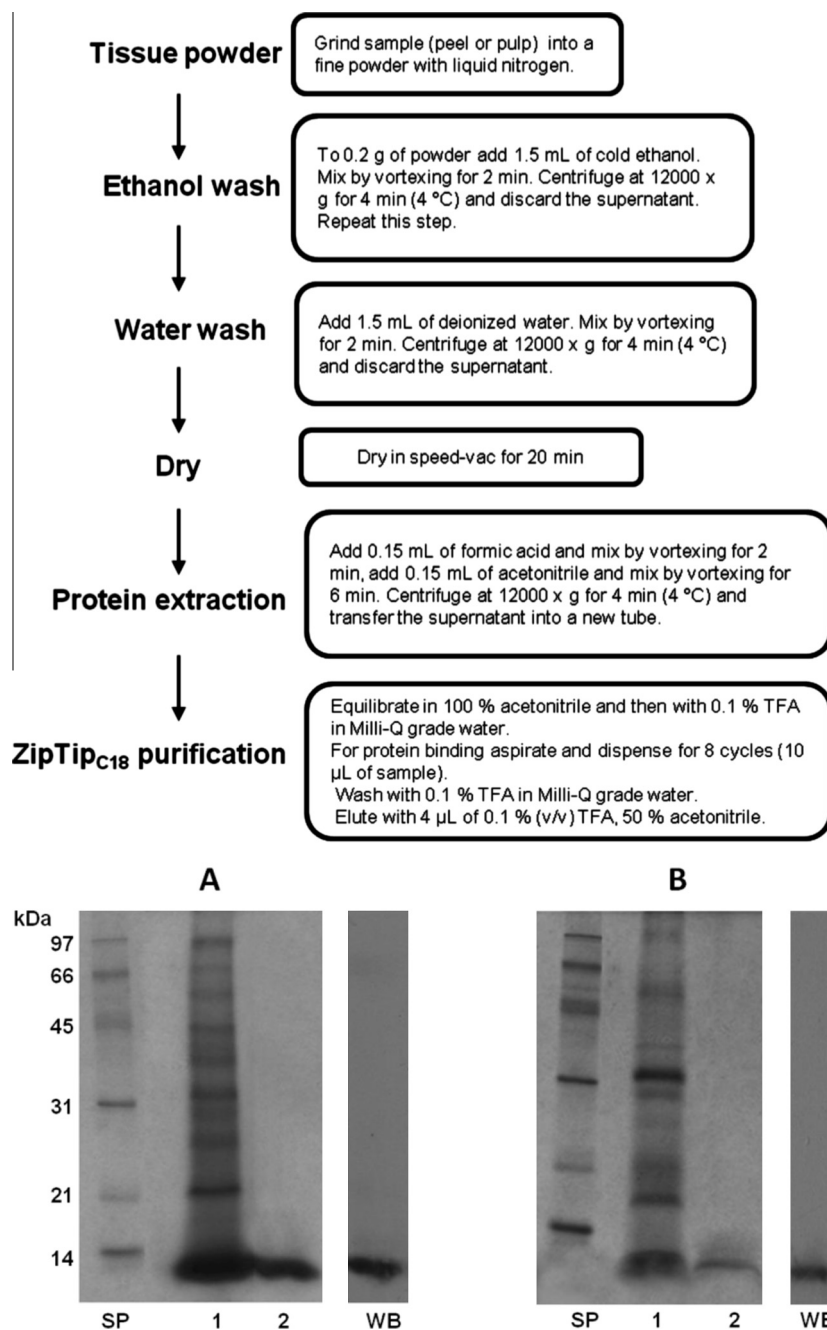


Fig. 2. Procedure scheme of purification of LTP, SDS-PAGE and immunoblot analysis. The protocol includes: ethanol wash of dry powder tissue to remove non-protein contaminants, acid extraction in the presence of acetonitrile and one-step LTP purification by miniature reversed-phase chromatography. The protocol allows LTP purification within 1 h. SDS polyacrylamide gel electrophoresis of peach extract (lanes 1) and LTP purified fractions (lanes 2) obtained from peel (gel A) and pulp (gel B) of Gladys variety. Lanes WB, showed the immunoblot analysis after electro-transfer onto nitrocellulose membrane of purified LTP incubated with a pool of sera. SP: low range standard proteins Bio-Rad.

3.5. 2-DE analysis

In order to assess the purity of LTP fractions, aliquots of LTP Gladys peach peel were analysed by 2-DE, in the first dimension (IEF) according to its pI in linear pH gradient of 3–10, and in the second dimension by its Mr. in a 16% polyacrylamide gel (SDS-PAGE).

In the silver stained gels (Fig. 4) a single spot with apparent molecular mass of 9 kDa and pI of 9.35 was detected. The image has intentionally been over-stained in order to visualise the eventual presence of contaminant spots. In the right panel (WB), is shown the result after electro-transfer to nitrocellulose membrane of the replica gel incubated with a pool of sera.

3.6. Estimation of the molecular mass by mass spectrometry

All peach varieties showed similar mass spectra. Peels spectra were characterised by the presence of two peaks corresponding to LTP, whereas pulp spectra showed an additional signal at m/z 6905. The detection of the same molecular mass indicates the presence of the same protein with identical amino acid sequence in all genotypes. The signals of peel-LTP were much higher than those of the corresponding pulp-LTP.

MALDI-TOF mass spectra of purified peel-LTP showed two peaks of around 9136 and 4569 Da. Fig. 5 shows the spectra of the peel-LTP fraction from the California variety.

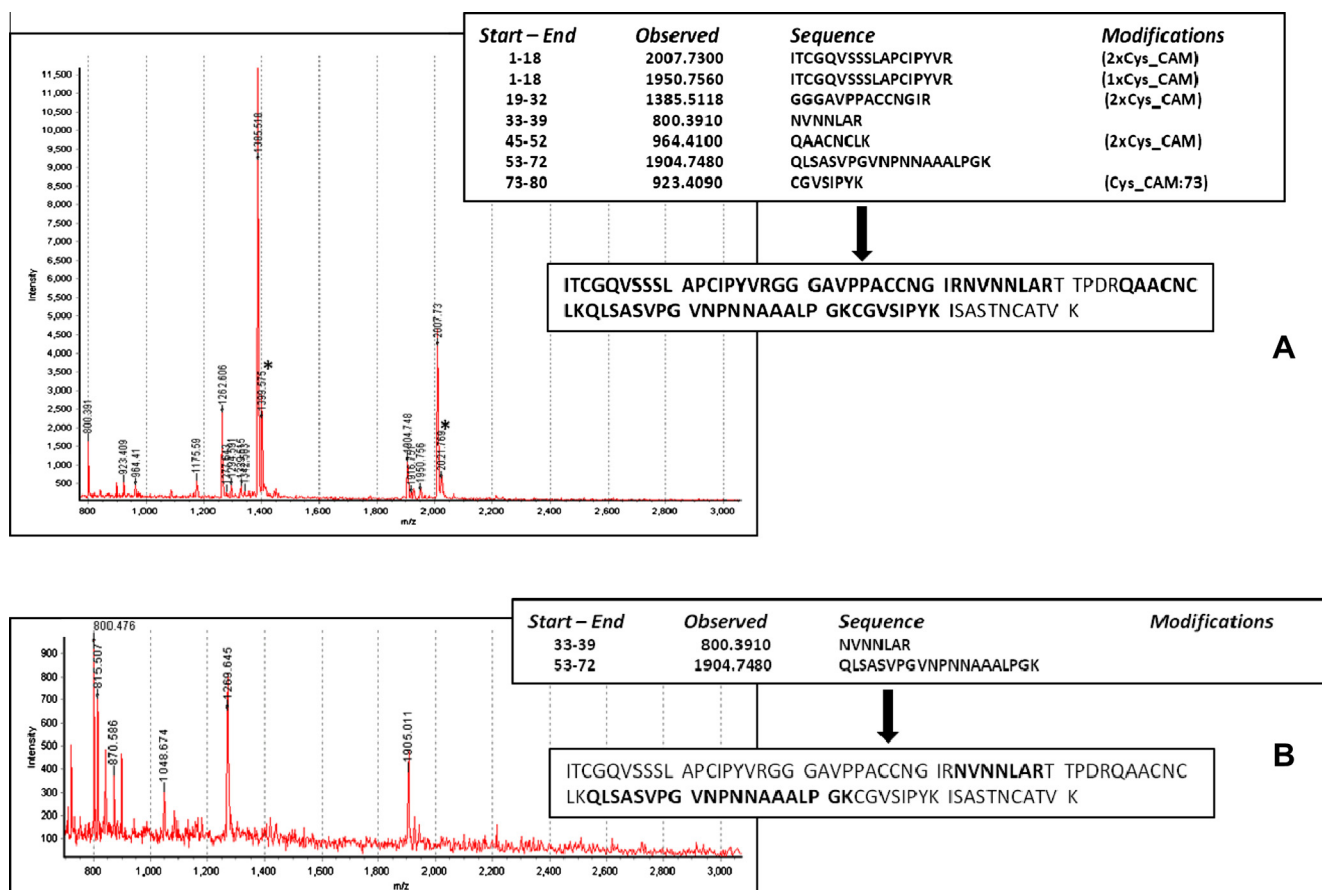


Fig. 3. Identification of purified LTP by MALDI-TOF MS analysis. Bands were excised from the gel, digested with trypsin and applied to MALDI-TOF MS. Panels show the mass spectra, the identified peptides and the covered sequence, corresponding to LTP purified fractions obtained from peel (A) and pulp (B) of Gladys variety, respectively. Protein bands correspond to peach LTP (Swiss-prot/TrEMBL accession number: Q9LED1). Methylated peptides are indicated with asterisk.

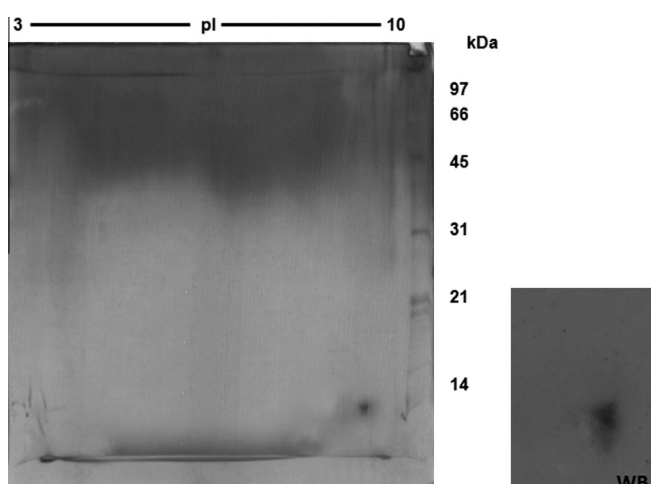


Fig. 4. Bidimensional electrophoresis (2-DE) of purified LTP from peach peel. 2-DE analysis (IEF: linear pH gradient of 3–10; SDS–PAGE: 16% polyacrylamide). Gel was silver stained, 25 µg of purified protein from peel of Gladys variety were applied on the gel. The small panel (WB) represents the result after the transfer on nitrocellulose membrane of the replica gel incubated with a pool of sera.

The mass of 9136 is in accordance with the theoretical mass of the LTP sequence Q9LED1 (Diaz-Perales et al., 2002) with four disulphite bridges, and with the LTP mass measured from Cavatorta et al. (2009), whereas the 4569 Da mass corresponds to the doubly charged peak. Actually, in all LTP spectra obtained from peels, in correspondence of the two peaks, two additional masses of 4583

and 9164 Da were detected, as can be observed in the enlarged image of the spectra showed in Fig. 5 (lower panel). The mass signal at m/z 9164 and 4583 corresponds to LTP masses of 9136 and 4569 Da increased of 28 and 14 Da, respectively. The increased masses were perfectly consistent with a dimethylation of LTP, in agreement with what was observed by peptide mass fingerprint analysis (Fig. 3A).

4. Discussion

In this work we describe an easy, reproducible and rapid procedure for extracting and purifying the LTP allergen for proteomic analysis. The protocol is not expensive and allows LTP purification within 1 h from both peel and pulp of the four different varieties of peaches analysed in this study.

In order to remove interfering agents such as pigments, phenol compounds and carbohydrates, the powdered samples were washed with ethanol. This step is also important for the separation from raw extract of lipids and other hydrophobic contaminants, which may bind to the ZipTip.

The presence of several non-proteic compounds such as phenols, lipids, pigments and carbohydrates in vegetal matrices is a common problem because they may interfere in the analytical procedures. Often complex procedures are needed to remove the interfering agents, and different protocols have been developed to obtain samples suitable for proteomic analysis. Polyvinylpyrrolidone (PVPP) was normally used to reduce amount of phenolic compounds during protein or allergen extraction from vegetable food (Björkstén, Halmepuro, Hannuksela, & Lahti, 1980), and

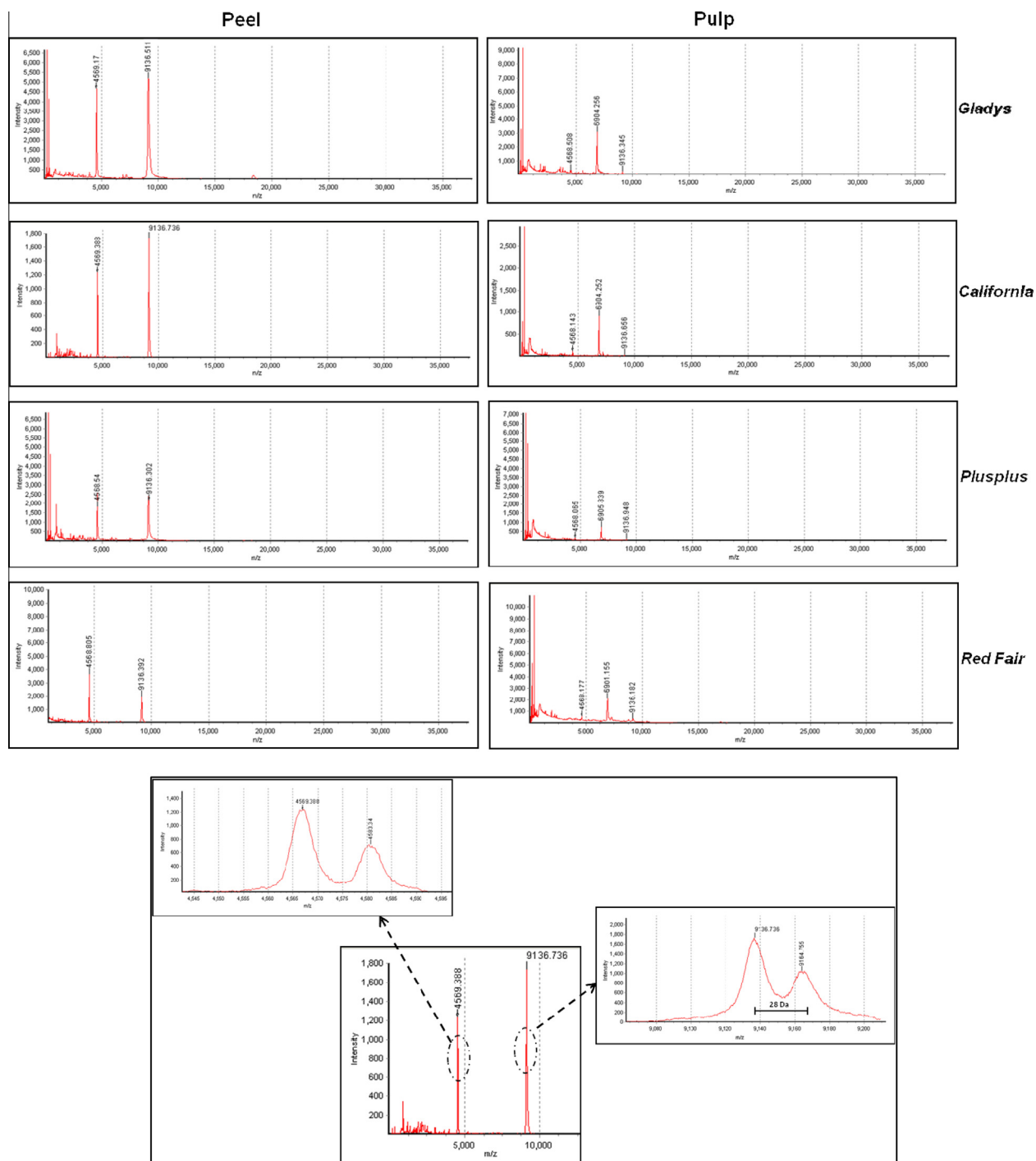


Fig. 5. MALDI-TOF mass spectra of purified LTP. Mass spectra of purified LTP obtained from peel (left panels) and pulp (right panels) of the four genotypes analysed. Spectra were acquired in positive linear mode. In the lower panel is shown the enlarged image of two LTP peaks. In the right panel are shown the mass signals at m/z 9136 and 9164 corresponding to the theoretical monoisotopic mass of LTP and to the dimethylated form, respectively.

several procedures based on TCA-acetone precipitation and phenolic extraction were used for the 2-DE analysis of proteins extracted from fruit (Carpentier et al., 2005; Wang, Vignani, Scali, & Cresti, 2006; Zheng, Song, Doncaster, Rowland, & Byers, 2007).

LTP was purified by extraction with organic acid followed by miniature reversed-phase chromatography using ZipTip_{C18} micro pipette tips. The strategy used for the one-step purification of LTP was based on its small size – LTP is little more than a peptide – and its cationic and hydrophobic nature. The application of ZipTips has proved very useful, because it allows purification and concentration within 1 h, when compared with the previously

applied procedures, based on at least on two chromatographic steps (Carnès et al., 2002; Cavatorta et al., 2009).

The percentages of LTP content found by us in peach peels (27–30% of the total protein), correspond to those described by other authors in other cultivars (Botton et al., 2006; Carnès et al., 2002). These values confirm that LTP content in peel is greater than in pulp, as peel extracts account for a content of LTP, which is about 9.5 times greater than in pulp extracts.

Proteomic analysis, carried out by 2-DE and mass spectrometry, reveals that the purified LTPs are the same in all genotypes, and have identical amino acid sequences independent of the source.

With regard to post-translational modifications, only the presence of four disulphide bridges, useful against thermal denaturation or enzymatic digestion, has been described up to now. Instead, our results indicate for the first time, the presence of two methylated sites on the arginine residues R¹⁸ and R³², in all LTPs purified from peel, independently from the genotype.

Modification of arginine residues in proteins can readily modulate their binding capability and therefore may regulate their biological functions. It may be suggested that the methylation of arginine residues might increase their affinity to aromatic rings and in general to hydrophobic residues. This is consistent with the biological functions of LTP. In fact LTPs are small proteins whose main activity is to facilitate the transfer of fatty acids, phospholipids, glycolipids, and steroids between membranes.

With regard to the allergenic properties of LTP and other dietary proteins in general, the possible effects of arginine dimethylation are still unknown. However, it should be considered that the presence of dimethylarginines in plants and vegetables is only now being reported, both by us in this paper and by Servillo, Giovane, Cautela, Castaldo, and Balestrieri (2013) in their very recent work.

On the other hand, dietary methylated proteins may also have indirect effects. Dimethylarginines may indeed derive from the hydrolysis of dietary methylated proteins, and compete with arginine for nitric oxide synthase (NOS), thus reducing nitric oxide (NO) formation. Inhibition of NOS might increase risk factors, as it has been reported that reduced availability of substrate for the nitric oxide synthase (NOS) isozymes may have a role in asthma airways (Scott et al., 2011). From another point of view, dimethylarginines can be considered as potential donors of methyl groups and this may favours DNA methylation. In conclusion, further studies are required to clarify the role of methylation in LTP and, in general, in protein allergens.

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