Food Chemistry 141 (2013) 2765–2771

Contents lists available at SciVerse ScienceDirect

Food Chemistry

journal homepage: www.elsevier.com/locate/foodchem

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

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ARTICLE INFO

Article history:
Received 18 January 2013
Received in revised form 7 March 2013
Accepted 23 April 2013
Available online 3 May 2013

Keywords:
LTP
Allergens
Protein purification
Reversed-phase chromatography
MALDI-TOF
Methylation

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 (Fernandez-Rivas & Cuevas, 1999). This may be ascribed to the more abundant presence of the allergenic protein in the epidermal tissues of the fruit (Carnés, Fernandez-Caldas, Gallego, Ferrer, & Cuesta-Herranz, 2002), or to until now, unknown structural differences between peel and pulp LTP.

Several LTP-sensitised 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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http://dx.doi.org/10.1016/j.foodchem.2013.04.082

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, Policoro, 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×*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×*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 focussing) steps were performed on IPG Dry-Strips of 13 cm in linear pH gradient of 3–10 (GE-Heathcare). 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 ($M+H^+$ 842.509 (monoisotopic) and $M+H^+$ 2211.104 (monoisotopic)). Protein identification was performed by the MASCOT search engine (http://www.matrixscience.com), against the NCBInr 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) carboamidomethylation 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 ($M+H^+$ 12,231) and hACTH 18–39 ($M+H^+$ 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

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