

Complete Amino Acid Sequence Determination of the Major Allergen of Peach (*Prunus persica*) Pru p 1

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The major protein allergen of peach (*Prunus persica*), Pru p 1, has recently been identified as a lipid transfer protein (LTP). The complete primary structure of Pru p 1, obtained by direct amino acid sequence and liquid chromatography-mass spectrometry (LC-MS) analyses with the purified protein, is described here. The protein consists of 91 amino acids with a calculated molecular mass of 9178 Da. The amino acid sequence contains eight strictly conserved cysteines, as do all known LTPs, but secondary structure predictions failed to classify the peach 9 kDa protein as an 'all-alpha type', due to the high frequency of amino acids (nine prolines) disrupting alpha helices. Although the sequence similarity with maize LTP is only 63%, out of the 25 amino acids forming the inner surface of the tunnel-like hydrophobic cavity in maize ns-LTP 16 are identical and 7 similar in the peach homolog, supporting the hypothesis of a similar function.
Key words: Lipid transfer protein / Primary structure / Rosaceae.

Introduction

Direct amino acid sequencing, with the aim of primary structure determination, is crucial in understanding the biological characteristics of functionally important proteins (Fisher, 1997). This is particularly true in the case of protein allergens, where co- and post-translational modifications, such as glycosylation, or the modification of single amino acids, may be physiologically significant (Hiller *et al.*, 1997; Petersen *et al.*, 1997). Unexpected cross-reactivities at the IgE level between unrelated glycoproteins

are now known to be due to the presence of common epitopes involving the carbohydrate moiety (Batanero *et al.*, 1996; Garcia-Casado *et al.*, 1996; Pike *et al.*, 1997; Fuchs *et al.*, 1997) and are thus not predictable by comparison of the structures obtained by translation of the corresponding genomic sequences. Knowledge of the overall structure of a protein allergen is thus a prerequisite for mapping and characterization of the epitopes involved in antigen recognition by either B cells (i.e. antibodies) or T cell receptors (Atassi, 1984; Chen *et al.*, 1996; Fedorov *et al.*, 1997). Moreover, the use of allergen-derived peptides in specific immunotherapy of type I allergy (Hoyne *et al.*, 1996), as an alternative to antigen/allergen immunotherapy (Hoyne and Lamb, 1996; Weber, 1997), must be based on a knowledge of the native structure of the protein's antigenic determinants (Hiller *et al.*, 1997; Atassi, 1984).

We recently identified the major allergen of peach (Pru p 1) as a 9 kDa protein belonging to the lipid transfer protein family (LTP) (Pastorello *et al.*, 1999). Plant LTPs are proteins of about 90 residues of unknown subcellular location. Since they are able to bind a wide variety of lipids *in vitro* they are assumed to have a role in membrane biogenesis. Some of these proteins show narrow substrate specificities, whereas others are non-specific (Rueckert and Schmidt, 1990). A defensive role for plant non-specific LTPs (ns-LTPs) against bacterial and fungal pathogens has also been proposed (Molina *et al.*, 1993), but very little is known about their allergenicity.

We describe here the complete primary structure determination of the principal protein allergen of peach, achieved by the combination of automated Edman degradation of the native and chemically modified protein and its peptides, and electrospray ionization-mass spectrometry (ESI-MS) techniques.

Results

The N-terminal sequence (32 residues) was determined on the reduced and pyridylethylated 9 kDa protein allergen; it was compared with the most similar amino acid sequences already known for plant LTPs and the sequence strategy planned. All the known sequences showed several basic residues (Arg and Lys), very few acidic residues (Asp and Glu) and rare methionines; except for some Arg and Lys, among the conserved residues useful for protein cleavage at specific peptide bonds there were only one aspartic acid and eight cysteines (forming four disulphide bridges). As a consequence, a tryptic digestion on the reduced and pyridylethylated protein was performed first. The fractions obtained by RP-HPLC separation of the tryptic peptides (Figure 1, panel A) were used for the amino

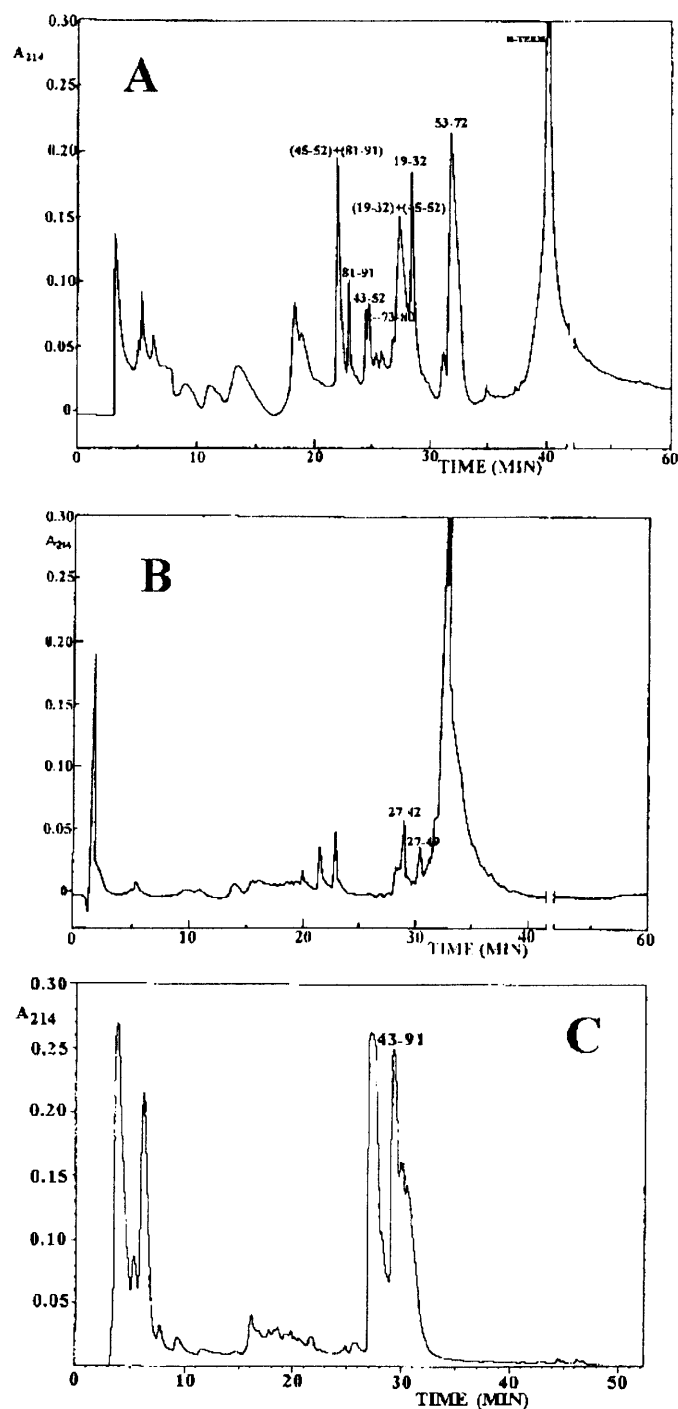


Fig. 1 RP-HPLC Separation of Protein Digests.

Panel A: tryptic peptides; panel B: Asp-N peptides from oxidized protein; panel C: Asp-N peptides from reduced and pyridylethylated protein. Labelled fractions correspond to the peptides indicated in Figure 2.

acid sequence determination; the results are summarized in Figure 2.

Since the protein lacked other common enzymatic cleavage sites and because of the abundance of cysteine residues, further digestion of the protein with endoproteinase Asp-N under oxidative conditions was performed. When reacting with the oxidized protein, this enzyme hy-

drolyzes both X-CysA (cysteic acid) and X-Asp peptide bonds. The fractions obtained by RP-HPLC separation of the Asp-N peptides (Figure 1, panel B) were used for the amino acid sequence determination, but most of the peaks tested were found to be N-terminally blocked. Only the two peaks labelled in Figure 1, panel B gave useful sequence information, but both sequences showed two

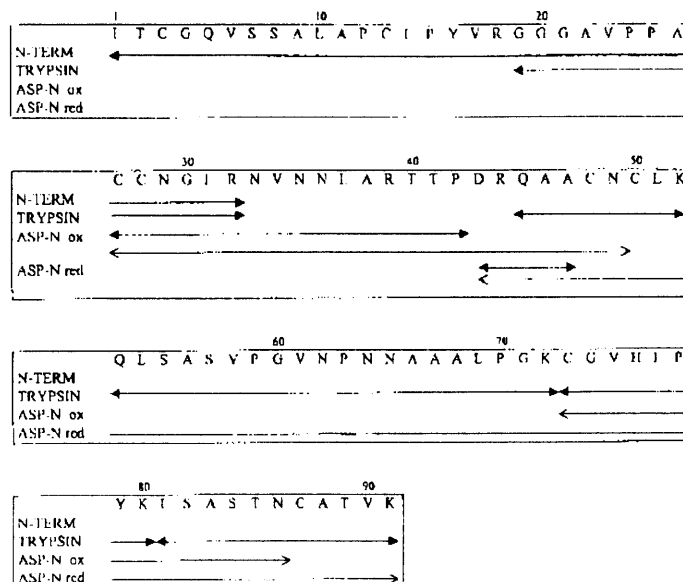


Fig. 2 Amino Acid Sequence of Peach 9 kDa Allergen.

Peptide sequences determined by Edman degradation chemistry are indicated with bold arrows, while those confirmed by mass spectrometry analyses are indicated with thin arrows. The broken line indicates blank cycles in amino acid sequencing reactions. N-TERM: N-terminal sequence determined on the reduced and pyridylethylated protein; TRYP SIN: tryptic peptides sequenced, corresponding to the labelled fractions in Figure 1A; ASP-N ox: sequences of the labelled peptides in Figure 1B, obtained after Asp-N digestion of the oxidized protein; ASP-N red: sequence of the labelled peptide in Figure 1C, obtained after Asp-N digestion of the reduced and pyridylethylated protein.

blank cycles for the first and second residue, followed by a readable sequence that began with residue No. 3 with Asn (Asn₂₉ in Figure 2). The former peak was sequenced to the end (Pro₄₂) while the latter, showing the same amino acid sequence, was stopped after a few residues, and mass spectrometry determination on both peptides was performed. The resulting masses, reported in Table 1, show that they are two different peptides, both beginning at Cys₂₇ but ending at Pro₄₂ and Asn₄₉ respectively, because of an incomplete cleavage at Asp₄₃. From the mass spectrometry calculation on the Cys₂₇-Pro₄₂ peptide, it was deduced that Cys₂₇ and Cys₂₈ were in cystine form, forming one intra-chain S-S bridge. Also in the Cys₂₇-Asn₄₉ peptide, all three Cys residues (Cys₂₇, Cys₂₈ and Cys₄₈) were in the cystine form, leading in this case to a homodimeric molecule with two intra- and one inter-molecular bridge, the latter linking two identical peptides; this was confirmed by mass spectrometry analysis (Table 1). This constellation would also explain the two blank cycles at the beginning of the two sequences, due to two cysteine residues formed under the strong reducing conditions, because of the DTT content in the sequencer's reagents.

To confirm the residues Asp₄₃ and Arg₄₄ by direct sequencing, and to obtain the overlaps at position 72–73 and 80–81, endoproteinase Asp-N digestion on the reduced and pyridylethylated protein was performed. Because of the presence of only one aspartic acid residue at position 43, the digestion was expected to give two peptides (1–42 and 43–91). The peak labelled 43–91 in Figure 1, panel C, was sequenced for a few steps, confirming

Table 1 ESI-MS Analysis of Endoproteinase Asp-N Digest of Peach 9 kDa Protein.

Asp-N peptides	Molecular mass (Da)		Δ Mass measured-expected
	measured	expected	
Aox 27-42	1742.36 $\pm$ 0.6	1742.85	-0.49
AoxD 27-49	4999.52 $\pm$ 0.8	5000.36	-0.84
Ared 43-91	5211.50 $\pm$ 1.1	5210.96	+0.54

The Aox peptides derive from the digestion of the oxidized protein while the Ared peptide derives from the digestion of the reduced and pyridylethylated derivative. AoxD is a dimeric form of peptide 27–49 linked by a disulphide bridge on Cys₄₈.

Asp₄₃ and Arg₄₄ in their expected positions. ESI-MS analysis on this peak also confirmed the amino acid sequence obtained by Edman degradation from residue 43 to 91: the mass deduced from the $[M+5H]^{5+}$ ion fitted well with the expected mass (Table 1).

The major allergen extracted from fresh peach peel (*Prunus persica*) is thus a 91 amino acid protein (Figure 2) with a calculated molecular mass of 9178 Da. Neither co- nor post-translational modifications of the protein were detected.

Discussion

Pru p1 is the first LTP allergen from the Prunoideae sub-family to be fully characterized. The structural characteris-

Table 2 Degree of Sequence Identity of Peach 9 kDa Allergen with Other Lipid Transfer Proteins.

Organism	Taxonomy			Peach Identity
ALMOND NLT1 (<i>Prunus dulcis</i>)	Dicotyledoneae	Rosales	Rosaceae	94%
SUNFLOWER (<i>Helianthus annuus</i>)	Dicotyledoneae	Asterales	Compositae	65%
CHICKPEA (<i>Cicer arietinum</i>)	Dicotyledoneae	Fabales	Fabaceae	64%
FRENCH BEAN (<i>Phaseolus vulgaris</i>)	Dicotyledoneae	Fabales	Fabaceae	63%
MAIZE (<i>Zea mays</i>)	Monocotyledonae	Cyperales	Gramineae	63%
COTTON LTP (<i>Gossypium hirsutum</i>)	Dicotyledoneae	Malvales	Malvaceae	61%

PEACH	- I T C G Q V S S A I A P C I P Y V R - - G G G A V P P A C
ALMOND lip1	- I T C G Q V S S N I A P C I P Y V R - - G G G A V P P A C
SUNFLOWER	A I S C G Q V S S S L A P C I S Y L T - - K G G A V P P A C
FRENCH BEAN	A I S C G Q V T S S I A S C I P P I T - - K G G A V P P A C
COTTON	A V T C G Q V T S S L A P C I G Y L T G N G A G G V P P G C
CHICKPEA	A I T C G R V D I A L A P C L G Y L Q - - G G P G P S A Q C
MAIZE	A I S C G Q V A S A I A P C I S Y A R G Q G S G P S - A G C
PEACH	C N G I R N V N N I A R T T P D R Q A A C N C L K Q L S A S
ALMOND lip1	C N G I R N V N N L A R T T P D R Q A A C N C L K Q L S A S
SUNFLOWER	C S G V K S I N S A A K T T P D R Q A A C G C L K S A Y N S
FRENCH BEAN	C S G V R S L N A A A K T T P D R Q V - C N C L K S A A G A
COTTON	C G G I K S L N S A A Q T T P D R Q A A C K C I K S A A A G
CHICKPEA	C G G V R S L N S A A V T T P D R Q A A C N C L K S A A G S
MAIZE	C S G V R S L N N A A R T T A D R R A A C N C L K N A A A G
PEACH	V P G V N P N N A A A L P G K C G V H I P Y K I S A S T N C
ALMOND lip1	V P G V N P N N A A A L P G K C G V N I P Y Q I S P S T N C
SUNFLOWER	I S G V N A G N A A S F P G K C G V S I P Y K I S P S T D C
FRENCH BEAN	I P G F N A N A G I L P G K C G V S I H Y N I S T S T N C
COTTON	I S G I N Y G I A S G L P G K C G V N I P Y K I S P S T D C
CHICKPEA	I S R L N A N N A A A I P G K C V V N I P Y K I S I S T N C
MAIZE	V S G L N A G N A A S I P S K C G V S I P Y T I S T S T D C
PEACH	A T V K
ALMOND lip1	A N V K
SUNFLOWER	S K V Q
FRENCH BEAN	A T I K F
COTTON	N V S K
CHICKPEA	A I R V
MAIZE	S R V N

Fig. 3 Sequence Alignment of Homologous Plant LTPs.

Peach (*Prunus persica*): SW P81402; almond (*Prunus dulcis*): SW Q43017; sunflower (*Helianthus annuus*): SPTREMBL Q39950; french bean (*Phaseolus vulgaris*): SPTREMBL Q24440; cotton (*Gossypium hirsutum*): SPTREMBL Q49200; maize (*Zea mays*): SW P19656; chickpea (*Cicer arietinum*): SPTREMBL Q23758. Secondary structure elements of maize LTP are indicated: bold lines = α -helices; thin lines = turns. Boxed amino acids = amino acids comprising the hydrophobic cavity (Shin *et al.*, 1995) (see text).

tics determined for this LTP of 91 amino acid residues are in line with those of the other LTPs described so far: the primary amino acid sequence determined for maize, castorbean, wheat and rice LTPs have also been shown to consist of 91–95 amino acid residues, with different sequences which, however, contain 8 strictly conserved cysteines, forming 4 disulphide bridges. Furthermore, all these LTPs share some biochemical properties, such as the small size, varying from 7 to 10 kDa, and the basic character, the isoelectric points being around 9. Our peach LTP has a molecular mass of 9 kDa and an isoelectric point above 9 (Pastorello *et al.*, 1999).

The search of data bases for homologies revealed a high degree of similarity with several other plant LTPs, as shown in Table 2. Currently, almond LTPs are the only available protein sequences from the Rosaceae (botanical

family of peach). None of these proteins have been reported to be allergens.

The 3D structures of only a few plant LTPs have been elucidated to date: wheat (Gincel *et al.*, 1994), maize (Shin *et al.*, 1995) and barley (Heinemann *et al.*, 1996). Together with soybean inhibitor they form a new protein family of an 'all-alpha' type. They consist of four α helices connected by three loops (indicated in Figure 3) and a C-terminal tail without a regular secondary structure. In the inner part they have a hydrophobic tunnel-like cavity to accommodate acyl chains.

Secondary structure predictions failed to classify the peach 9 kDa protein as an all-alpha type; this is not surprising in view of the high frequency of amino acids disrupting α -helices in this protein, i.e. four proline residues in the maize protein and nine in the peach protein.

Nevertheless, out of the 25 amino acids forming the inner surface of the tunnel-like hydrophobic cavity in maize ns-LTP (Shin *et al.*, 1995), 16 are identical and 7 similar in the peach homolog (as indicated in Figure 3), supporting the hypothesis of a similar function.

Most LTPs are located extra-cellularly, and this has led to the hypothesis that they play a role in processes involved in the transfer of lipids from the cell wall. For example, LTPs may participate in the biosynthesis of the cutin layer and the surface wax. In line with these findings, even though it is not known where peach LTP genes are mainly expressed, a localization in the epidermis may be postulated; indirect evidence lies in the allergic symptoms that arise in sensitive subjects immediately after skin contact with peaches. Furthermore, since we found a higher concentration of peach LTP in the peach peel than in the pulp, we may suppose this location to be connected with a role in surface composition, and especially with cutin production or defense from environmental factors, as appears to be suggested by recent findings indicating a defensive role for this class of proteins (Garcia-Olmedo *et al.*, 1995; Kader, 1996).

It has been demonstrated that several LTP genes in barley are upregulated in response to infections by various strains of fungal pathogens (Garcia-Olmedo *et al.*, 1995). The idea of a defense-protein role is supported by the finding that LTP mRNA levels in barley leaves increase significantly when inoculated with a fungus (Molina and Garcia-Olmedo, 1993). These properties of LTPs might also be linked to their interaction, as cationic proteins, with fungal membranes; this could be particularly important for peach, whose major pathogens are fungi of the *Candida* genus (Kader, 1997).

Since barley LTPs have recently been successfully used to treat tobacco leaves infected with *Pseudomonas syringae* (Molina and Garcia-Olmedo, 1997), it is possible that LTPs genes might be used to produce more resistant transgenic foods. However, the existence of an LTP acting as a potent allergen, as is the case with this peach LTP allergen, suggests that other LTPs may also act as allergens. Moreover, as an increasing accumulation of molecular and clinical data suggest that structurally related proteins are often responsible for allergic associations, it might be worthwhile to investigate the allergenicity of peach-homologous LTPs, mostly from the *Prunoideae* subfamily, which includes a variety of fruit trees (i.e. cherry, apricot, plum) important in the human diet. Further characterization of these LTPs should determine both their B and T cell epitopes for a better understanding of *in vitro* and *in vivo* cross-reactivity phenomena.

Materials and Methods

Purification of the Protein

Extraction and purification of the peach 9 kDa allergen has been described elsewhere (Pastorello *et al.*, 1999).

Reduction and Pyridylethylation

250 µg of the protein were dissolved in 450 µl of a solution containing 6 M guanidinium chloride, 0.1 M Tris-HCl, 1 mM EDTA, pH 8.3; dithiothreitol (DTT) was added to a final concentration of 2 mM.

The solution was incubated for 1 h at 37 °C under an oxygen-free N₂ atmosphere. 1 µl of 4-vinylpyridine was added and the solution incubated in the dark overnight (O/N) at room temperature (RT).

Oxidation with Performic Acid

1 volume of H₂O₂ (30% w/v) was mixed with 10 volumes of formic acid and the solution left for 1 h at 4 °C. 100 µl of this reagent were added to 200 µl of protein solution and the mixture incubated for 10 min. at RT. The sample was then frozen and dried in a Speed Vac (Labconco, Kansas City, Missouri, USA) concentrator.

Trypsin Digestion

250 µg of reduced and pyridylethylated protein were dissolved in 400 µl of N-ethylmorpholine-acetate pH 8.3; the reaction was performed with trypsin 1% (w/w) (Promega, Madison, WI, USA) (stock solution 1 mg/ml in 0.1 M HCl) in a slow N₂ stream, for 1 h at 37 °C. The procedure was repeated twice to increase the yield. The reaction was stopped by freezing.

Asp-N Digestion

200 µg of protein were dissolved in 200 µl 10 mM Tris-HCl, pH 8. Oxidized protein was used for cleavage at cysteine/aspartic acid sites, while the reduced form was used for cleavage at aspartic acid sites only.

2 µg of endoproteinase Asp-N (Sigma Diagnostic, St. Louis, MO, USA) dissolved in 50 µl 10 mM Tris-HCl, pH 8 were added to the protein solution and allowed to react for 5 h at 37 °C, then stopped by freezing.

Separation of Peptide Mixture by HPLC

Tryptic peptides were separated by RP-HPLC on a C18 column (4.6 mm × 15 cm, Vydac 218TP5415, Hesperia, CA, USA), in 0.1% trifluoroacetic acid (TFA) with a linear gradient of 0–40% acetonitrile (ACN) in 1 h, and 40–70% ACN in 10 min., at a flow rate of 0.7 ml/min.

Asp-N peptides were separated by RP-HPLC on a C18 column (4.6 mm × 15 cm, Vydac 218TP5415), in 0.1% TFA with a linear gradient of 0–70% ACN in 1 h, at a flow rate of 0.7 ml/min.

Amino Acid Sequencing

The intact protein and purified peptides were sequenced on a Perkin Elmer-Applied Biosystems 470A gas-phase sequencer (Foster City, CA, USA) and PTH-derivative amino acids were identified by RP-HPLC (Perkin Elmer-Applied Biosystems 120A).

Mass Spectrometry Determination

The mass spectra were produced by LCMS-QP8000, a single quadrupole mass spectrometer equipped with an electrospray interface coupled to a LC-10Adv series HPLC system (Shimadzu, Kyoto, Japan).

Flow injection analysis was used to check some fractions after their HPLC separation. 15 µl portions of samples of interest were dried under a nitrogen stream; 15 µl of distilled water were added, dried again under a nitrogen stream and reconstituted with 15 µl of carrier solvent (acetonitrile: 1% formic acid = 50/50 v/v). Samples were introduced into the ion source via a Rheodyne injector

at a flow rate of 200 μ l/min. The potential of the electrospray probe was 4.5 kV, the curve desolvation line temperature was 230 °C and its voltage was -4 V; the deflector lens voltage was set at +40 V.

The scan time was 3 s and mass spectra were acquired over the range m/z 250–1950 operating in the scan mode. Control of LCMS-QP8000 and process data were both managed by CLASS-8000 software (Shimadzu, Kyoto, Japan).

Acknowledgement

This work was supported from a grant of European Commission under the program FAIR CT 97-3224.

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Received June 1, 1999; accepted August 20, 1999