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Determination of the primary structure of two lipid transfer proteins from apricot (*Prunus armeniaca*)

A. Conti[a,*], D. Fortunato[a], C. Ortolani[b], M.G. Giuffrida[a], V. Pravettoni[c], L. Napolitano[a], L. Farioli[d], L. Perono Garoffo[a], C. Trambaioli[c], E.A. Pastorello[c]

[a]*Centro Studio Alimentazione Animali, CNR, c/o BioIndustry Park Canavese, Via Ribes 5, 10010 Colleretto Giacosa (Turin), Italy*
[b]*Bizzozzero Division, Riguarda Ca' Granda Hospital, Milan, Italy*
[c]*Allergy Center, Department of Internal Medicine, Ospedale Maggiore IRCCS, Milan, Italy*
[d]*UOOML (Unità Operativa Ospedaliera di Medicina del Lavoro), Milan, Italy*

Abstract

It has been recently demonstrated that the major allergen of apricot is a protein of molecular mass (M_r) 9000 belonging to the family of Lipid Transfer Protein. The aim of this study was the determination of the primary structure of apricot LTP by micro-sequencing and mass spectrometric analyses. Apricot LTP is a 91 amino acids protein like peach and almond LTPs with a sequence identity of 91% and 94%, respectively. Like for the peach LTP, 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 apricot LTP, supporting the hypothesis of a similar function.

Keywords: Food allergy; Apricot; Lipid transfer proteins

1. Introduction

It has been recently demonstrated that the major allergen of apricot is a protein of molecular mass (M_r) 9000 belonging to the family of lipid transfer proteins (LTPs) [1]. The lipid transfer proteins represent a family of defence-related proteins against adverse environmental factors or infections. Their location in the epidermal layer could give reasons both for their defensive biological function and for the allergenic problems they give rise to. In fact, homologous LTPs of M_r 9000 were found to be not only the major allergen of apricot but also of peach [2] and plum [3] which are frequent causes of food allergic reactions at the oral mucosal site such as Oral Allergy Syndrome. The presence of peach LTPs in the fruit peel, from which it has been easily isolated and successively sequenced [4], might well explain the characteristics of those symptoms which appear just few seconds upon the contact.

The determination of the primary structure is still an important step in the allergenic characterization as it allows, by comparing the sequences, to explain or predict in vivo and in vitro cross-reactivities, thus aim of this study has been the determination of the primary structure of apricot LTPs.

2. Material and methods

2.1. Protein purification

Extraction and purification of the apricot M_r 9000

*Corresponding author. Tel.: +39-0125-564-035; fax: +39-0125-564-942.
E-mail address: am.conti@csaapz.to.cnr.it (A. Conti).

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allergen was performed as described elsewhere [1]. Briefly, the apricot extract was prepared according to the method of Bjorksten et al. [5] by using apricot peels homogenized in phosphate-buffered saline (PBS). The concentrated raw extract was then loaded on a Resource S column (column dimensions 6.4×30 mm, particle size 15 μm, pore size 200–10 000 Å; Pharmacia Biotech, Uppsala, Sweden) equilibrated with 50 m*M* sodium acetate buffer, pH 5, and eluted with salt gradient 0 to 1 *M* NaCl in the same buffer. The eluted fraction, which was analyzed by SDS–PAGE, contained the M_r 9000 protein with other high-molecular-mass impurities and, therefore, further resolution was achieved by gel filtration. Cationic-exchange concentrated fractions were separated on a Superdex 75 HR 10/30 column (bed dimensions 300–310×10 mm, bead diameter 13 μm; Pharmacia Biotech) equilibrated and eluted with 50 m*M* sodium acetate buffer, pH 5, 150 m*M* NaCl. The purity of the M_r 9000 fraction was assessed by SDS–PAGE-immunoblotting with sera from patients allergic to apricot, according to the methodology previously reported [1].

2.2. Reduction and carboxamidomethylation

A 200-μg amount of protein was dissolved in 450 μl of a solution containing 6 *M* guanidinium chloride, 0.001 *M* EDTA, 0.1 *M* Tris–HCl, pH 8.3; 0.002 *M* dithiothreitol (DTT) was added and the reduction proceeded under nitrogen, in the dark, at 37°C for 1 h. *S*-Carboxamidomethylation was performed with 0.1 *M* iodoacetamide under the same conditions as for the reduction of the protein.

2.3. Trypsin digestion

The reduced and carboxamidomethylated protein was dissolved in 200 μl of *N*-ethylmorpholine-acetate, pH 8.3; the reaction was performed with 2 μg of trypsin (Promega, Madison, WI, USA) in 10 μl 50 m*M* acetic acid, in a slow nitrogen stream, for 2 h at 37°C. The procedure was repeated twice to increase the yield. The reaction was stopped by freezing.

2.4. Separation of peptide mixture by HPLC

Tryptic peptides were separated by RP-HPLC on a C_{18} column (250×2 mm; Phenomenex, Torrance, CA, USA), in 0.1% trifluoroacetic acid (TFA) with a linear gradient of 0–40% acetonitrile (ACN) in 50 min, and 40–80% ACN in 10 min, at a flow-rate of 0.2 ml/min.

2.5. Amino acid sequencing

The intact protein and purified peptides were sequenced on a Perkin-Elmer Applied Biosystems 492 pulse-liquid sequencer.

2.6. Mass spectrometry determination

Mass spectrometric analyses were carried out using a LCQ Finnigan MAT ThermoQuest (San Jose, CA, USA) ion trap mass spectrometer. An aliquot of fraction obtained in gel filtration was dialyzed against water and dried under a nitrogen stream, then reconstituted with 50 μl of carrier solvent (1% formic acid in 50% methanol). The data were managed by the Xcalibur software (Finnigan MAT ThermoQuest, San Jose, CA, USA).

3. Results

Mass spectrometric analyses of the purified apricot allergen revealed the presence of two components differing in their M_r values by about 1932 (Fig. 1). The higher M_r component had a molecular mass (9170.4) in agreement with the apricot M_r 9000 allergen characterized in a previous paper [1], while the smaller component showed a molecular mass of 7238.0. In agreement with this finding, two N-terminal sequences were obtained from the same sample in native condition. Due to the great difference in the concentration of the two components in the sample (about 80% of the total protein content for the M_r 9000 component and 20% for the M_r 7000 component), it was decided to sequence the two proteins without any further purification.

The sequence of the first 25 N-terminal amino acids of the apricot M_r 9000 allergen, determined on

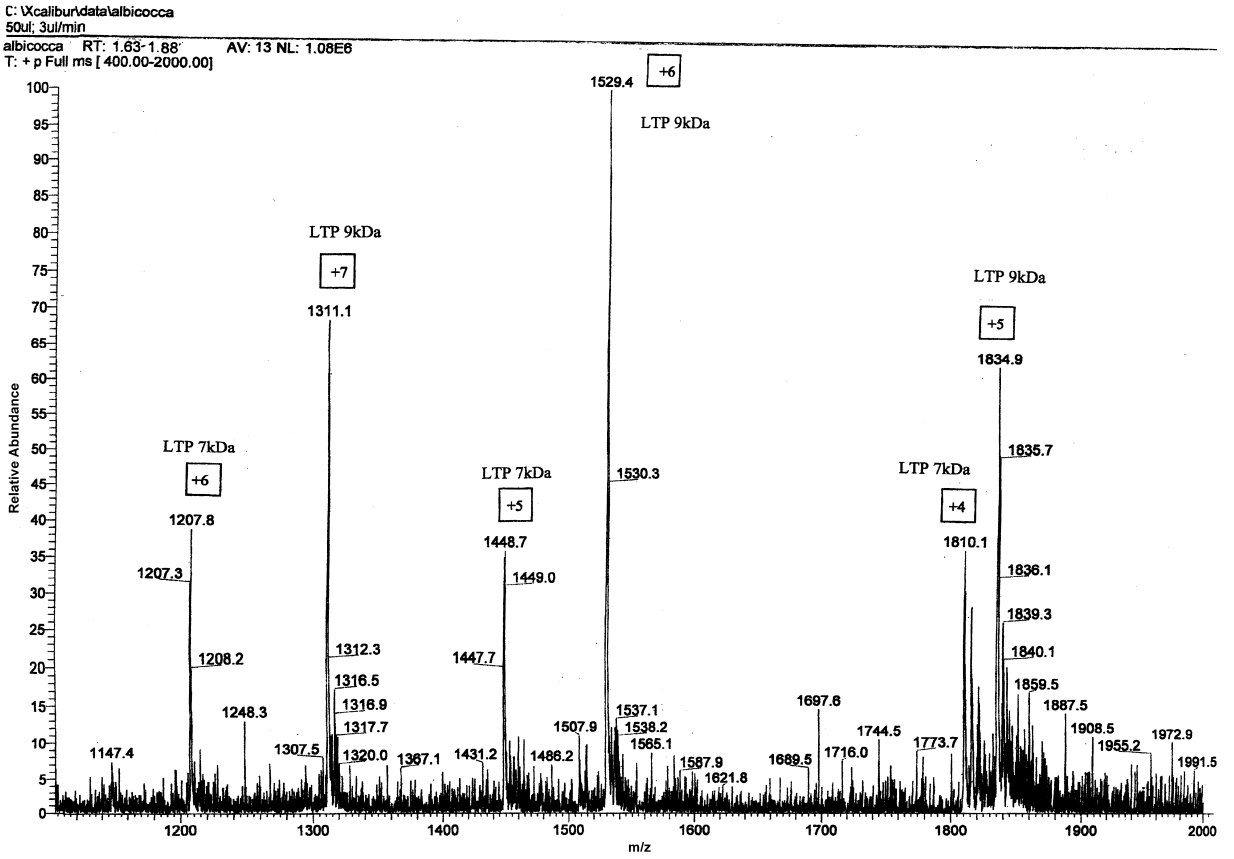

Fig. 1. Electrospray mass spectroscopic analysis of purified apricot sample. Molecular masses of the two components were deduced from the m/z ratio of multiply charged species of each protein.

the reduced and carboxymethylated protein, showed to be 92% homologous with the corresponding peach protein already sequenced [4]. Based on this high degree of homology between the two allergens, just a tryptic digestion of the apricot M_r 9000 allergen was performed to achieve its whole amino acid sequence. Fig. 2 shows the purification of the tryptic peptides obtained by RP-HPLC on a C_{18} column; amino acid sequencing of some of the fractions in Fig. 2 gave, by alignment with the peach homologue, the complete sequence of the apricot M_r 9000 protein (Fig. 3).

The M_r 9000 apricot major allergen resulted in a 91 amino acid protein with a deduced molecular mass of 9170.6.

Table 1 shows the percentage of identity of the apricot M_r 9000 LTP with other LTPs, where the very high degree of homology among the prunoidae fruits is evident, while a minor, but still significant homology, with the LPTs of other botanical families is shown.

A database search for homologues of the peptides of the minor component gave an average identity bigger than 50% with the LTP of *Arabidopsis thaliana* (TrEMBL: Q42158), a 68 amino acid protein with a deduced molecular mass of 7323.6. Referring to the sequence of this protein was it then possible to align the tryptic peptides of the M_r 7000 apricot LTP (Fig. 2), to obtain its complete amino acid sequence (Fig. 4). Like the *Arabidopsis thaliana* LTP, also the M_r 7000a apricot LTP molecule consists of 68 amino acids with a molecular

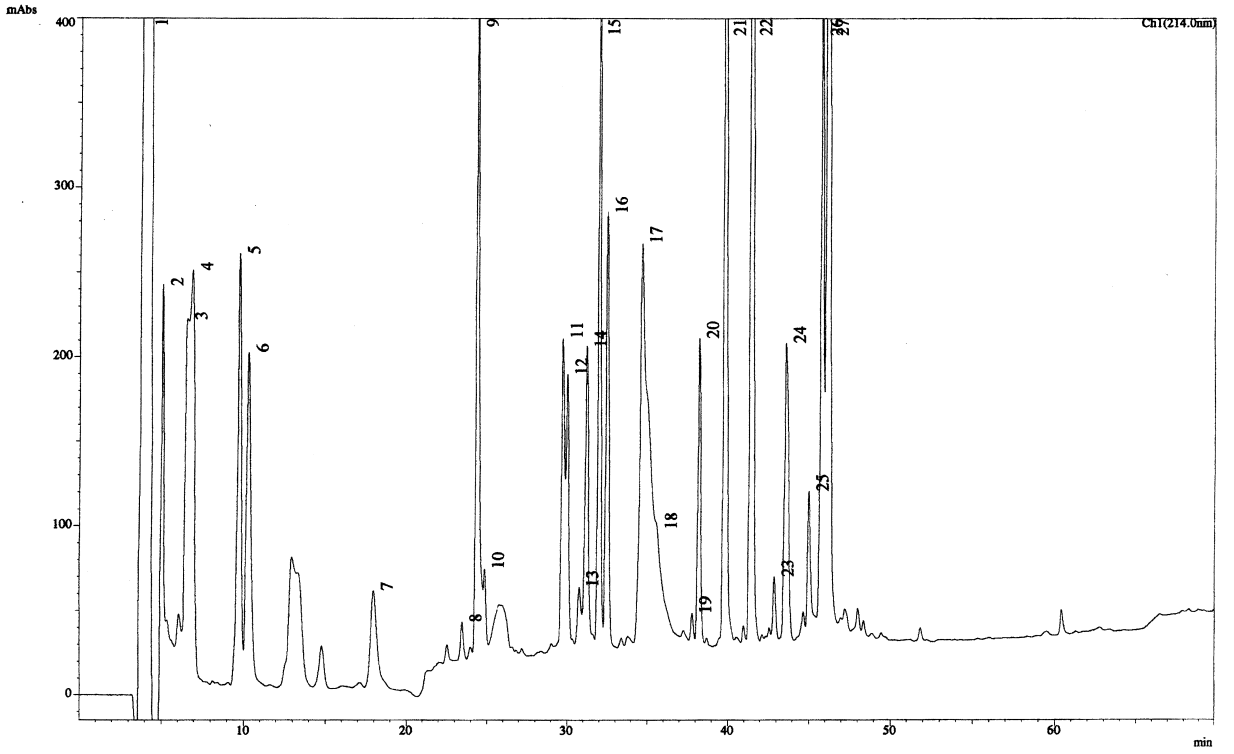

Fig. 2. RP-HPLC separation of tryptic peptides from reduced and carboxamido methylated apricot allergen purified as described in the text. The whole amino acid sequences of the apricot M_r 9000 and 7000 LTPs were obtained by alignment of the sequences of fractions 9 (T40–K91), 11 (T46–K52), 15 (I81–K91), 16 (N33–R39), 17 (G19–R32), 21 (C73–K80), 22 (Q53–K72), 27 (I1–P25) for the M_r 9000 LTP and fractions 14 (N42–R55), 20 (K56–C68), 24 (L29–K41), 26 (V1–K28) for the M_r 7000 protein.

mass of 7237.5 deduced from the amino acid sequence, which is in good agreement with the mass calculated by ESI-MS (7238.0).

Neither for the M_r 9000 nor for the M_r 7000 apricot LTPs were post-translational modifications detected.

	1								10								20								30		
APRICOT	I	T	C	G	Q	V	S	S	S	L	A	P	C	I	G	Y	V	R	G	G	G	A	V	P	P	A	C	C	N	G	I
PEACH	I	T	C	G	Q	V	S	S	A	L	A	P	C	I	P	Y	V	R	G	G	G	A	V	P	P	A	C	C	N	G	I
ALMOND	I	T	C	G	Q	V	S	S	N	L	A	P	C	I	P	Y	V	R	G	G	G	A	V	P	P	A	C	C	N	G	I

	40								50								60										
APRICOT	R	N	V	N	N	L	A	R	T	T	P	D	R	R	T	A	C	N	C	L	K	Q	L	S	G	S	I	S	G	V	N
PEACH	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	V	P	G	V	N
ALMOND	R	N	V	N	N	L	A	R	T	I	P	D	R	Q	A	A	C	N	C	L	K	Q	L	S	A	S	V	P	G	V	N

	70								80								90										
APRICOT	P	N	N	A	A	A	L	P	G	K	C	G	V	N	I	P	Y	K	I	S	A	S	T	N	C	A	T	V	K		
PEACH	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	A	T	V	K		
ALMOND	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	A	N	V	K		

Fig. 3. Amino acid sequence of the M_r 9000 apricot component and alignment of homologous M_r 9000 LTPs from the Prunoidae subfamily. Apricot (*Prunus armeniaca*): SW P81651; peach (*Prunus persica*): SW P81402; almond (*Prunus dulcis*): SW Q43017.

Table 1
Degree of sequence identity of apricot M_r 9000 allergen with other lipid transfer proteins

Organism	Taxonomy			Apricot identity
Almond (*Prunus dulcis*)	Dicotyledoneae	Rosales	Rosaceae	94%
Peach (*Prunus persica*)	Dicotyledoneae	Rosales	Rosaceae	91%
Sunflower (*Helianthus annuus*)	Dicotyledoneae	Asterales	Compositae	65%
French bean (*Phaseolus vulgaris*)	Dicotyledoneae	Fabales	Fabaceae	63%
Maize (*Zea mays*)	Monocotyledonae	Cyperales	Gramineae	63%

4. Discussion

Aim of this study was the determination of the whole amino acidic sequence of the major allergen of apricot. This allergen was already identified and characterized by N-terminal sequence in a previous study [1], in which the purification protocol was assessed and the clinical importance of the allergen was evaluated in depth.

In the present study the results of sequencing showed the presence, in the same chromatographic fraction, not only of the expected M_r 9000 allergen but also of a second M_r 7000 LTP which co-eluted with the allergenic protein and was present in remarkably lower amounts.

The first fraction was corresponding to the major apricot allergen, Pru ar 3 [1], which is the second M_r 9000 LTP purified from a fruit of the Prunoideae subfamily to be fully characterized, after Pru p 3, the major peach allergen [4]. Despite the extremely high degree of homology between these two proteins – 83 identical residues out of 91 – exchanges at positions 15 (G/P), 56 (G/A) and 59 (S/P), respectively, could determine some slight differences in the secondary structure of the two LTPs, being the apricot LTP molecule possibly more flexible. These few molecular differences seem however enough to justify a different rate of sensitisation to peach and apricot. In fact, all the patients allergic to apricot [1] are allergic to peach, while not all peach allergic patients are allergic to apricot [2,6], which seems to be a little bit less allergenic. This could be due to the minor stability of apricot LTP, with respect to the peach LTP. Nevertheless, like for the peach LTP, out of the 25 amino acids forming the inner surface of the tunnel-like hydrophobic cavity in maize ns-LTP [7], 16 are identical and seven similar in the apricot M_r 9000 LTP, supporting the hypothesis of a similar

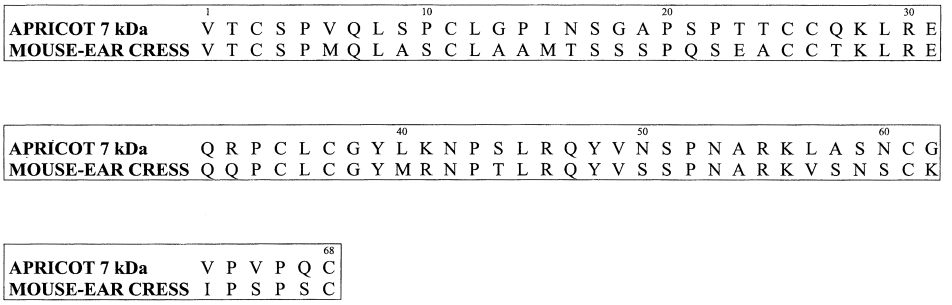

Fig. 4. Amino acid sequence of the M_r 7000 apricot component and alignment with *Arabidopsis thaliana* (mouse-ear cress) LTP (TrEMBL: Q42158). Sequence identity between the two proteins is 60.3%.

function. This role could be defensive as this cavity should hold acyl chains which are involved in cutin layer formation, of which synthesis is stimulated in response to infection by phytopathogens [8]. This internal sequence seems to be very characteristic of the different plant LTPs and thus could also be very important from an immunological point of view.

The second component found in the purified fraction co-eluted with the major apricot allergen resulted in an M_r 7000 LTP not described in the apricot fruit so far. A common feature of the two apricot LTPs here characterized is the presence of eight conserved cysteines with quite a similar distribution along the molecule; Fig. 5 shows a possible alignment of the two molecules based on the eight cysteines: insertions have been added to compensate for the different length of the two proteins. A common origin of the two proteins can be inferred counting 28 amino acids identical and 19 isofunctional.

This second LTP has an average identity greater than 50% with the LTP of *Arabidopsis thaliana*, which was isolated from the seeds of the plant. It is important to note that almost all of the known LTPs are isolated from seeds or from other tissues, above all from the epidermal cell of the aerial portions of the plants [9], while we isolated LTPs from fruit peels of apricot, and also from peels of peach [2], plum [3] and apple [10], thus evidencing the possible specific expression of LTP genes in the epidermal layer of fruits in the Rosaceae botanical family. In *A. thaliana* three different LTP genes encoding three different proteins have been identified, and LTP3 (homologous to the apricot M_r 7000 molecule) was the latest to be discovered in 1999 [11], its biological function being still unknown.

Regarding apricot LTPs, it is important to note that in our previous study concerning the identification and purification of the major allergen of apricot [1] we detected only the M_r 9000a protein; this molecule has specific immunoglobulin E (IgE) binding capacity, while the M_r 7000 protein seems to be not allergenic and its significance is not clear. With regard to this last molecule, different hypothesis can be done, as it could be expressed selectively in some cultivars and not in others, so that a fail in randomization of apricots used in our present study caused an accidental selection of M_r 7000-expressing varieties; it could otherwise be expressed in plants subjected to environmental stress (drought, cold or salt stresses, as reported for barley and tomato [9]) or to pathogens, as it is known that apricot plants are more frequently prone to phytopathogens attacks with respect to other fruit trees. A different temporal expression of some LTP genes in one fruit species, could thus be the result of environmental changes.

5. Nomenclature

SDS–PAGE	Sodium dodecyl sulfate–polyacrylamide gel electrophoresis
M_r	Molecular mass
RP-HPLC	Reversed-phase high-performance liquid chromatography

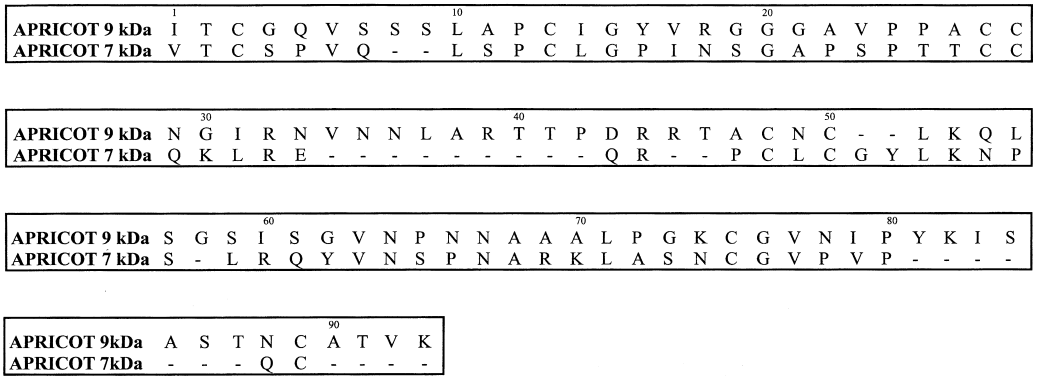

Fig. 5. Amino acid alignment of the two LTPs from apricot fruit.

ESI-MS	Electrospray ionisation mass spectrometry
ns-LTP	Non specific-lipid transfer protein

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