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Crystal Structure of Peach Pru p 3, the Prototypic Member of the Family of Plant Non-specific Lipid Transfer Protein Pan-allergens

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This study describes the three-dimensional crystal structure of a non-specific lipid transport protein (ns-LTP) from *Rosaceae*. Whilst ns-LTPs from species other than *Rosaceae*, such as nuts, cereals, grape, oranges and vegetables are also responsible for plant food allergies, this is less frequent compared with ns-LTPs from *Rosaceae* in the Mediterranean area. In this heterologously expressed peach Pru p3, a ligand is present inside the central cavity of the protein, presumably a fatty acid that was present or produced in the culture medium of the expression organism *Escherichia coli*. Moreover, the two molecules of ns-LTP present in the asymmetric unit bind this ligand in a different way, suggesting a significant degree of plasticity for the peach ns-LTP binding cavity, despite the presence of four disulphide bridges. Two molecules are present in the asymmetric unit: molecule A is a fully liganded protein, while molecule B apparently represents a partially liganded state. Also, molecular dynamics simulation, along with other evidence, suggests that these two molecular conformations represent different states in solution. Comparison of the 3D models of different ns-LTPs justifies the evidence of a high degree of conservation of the putative IgE binding epitopes among proteins of the *Rosaceae* family and the presence of significant amino acid replacements in correspondence of the same regions in ns-LTPs of botanical species unrelated to *Rosaceae*.

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Introduction

Non-specific lipid transfer proteins (ns-LTPs) constitute a family of small (~9 kDa) and ubiquitous plant binding proteins. Their importance in medicine has recently emerged as they represent relevant food allergens, e.g. in the Mediterranean countries. They are characterized by non-specific ligand binding, being capable of binding *in vitro* a broad range of amphiphilic molecules, such as fatty acids,

acyl-coenzyme A, phospholipids and glycolipids.^{1,2} Moreover, they have been named “lipid transfer proteins” due to their ability to transfer lipid molecules between membranes *in vitro*, a property suggestive of a possible involvement of these binding proteins in the intracellular trafficking of lipids. However, this role is not consistent with their secretion and extracellular location in plant tissues.^{1,3} Hence, it has been proposed that other roles are played by ns-LTPs, such as their participation in cutin formation, in which they might function as carriers of acyl monomers,⁴ and in defense mechanisms against phytopathogens.^{3,5,6} These latter roles are consistent with the main location of ns-LTPs in the outer epidermal cell layers of plant aerial organs.^{7,8} Moreover, evidence has been

Abbreviations used: r.m.s.d., root-mean-square deviation; ns-LTPs, non-specific lipid transfer proteins; PEG, polyethylene glycol.

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obtained recently suggesting that plant ns-LTPs may be involved in the process of cell wall extension.⁹

Previous structural studies carried out by X-ray crystallography and NMR on ns-LTPs from cereals have basically shown that the structure of ns-LTPs involves four helices packed against a C-terminal region and is stabilized by four disulfide bridges.^{10–15} This structure type is characterized by a tunnel-like hydrophobic cavity running through the whole molecule and able to accommodate ligands, as shown by NMR and X-ray analysis of ns-LTPs in complex with different compounds, such as phospholipids, acyl-coenzyme A and an array of fatty acids, ranging from C10 to C18.² However, the binding mode of ligands established by NMR has been reported to be different from that determined by X-ray analysis,^{2,11,13,16–18} which suggests that different orientations are possible for hydrophobic ligands bound to plant ns-LTPs. Moreover, evidence has been presented to indicate that the flexibility of the ligand-binding cavity, together with the involvement of non-specific van der Waals interactions with ligand hydrophobic moieties, can account for the lack of binding specificity of plant ns-LTPs.²

Regarding the involvement of ns-LTPs in allergies, several lines of evidence indicate that these proteins represent true food allergens, being responsible for primary sensitization, associated with the formation of specific IgE and the induction of systemic symptoms. These features have been correlated with a high level of structural stability against denaturing agents and the resistance to proteolytic digestion of ns-LTPs, which, due to these stability properties, have the potential to reach the gastrointestinal immune system where immunogenic and allergenic responses can be elicited in allergic subjects.¹⁹ While in the Mediterranean area ns-LTPs present in plant foods may act as primary sensitizers

and are more frequently responsible for plant food allergy, pollen-related food allergies, in which allergens present in pollens are the primary sensitizers, are most commonly found in the populations of Central and Northern Europe (for recent reviews on ns-LTPs as food allergens, see Pastorello & Robino²⁰ and Salcedo *et al.*²¹). Peach ns-LTP (Pru p 3), which is especially involved in plant food allergy in the Mediterranean area, was the first member of the family of ns-LTPs to be recognized as a plant food allergen,^{22,23} thus serving as a sort of “prototype” or reference for the other members of the ns-LTP family subsequently recognized as plant food allergens. In this respect, it should be pointed out that the ns-LTPs present in peach and in the other fruits belonging to the *Rosaceae* family are, in general, major allergens. In addition, the wide distribution of ns-LTPs among plant foods and recent evidence for food allergies induced by ns-LTPs from species distinct from *Rosaceae* indicate that these proteins represent plant pan-allergens.

We report here the crystal structure of peach ns-LTP. The aim of this work was to characterize structurally an ns-LTP distinct from cereal ns-LTPs and representing the prototypic member of a family of pan-allergens. The main focus here is on the structural features of its ligand-binding cavity and of its protein surface, which encompasses the IgE epitopes associated with the allergic response.

Results and Discussion

Structural model of peach ns-LTP

Crystals of peach ns-LTP were obtained in two different conditions, hence two structural models could be obtained: structural model 1 (Table 1) is

Table 1. Statistics on data collection, processing and refinement

	Data set I (Elettra)	Data set II (ESRF)	Xenon anomalous data	Data set III (APS)
Precipitants, conditions	PEG monomethylether 5000 37.5% (w/v) 0.1 M Mes (pH 6.5) ammonium sulfate 0.20 M	Ammonium sulfate 2.3 M, sodium acetate 0.1 M (pH 4.6)	Ammonium sulfate 2.3 M, sodium acetate 0.1 M (pH 4.6)	Ammonium sulfate 2.3 M, sodium acetate 0.1 M (pH 4.6)
Resolution (Å)	44.28–2.21 (2.33–2.21)	51.30–2.47 (2.60–2.47)	42.35–2.80 (2.95–2.80)	44.32–2.35 (2.43–2.35)
Total reflections	106,130 (9527)	129,907 (13,809)	97,314 (14,234)	118,968
Independent reflections	10,732 (1328)	9176 (1296)	6340 (893)	10,082
Multiplicity	9.9 (7.2)	14.2 (10.7)	15.3 (15.9)	11.8 (11.0)
Completeness (%)	87.1 (87.1)	99.9 (99.9)	100 (100)	99.9 (99.5)
$\langle I/\sigma(I) \rangle$	4.6 (1.4)	4.2 (1.7)	5.3 (1.5)	37.8 (4.1)
R_{sym}	0.137 (0.477)	0.138 (0.414)	0.123 (0.488)	0.077 (0.663)
R_{anom}	–	–	0.056 (0.112)	–
	Data set I	Data set II	Data set III	
Resolution range (Å)	51.19–2.30	51.30–2.47	44.32–2.35	
Protein atoms	642	642	642	
Water molecules/sulfate ions/ligands	147/1/3	154/1/3	149/1/3	
$R_{\text{cryst.}} (\%) / R_{\text{free}} (\%)$	20.4/26.2	21.1/26.4	21.6/26.3	
r.m.s. on bond distances (Å)	0.006	0.005	0.009	
r.m.s. on bond angles (deg.)	1.3	1.3	1.4	

Numbers in parentheses refer to the last resolution shell.

that derived from a crystal grown in polyethylene glycol (PEG) monomethylether as precipitant at pH 6.5, while structural model 2 is that derived from a crystal grown at pH 4.6 in the presence of ammonium sulfate as precipitant. Both crystals belong to the same space group $P6_522$ and diffract to a maximum resolution of 2.3 Å. In both forms two independent molecules, labeled A and B, are present in the asymmetric unit. The presence of only minor structural differences between the two models, which are likely to be due to the different crystallization conditions and will be considered later, suggests that the structural details reported here pertain to the protein in solution as well.

As for other structurally characterized ns-LTPs, the main structural motif of peach ns-LTP is represented by an α -helical compact domain, where four helices are connected by short loops (Figure 1). Eight cysteine residues, which constitute about 9% of the entire amino acid sequence of the

protein, form a network of four disulphide bridges (connecting Cys residues 3–50, 13–27, 28–73, 48–87). In addition to disulphide bridges, a large number of main-chain and side-chain intramolecular H-bonds (44 and 46 in molecules A and B, respectively) contribute to the structural stabilization and rigidity of the protein.

In the case of model 1, the electron density for molecule A is very well defined for both main chain and side-chains, with the exception of the -OH group of Ser76 and the side-chain of the C-terminal Lys91. In molecule B, some disorder is found for the side-chains of Arg39, Lys80, Thr85 and Lys91. Moreover, there is a small break of electron density corresponding to the bond C²–C of Pro78 and some disorder for residues 81–82. Superposition of the backbones of molecules A and B gives an average r.m.s.d. of 0.932 Å for C² atoms, which increases to 1.22 Å for all atoms. The most significant differences are present in two regions: residues 52–58 and

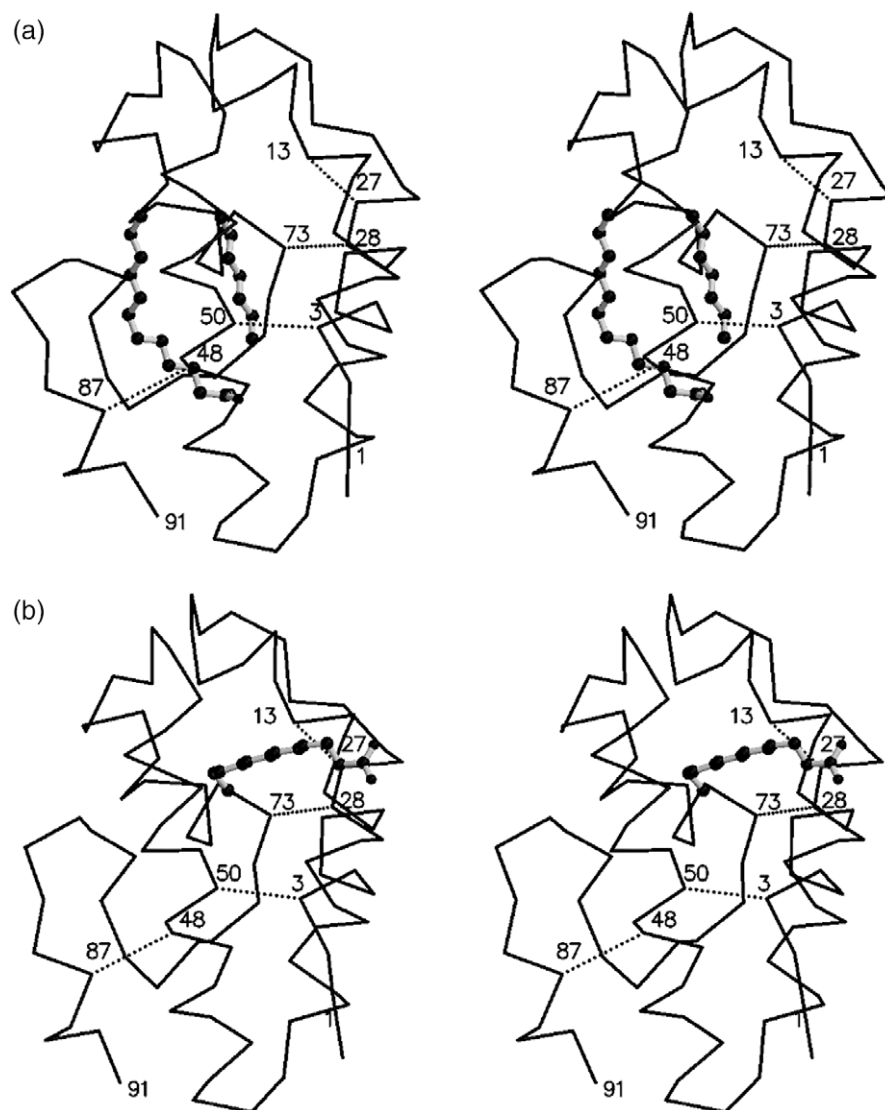


Figure 1. (a) Stereo view of the C^α chain trace of molecule A of peach ns-LTP. The two hypothetical ligands (or the two orientations of the ligand) inside the cavity are shown (ball-and-stick model). The disulphide bridges are denoted by broken lines. (b) Same as (a), for molecule B.

76–85. The former corresponds to the final part of the α -helix C and the loop connecting it to helix D, the latter is close to the C terminus, after helix E. In particular, strand 76–85 of molecule B collapses towards the core of the molecule, inducing a reduction in size of the central lipophilic cavity. The different conformation, especially for residues from 78 to 80, can be correlated with differences in ligand binding (see below). In this area, a significant role is played by Tyr79, the side-chain of which is on the external surface in the case of molecule A, whilst in molecule B it is rotated and points towards the interior of the cavity, occupying part of the space of the ligand bound to molecule A (Figure 1, and see next paragraph).

A very similar situation is present in model 2. In fact, the r.m.s.d. for C $^{\alpha}$ atoms are 0.15 Å and 0.17 Å comparing pairs of molecules A and B of models 1 and 2, respectively, and the r.m.s.d. for all atoms are likewise close at 0.28 Å and 0.29 Å. The finding that in the presence of different precipitating agents and at different pH values quite similar situations are obtained for protein molecules A and B supports the hypothesis that two populations of molecules exhibiting different modes of ligand binding (see below) and conformational differences involving Tyr79 are present in solution.

In molecule A two continuous electron densities are visible inside the lipophilic cavity formed by the packing of the four helices. Since their chemical nature can only be hypothesized, two chains were fitted into them, a lauric acid molecule and a linear chain of seven C atoms. The putative lauric acid was oriented with the carboxylic end pointing towards the interior of the cavity, its negative charge being neutralized by the positive Arg44 (Lau O–Arg44 NH₂, 3.8 Å). Since there is no space for two fatty acids of the same length, we believe that only one fatty acid molecule assuming two different orientations is present inside the binding cavity. The second orientation is likely to be represented by the position of the shorter hydrophobic chain we have fitted inside the cavity, whilst the two orientations share the same negatively charged head. The rest of the cavity is lipophilic, being lined by apolar side-chains. The cavity of molecule B is definitely smaller, due to the previously described conformational differences between molecules A and B. It contains only one piece of electron density, revealing a different mode of ligand binding as compared to that of the ligand bound to protein molecule A, and with a length approximately corresponding to that of lauric acid. However in this case the ligand is only partially inserted into the protein cavity, with the carboxylic end totally exposed to the solvent. In the crystal, this negative charge is neutralized by Arg18 of a symmetry-related protein molecule B (Lau O–Arg18 NH₂, 2.6 Å). The same situation for the ligands has consistently been found in several crystals examined at a variety of different synchrotron radiation (SR) sources (ELETTRA, ESRF, SRS and APS) used to achieve the best possible diffraction resolution.

Comparison of the structural models of peach and cereal ns-LTPs

Although all the ns-LTP X-ray structures determined till now are characterized by a very well conserved overall fold, there are significant differences between them, as illustrated by the relatively high r.m.s.d. values for C $^{\alpha}$ atoms between pairs of models (Table 2). In particular, ns-LTPs from rice, barley and wheat form a cluster, with the latter being slightly less similar to the other two. Maize and peach ns-LTPs are most different from the other ns-LTPs and are also different from each other, with the lowest r.m.s.d. for their C $^{\alpha}$ atoms being 3.3 Å. In general, the region that is most different between the different models is around the C terminus, in particular around position 79, but some significant differences are also present in other areas.

In maize ns-LTP (PDB ID 1FK1), a lauric acid molecule is bound in the same position corresponding to one of the two orientations of the ligand bound in molecule A of peach ns-LTP. In rice ns-LTP (PDB ID 1UVC) a longer stearic acid molecule occupies the position of the other orientation of the ligand of molecule A and, at the same time, protrudes from the cavity in a way similar to that observed for molecule B. Two much longer ligand molecules are concomitantly present in the cavity of wheat ns-LTP complexed *in vitro* with lyso-myristoyl-phosphatidyl-choline (PDB ID 1BWO): both occupy the cavity and protrude from the surface of the molecule towards the solvent. Inside the cavity, they superimpose on the two orientations of the ligand in molecule A of peach ns-LTP, and the external moiety of one of them mimics the position of the ligand bound to molecule B of peach ns-LTP (Figure 2). The side-chain of Tyr79 (the numbering system used in the following discussion is that of peach ns-LTP) in maize and rice ns-LTPs has the same conformation that we observe in molecule B of peach ns-LTP, i.e. it points towards the interior of the cavity, partially occupying the position held by one of the ligand orientations present in molecule A. In both maize and rice ns-LTPs, in fact, only one ligand is present inside the cavity. In wheat ns-LTP Tyr79 again points towards the interior of the cavity, but the latter is larger compared to peach ns-LTP, since the coil from 76 to 79 is more exposed to the

Table 2. The r.m.s.d. (Å) between corresponding C $^{\alpha}$ atoms of models of ns-LTP whose structure has been determined by means of X-ray diffraction

	Maize	Wheat	Rice	Barley
Peach (A)	3.37	4.24	3.87	3.83
Peach (B)	3.28	4.40	3.95	3.98
Maize	0	5.04	5.14	5.13
Wheat		0	2.25	2.16
Rice			0	1.06
Barley				0

Molecules A and B of peach ns-LTP were compared with molecule A of the other models using the program TOPP.⁴¹

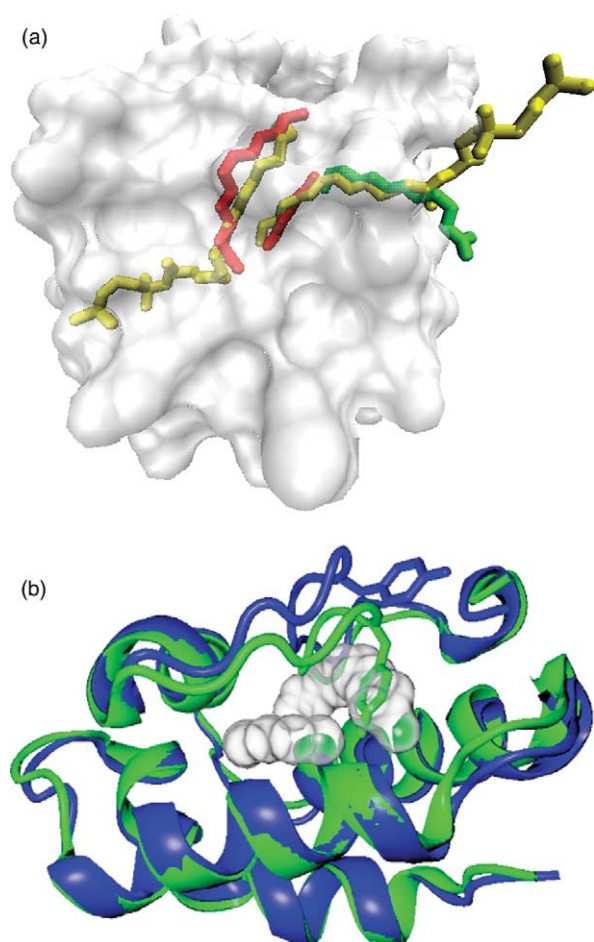


Figure 2. (a) Superposition of the ligands of wheat ns-LTP A molecule (1BWO PDB code) on the ligand of peach ns-LTP A and B molecules. The image shows the following elements: transparent van der Waals surface of peach ns-LTP molecule A; stick view of the peach protein A (red) and B (green) ligands and stick representation of the two wheat ns-LTP lyso-palmitoyl-phosphatidylcholine ligands (yellow). (b) Superposition of ribbon models of molecules A (blue) and B (green). Ligands of molecule A are shown as CPK spheres. The Tyr79 side-chain of molecule B partially occupies the position of one of molecule A ligands (see the text for details).

solvent. Finally, in barley ns-LTP (PDB ID 1MID), whose cavity contains ligands, Tyr79 is oriented exactly as in molecule A of peach ns-LTP. Moreover, in rice ns-LTP (PDB ID 1RZL), whose cavity is empty, Tyr79 points towards the cavity. The different modes of binding of the ligand, the different orientations of Tyr79 and the different size of the cavity that we have established for molecules A and B of recombinant peach ns-LTP represent additional evidence for a high degree of flexibility of the ns-LTP structure corresponding to its binding cavity.

A further demonstration of the conformational difference between molecules A and B is represented by the anomalous data set measured on a crystal previously exposed to a high pressure of Xe gas (see Materials and Methods). An anomalous

Fourier-difference map calculated using the phases of the final model shows the presence of three maxima, greater than 4σ , in the internal cavity of molecule B (Figure 1, Supplementary Data), whilst no maxima are visible inside the cavity of molecule A. The maxima are proximal to hydrophobic zones surrounded by lipophilic side-chains. These three xenon atoms define the hydrophobic cavity of molecule B, whilst they do not fill molecule A, which is already fully occupied by the ligands.

Molecular dynamics of the ligands

In order to check if the position of the fatty acid that we observe in molecule B in our crystal structure does represent a stable or transient state of the molecule in solution, we performed a molecular dynamics simulation, using a protocol described in Materials and Methods. The simulation indicates that the time-evolving structure ensemble, representing the solution conformation of the protein, remains in a state very similar to that of the starting model. During the first 8 ns, the r.m.s.d. calculated over the backbone atoms stabilizes after 2 ns reaching an average of 1.1 Å in the time window between 2 and 8 ns (Figure 3(a)). The lauric acid molecule remains well positioned within its binding site, showing an r.m.s.d. that rapidly settles to about 1.1 Å (Figure 3(a)) for the whole simulation. No water molecules were observed within the hydrophobic cavity throughout the whole simulation.

The major differences observed during the dynamics involve the position of Tyr79. The side-chain of this residue experiences a rotation towards the interior of the hydrophobic cavity. The trajectory of the torsional dihedral angle around the $C^\alpha-C^\beta$ bond oscillates at the beginning of the dynamics (Figure 3(b)), then remains in a position near 58° (10°), compared with the 42° (20°) observed in the crystal structure (molecule B).

Comparison of the pseudo-temperature factors derived from the molecular dynamics (MD) atomic fluctuation motion during dynamics with those obtained from the crystal structure refinement shows a reasonable if not exact agreement. Figure 3(c) shows that the region from Arg18 to Val23, which comprises the loop connecting the first and the second α -helices, exhibits fluctuations significantly larger relative to the experimental B factors determined for the X-ray structure. This discrepancy may be due to the lack in solution of the stabilizing interactions of Arg18 with the carboxylic head of lauric acid bound to the molecule B of a neighbouring asymmetric unit in the crystal, which could be responsible for the destabilization of the whole local structure of the loop. The good match between simulated and experimental averaged B -factors of the lauric acid heavy atoms suggests that the position of the fatty acid within the binding site is also stable under solution conditions. Since the stability of the bound ligand that we have observed might be limited by the short

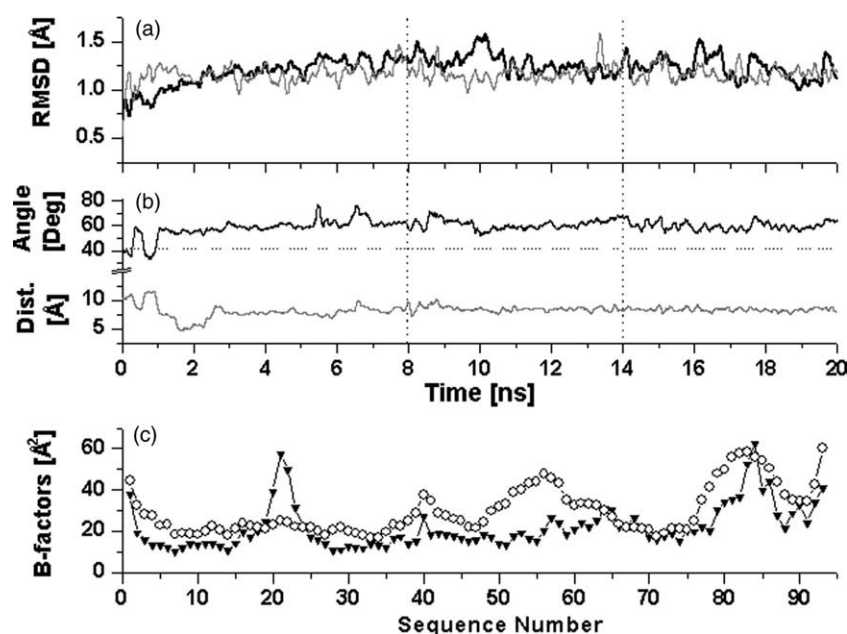


Figure 3. Structural and dynamical data from MD simulation. (a) r.m.s.d. on the backbone atoms of the protein (black) and on the non-hydrogen atoms of lauric acid (grey) against simulated time. The vertical broken line indicates the point at which the temperature was raised by 33 K and 30 K, respectively. (b) Trajectory of the torsional angle around the C^α-C^β of Tyr79 (black) and the distance between the last methyl carbon atom of the lauric acid tail and the C^ε atom of Tyr79 (grey). (c) Comparison between the B-factors of the C^α atoms as obtained from the crystal structure (grey open circles) and from the MD simulation (black triangles). The last point corresponds to the B-factors of lauric acid averaged over all the non-hydrogen atoms.

time-scale explored in the simulation, we supposed that an increase in the temperature could facilitate the release of the ligand. Hence, after 8 ns we raised the temperature of the system by 33 K and kept it at 333 K for 6 ns. Successively, the temperature was raised again by 30 K reaching 363 K and MD simulation was continued for 6 ns up to 20 ns. However, no significant change in the global structure or in the dynamic behavior of both protein and ligand was observed, which further supports the notion of a high degree of stability of the ligand-protein complex (Figure 3).

The dynamics simulation suggests that the two molecules that we observe in the crystal do represent different states in solution and that they are not induced by crystal packing forces. This is supported by other lines of evidence: (a) a similar situation is present in crystals obtained under different crystallization conditions; and (b) a small but significant difference in the main-chain conformation of the protein is induced by the presence of ligands.

Potential IgE-binding epitopes in peach ns-LTP

The high level of amino acid sequence identity (higher than 80%) shared by ns-LTPs from *Rosaceae* fruits together with the high degree of immunological cross-reactivity between *Rosaceae* ns-LTPs^{23–29} strongly suggests the participation of identical or nearly identical ns-LTP IgE epitopes in allergic responses caused by different *Rosaceae* fruits. Regarding the role of ns-LTPs as pan-allergens, several reports have recently appeared in the literature indicating that ns-LTPs from species other than *Rosaceae*, such as nuts,^{30,31} cereals,²⁷ grapes,^{32,33} oranges³⁴ and vegetables,^{35,36} are also responsible, although less frequently as compared

to ns-LTPs from *Rosaceae*, for plant food allergies. The finding of immunological ns-LTP-associated cross-reactivity between peach and plant foods from species other than *Rosaceae*^{26,30,32–38} is consistent with homologous IgE epitopes being shared by ns-LTPs from botanically unrelated species and with ns-LTPs thus being pan-allergens.

Despite the overall structural similarity of ns-LTPs, a significant difference between the various ns-LTPs is represented by their electrostatic potential surface: substantially different patterns of positive and negative charges on the protein surface are present in the known ns-LTP structures. With regard to peach ns-LTP, only the side-chain of Asp43 and the C terminus confer to the surface of this ns-LTP a very limited negative character (Figure 4). Based on a hypothetical 3D structural model of peach ns-LTP, it had been suggested that charged zones on the protein surface may represent potential epitopes.²¹ In particular, it had been proposed that five residues, mostly positively charged, are likely candidates involved in epitope formation: Arg39, Thr40, Arg44, Lys80 and Lys91. The IgE-binding regions of peach ns-LTP have also been explored using a library of 10-mer synthetic peptides, which screened the whole protein sequence.³⁹ That investigation led to the identification of potential epitope zones corresponding to three peptides (Figure 5). The amino acid sequences of these peptides are, in general, very well conserved among the *Rosaceae* ns-LTPs, whilst significant amino acid replacements are present in ns-LTPs from cereals. The same holds for several amino acid residues that are exposed on the protein surface in peach ns-LTP (Figure 5). Moreover, a mutational analysis was performed and the immunoreactivity of site-directed mutants evaluated.³⁹ Whereas the single Lys80Ala and Lys91Ala ns-LTP mutants exhibited an immunoreactivity similar to

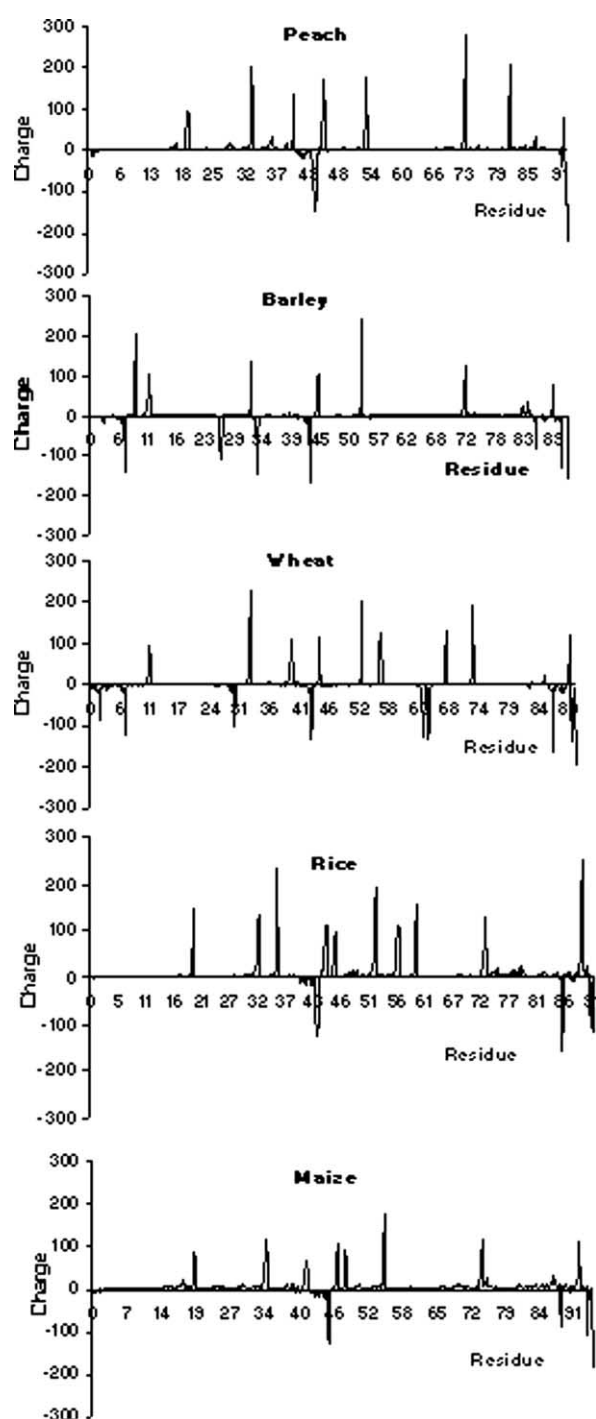


Figure 4. Plots showing the contribution of charged residues to the total charge on the protein surface, for the four ns-LTP structures known. Data were obtained using software GRASP.⁵¹

that of the wild-type protein, a drastic reduction of IgE binding capacity was found for the triple mutant Arg39Ala/Thr40Ala/Arg44Ala, which suggests that the zone encompassing these three latter residues represents a relevant epitope of Pru p 3.³⁹ Residue Arg44 is fully conserved in all the 3D models, but it has a low exposure to the solvent,

being involved in interactions with the carboxy C terminus and other polar side-chains. On the contrary, Arg39 and Thr40, which are highly exposed to the solvent, are well conserved in *Rosaceae* ns-LTPs and only partially conserved in cereal ns-LTPs. Since they are present in an immunoreactive peptide, they are likely to participate in one of the specific IgE epitopes of ns-LTPs (Figure 5).

Materials and Methods

Cloning, expression and purification of peach ns-LTP

The clone containing the EST sequence BU047210 in plasmid pBluescript II SK(-) was purchased from the Genomics Institute of Clemson University (South Carolina, USA). The region of plasmid corresponding to the mature peach LTP coding sequence (276 bp) was PCR-amplified using a thermostable DNA polymerase (MasterTaq DNA polymerase, Eppendorf) and two sequence-specific primers: an NdeI-tailed upstream primer (5'-CATATGATAACATGTGGCCAAGTG-3') and a BamHI-tailed downstream primer (5'-GGATCCTCACTTCACGGTGGC-3'). The amplification product was inserted into the pGEM vector (Promega) to generate the intermediate vector pGEM-LTP. The restriction fragment obtained from NdeI/BamHI digestion of plasmid pGEM-LTP was then ligated into the NdeI and BamHI sites of the expression vector pET11b (Novagen). The resulting plasmid (pET-LTP) was sequenced on both strands and electroporated into *E. coli* BL21 Origami (DE3) cells. The expression of peach LTP was induced by adding 1 mM isopropyl-1-thio- β -D-galactopyranoside; after an overnight incubation at 28 °C, cells were lysed by 20 bursts (30 s each) of sonication. LTP was purified to homogeneity using a two-step procedure, consisting of cation-exchange chromatography and gel-filtration, with a final yield of approximately 1.3 mg/l of cell culture.

Crystallization and data collection

The purified recombinant peach ns-LTP was concentrated to 11 mg/ml in 20 mM Tris-HCl buffer (pH 7.5), and crystallized by hanging-drop vapour-diffusion. Very small crystals (less than 0.050 mm in each dimension) were originally obtained. After optimizing conditions, crystals of typical size of about 0.15 mm $\times$ 0.15 mm $\times$ 0.10 mm were obtained in one month, at 20 °C, using two different precipitant conditions: (i) 37.5% (w/v) PEG monomethylether 5000, 0.1 M Mes (pH 6.5), 0.20 M ammonium sulphate; and (ii) 2.3 M ammonium sulphate, 0.1 M sodium acetate (pH 4.6). Both conditions produced the same crystal morphology, but the crystals obtained in the presence of PEG were often of slightly better diffraction resolution. Several datasets were collected on single crystals, under cryogenic conditions. The first small crystals available were measured at the ID13 beamline of ESRF (Grenoble, France). When bigger crystals became available, data were measured at ELETTRA (Trieste, Italy, XRD1 beamline), APS (SBC CAT beamline ID19-1, APS, Chicago, US) and SRS (MAD 10, SRS, Daresbury, UK⁴⁰) to obtain the highest possible diffraction resolution afforded by state-of-the-art SR beamlines. In particular at SRS, UK, two data sets were collected at a softer X-ray wavelength of 2.07 Å to optimize the LI anomalous signal from xenon

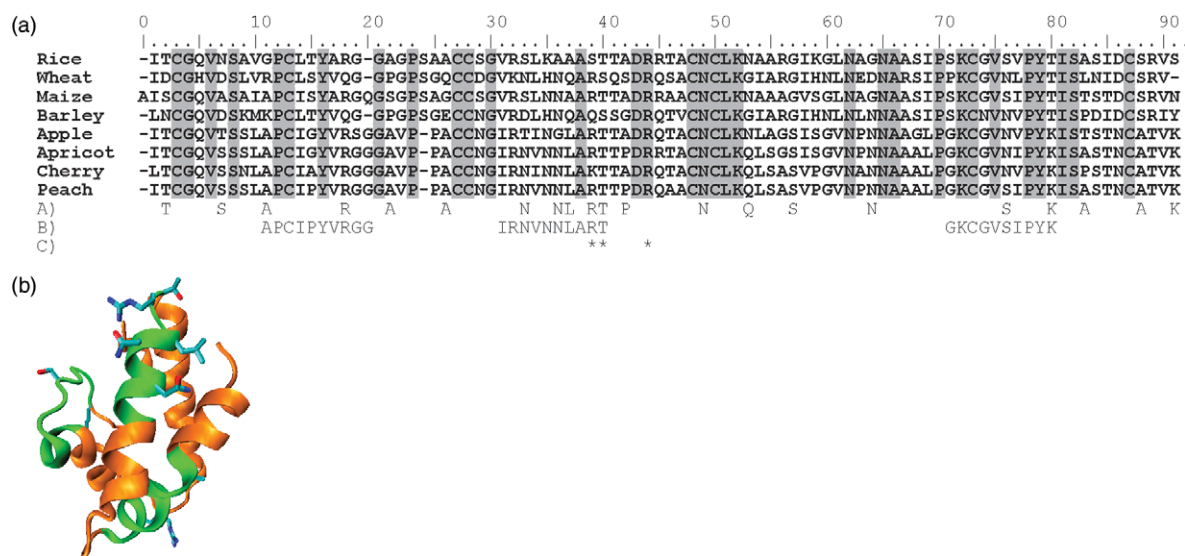


Figure 5. (a) Alignment of the peach ns-LTP amino acid sequence with those of other *Rosaceae* ns-LTPs and of five cereal ns-LTPs whose 3D structures have been determined by X-ray diffraction. Fully conserved residues are shown on a grey background. The numbering for amino acid sequences is that of peach ns-LTP. Bottom lines: A, residues corresponding to the most exposed side-chains, based on the evaluation of the solvent accessibility per residue of our model, performed using a probe radius of 1.4 Å; B, peptides corresponding to three potential epitopes;³⁹ C, the positions of three amino acid residues that have been replaced by A residues in a triple peach ns-LTP mutant are indicated³⁹ (see the text for details). (b) Cartoon view of peach ns-LTP showing the positions of the three immunoreactive peptides³⁹ in the 3D structure (green) and of the exposed amino acid residues (stick representation).

and also enhance the anomalous signal from the sulphur K edge. The xenon derivative was prepared with an Oxford Instrument Xenon Pressure Chamber at a pressure of 20 atm for 15 min.

Data processing and structure determination

The data were processed and scaled with MOSFLM and SCALA software, respectively.⁴¹ Possible space groups were those belonging to trigonal and hexagonal lattices, with $a=b=102.38$ Å and $c=77.30$ Å unit cell dimensions (for differences in cell parameters of different data sets, see Table 1 of Supplementary Data). Systematic absences along the c axis suggested the presence of a 6_1 or 6_5 screw-axis, but other screw axes could not be definitely excluded. Analysis of the self-rotation function, despite indicating the group 622, did not allow a definitive choice between the $P6_122$ and $P6_522$ space groups, both in agreement with the previous analyses. Viable Matthews' volume coefficients were obtained locating either two or three protein molecules in each unit cell, with corresponding solvent contents of 59% or 39%, respectively.⁴² Because of the high level of sequence homology between peach ns-LTP and the other structurally characterized ns-LTPs (for example, peach and maize ns-LTPs share an amino acid sequence identity of 61%), the phase problem was first approached with the molecular replacement technique. Models used as templates were either those of ns-LTP from maize or a hypothetical molecular model of peach ns-LTP built with the Swiss-Model server.^{43,44} However, no reliable solutions were found using either the programs AMoRe⁴¹ or CNS even when attempted in different resolution ranges. Since the ratio between the number of S atoms and the total number of protein atoms is 8/1284, the measurement of the anomalous signal at a softer X-ray wavelength was also attempted. Anomalous data were collected using a

wavelength of 2.07 Å (SRS MAD 10, Daresbury). Unfortunately, the weak anomalous signal coupled with the uncertainty about the correct space group choice and the unknown number of protein molecules per asymmetric unit limited this approach. Anomalous data measured at the Xe edge produced similarly inconclusive results for the structure determination. Finally, a data set at the APS synchrotron SBC Sector undulator ID, was measured, in order to improve the resolution for the native protein (see Table 1 for details about different data sets collected). At the same time, the molecular replacement technique was tested using the software PHASER.⁴⁵ The advantage of the latter program is that the rotation and translation functions can be tested on different space groups with limited intervention, because only the point group needs to be assigned at the outset. Calculations were performed in the resolution range 20–2.5 Å for point groups 312 and 622. An apparently correct solution was found for space group $P3_212$, with four molecules per asymmetric unit. After some refinement cycles with the CNS package the model reached an encouraging $R=0.31$ and $R_{\text{free}}=0.39$ but which plateaued at these values. A visual inspection of this solution showed the presence of a 6-fold screw axis correlating pairs of molecules in the asymmetric unit. A change of origin finally allowed the reduction of the $P3_212$ space group into the $P6_522$ space group, with two molecules in the asymmetric unit.

Structure refinement

After locating the model in the final correct hexagonal space group, the two molecules were optimised in the electron density by rigid body positional minimization. The detailed model refinement was carried out using the CNS package programs.⁴⁶ In the initial stage, the refinement was performed imposing non-crystallographic restraints; they were removed in the final

refinement cycles, since some significant differences were present in the two independent molecules. A total of 146 solvent molecules were added to the model according to H-bond criteria, both by the CNS package and manually. A density inside the internal cavity of the molecule was visible from the beginning, but a ligand was included only in the last cycles of refinement. Because no fatty acids or lipophilic molecules were added during protein purification and crystallization processes, we can assume the presence inside the binding cavity of an hypothetical ligand, originating during the heterologous expression of ns-LTP, and resembling lauric acid according to shape and length of the electron density. A continuous relatively long piece of electron density outside the molecule was interpreted as an ordered PEG fragment. The dihedral torsion angles of all the amino acid residues fall in the most favored (95%) or additionally allowed (5%) regions of the Ramachandran plot. The overall *G*-factor is 0.3. Some other small molecules, besides the protein cavity bound molecules, and other than water, were observed and fitted in the electron density map. In model 1, a PEG monomethylether chain was fitted into a long continuous electron density located between the N-terminal residues of the molecule A and the residues Arg32, Asn35, Asn36 of a symmetry related B molecule. Another large electron density was present, close to the residues Met0, Thr40, Asp43 of molecule A and Pro25 of molecule B. It probably represents another PEG molecule, but its proximity to a crystallographic 2-fold axis made the interpretation difficult and water molecules were set into it. Finally, a sulphate molecule was positioned in a large electron density present in both crystal forms in an external part of the molecule, close to the C terminus of molecule A and two symmetry-related molecules.

Crystal packing

The structure consists of two molecules of peach protein in the asymmetric unit organized around the 6-fold screw axis. This, as one expects, left a large zone close to the axis completely empty. The buried area of each molecule due to the formation of the dimer is 507 Å² (about 10% of the total surface of each molecule), a value relatively high, but not enough to suggest the presence of a dimer in solution. In fact, in solution the protein is eluted as a monomer in gel-filtration chromatography (not shown). It is worth noting that large parts of the molecular surfaces are involved in interactions with other symmetry-related molecules within the crystal; 46% and 63% of the surface of molecules A and B, respectively, are in fact involved in these interactions. In particular, a large buried area of 949 Å² can be estimated for molecule A in its interaction with a symmetry-related molecule B. Nevertheless, none of the hypothetical dimers present in the crystal correspond to that found in the crystals of rice or wheat ns-LTPs.

Computational methods

The starting structure used for MD simulations was that of the isolated ns-LTP with bound fatty acid (lauric acid) corresponding to the protein molecule B determined in this work. MD calculations were carried out with the SANDER module of AMBER 8.0 suite of programs.⁴⁷ The complex was immersed in a simulation box of ~52 Å × 63 Å × 55 Å edges containing 5271 water molecules and six neutralizing Cl⁻ counterions. Periodic rectangular boundary conditions were used. The particle mesh Ewald

summation method⁴⁸ was used for long-range electrostatic interactions. A 10 Å cutoff was adopted for short-range non-bonded interactions. A dielectric constant of 1 was assumed. All chemical bonds were constrained using the SHAKE algorithm.⁴⁹ A time-step of 2 fs was used. Temperature and pressure were kept constant at 300 K and 1 atm, respectively, by coupling the system to external baths with coupling constants of $\tau_t=0.5$ ps and $\tau_p=1.0$ ps, respectively. For the protein and the counterions, the AMBER99 force field parameters were used, respectively. The TIP3P water model⁵⁰ was chosen. The parameterization and topology of the lauric acid was generated with the module *Antechamber* of AMBER8 using the *divcon* semi-empirical program. The computational system underwent first energy minimization and gradual heating passing from 0 K to 300 K in 100 ps. After stabilization, 8 ns of MD were performed for analysis. Subsequently, the temperature was raised to 333 K and 6 ns were simulated for a total simulation time of 14 ns. The r.m.s.d. from the starting positions were calculated on the backbone atoms. Pseudo-*B*-factors were calculated from the atomic positional fluctuations of the C α atoms multiplied by $(8/3)\pi^2$. Likewise the pseudo-*B*-factors of lauric acid were averaged over all its non-hydrogen atoms to compare them with the corresponding crystallographic *B*-factor values.

Protein Data Bank accession codes

Coordinates have been deposited at the Protein Data Bank, Research Collaboration for Structural Bioinformatics† for immediate release with accession numbers 2ALG and 2B5S.

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Supplementary Data

Supplementary data associated with this article can be found, in the online version, at [doi:10.1016/j.jmb.2005.11.063](https://doi.org/10.1016/j.jmb.2005.11.063)

† <http://www.rcsb.org/pdb>

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