



Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (*Oryza sativa*)[☆]

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Received 13 May 2002

Abstract

A novel 7-kDa non-specific lipid transfer protein-2 (nsLTP2) has been isolated from rice (*Oryza sativa*) seeds. In contrast to nsLTP1s, few nsLTP2s have been purified and characterized. Complete amino acid sequence of rice nsLTP2 was determined by N-terminal Edman degradation of the intact protein as well as the peptide fragments resulted from trypsin digestions. Rice nsLTP2 consists of 69 amino acid residues with eight conserved cysteines forming four disulfide bonds. The secondary structure of rice nsLTP2 is predominately α -helical as determined by circular dichroism spectroscopy. Cysteine pairings of nsLTP2 have one mismatch at Cys³⁵–X–Cys³⁷ motif compared to nsLTP1. Primary structure analysis of various plant nsLTP2s revealed an interesting conservation of sequence features among nsLTP2 family. © 2002 Elsevier Science (USA). All rights reserved.

Keywords: Novel protein; Plant nsLTP2; Proteolytic digestion; Trypsin; Thermolysin; Protein sequencing; Disulfide bond pattern; Lipid transfer; All α -helical protein

Plant non-specific lipid transfer proteins (nsLTPs) have been isolated from many plants including wheat, rice, barley, maize, peach, and apricot [1]. nsLTPs bind to a variety of lipid molecules and catalyze their transfer across membranes in vitro [2]. nsLTPs also have additional biological functions, including biosynthesis of cutin, involvement in defense against pathogens, and managing abiotic stress conditions imparted by temperature or drought [2,3]. The nsLTP superfamily possesses eight highly conserved cysteine residues forming four disulfide bonds [1,4]. nsLTPs are subdivided into two subfamilies that differ in molecular mass, nsLTP1 (9 kDa), and nsLTP2 (7 kDa) [1].

Three-dimensional structures of nsLTP1 from various sources were determined by X-ray and NMR spectroscopic techniques [5–7]. The common feature of nsLTP1's structure shows a hydrophobic cavity surrounded by four helices connected through disulfide

bonds. The hydrophobic cavity is the binding pocket for lipid or fatty acid molecules [7]. nsLTP1s are well characterized, whereas the structure and functionality of nsLTP2s are poorly understood [8,9]. Only the wheat nsLTP2's lipid transfer activity and disulfide bond pattern have been determined so far [4]. Interestingly, lipid transfer activity of wheat nsLTP2 is higher than that of wheat nsLTP1. Furthermore, the disulfide bond pattern of wheat nsLTP2 is different from that of nsLTP1 but similar to hydrophobic protein of soybean [4].

Although the nsLTP2 gene from rice was cloned, protein has not been purified [10]. In the present study, we report on the purification, primary sequence, disulfide bond pattern, and secondary structure of rice nsLTP2. We have also compared primary structure of rice nsLTP2 with nsLTP2s from different plant sources.

Materials and methods

Rice seeds (*cv. Japonica*) were obtained from the local supermarket. Cationic columns and resins were obtained from Pharmacia. Trypsin and thermolysin were purchased from Sigma chemicals. All the chemicals used were of high quality analytical grade. All experiments were performed at room temperature unless otherwise mentioned.

[☆] Note: The amino acid sequence reported in this paper (TrEMBL: P83210) corresponds to rice species, *Japonica*. The draft sequence of rice genome was published when this paper was under review (Goff et al., Science 296 (2002) 92–100).

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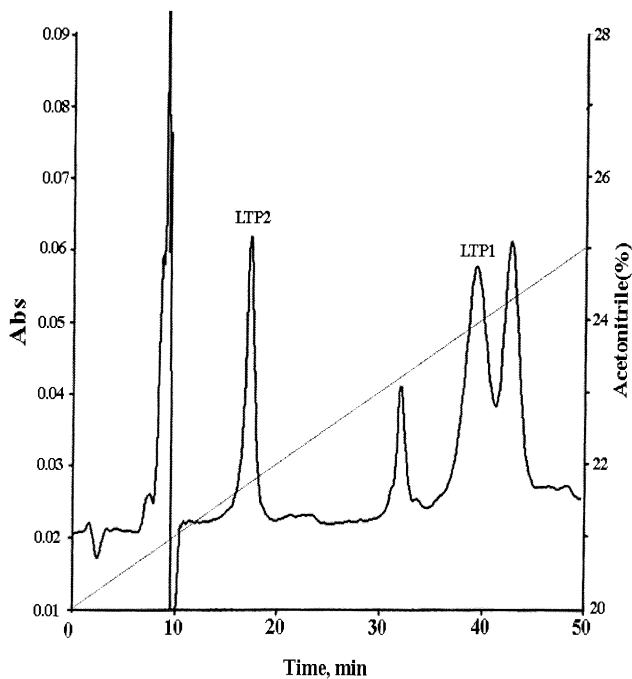


Fig. 1. HPLC profile of the separation of nsLTPs from rice. The absorption was monitored at 220 nm. The sample was eluted with a linear CH₃CN/H₂O gradient (buffered with 0.1% TFA) from 20–25% CH₃CN for 150 min.

Purification of rice nsLTP2. Rice nsLTP2 was purified from rice seeds with procedures similar to the rice nsLTP1 purification [11]. Rice seeds (100 g) suspended in 800 ml of 50 mM sulfuric acid were stirred for 4 h. The suspension was centrifuged at 9000g for 20 min to remove flocculent sediments. Supernatant was kept at 4°C for 12 h, after

adjusting the pH to 7. A clear solution was loaded onto a Sephadex column (C-25), prewashed with two bed volumes of 25 mM sodium phosphate buffer (pH 7.2). Low-affinity proteins were eluted with the same wash buffer. The proteins of interest, eluted with wash buffer containing 1 M NaCl, were desalted and concentrated using an Amicon concentrator. The concentrated protein solution was loaded onto a Source 15S cation exchange column (Pharmacia) to elute nsLTPs using a 0.2–0.3 M NaCl gradient. NsLTPs were separated with a reverse-phase HPLC using C₁₈ semi-preparative column (Cosmosil) to get pure rice nsLTP2. The mobile phase used was acetonitrile containing 0.1% (v/v) trifluoroacetic acid. NsLTP2 was eluted at 20–25% acetonitrile while using linear acetonitrile–water gradient.

Amino acid sequencing. NsLTP2 was reduced and alkylated before the enzymatic digestion for sequence analysis. Two mg of rice nsLTP2 was incubated at 37°C for 60 min in 100 mM Tris buffer (pH 8.7) containing 6 M guanidine hydrochloride, 100 mM dithiothreitol, and 1 mM EDTA. The free thiols groups of nsLTP2 resulted from above reaction were alkylated with 100 mM iodoacetamide, in dark, for 10 min at room temperature. The alkylated protein was purified by RP-HPLC with water:acetonitrile eluent system using a linear gradient of 0–25% acetonitrile in 150 min. To determine amino acid sequence, nsLTP2 was cleaved with proteolytic enzyme, trypsin. The digestion protocol was followed as reported elsewhere except that the incubation time was increased to 7 h [12]. Various peptide fragments were analyzed by Edman degradation methods using MilleGen 6600 protein sequencer. In the case of thermolysin digestion, 10 µg thermolysin was added to 1 mg of native nsLTP2 in 1 ml of 25 mM Tris buffer (pH 7.4) and the digestion was allowed to proceed at 50°C for 2 h. Amino acid compositions of the polypeptide fragments, purified from RP-HPLC, were determined by Pico-Tag method with Waters Picotag amino acid analysis system. The fragments were hydrolyzed in 6 M HCl at 115°C for 24 h. The masses of the polypeptide fragments of nsLTP2 were analyzed using Micromass Quattro Ultima with electron spray ionization (ESI-MASS) method.

Circular dichroism experiments. Circular dichroism spectra were recorded with an Aviv 202 spectropolarimeter using a 1 mm pathlength cuvette. Protein concentrations were determined from the tyrosine

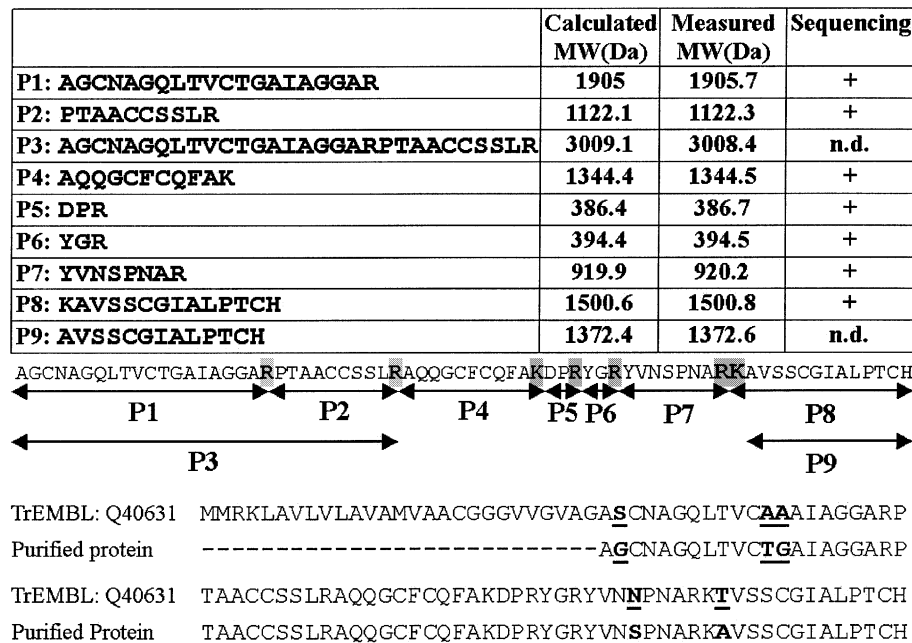


Fig. 2. (A) Tryptic fragments of cysteine-alkylated nsLTP2 and their masses. (B) Amino acid sequence comparison between nsLTP2 purified from rice and the theoretical translational product of nsLTP2 gene in the database.

absorbance at 280 nm in 6 M guanidine hydrochloride [13]. Circular dichroism measurements were performed on 26 μ M protein in 30 mM phosphate buffer (pH 7.2). The CD spectra were average of three scans with a 0.5 nm step size. Fraction of helix (f) was calculated using the equation " $f = [-[\theta]_{\text{obsd}}/40,000(n-4)/n] \times 100\%$ " [14].

Results and discussion

Sequencing of rice nsLTP2

Rice nsLTPs were purified by RP-HPLC using water/acetonitrile eluent system. NsLTP2 was eluted at 21.5% of acetonitrile, whereas two isoforms of nsLTP1 were eluted at 24% of acetonitrile (Fig. 1). Molecular mass of the purified nsLTP2, 7001.8 Da, exactly matched with

theoretical transcriptional product obtained from nsLTP2 gene reported in the database (TrEMBL: Q40631) [10]. The theoretical mass was calculated as 7009 Da with eight cysteines in the free form. Surprisingly, the N-terminal sequencing of purified nsLTP2 showed three mismatches with TrEMBL: Q40631. Thus, the complete amino acid sequence determination of nsLTP2 was necessary. Proteolytic digestion of cysteine-alkylated nsLTP2 with trypsin resulted in nine peptide fragments (designated P1–P9 in Fig. 2). The molecular masses of alkylated peptide fragments were determined by ESI-MASS. Amino acid sequences of the polypeptides fragments were determined by Edman degradation method. Theoretical masses of the polypeptides fragments derived from sequence data matched with exper-

A

	Sequence	Positions	Cys–Cys	MW (Da)
1.	VNSPN	49–53	None	530.1
2.	VNSPNARK	49–56	None	885.5
3.	VNSPNARKA	49–57	None	956.5
4.	YVNSPNARKA	48–57	None	1136.4
5.	YGR or GRY	45–47 or 46–48	None	394.9
6.	YGRY	45–48	None	558.2
7.	IAGGARPT	15–22	None	742.2
8.	AGCNAGQ LRAQQGC	1–7 29–35	3, 35	1392.7
9.	FAKDPR	39–44	None	733.3
10.	AKDPRY	40–45	None	749.3
11.	FAKDPRY	39–45	None	896.5
12.	LTVCTG AACCSS AVSSCG	8–13 23–28 57–62	11, 25, 26, 61	1651.7
13.	LTVCTGA AACCSS AVSSCG	8–14 23–28 57–62	11, 25, 26, 61	1722.9
14.	FCQ IALPTCH	36–38 63–69	37, 68	1148.5
15.	AGCNAGQ LRAQQGCFCQ IALPTCH	1–7 29–38 63–69	3, 35, 37, 68	2523.4

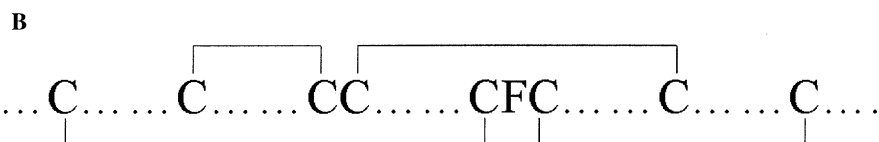


Fig. 3. (A) Biochemical analysis of various polypeptide fragments of rice nsLTP2 obtained from RP-HPLC after thermolysin treatment. (B) Schematic representation of the disulfide bond pattern of nsLTP2.

imental results. Theoretical protein sequence of the reported gene (TrEMBL: Q40631) was used as template to assemble the nine peptide fragments. The amino acid sequence of nsLTP2 was further confirmed by peptide fragmentation studies with another proteolytic enzyme, thermolysin. The sequence of purified nsLTP2 differed at five positions compared to the template (Fig. 2). Except the mutation at position 51, where a well-conserved serine is replaced by an asparagine, the other differences are in non-conserved regions of the polypeptide.

Disulfide bond pattern

The nsLTPs superfamily consists of eight well-conserved cysteine residues forming four disulfide bonds. Trypsin digestion of native nsLTP2 did not resolve the disulfide bond pattern. We therefore used thermolysin to produce 15 different peptide fragments. These peptide fragments were subjected to amino acid composition analysis. The biochemical properties of the fragments purified from RP-HPLC are given in Fig. 3A. Five of these fragments provided useful information on the disulfide bond pattern (Fig. 3A). The disulfide bond pattern of nsLTP2 could be derived as Cys³–Cys³⁵, Cys¹¹–Cys²⁵, Cys²⁶–Cys⁶¹, and Cys³⁷–Cys⁶⁸. Schematic representation of the disulfide bond pattern of nsLTP2 is given in Fig. 3B. Major advantage of using thermolysin is that it cuts at the Cys³⁵–Phe³⁶–Cys³⁷ motif. The two fragments obtained by a cleavage between Cys³⁵ and Phe³⁶ were found in fractions 8 and 14 of Fig. 3A. The disulfide bond pattern in nsLTP2 differs from nsLTP1 at the –CXC– motif. In the case of nsLTP2, central residue of the –CXC– motif, phenylalanine, is buried inside the molecule, whereas the hydrophilic residue of nsLTP1 projects at the surface of the molecule [7]. Since no protease can act on the Cys²⁵–Cys²⁶ motif, it is assumed to obey the pattern of the nsLTP family of proteins [4]. Interproton cross-peaks of various cysteines in 2D NMR NOESY spectrum further confirm the pattern of the disulfide bonds (data not shown). The disulfide bond pattern determined for rice nsLTP2 together with the finding in wheat nsLTP2 substantiates cysteine pairing of the plant nsLTP2 subfamily [4].

Secondary structure of nsLTP2

The CD spectrum of nsLTP2 showed a characteristic α -helical signal, double minima at 222 and 208 nm, and a maximum at 195 nm (Fig. 4). We can assume that nsLTP2 preserves the structural features of plant nsLTP's family, an all α -helix fold stabilized by four disulfide bonds enclosing a hydrophobic cavity. Since molecular size of nsLTP2 is relatively small, the polypeptide chain should be flexible enough to accommodate of lipid molecule. The percentage of helicity of nsLTP2

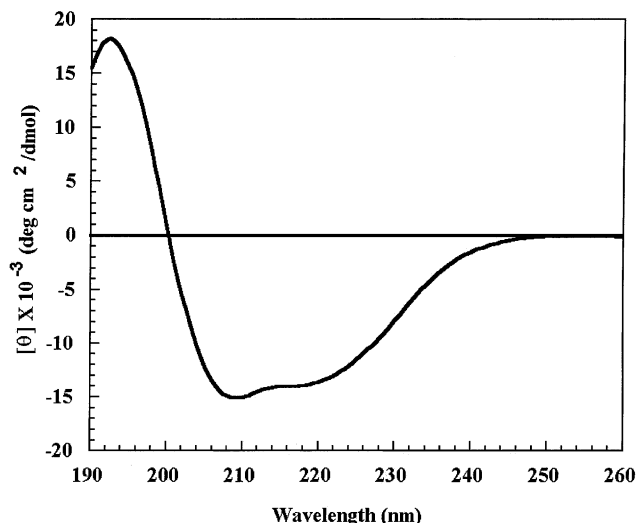


Fig. 4. Far-UV CD spectrum of rice nsLTP2. A solution containing 26 μ M of nsLTP2 in 30 mM phosphate buffer (pH 7.2) was used to record the spectrum at 25 °C using 1 mm path length cuvette.

(37%) measured by its mean residue ellipticity value ($-14,000 \pm 500$ deg cm²/dmol at $[\theta]_{222}$) shows a comparatively less helical content than nsLTP1 (50%). The lower helical percentage of nsLTP2 implies that more of turns and loops are present in the molecule. These turns and loops may help to maintain its biological role of transferring the lipid molecules upon binding to its hydrophobic cavity.

Sequence comparison of rice nsLTP2

The sequence comparisons of rice nsLTP2 with other plant nsLTP2s show 40–60% of sequence identity (Fig. 5). Although sequence similarity is not high, all the nsLTP2s contain eight well-conserved cysteines and a hydrophobic residue at the center of the Cys–X–Cys motif. The proline residues present at positions 22, 44, 52, and 66 are also highly conserved. These prolines may constitute the necessary turns to bring different helices together to fold into its three-dimensional structure. nsLTP1s' binding and transportation activities are facilitated by the presence of the hydrophobic cavity inside the molecule [1]. In the case of LTP2, highly conserved Leu⁹, Ile¹⁶, and Leu³⁰, along with residues Leu³⁷, Phe⁴⁰, Tyr⁴⁹, Val⁵⁰, Leu⁶⁴, and Ile⁶⁶ may form the hydrophobic core. The highly conserved basic residues at positions Arg³¹, Lys⁴², Arg⁴⁸, and Arg⁵⁶ may be helpful in maintaining its biological role such as the membrane recognition or the plant defense activity [1]. A notable difference was observed in the number of glycine residues. It ranges between one and eight for these six plant nsLTPs. The conformationally important glycine residues provide more flexibility to the polypeptide chain, especially to accommodate the backbone constraints imparted by four disulfide bonds.

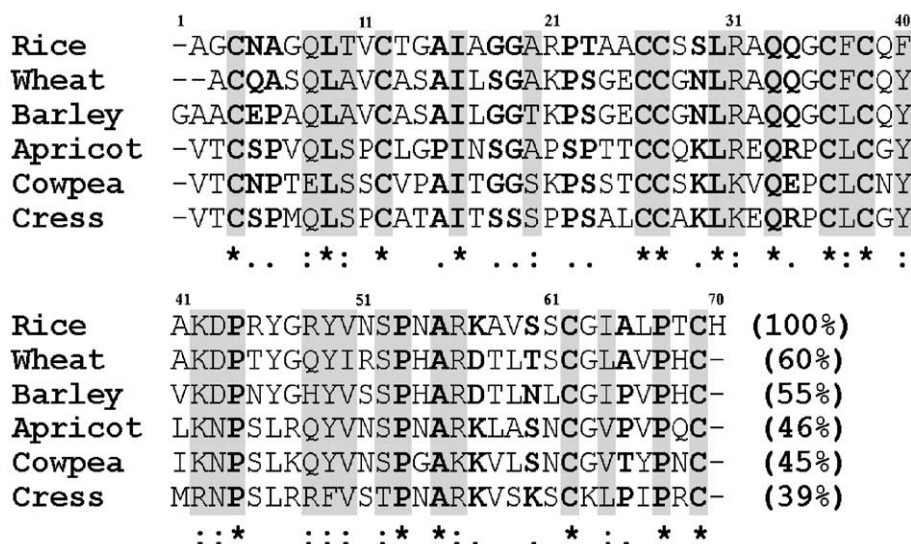


Fig. 5. Sequence comparisons of nsLTP2s from rice, wheat, barley, apricot, cowpea, and mouse-ear cress. The numbering is made to represent every residue in the nsLTP2 family. The symbol "*" denotes identical or conserved residues in all sequences. The conserved substitutions and semi-conserved substitutions are indicated with ":" and ".", respectively. CLUSTAL W program was used for the sequence alignments [15].

Comparative modeling for nsLTP2 is not possible since it shares less than 30% sequence identity with any known structures. The three-dimensional structure determination of rice nsLTP2, currently under progress in our laboratory, will provide new insights into the biological function and significance of nsLTP2. Disulfide bond pattern of plant nsLTP2 subfamily has been substantiated in this report. The influence of various amino acid substitutions at central residue of the -CXC- motif on disulfide bond pattern is presently under study.

Acknowledgments

This work is supported in part by a research grant (NSC-892113M007046) from the National Science Council and Program for Promoting Academic Excellence of Universities grant (89-B-FA04-1-4) from the Ministry of Education, Taiwan. The authors wish to acknowledge the support of the Regional Instrument Center, Department of Life Sciences for the sequencing and composition experiments. We thank Prof. Albert H. Markhart III, Department of Horticultural Science, University of Minnesota for the suggestions and help in proofreading the manuscript.

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