ISSN 0006-2979, Biochemistry (Moscow), 2007, Vol. 72, No. 4, pp. 430-438. © Pleiades Publishing, Ltd., 2007.
Published in Russian in Biokhimiya, 2007, Vol. 72, No. 4, pp. 533-543.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM06-310, February 18, 2007.

# Purification and Primary Structure of Novel Lipid Transfer Proteins from Germinated Lentil (*Lens culinaris*) Seeds

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Received November 23, 2006
Revision received December 18, 2006

**Abstract**—A subfamily of eight novel lipid transfer proteins designated as Lc-LTP1-8 was found in the lentil *Lens culinaris*. Lc-LTP2, Lc-LTP4, Lc-LTP7, and Lc-LTP8 were purified from germinated lentil seeds, and their molecular masses (9268.7, 9282.7, 9121.5, 9135.5 daltons) and complete amino acid sequences were determined. The purified proteins consist of 92-93 amino acid residues, have four disulfide bonds, and inhibit growth of *Agrobacterium tumefaciens*. Total RNA was isolated from germinated lentil seeds, RT-PCR and cloning were performed, and the cDNAs of six LTPs were sequenced. Precursor 116-118-residue proteins with 24-25-residue signal peptides were found, and two of them are purified proteins Lc-LTP2 and Lc-LTP4.

**DOI**: 10.1134/S0006297907040104

*Key words*: lipid transfer proteins, lentil, germinated seeds, primary structure, antibacterial activity, cDNA

During their evolution, plants have developed different mechanisms to protect themselves against pathogens, pests, and other assaults. One of them includes biosynthesis of a suite of pathogenesis-related (PR) proteins specifically induced in response to stress, microbial or viral infections, and abiotic stimuli [1]. The PR-proteins are classified into 14 families comprising such proteins as chitinases, glucanases, endoproteinases, peroxidases, as well as small proteins like defensins, thionins, proteinase inhibitors, and lipid transfer proteins (LTPs) [2].

LTPs are generally small basic proteins divided into two subfamilies with molecular masses of 9 kD (LTP1s) and 7 kD (LTP2s) [3, 4]. They contain eight cysteine residues forming four disulfide bonds. The three-dimensional structure of LTPs has shown the presence of an internal hydrophobic cavity surrounded by four α-helices and a long C-terminal tail [4-8]. Despite common structural features, LTP1s and LTP2s strongly differ from each other.

Biological role of plant LTPs is still obscure, despite the fact that a number of possible functions have been proposed for them [9]. The expression of LTPs can be induced by environmental stress factors, such as pathogen invasion [6], high or low temperature [10, 11], drought, and chemical treatment [12, 13]. LTPs are thought to be involved in plant defense against pathogens [4-7, 9, 14, 15]. LTPs bind to a receptor involved in the control of plant defense responses. Binding and *in vivo* competition experiments showed that the binding sites are common to LTP1s and elicitins, which are known as elicitors of plant defense mechanisms [16].

Indirect proofs have been obtained regarding a possible role of LTPs in the biosynthesis of epicuticular wax, cutin and suberin layers, embryogenesis [6, 9], and pollen adhesion [17]. Recently LTPs were proposed to be involved in recycling of endosperm lipids and in protecting growing cotyledons as inhibitors of proteases released during programmed cell death [18]. The ability of plant

*Abbreviations*: FPLC) fast protein liquid chromatography; LTP) lipid transfer protein; MALDI-TOF-MS) matrix-assisted laser desorption ionization time-of-flight mass spectrometry; PR-proteins) pathogenesis-related proteins; RACE) rapid amplification of cDNA ends; RT-PCR) reverse transcription polymerase chain reaction.

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LTPs to initiate the apoptotic cascade was investigated with mammalian mitochondria. The results show that maize LTP is able to induce cytochrome *c* release from the intermembrane space of mouse liver mitochondria without significant mitochondrial swelling, similarly to mouse full-length Bid, a pro-apoptotic protein of the Bcl-2 family [19].

LTPs have raised increasing interest as drug carriers and delivery systems in connection with the ability of these proteins to transfer phospholipids and bind fatty acids *in vitro* [20-22]. LTPs are identified as major allergens responsible for development of food allergy for vegetables, fruits, nuts, cereals, and other foods [2, 23, 24]. Recent experiments have shown that LTPs may be useful tools in component-resolved diagnosis of food allergy [25].

Plant seeds contain many PR-proteins that are involved in protection of dormant seeds and developing seedlings. We have discovered a subfamily of eight novel LTPs designated as Lc-LTP1-8 in the germinated lentil seeds. Here we describe their purification, complete amino acid sequence determination, and antimicrobial activity evaluation.

## MATERIALS AND METHODS

**Purification of LTPs.** Lentil seeds (100 g) were germinated for three days, homogenized in 300 ml extraction buffer (10 mM $Na_2HPO_4$, 15 mM $NaH_2PO_4$, 100 mM KCl, 2 mM EDTA, 1.5% polyvinylpolypyrrolidone, protease inhibitor cocktail (Sigma, Germany)) and stirred at 4°C for 3 h. Centrifugation (10,000*g*, 50 min, 5°C) was used to clarify the supernatant, which was taken for further purification procedure. Solid ammonium sulfate was added to the supernatant to obtain 75% relative saturation, and the mixture was stored overnight at 4°C. The resulting precipitate was collected by centrifugation (10,000*g*, 50 min, 5°C) and dissolved in 50 mM ammonium acetate with 2 M urea, pH 7.0. The clear solution was loaded onto a Sephadex G-75 column (100 × 2.5 cm) equilibrated with the same buffer. The column was eluted at flow rate 7 ml/h. The fractions were analyzed by Tris-Tricine-SDS-PAGE, and those containing 7-9 kD proteins were pooled and dialyzed against 50 mM ammonium acetate, pH 6.0, for 24 h at 4°C. The resulting solution was loaded onto a Mono S HR 5/5 cation-exchange column (Pharmacia, Sweden). Proteins were eluted with a linear gradient from 50 to 288 mM ammonium acetate (2.8 mM/min), pH 6.0, at a flow rate of 1 ml/min. The LTP-containing fraction was then applied to HPLC on an Altex (USA) system equipped with a Luna $C_{18}$ reverse-phase column (5 μm, 250 × 4.6 mm; Phenomenex, USA) using a linear gradient from 5 to 65% acetonitrile (1.33%/min) with 0.1% TFA at a flow rate of 0.5 ml/min.

**Gel electrophoresis.** Tris-Tricine-SDS-PAGE with urea, a suitable method for resolution of peptides, was used to monitor the homogeneity of the fractions [26]. The electrophoresis run was performed in 0.75-mm slab gels using a SE 250 Mighty Small mini-vertical unit (Hoefer, USA). Proteins were fixed in 50% methanol, 10% acetic acid and visualized by Coomassie G-250 staining.

**Mass-spectrometry.** The molecular masses of the purified LTPs were determined using a Reflect III MALDI-TOF mass spectrometer (Bruker, Germany) with UV-laser 336 nm. 2,5-Dihydroxybenzoic acid (Sigma) was used as a matrix in 20% acetonitrile and 0.1% TFA at a concentration of 10 mg/ml.

**N-Terminal protein sequencing.** The amino acid sequence was determined using the Procise cLC 491 protein sequencing system (Applied Biosystems, USA). Phenylthiohydantoin derivatives of the amino acids were identified using a 120A PTH Analyzer (Applied Biosystems).

**Determination of C-terminal amino acids using carboxypeptidase A and B.** C-Terminal amino acids were cleaved by a mixture of carboxypeptidase A (70.5 U/mg protein) and carboxypeptidase B (133 U/mg protein) from Fluka (Germany). The purified LTPs (0.2 nmol) were dissolved in 20 μl of 0.2 M N-ethylmorpholine acetate buffer, pH 8.5, containing 0.1 μg carboxypeptidase A and 0.05 μg carboxypeptidase B. After incubation for 2 h at 37°C, the samples were dried. Released amino acids as dansyl derivatives were analyzed by two-dimensional thin-layer chromatography on 5 × 5 cm silica gel plates (Merck, Germany) as described in [27]. The plates were viewed under a UV-lamp at 366 nm. Identification and semi-quantitative analysis of released amino acid was done by comparing their mobilities and fluorescence intensities with those of a standard mixture of dansyl amino acids.

**Reactions of reduction and alkylation.** Cysteine residues number was determined in the purified proteins as described [28]. The S-pyridylethylated derivatives were desalted immediately by reverse-phase HPLC on an Altex system equipped with a Luna $C_{18}$ column (Phenomenex) with a linear gradient of acetonitrile concentration from 10 to 65% (1.38%/min) in 0.1% TFA at a flow rate of 0.5 ml/min. The presence of disulfide bonds was demonstrated by the same scheme but without prior reduction of the protein.

**Total RNA isolation.** Germinated lentil seeds were homogenized in liquid nitrogen. Intact total RNA was isolated using an SV Total RNA Isolation System (Promega, USA) according to Promega's protocol.

**Reverse transcription polymerase chain reaction (RT-PCR).** The complete sequences of the LTPs were determined using the RACE (rapid amplification of cDNA ends) strategy. RT-PCR experiments were conducted with the SMART™ RACE cDNA Amplification Kit (BD Biosciences Clontech, USA). Four degenerate gene-specific primers for 3′-RACE were designed corresponding to the N-terminal amino acid sequence of the LTPs:

No. 1, 5′-GCT ATC ACT TGC GG(A,T) GC(A,C,T) GT(G,T) AC-3′;

No. 2, 5′-GCT ATC ACT TGC GG(A,T) GC(A,C,T) GT(A,C) AC-3′;

No. 3, 5′-GAC CTG ACT CCA TGC CT(A,G) AC(A,C) TA(C,T) (C,T)T-3′;

No. 4, 5′-GAC CTG ACT CCA TGC (C,T)T(G,T) ACT TA(C,T) (C,T)T-3′.

The following thermal cycle profile was used for 3′-RACE PCR: 95°C for 3 min, 37 cycles of 94°C for 30 sec, 55°C for 40 sec, 68°C for 2 min. The 5′-cDNA ends were amplified with five gene-specific primers complementary to the 3′-untranslated regions of cDNA:

No. 5, 5′-ACT TTC TTC CTC TCA TGC AAA CTA T-3′;

No. 6, 5′-ACT GAG AAG ATT CCA TCT AAT AG-3′;

No. 7, 5′-GTG AGA AGT TTC CAT ATG ATA A-3′;

No. 8, 5′-ACT GAA TAC AGT ATA TAG ATA CAA GAG-3′;

No. 9, 5′-ACA ACA CAT TAA GTA ATA GAA AAT CCA AA-3′.

In this case, step-down PCR was applied: 95°C for 3 min, 25 cycles of 94°C for 30 sec, 65...57°C for 40 sec (the temperature was decreased by 2°C every 5 cycles), 68°C for 2 min, and 25 cycles of 94°C for 30 sec, 55°C for 40 sec, 68°C for 2 min. Products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and viewed with a UV transilluminator.

**Cloning and sequencing of RT-PCR products.** The PCR products were eluted from low melting point agarose gel and cloned in pGEM-T vector (Promega). Basic recombinant DNA techniques were exploited as described [29]. *Escherichia coli* strain DH-10B (Life Technologies, USA) competent cells were transformed and a mini-scale preparation of plasmid DNA was carried out using the alkaline lysis method. Nucleotide sequence was analyzed by the dideoxy chain termination technique in double stranded vector using the fluorescence-labeled dye terminator method and an ABI PRISM 3100-Avant automatic sequencer (Applied Biosystems).

**Tryptic digestion.** Samples of the reduced and S-pyridylethylated LTPs (5 μg) were dissolved in 10 μl of 50 mM $NH_4HCO_3$, pH 8.0, containing 10 μg/ml of modified trypsin (Promega). The samples were digested at 56°C for 2 h and stopped by addition of 10 μl 0.5% TFA in 10% acetonitrile. Mass spectra of the polypeptide fragments of LTPs were obtained in positive ion mode using a reflectron.

**MALDI-TOF/TOF mass spectrometry.** MS/MS spectra of tryptic LTPs fragments were acquired on an Ultraflex MALDI-TOF/TOF mass spectrometer (Bruker) with UV-laser 337 nm operated in the positive ion mode.

**Antibacterial activity.** Antibacterial activity of LTPs towards the *Agrobacterium tumefaciens* strain A281 was measured microspectrophotometrically using 96-well microplates. Bacteria were precultured overnight at 30°C on sterile Petri dish with LB agar. Then fresh biomass of the test-microorganism was inserted in 3 ml 1/2LB and cultured at 30°C to cell concentration of $2 \cdot 10^8$ cell/ml (optical density 1-1.5). Filter-sterilized protein solutions (10 μl) of different concentrations in 0.1% TFA were added to aliquots (110 μl) of tenfold diluted by 1/2LB exponential culture and incubated in a 30°C shaker. Bacterial growth is evaluated by measuring the culture absorbance at 620 nm a using microplate reader (Multiscan EX; Thermo, Finland). As a negative control, 0.1% TFA was added.

## RESULTS

**Purification and molecular characterization of the lentil LTPs.** A crude protein preparation was obtained from the homogenized germinated lentil seeds. After initial purification steps, including extraction, centrifugation, and ammonium sulfate precipitation, gel filtration on Sephadex G-75 column was performed. To prevent protein aggregation and precipitation on the column, buffer with 2 M urea was used. The eluted fractions were analyzed by Tris-Tricine-SDS-PAGE, and those containing proteins with approximate molecular mass of 7-9 kD were pooled, dialyzed, and applied onto a Mono S HR 5/5 FPLC column. A linear salt gradient was applied to elute the proteins. The proteins with approximate molecular mass of 9 kD were detected in the major peak *3* (Fig. 1a), which was separated by RP-HPLC (Fig. 1b). Mass spectrometric analysis revealed the presence of two molecular ion masses at *m/z* of 9121.9 and 9135.9 in fraction *1* and two additional molecular ion masses at *m/z* of 9269.1 and 9283.1 in fraction *1′*. The N-terminal amino acid sequences of the purified proteins were determined. The first 24 amino acid residues of four purified proteins turned out to be identical. The partial amino acid sequences demonstrated homology of the purified lentil proteins to the LTP1s family and were deposited in the SWISS-PROT and TrEMBL knowledgebase under accession number P84255.

The C-terminal amino acid residues were determined using hydrolysis by mixture of carboxypeptidases A and B. The COOH-terminal amino acids Lys>Val>Thr>Asn were identified in hydrolyzate of the LTPs from fraction *1*. The

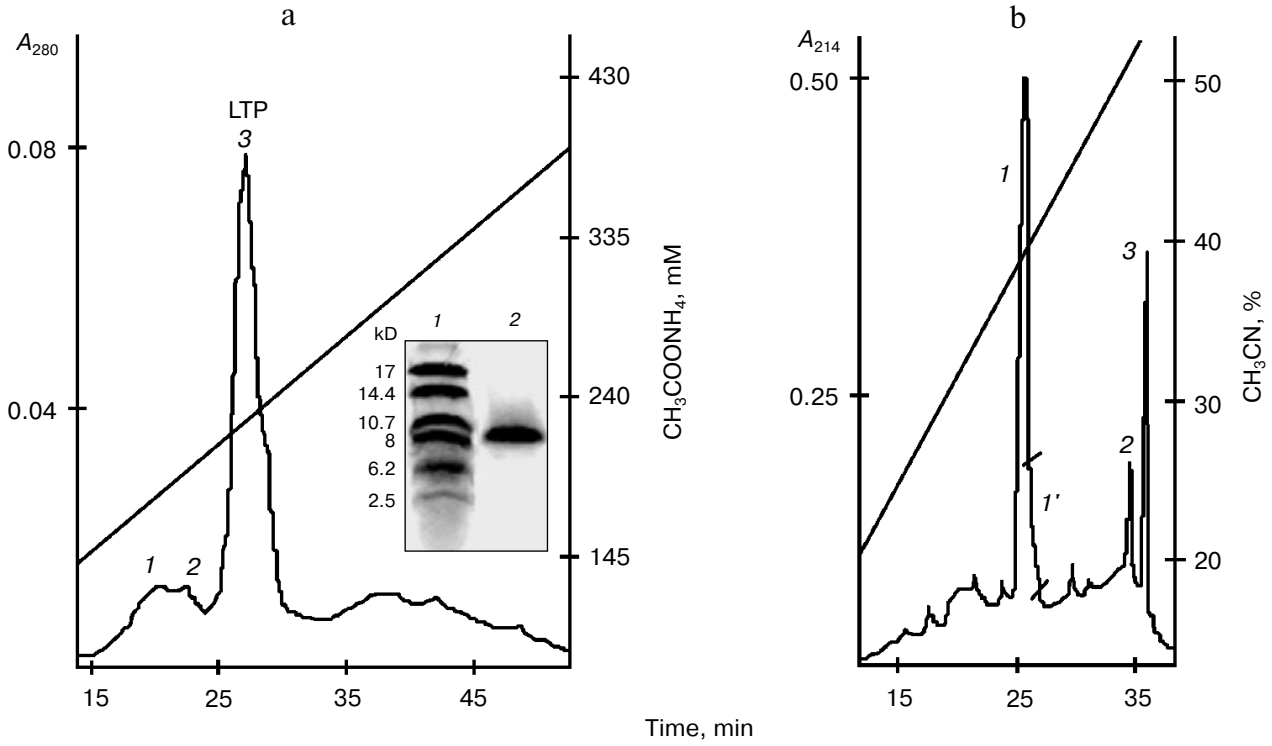


**Fig. 1.** a) Cation-exchange chromatography on a Mono S column and (insert) SDS-PAGE of fraction *3* (*2*); *1*) St, molecular mass standards (Pharmacia Biotech, Sweden). b) HPLC elution profile of fraction *3* from Mono S; LTPs were present in fractions *1* and *1′*.

COOH-terminal amino acids Phe>Lys>Val>Thr>Asn were detected in hydrolyzate of the LTPs from fraction *1′*. C-Terminal Phe is a typical amino acid for LTP1s.

The number of cysteine residues in each purified protein was determined using 4-vinylpyridine after reduction with dithiothreitol. Reduction and alkylation of the LTPs lead to molecular mass changes by 848.0-849.5 daltons. These results demonstrated that each lentil LTP contains eight cysteine residues (Table 1). Alkylation of the purified LTPs without prior reduction did not lead to any molecular mass changes (results not shown), showing that the eight cysteine residues of each protein are engaged in four disulfide bridges.

The *m/z* difference in each pair of the purified proteins (9269.1/9121.9 and 9283.1/9135.9) constitutes 147.2 daltons, which corresponds well with molecular mass of a phenylalanine residue. Based on this observation, we proposed that proteins with *m/z* of 9121.9 and 9135.9 are the shortened proteins with *m/z* of 9269.1 and 9283.1 without C-terminal Phe, correspondingly. In order to prove this proposal, we decided to determine the complete lentil LTP sequences and performed molecular cloning and cDNA sequencing.

**Total RNA isolation, RT-PCR, cloning, and cDNA sequencing.** Total RNA was obtained by solubilization of the tissue homogenate in guanidine thiocyanate contain-

**Table 1.** Comparison of measured and calculated molecular masses of native and S-pyridylethylated LTPs

| LTPs | Mass, daltons | | | |
|---|---|---|---|---|
| | native LTPs | | S-pyridylethylated LTPs | |
| | measured | calculated | measured | calculated |
| Lc-LTP2 | 9283.1 | 9282.7 | 10131.1 | 10131.9 |
| Lc-LTP4 | 9269.1 | 9268.7 | 10118.6 | 10117.9 |
| Lc-LTP7 | 9135.9 | 9135.5 | 9984.0 | 9984.7 |
| Lc-LTP8 | 9121.9 | 9121.5 | 9970.1 | 9970.7 |

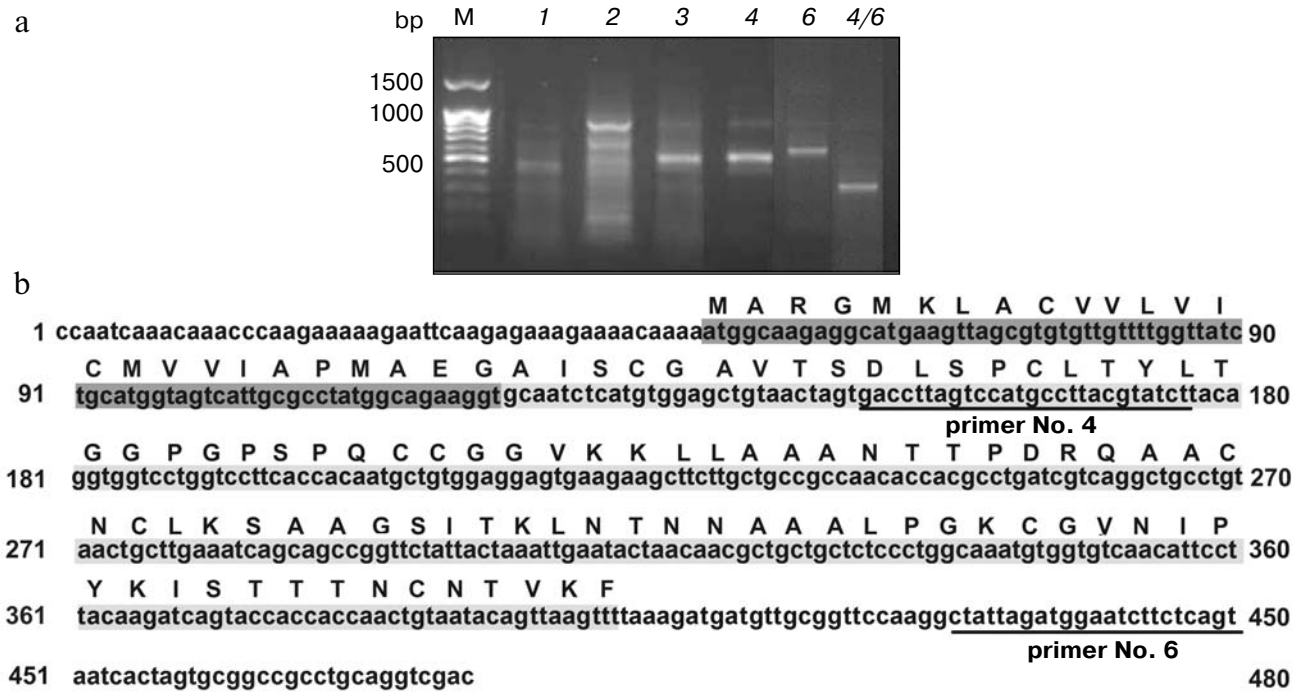


**Fig. 2.** a) Results of 3′-RACE with primers Nos. 1-4 and 5′-RACE with primer No. 6. b) Sequence of the mRNA encoding Lc-LTP2. The open reading frame is represented with the following individual components: signal peptide (dark gray), mature peptide (light gray).

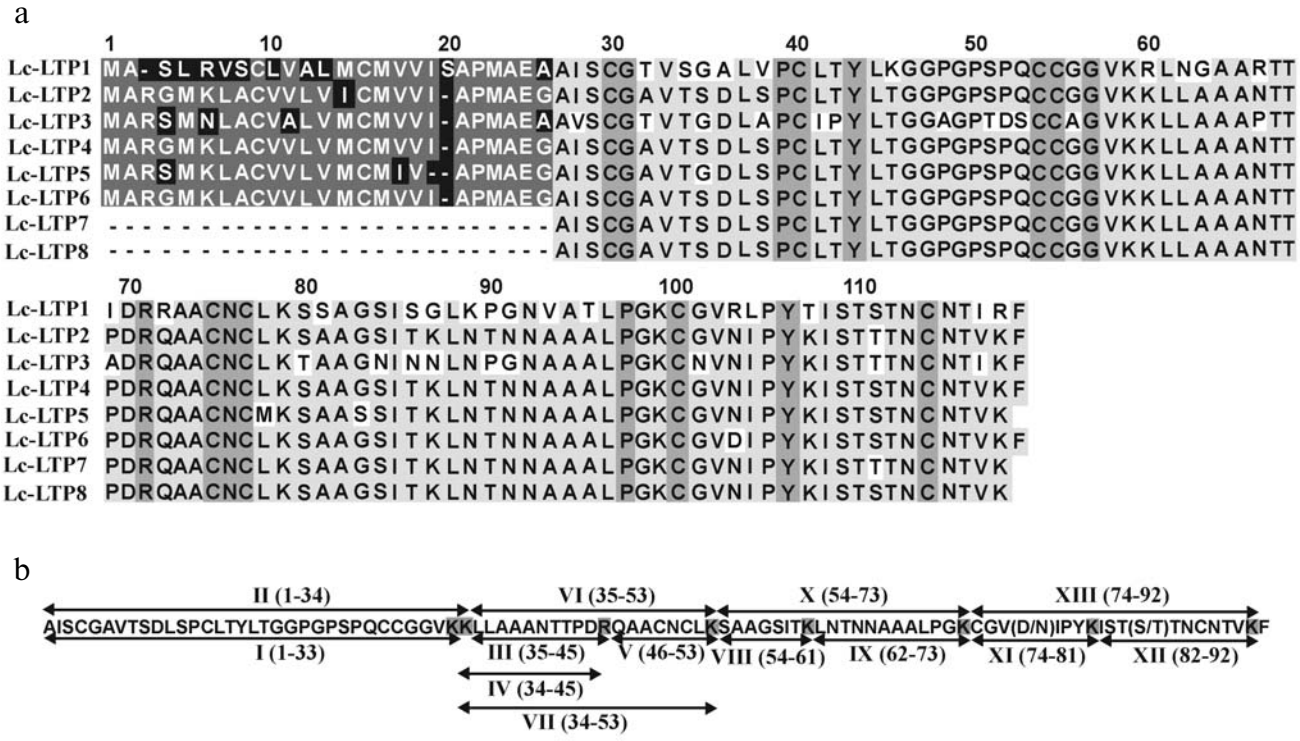


**Fig. 3.** a) Amino acid sequences of Lc-LTP1-8. Signal peptides are shown in dark gray, mature peptides in light gray, amino acid replacements in black and white. b) Tryptic fragments of the mature Lc-LTP2, -4, -6 proteins.

ing buffer and subsequent purification on silica gel spin columns. The cDNA structure was determined using a modification of the RACE protocol [30]. A set of 3′-RACE reactions using degenerate primers Nos. 3 and 4, specific for the N-terminal part of the purified lentil LTPs, showed a band of approximately 550 bp (Fig. 2a). Cloning and sequencing of the fragment acquired with primer No. 4 revealed five different C-terminal sequences and the 3′-UTR of cDNA of LTP homologs. The full-length cDNA of these proteins were determined by 5′-RACE experiments (Fig. 2, a and b). At this stage, the cDNA coding the sixth LTP was also revealed (Fig. 3a). Five nucleotide sequences (GenBank Accession Nos.: AY793553 (Lc-LTP1), AY793554 (Lc-LTP2), AY793555 (Lc-LTP3), AY793558 (Lc-LTP4), AY793557 (Lc-LTP6)) include a 354-bp open reading frame encoding protein precursors of 118 amino acid residues (Fig. 3a). The sixth sequence contains a 348-bp open reading frame encoding a protein precursor, which is shorter by two amino acid residues including C-terminal phenylalanine (Lc-LTP5, GenBank Accession No.: AY793556).

BLAST search of protein and nucleic acid databases (http://www.ncbi.nlm.nih.gov) revealed homology of these sequences to the LTP1s family. The sequence analysis using SignalP v.3.0 (http://www.cbs.dtu.dk) showed that the most probable cleavage sites are: the Ala25–Ala26 bond in the case of Lc-LTP1 and Lc-LTP3; the Gly25–Ala26 bond in the case of Lc-LTP2, Lc-LTP4, and Lc-LTP6; and the Gly24–Ala25 bond in the case of Lc-LTP5. To correlate the determined sequences with those of the purified LTPs, tryptic digestion with following microsequencing of the obtained fragments was performed.

**Molecular characterization of the purified LTPs by tryptic digestion.** Tryptic digestion of the reduced and S-pyridylethylated LTPs resulted in 13 peptide fragments (Table 2 and Fig. 3b). The calculated molecular masses of the Lc-LTP2 and Lc-LTP4 tryptic fragments (Protean, DNASTAR) matched the measured masses by MALDI-TOF-MS (Fig. 4a). The C-terminal fragment XII of Lc-LTP4 with molecular mass of 1166.2 daltons was not found. At the same time, the Lc-LTP4 fragment XIII consisting of fragments XI and XII was revealed and gave evidence about unusual stability of the Lys80–Ile81 bond. Taking into account that the molecular mass difference between Lc-LTP4 and Lc-LTP6 is about 1.0 dalton, microsequencing of the S-pyridylethylated fragment XI CGV($N$,$D$)$^{77}$IPYK was performed by MALDI-TOF/TOF-MS (Fig. 4b). The selection and collision-induced dissociation of an ion at $m/z$ of 998.5 showed a series of $y$ ions at $m/z$ of 520.28 and 634.32, and of $b$ ions at $m/z$ of 365.17 and 479.12 in the MS/MS spectra. Thus the amino acid residue Asn77 was identified.

As a result, we established that the purified lentil LTPs with molecular ion $[M + H]^+$ masses at $m/z$ of 9269.1 and 9283.1 correspond to the mature proteins of

**Table 2.** Comparison of measured and calculated masses of reduced and alkylated LTPs tryptic fragments

| Fragment | | Cys number | Mass, daltons | | | |
|---|---|---|---|---|---|---|
| | | | reduced LTPs | | S-pyridylethylated LTPs | |
| | | | measured | calculated | measured | calculated |
| I | | 4 | 3139.4 | 3139.6 | 3559.6 | 3560.0 |
| II | | 4 | 3267.4 | 3267.8 | 3687.7 | 3688.2 |
| III | | — | 1141.6 | 1141.2 | 1141.7 | 1141.2 |
| IV | | — | 1269.8 | 1269.4 | 1269.8 | 1269.4 |
| V | | 2 | 849.5 | 849.0 | 1059.5 | 1059.2 |
| VI | | 2 | 1972.9 | 1973.3 | 2183.1 | 2183.5 |
| VII | | 2 | 2101.0 | 2101.4 | 2311.2 | 2311.6 |
| VIII | | — | 733.4 | 732.8 | 733.4 | 732.8 |
| IX | | — | 1182.7 | 1182.3 | 1182.7 | 1182.3 |
| X | | — | 1898.0 | 1898.1 | 1898.0 | 1898.1 |
| XI | | 1 | 892.4 | 892.0 | 997.5 | 997.2 |
| XII | Lc-LTP2 | 1 | 1180.6 | 1180.3 | 1285.7 | 1285.4 |
| | Lc-LTP4 | | — | 1166.3 | — | 1271.4 |
| XIII | Lc-LTP2 | 2 | 2054.9 | 2055.4 | 2265.2 | 2265.6 |
| | Lc-LTP4 | | 2040.9 | 2041.3 | 2251.1 | 2251.5 |

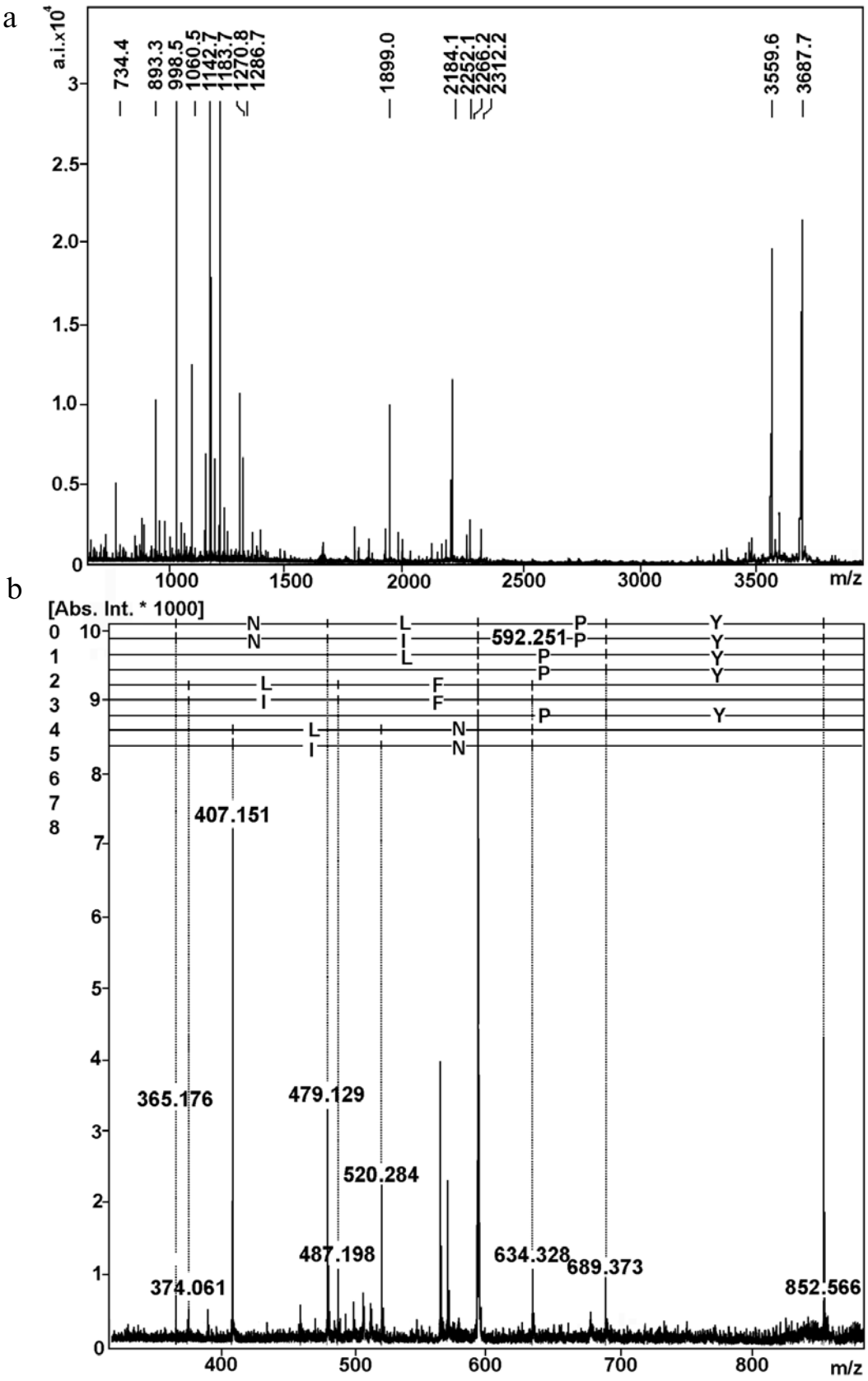


**Fig. 4.** a) Mass spectrometry of tryptic digest of the cysteine-alkylated lentil LTPs. b) MALDI-LIFT-TOF/TOF mass spectra of the cluster signal at *m/z* 998.5 recorded from a tryptic digest of the S-pyridylethylated lentil LTPs.

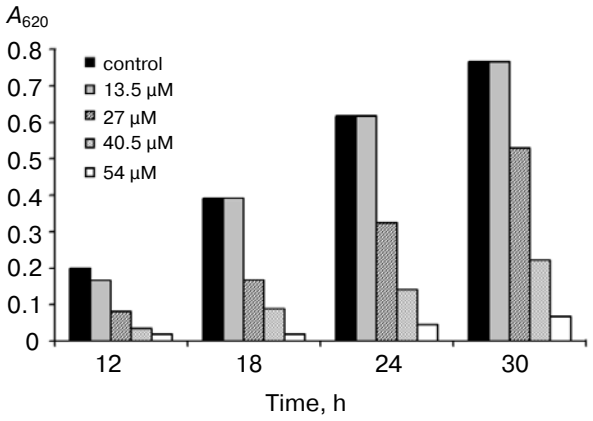


**Fig. 5.** Effects of Lc-LTP2/4 on *A. tumefaciens* growth.

Lc-LTP4 and Lc-LTP2 (calculated molecular masses of 9268.7 and 9282.7 daltons, correspondingly; EditSeq, DNASTAR; Table 1). The purified proteins with molecular ion $[M + H]^+$ masses at *m/z* of 9121.9 and 9135.9, designated as Lc-LTP8 and Lc-LTP7, correspond to the Lc-LTP4 and Lc-LTP2 shortened isoforms without C-terminal Phe (calculated molecular masses of 9121.5 and 9135.5 daltons, respectively; EditSeq, DNASTAR; Table 1 and Fig. 3a).

**Biological activity of the LTPs.** Antibacterial activity of the LTPs against the soil bacterium *A. tumefaciens*, which causes plants to swell, was studied. We found that *A. tumefaciens* growth was reduced in the presence of the Lc-LTP2 and Lc-LTP4 mixture. Inhibition of the bacterial cell growth was 92% in 24 h at the LTPs concentration of 54 μM and 48% at 40.5 μM. However, the LTPs at concentration of 13.5 μM had no inhibitory effect on the bacterial cell growth (Fig. 5).

## DISCUSSION

Presence of several isoforms in one plant is typical for the LTP family [6, 9]. For example, three LTP1s were isolated from rape seedlings and three additional germination-specific LTP1s were found by cDNA sequencing [31, 32]. Various LTPs isoforms are assumed to perform different functions in a plant, and their expression level may depend upon environmental conditions [6, 10-13]. In the present study, we confirmed that multiple LTP isoforms are present in plants and described a subfamily of eight novel lipid transfer proteins Lc-LTP1-8 found in lentil *L. culinaris* seeds.

Six 116-118-residue precursor proteins of Lc-LTP1-6 were found. All the precursor proteins contain typical for LTPs 24-25-residue signal peptides [6, 7]. The novel LTPs show sequence similarity to the LTP1s family. The lentil proteins have the most obvious similarity with LTP from *Cicer arietinum* seeds: 76% homology for Lc-LTP2 and 77% for Lc-LTP4 (Fig. 6). Three lentil LTPs (Lc-LTP2, -4, -6) are very similar to each other. There are two

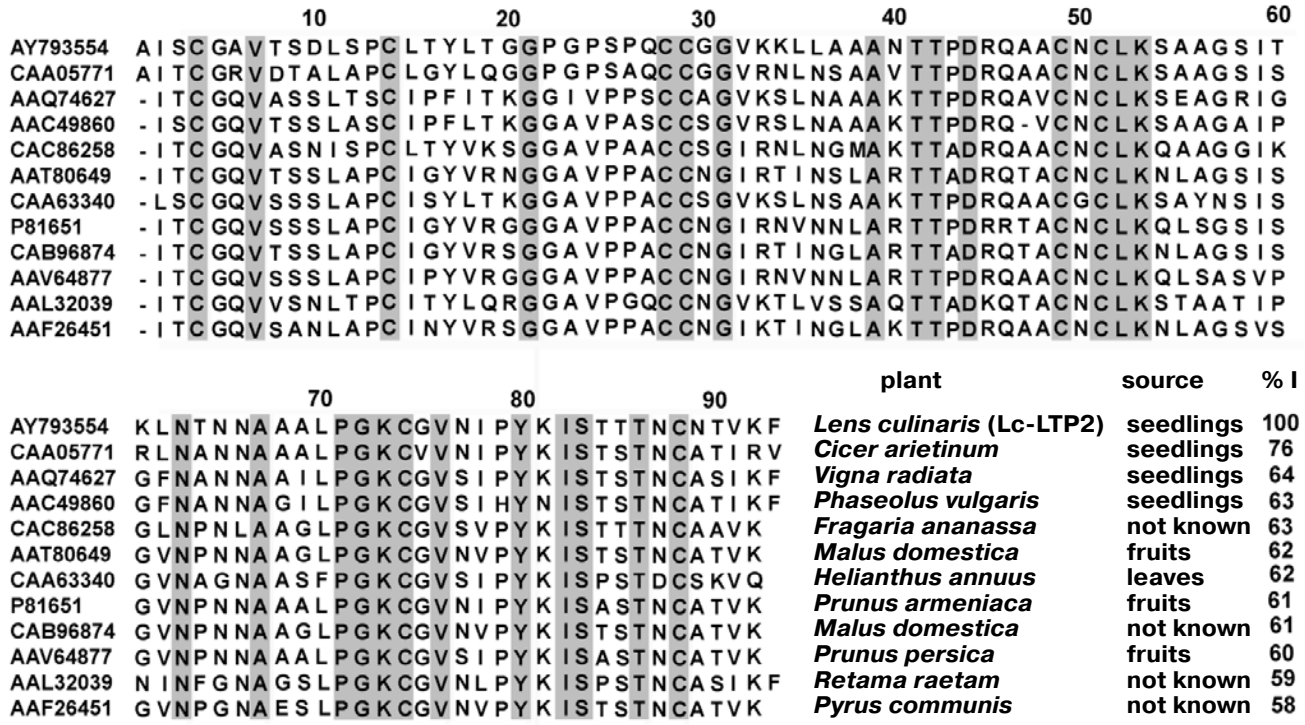


**Fig. 6.** Comparison of the Lc-LTP2 amino acid sequence with those of other plant LTP1s. % I, percentage of sequence identity.

differences in Lc-LTP2 and Lc-LTP4 amino acid sequences: Ser instead of Thr85 in the mature protein and Ile instead of Met14 in the signal peptide. Lc-LTP4 and Lc-LTP6 have the only replacement Asn77Asp. Each of the purified LTPs has eight cysteine residues forming four disulfide bridges.

Plant LTP1s contain a number of essential for their biological activity amino acid residues. Locations of eight cysteines, Tyr17, Tyr80, Gly5, Pro13, Gly31, and Pro71, and some important positively charged residues, for example Arg45, are well conserved. Thus, replacement Tyr17Ala, Arg45Ala, or Pro71Leu destroyed both the spatial structure and antifungal activity of LTP110 from rice [5, 33, 34]. The central hydrophilic residue in the CysXCys motif may govern the cysteine pairing and influence the overall folding of LTP1s [8]. All the mentioned amino acid residues are present and highly conserved in the discovered lentil LTPs (Fig. 3a).

The purified LTPs show antibacterial activity towards *A. tumefaciens*. It is necessary to note that the LTPs inhibit bacterial growth in high concentration. Obviously *A. tumefaciens* is not the main target of lentil LTPs. As is known, activity of representatives of LTPs family can strongly depend on pathogen species and serves as showing of specificity [4, 14].

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