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Tetraploid and Hexaploid Wheats Express Identical Isoforms of nsLTP1

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Nonspecific lipid-transfer proteins (nsLTPs) have been recognized as allergens in several plant species among which are cereals important in human nutrition. In this report, we purified a 9600 ± 1 Da protein from both soft wheat and farro bran. Mass spectrometric analyses revealed that these proteins are identical, belong to the nsLTP1 class, and have high sequence homology with nsLTP1 isolated from other cereal species. Their identification was further supported by the ability of the soft wheat nsLTP1 to transfer pyrene-labeled lipids between donor and acceptor membranes. The results are discussed in view of the increasing diffusion on the markets of bran-rich products.

KEYWORDS: Nonspecific lipid-transfer protein; lipid-transfer assay; farro; cereal allergens; direct sequence verification; mass spectrometry; *Triticum aestivum*; *Triticum dicoccon*

INTRODUCTION

In our previous paper (*1*), we demonstrated the presence of several nsLTP1 and nsLTP2 isoforms in *Triticum aestivum* cv. Centauro and *Triticum dicoccon* (emmer or Italian farro) bran and showed that some of their average molecular masses and number of cysteine residues are identical to those of nonspecific lipid-transfer proteins (nsLTP1s) already purified and characterized from soft and durum wheats (*2*). nsLTP1s are basic, soluble proteins of 9−10 kDa, consisting of 90−95 amino acid residues. Their primary structure is characterized by a conserved pattern of cysteine residues involved in intramolecular disulfide bonds, whose connections have been strictly conserved among nsLTP1s (*3*). The three-dimensional structure of these proteins has been determined from NMR and X-ray crystallography data (*4*, *5*). The fold reveals a very compact structure consisting of four α-helices connected by eight cysteine residues forming four disulfide bridges. The most interesting feature of this fold is the presence of a large internal cavity whose surface is covered with the side chain of hydrophobic residues; this cavity offers a potential binding site for fatty acids, acyl-CoA, or phospholipids (*6*). A detailed analysis of maize nsLTP1/fatty acids complexes suggests that the structural flexibility of the ligand-binding cavity and the predominant involvement of van der Waals interactions are responsible for the nonspecific binding (*7*). The nsLTPs capacity of binding spontaneously lipids can be evidenced by lipid-binding assays based on radiolabeled or fluorescent lipids (*8*, *9*). The role of nsLTPs in plants is still unclear; several hypotheses have been formulated to explain their function (*10*), and among these, a role in the deposition of apoplastic hydrophobic material has received convincing arguments, especially in view of the localization of nsLTP1 in the peripheral cell layers (*11*−*13*). Although most of the nsLTP1s studied so far are mainly located in the cell wall (*11*, *12*), there are examples of intracellular localization such as the castor bean glyoxysomes (*14*), cowpea cotyledons and embryonic axes protein storage vacuoles (*15*). nsLTP1 has intracellular localization in wheat seeds, too; immunogold electron microscopy specifically labels the aleurone grain inclusions but not the cell walls (*16*). Such a localization is not consistent with a role in the deposition of extracellular hydrophobic material; it is not consistent either with a role in aleurone lipids β-oxidation during germination, since the mobilization of aleurone triglycerides is slower than the degradation of aleurone nsLTP1 (*17*).

The aim of this work was to purify and characterize the most abundant nsLTP1 isoform present in soft wheat and farro bran (*1*). The proteins were purified using a combination of chromatographic procedures and further characterized by mass spectrometric techniques; their lipid-binding activity was investigated using a pyrene-labeled fatty acid.

MATERIALS AND METHODS

Materials. The bran samples from soft wheat and farro seeds were obtained and stored as already described (*1*). High-performance liquid chromatography (HPLC) grade water and CH_3CN were provided by Lab-Scan (Dublin, Ireland). Trifluoroacetic acid (TFA) was purchased from Sigma (St. Louis, MO). All chemicals were of the highest purity commercially available and were used without any further purification.

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Purification of Bran nsLTP1. The extraction and partial purification of nsLTP1 from bran samples were performed as already described in Capocchi et al. (*1*). Purification of nsLTP1 from the freeze-dried soft wheat and farro gel filtration pools was achieved by reversed phase (RP)-HPLC on semipreparative and analytical C-18 columns (Nucleosil, 10 mm × 250 mm, 300 Å, 7 μm; and Nucleosil, 4 mm × 250 mm, 300 Å, 5 μm; Macherey-Nagel GmbH & Co. KG, Düren, Germany).

For the semipreparative separation, the lyophilized pools (about 10 mg of protein) were dissolved in 20% solvent B (CH_3CN−0.05% TFA) and 80% solvent A (H_2O−0.05% TFA) and injected on the column equilibrated with a solution containing 80% solvent A and 20% solvent B. The separation was achieved with a linear gradient from 20 to 50% of solvent B in 60 min, at 50 °C, at a flow rate of 3 mL/min. As individual absorbing fractions eluted, they were collected and analyzed by 15% sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) (*1*).

The lyophilized fractions (about 1 mg) containing proteins of molecular mass around 9 kDa were injected onto the analytical column equilibrated with 80% solvent A and 20% solvent B. The elution was performed at a flow rate of 1 mL/min, with a linear gradient from 20 to 50% solvent B in 120 min, at 50 °C. Fractions exhibiting a single band with M_r around 9 kDa in 15% SDS−PAGE were collected, freeze-dried, and used for further characterization. Duplicate 15% SDS−PAGE gels were run for Western blotting experiments, as already described (*1*).

Electrospray Ionization (ESI)-MS Analyses of the Intact nsLTP1. ESI-MS analyses were performed with a LCQ-Deca instrument (ThermoFinnigan, San Jose, CA). Protein solutions were infused at 3 μL/min. The capillary temperature was maintained at 200 °C. Mass spectra were acquired in positive mode scanning from *m/z* 700 to 2000. Samples for mass spectrometric analysis were prepared dissolving the proteins in 50% CH_3CN−0.1% TFA at a concentration of approximately 10^{-5} M.

Tryptic Digestion of nsLTP1. nsLTP1s isolated from soft wheat and farro were reduced and carbamidomethylated as previously described (*1*). Reduced and carbamidomethylated proteins were dissolved at a concentration of 1 μg/μL in 50 mM ammonium acetate buffer, pH 8.2. Modified porcine trypsin (Promega, Madison, WI) dissolved in the same buffer was added to the proteins at a molar enzyme/substrate ratio of 1:50 and incubated at 37 °C for 4 h. The digestion was stopped by cooling in liquid nitrogen, and the mixture was immediately freeze-dried.

RP-HPLC/ESI-MS of the Tryptic Digests of the nsLTP1. Aliquots of the freeze-dried tryptic digests of nsLTP1s were dissolved (1 μg/μL) in CH_3CN−0.05% TFA/H_2O−0.05% TFA (20:80, v/v), filtered through a 0.45 μm Micro-spin filter (Alltech, Milan, Italy), and subsequently analyzed by RP-HPLC/ESI-MS. RP-HPLC/ESI-MS of the nsLTP1 tryptic digests was performed analyzing 30 μL of these solutions, corresponding to 30 μg of protein, onto a C-12 narrow-bore RP column (2.0 mm × 150 mm, 90 Å, 4 μm; Phenomenex, Labservice, Bologna, Italy). The column was first eluted at room temperature with H_2O−0.05% TFA for 5 min at a flow rate of 200 μL/min and then with a linear gradient of CH_3CN−0.05% TFA/H_2O−0.05% TFA from 0 to 55% in 50 min at a flow rate of 200 μL/min. The HPLC system was interfaced to an ion trap electrospray mass spectrometer (ThermoFinnigan LCQ-DECA) operating under the following conditions: source temperature, 220 °C; gas flow rate, 80 u.a. (corresponding to 1.2 L/min). Repetitive mass spectra were scanned in the *m/z* range 600−2000 and acquired in positive ion mode.

The amino acid sequences of the peptides of interest detected during RP-HPLC/ESI-MS were determined by MS/MS. The operating conditions for MS/MS analysis were as follows: isolation width, 3; normalized collision energy, 26 au; and activation Q, 0.250.

Lipid Transfer Activity of the Purified Soft Wheat nsLTP1. Donor membranes were made by injecting 10 μL of 0.1 mM Pyr-PC (in spectroscopic grade ethanol) into a quartz cuvette containing 2 mL of 20 mM Tris-HCl buffer, pH 7.4, and were equilibrated for 2 min prior to the start of the lipid transfer experiments (*18*). Acceptor vesicles were prepared as described in ref *19*.

Fluorescence measurements were made with a Perkin-Elmer LS 50B spectrophotometer. The excitation and emission wavelengths were 343 and 378 nm (slit width, 2.5 and 5 nm, respectively). The fluorescent phospholipid transfer assay was performed by injecting 10 μL of Pyr-PC (0.5 μM final concentration) in a thermostated (37 °C) quartz cuvette containing 2 mL of HBS buffer, under continuous stirring; after 2 min of incubation, SUVs were added to a 25 μM final concentration. The transfer activity was started by adding increasing aliquots (from 10 to 60 μM, final concentration) of purified soft wheat nsLTP1 in HBS buffer (1 μg/μL). The increase in fluorescence intensity was followed for 6 min. Transfer rates were corrected for the spontaneous transfer of Pyr-PC. All of the experiments were performed in triplicate.

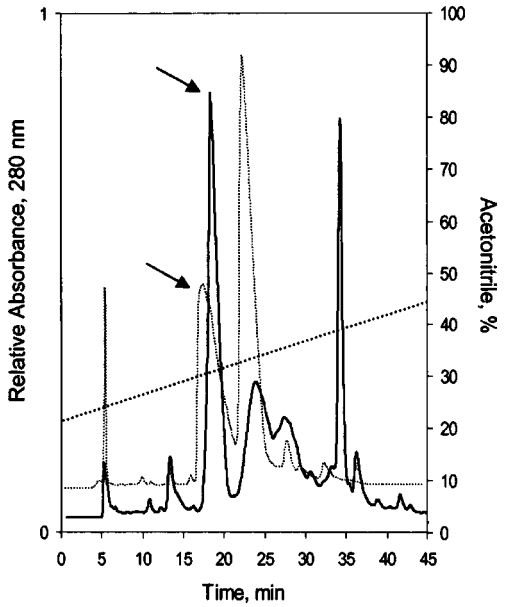

Figure 1. Semipreparative RP-HPLC of farro and soft wheat gel filtration pools. The continuous and dotted lines indicate the farro and the soft wheat sample elution, respectively. The arrows point to those peaks that were recovered and subjected to further purification.

RESULTS AND DISCUSSION

Purification and Molecular Mass Determination of Soft Wheat and Farro nsLTP1. The results obtained by the cation exchange and gel filtration steps on soft wheat and farro bran extracts have been already discussed (*1*). The fractions obtained by semipreparative RP-HPLC (**Figure 1**) showed on 15% SDS−PAGE (data not shown) that both 9 and 7 kDa proteins were present in the farro and soft wheat peaks that eluted at 28.8 and 28.3% CH_3CN, respectively (**Figure 1**).

These two fractions were then separated on a C-18 analytical RP-HPLC column. Flattening the elution gradient with respect to the previous separation resulted in the splitting of the 28.8 and the 28.3% CH_3CN fractions in two well-resolved peaks that were collected individually (**Figure 2**). SDS−PAGE analyses (data not shown) revealed that in both plant materials the first eluting peak (24.2% CH_3CN) contained exclusively 7 kDa proteins, while the second eluting peak (26.7% CH_3CN) contained 9 kDa proteins. The SDS−PAGE analyses of the soft wheat and farro 26.7% CH_3CN peaks demonstrated the presence of one protein band with an apparent molecular mass of about 9 kDa; when duplicate gels were carried out in Western blotting experiments, the soft wheat and farro 9 kDa proteins gave a strong immunoreaction against barley anti-nsLTP1 IgG (**Figure 3**). The alignment of the amino acid sequences of mature nsLTP1 from cereal genera such as *Triticum*, *Hordeum*, *Zea*, *Oryza*, and *Sorghum* demonstrated that *Triticum* nsLTP1 shares the highest amino acid identity with *Hordeum* nsLTP1, explaining the strong reaction with polyclonal antibodies obtained with

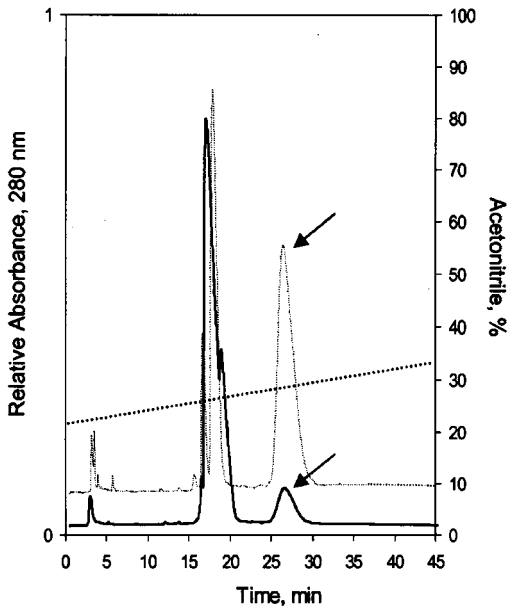

Figure 2. Analytical RP-HPLC of the fractions recovered from semi-preparative RP-HPLC of the farro and soft wheat samples. The continuous and dotted lines indicate the farro and the soft wheat sample elution, respectively. The arrows point to the LTP1 peaks that were recovered and subjected to protein identification by mass spectroscopy analyses.

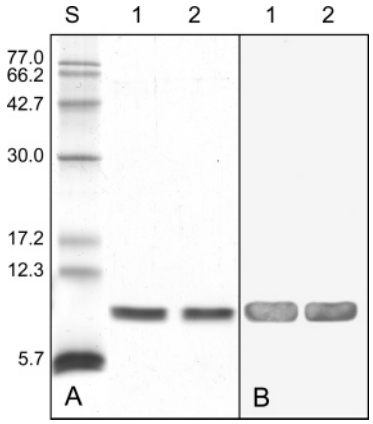

Figure 3. 15% SDS–PAGE and Western blotting of the purified LTP1 fractions. (**A**) 15% SDS–PAGE of the soft wheat and farro LTP1, stained with Coomassie Brilliant Blue-R. (**B**) A duplicate of the gel in box **A**, blotted onto nitrocellulose and immunoreacted with barley anti-LTP1 IgG. S, Sigma low molecular weight standards; 1, soft wheat LTP1 sample (2.5 μg/lane); and 2, farro LTP1 sample (2.5 μg/lane).

barley nsLTP1 (*2*). On the other hand, anti-maize nsLTP1 antibodies do not cross-react with *Triticum* nsLTP1 (*20*), due to a significantly lower percentage of amino acid identity.

The ESI-MS of the 9 kDa proteins isolated from soft wheat and farro bran are reported in **Figure 4A**,**B**. Deconvolution of these multicharged spectra confirmed that the purified 9 kDa proteins had molecular masses of 9600 ± 1 Da in both *Triticum* species. A nsLTP1 protein with a molecular mass of 9599 Da has already been identified from *Triticum aestivum* cv. Camp Remy seeds (*21*). Dieryck et al. (*22*) isolated a cDNA clone from a *Triticum durum* midmaturation (22 DAF seeds) cDNA library, encoding a 9 kDa nsLTP. The deduced primary structure of the mature *T. durum* nsLTP1 was identical to the nsLTP1 purified from *T. aestivum* by Désormeaux et al. (*21*).

Amino Acid Sequence Analysis. The amino acid sequences of the purified nsLTP1s were determined by RP-HPLC/ESI-MS analysis of their tryptic peptides. **Figure 5A**,**B** illustrates the total ion currents (TICs) of the tryptic hydrolizates from soft wheat and farro nsLTP1, respectively.

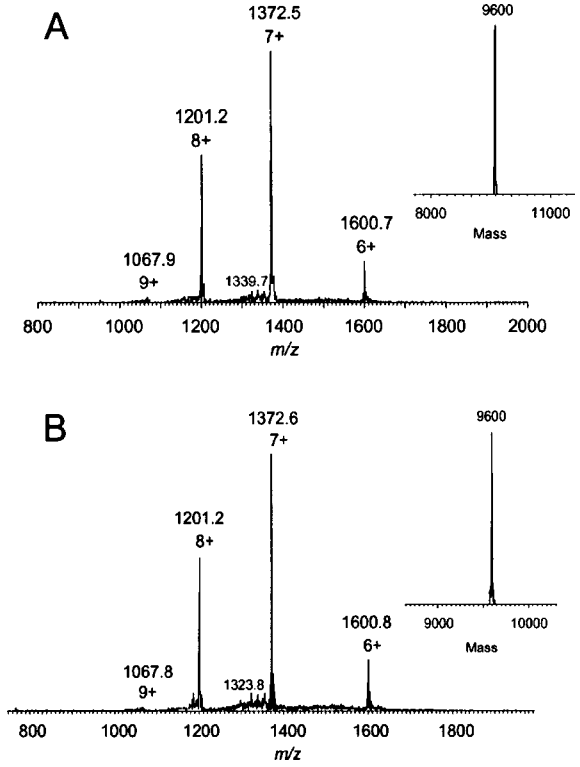

Figure 4. Multicharged and deconvoluted mass spectra of the of the soft wheat (**A**) and farro (**B**) 26.7% CH_3CN peaks in the analytical separation.

The sequence reported by Désormeaux et al. for the 9599 Da nsLTP1 isolated from *T. aestivum* (*21*) shows six Arg and three Lys residues. Because of the presence of an Arg-Pro sequence that is not cleaved, or cleaved to a very limited extent by trypsin, nine tryptic peptides can be predicted for this sequence. Sequences and calculated molecular masses for these peptides are reported in **Table 1**. All of these peptides were identified in the RP-HPLC/ESI-MS chromatograms by their ESI mass spectra (**Figure 5A**,**B** and **Table 1**). In addition, some subfragments were also detected as minor peaks in the chromatograms. These subfragments originated by partial or nonspecific cleavages. One of these subfragments, having an MH^+ of 1207.5, corresponds to the Ile^1-Arg^{11} subfragment of T1. This subfragment originated by peptide bond scission between Arg^{11} and Pro^{12}. This interpretation is confirmed by the presence of the complementary subfragment Pro^{12}-Lys^{32} (MH^+ 2223.5). These two subfragments are indicated in **Table 1** as $T1^1$ and $T1^2$, respectively. Nonspecific cleavage between carbamydomethyl Cys^{13}-Leu^{14} in fragment T1 originates from the subfragment $T1^3$ (Ile^1-Cys^{13}, MH^+ at *m*/*z* 1527.5) and its complementary $T1^4$ (Leu^{14}-Lys ^{32}, MH^+ at *m*/*z* 1965.5). Nonspecific cleavage of the Tyr^{79}-Thr^{80} peptide bond in fragment T8 produces the subfragment $T8^1$ (carbamidomethyl Cys^{73}-Tyr^{79}, MH^+ at *m*/*z* 822.3) and its complementary $T8^2$ (Thr^{80}-Arg^{89}, MH^+ at *m*/*z* 1178.5). The T9 fragment is formed by cleavage at the amino group of the last Val residue. This single amino acid cannot be detected under our experimental conditions. However, in both chromatograms (**Figure 5A**,**B**), it is present as a T8 + T9 fragment, originating by partial cleavage of the peptide bond between Arg^{89} and Val^{90}. This fragment confirms the presence of a Val residue as the C-terminal amino acid of the polypeptide chain (**Table 1**).

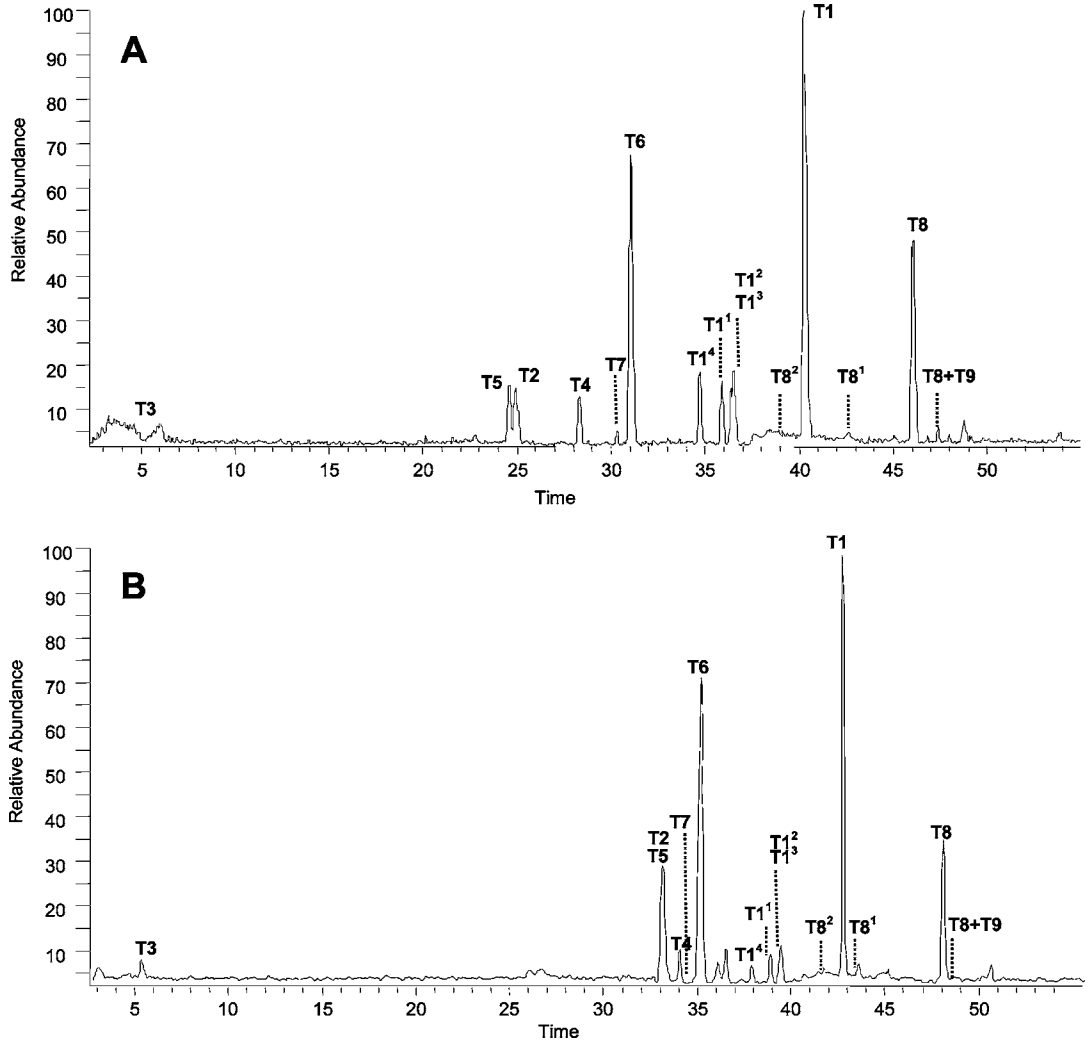

Figure 5. TIC chromatograms of the tryptic digest of the 9599 Da nsLTP from soft wheat (**A**) and farro (**B**) obtained by RP-HPLC/ESI-MS.

Table 1. Sequence, Position, Calculated Monoisotopic and Average MH^+, Experimentally Measured MH^+ of Tryptic Fragments of Soft Wheat and Farro nsLTP1

		calculated MH^+		ESI-MS measured MH^+		
fragment	position	monoisotopic	average	LTP1 C	LTP1 F	sequence
T1	**1–32**	**3474.6**	**3476.9**	**3476.3**	**3476.1**	**IDCGHVDSLVRPCLSYVQGGPGPSGQCCDGGVK**[a]
$T1^1$	1–11	1270.6	1271.4	1270.5	1270.5	IDCGHVDSLVR*[b]
$T1^2$	12–32	2223.0	2224.5	2223.5	2223.5	PCLSYVQGGPGPSGQCCDGGVK*
$T1^3$	1–13	1527.7	1528.7	1527.5	1527.6	IDCGHVDSLVRPC*
$T1^4$	14–32	1965.9	1967.2	1965.5	1965.3	LSYVQGGPGPSGQCCDGGVK*
T2	**33–39**	**852.4**	**852.9**	**852.4**	**852.4**	**NLHNQAR***
T3	**40–44**	**592.3**	**592.6**	**592.3**	**592.3**	**SQSDR***
T4	**45–52**	**980.4**	**981.1**	**980.4**	**980.3**	**QSACNCLK***
T5	**53–56**	**416.3**	**416.5**	**416.3**	**416.2**	**GIAR***
T6	**57–67**	**1252.6**	**1253.3**	**1252.6**	**1252.5**	**GIHNLNEDNAR***
T7	**68–72**	**541.3**	**541.7**	**541.3**	**541.3**	**SIPPK***
T8	**73–89**	**1981.9**	**1983.3**	**1981.7**	**1982.5**	**CGVNLPYTISLNIDCSR***
$T8^1$	73–79	822.4	822.9	822.3	822.3	CGVNLPY*
$T8^2$	80–89	1178.6	1179.3	1178.5	1178.5	TISLNIDCSR*
T9	90–90	118.1	118.2			V
T8 + T9	73–90	2081.0	2082.4	2081.7	2081.5	CGVNLPYTISLNIDCSRV*

[a] Theoretical tryptic fragments are reported in bold. [b] Peptide sequences confirmed by MS/MS are indicated by asterisks.

The peptide sequences of all of the fragments detected were confirmed by MS/MS spectra of their single or doubly charged ions, except for fragment T1, which could not be directly sequenced in MS/MS experiments because of its high molecular mass. However, the sequence of this peptide was indirectly confirmed by MS/MS sequence determination of its subfragments $T1^1$ and $T1^4$. In conclusion, the RP-HPLC/ESI-MS analysis of the tryptic hydrolizates of purified soft wheat and farro nsLTP1 demonstrates an identical amino acid sequence for both proteins (**Table 1**).

Phospholipid Transfer Activity Assays. Because of the amino acid sequence identity of the soft wheat and farro

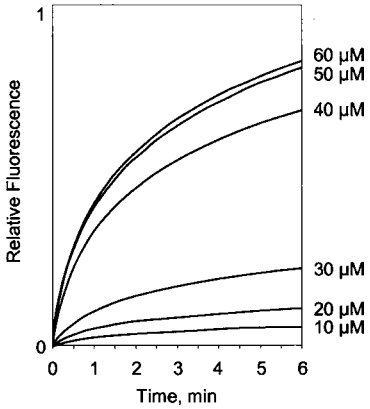

Figure 6. Soft wheat nsLTP1 lipid-transfer activity between donor and acceptor vesicles. The assay was carried out with various protein concentrations, as indicated next to each curve. The release of fluorescence upon transfer of pyr-PC to the acceptor vesicles was followed for 6 min.

nsLTP1s, we measured the phospholipid transfer activity of the soft wheat nsLTP1 alone. Upon addition of the purified soft wheat nsLTP1 to a mixture of acceptor and donor liposomes, the fluorescence emission increased as a function of time and nsLTP1 concentration (**Figure 6**). This result can be interpreted in terms of binding of fluorescent phospholipid to nsLTP1 and formation of increasing concentration of the lipid−protein complex (*23*). The nsLTP-mediated transfer of fluorescent phospholipid is easily corrected from the spontaneous transfer that, however, is particularly low when injected Pyr-PC vesicles are used as donors (*24*). Upon examination of **Figure 6**, the relative fluorescence intensifies when increasing amounts of purified nsLTP1 are added to the donor−acceptor mixtures. In particular, a 3-fold increase of the relative fluorescence is observed when the nsLTP1 concentration is raised from 20 to 30 μM. Further increases of nsLTP1 concentrations, namely, between 50 and 60 μM, gave identical curves of relative fluorescence.

The greatest amount of fluorescent phospholipids transferred from donor to acceptor represents approximately 55% of the initial concentration of fluorescent phospholipids. The plateau curves obtained with 50 and 60 μM nsLTP1 could be explained with data indicating that plant nsLTPs are able to catalyze exchange of lipids that involves only the external leaflet of donor vesicles; therefore, a 50 μM concentration of transferring protein could be sufficient for the depletion of the external monolayer of donor vesicles (*25*).

Our data on the purified soft wheat nsLTP1 agree with the results obtained by other authors who isolated corresponding nsLTP1 isoforms from *T. aestivum* and *T. durum* seeds (*2*, *22*). However, in our previous report (*1*), we also demonstrated for the first time the presence of nsLTP1 isoforms in farro bran, and in the present paper, we also report on the purification and sequence verification of one of these.

Triticum dicoccon (emmer; 28 chromosomes, AABB) is a tetraploid hulled wheat, and like the diploid *Triticum monococcum* (einkorn; 14 chromosomes, AA) and the hexaploid *Triticum spelta* (spelt; 42 chromosomes, AABBDD), it is among the most ancient cereal crops in Europe. Once very popular in the Mediterranean region and in the neighboring Near East, these cereals have been replaced by modern and high-yielding varieties of unhulled wheats, and their cultivation is restricted in marginal areas. In Italy, the traditional farro-growing areas are located, mainly, in the Central and Southern Apennine regions (*26*).

The last 15 years have witnessed a new interest in the use of ancient wheats, particularly in the organic agriculture and in the health food market. They are low-input crops, suitable for growth in harsh ecological conditions without the need for pesticides and fertilizers. However, the studies about the nutritional and technological characteristics of these wheats are very scanty and frequently divergent. In folk knowledge, *T. dicoccon* is believed to have positive effects in the treatment of coeliac and obesity-affected patients (*27*); in contrast, Kasarda and D'Ovidio (*28*) evidenced a 98.5% identity between *T. spelta* and *T. aestivum* α-gliadin and demonstrated that *T. spelta* contains sequences identical to those of common wheat active in coeliac disease. The close genetic relationship between hulled and unhulled wheats makes it unlikely that they will differ significantly in their processing and nutritional properties. The domestication of modern wheat species could have brought about an unintentional diminution of some antinutritional factors responsible for resistance to biotic and abiotic stresses. For these reasons, it could be useful to examine these ancient wheats for the possible presence of these antimetabolic or potentially allergenic compounds (tannins, protease inhibitors, phytic acid, lectins, nsLTPs, etc.); moreover, because these alternative cereals are mainly consumed as whole, antinutrients and potentially allergenic substances could be found in significantly high amounts, since some of these (e.g., phytic acid and nsLTPs) are located mainly in the aleurone layer that is part of the bran. Considering that farro is an important source of genetic variability and an increasingly used "healthy" food source, the scientific community has realized that much research work is needed in order to fully evaluate the nutritional and agronomic potential of this grain. Our work represents the first report on the presence of nsLTPs in farro bran and as such contributes to the knowledge about this ancient cereal species.

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