Identification of a New Form of Lipid Transfer Protein (LTP1) in Wheat Seeds

Jean-Paul Douliez,*,† Sandrine Jégou,† Christine Pato,† Colette Larré,† Daniel Mollé,‡ and Didier Marion†

Laboratoire de Biochimie et Technologie des Protéines, INRA, rue de la Géraudière, B.P. 1627, 44316 Nantes, France, and Laboratoire de Recherche en Téchnologie Laitière, INRA, 65 rue de St Brieuc, 35042 Rennes Cedex, France

Recently, this laboratory has isolated from barley and beer extract an isoform of lipid transfer protein (LTP1), which was not fully sequenced (Jégou, S.; Douliez, J. P.; Mollé, D.; Boivin, P.; Marion, D. *J. Agric. Food Chem*. **2000**, *48*, 5023−5029). It was named LTP1b and exhibited a molecular weight 294 Da higher than that of the known LTP1. This paper reports the finding of an LTP1 isoform in wheat that also exhibits an excess of 294 Da compared to the native protein. Amino acid sequencing, reduction and alkylation, and mass spectrometry showed that this new LTP1b possesses the same N-terminal sequence as the native LTP1, suggesting that the difference resides in the binding of an adduct which has a molecular weight of 294 Da. The aim of the present paper is to highlight various biophysical techniques that afford the identification of such an isoform-like LTP1 and to correlate this finding with other isoforms of LTP1 that were isolated from other plants but not fully sequenced.

Keywords: *Plant lipid transfer protein; wheat; isoform; adduct; post-translational modification*

INTRODUCTION

Plant lipid transfer proteins (LTP1) are ubiquitous lipid binding proteins of the plant kingdom. They are of great technological importance because they are involved in beer foam formation (*1*−*4*). They exhibit a molecular mass of ∼9 kDa and a basic p*I* and share a common cysteine motif forming four disulfide bridges (*5*). LTP1s fold in a four-helix bundle around a hydrophobic cavity (*6*−*10*) and can bind and transfer various lipids (*11*−*13*).

It has been shown that LTP1 can exhibit post-translational modification such as phosphorylation (*14*,*15*) and that different isoforms coexist in a given plant. These isoforms have been observed in spinach, sunflower, maize (*7*, *16*−*18*), carrot (*19*), gravepine (*20*), barley (*1*, *21*), onion (*22*), and castor bean (*23*). However, these were not fully sequenced and sometimes discarded from the purification. Recently, we reported on the finding of an isoform named LTP1b in barley seeds and beer extract (*4*), which exhibits a molecular weight 294 Da higher than that of the known LTP1 (*1*). It displays a comparable amino acid composition but was not fully sequenced. In fact, the finding of such a protein has been first reported in barley by Evans and Hejgaard (*3*). Such a low mass difference cannot be detected by SDS-PAGE, showing that care must be taken to evaluate the purity of the proteins at the end of the purification procedure. Moreover, we encountered difficulties in getting pure LTP1 from barley (*4*) because both LTP1 and LTP1b are eluted at analogous times in HPLC. This is of great importance because very pure proteins are required for structure determination and lipid binding studies.

In the present study, we report on the finding of an LTP1b in wheat having a molecular mass that also differs by 294 Da. The aim of this paper is to highlight various biophysical techniques that allow the identification of these LTP1b isoforms at the end of the purification procedure and correlate this finding with a few isoforms isolated from other plants.

MATERIALS AND METHODS

Purification of LTP. Cation-exchange chromatography was performed on a column (5 × 30 cm) packed with a resin from streamline SP (big beads), Pharmacia. The fractions were eluted by applying a gradient from 0 to 0.7 M NaCl for 2 h in 20 mM MES (pH 5.6) buffer at a flow of 30 mL/min. Analytical C18 RP-HPLC was done with a C18 reversed-phase (5 μm, 100 Å) bonded silica column (25 × 0.75 cm) using a gradient of water/acetonitrile/0.05% TFA (1% acetonitrile/min) at 50 °C at a flow of 1 mL/min. Semipreparative C18 RP-HPLC was performed on a 5 μm, 300 Å, bonded silica column (25 × 1 cm) using the same buffers and gradient as for analytic HPLC except that the flow was adjusted to 3 mL/min. In the case of size exclusion gel filtration, a column (3 × 100 cm) was packed with Sephadex G50 (Sigma) and eluted in 20 mM MES (pH 5.6) buffer.

Mass Spectrometry. Protein molecular mass was obtained using a Perkin-Elmer APIII (Sciex, Thornill, ON, Canada) triple-quadrupole mass spectrometer equipped with an atmospheric pressure ionization source (electrospray mass spectrometer, ES-MS) (*24*). The sample analysis (1 mg/mL) was achieved by an on-line coupling between MS and RP-HPLC (LC-MS). Elution was carried out on an RP-HPLC column (Symmetry C18 Waters, Milford, MA) at a flow rate of 0.25 mL/min (40 °C) with a split to the MS ionization source that was set at a flow rate of 0.3 mL/min. Ion detection was performed in positive mode and mass calculation with Biomultiview 1.2 (software package Sciex) from scan mass to charge *m*/*z*.

Amino Acid Sequencing. N-terminal amino acid sequencing was performed by Edman degradation on a model 477A

* Corresponding author (e-mail douliez@nantes.inra.fr; telephone 33 2 40 67 50 56; fax 33 2 40 67 50 25).

† Laboratoire de Biochimie et Téchnologie des Protéines.

‡ Laboratoire de Recherche en Téchnologie Laitiére.

10.1021/jf001327m CCC: $20.00
Published on Web 03/17/2001

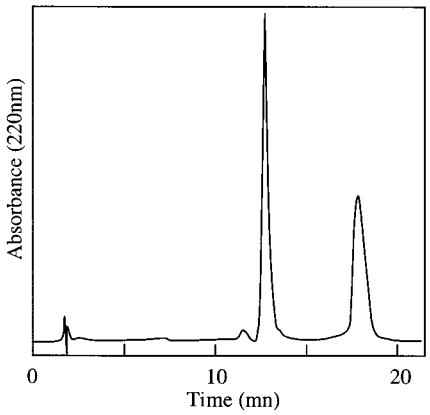

Figure 1. C18 RP-HPLC chromatogram of the 9 kDa wheat protein enriched fraction obtained after size exclusion gel filtration. Proteins were eluted according to a gradient of water/acetonitrile (see Materials and Methods). LTP1 is eluted at 33% acetonitrile (13 min) and LTP1b at 38% (18 min).

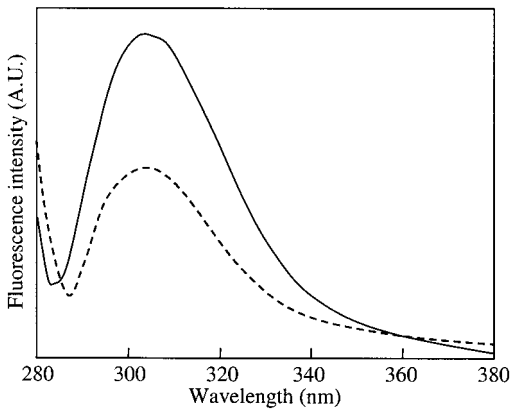

Figure 2. Fluorescence emission spectra of LTP1 (—) and LTP1b (- -). The excitation was set at 275 nm, and the protein concentration was 50 μM.

gas phase sequencer. The phenylthiohydantoin amino acids from the sequencer were identified on-line by RP-HPLC using a 120A analyzer (Applied Biosystems, Foster City, CA).

Capillary Electrophoresis. Electrophoresis was carried out using a Beckman P/ACE 5510 system. The separation was performed on a fused silica capillary (50 μm $\times$ 50 cm), and the sample (1 mg/mL) was introduced at the anode under high pressure for 3 s. Before each run, the capillary was rinsed with 0.1 M NaOH (1 min) and water (1 min) and then conditioned with the electrophoresis buffer (5 min). Separation was achieved with a constant voltage of 30 kV in the case of borate buffer and 20 kV for the Tris-HCl buffer at 25 °C and monitored by UV detection at 214 nm.

Fluorescence Spectroscopy. Fluorescence intensity was measured at 25 °C with a Fluoromax-Spex (Jobin et Yvon, France). Excitation was set at 275 nm, and emission spectra were recorded from 280 to 380 nm. The protein concentration was 0.5 mg/mL in a 50 mM phosphate buffer (pH 7).

RESULTS AND DISCUSSION

Purification of LTP1. Proteins were first extracted from 2 kg of wheat endosperm flour or 3 kg of bran with 10 L of distilled water. After centrifugation (flour) and filtration (bran), the aqueous extract was adjusted to 20 mM MES (pH 5.6) and directly loaded on a cation-exchange column. In contrast with previous purification procedures (*25*, *26*), no ammonium precipitation steps or reducing and chelating agents were used. All of the fractions that displayed the 9 kDa band by SDS-PAGE were pooled, dialyzed, and freeze-dried. This material was fractionated by size exclusion chromatography, and the eluted fractions that contained LTP1 were pooled. Analytical C18 RP-HPLC revealed two well-resolved peaks, H1 and H2, eluted at 33% (13 min) and 38% (18 min) of acetonitrile, respectively (Figure 1). It must be mentioned that this chromatogram pattern markedly differs from that obtained in the case of barley (*4*). In that case, LTP1b was eluted as a shoulder of the LTP1 peak.

Purification was completed by semipreparative C18 RP-HPLC, and the collected H1 and H2 fractions were diluted with distilled water and freeze-dried, affording 400 and 200 mg of dry protein powder, respectively.

Mass spectrometry returned masses of 9600 $\pm$ 1 and 9894 $\pm$ 1 Da for H1 and H2, respectively. From these data, H1 could be attributed to known LTP1 from wheat in agreement with the calculated mass (*25*). Amino acid sequencing of the first 15 N-terminal amino acids of H2 provided a sequence strictly identical to that of wheat LTP1. By comparison with barley (*4*), because H2 exhibited a 294 Da increase of molecular weight and analogous N-terminal, it was named LTP1b. Reduction and alkylation of LTP1 and LTP1b in the case of wheat yielded alkylated proteins with the same molecular weight (not shown). This suggests that LTP1b and LTP1 are analogous proteins; that is, they exhibit the same amino acid sequence. The difference resides in the binding by the LTP1b of an adduct having a molecular weight of 294 Da. This is emphasized by the fact that the same molecular mass excess is found in the case of barley (*3*, *4*).

It must be mentioned that both flour and bran extracts gave similar RP-HPLC patterns and yields for both LTP1 and LTP1b. However, the ratio of LTP1 to LTP1b was found to be slightly dependent on the *Triticum aestivum* cultivar (Florence Aurore or Etoile de Choisy) and was about 3/2 in case of *Triticum durum* wheat.

Physicochemical Characterization of LTP1b. Because both LTP1 and LTP1b exhibit close molecular weight, they cannot be differentiated by SDS-PAGE, so it was necessary to search for biophysical techniques that could afford the identification of LTP1b in the purified proteins. This is of great importance because, as outlined above, LTP1b and LTP1 can be eluted by chromatography in the same peak or as a shoulder so that one needs to check the purity of the proteins. Mass spectrometry is indeed the best way to highlight the presence of LTP1b in a peak, whereas SDS-PAGE is not efficient for such a task.

Another attempt to identify LTP1b was accomplished by using UV, fluorescence, circular dichroism (CD) spectroscopies, and capillary electrophoresis techniques. The use of fluorescent labeled phospholipids (*27*) showed that both LTP1 and LTP1b were able to transfer lipids with the same efficiency and no differences between the two proteins were observed in the absorption or far-UV CD spectra (not shown). This finding is of great importance because in most of the purification procedures of LTP1, fractions containing LTP1 are identified by their lipid transfer activity. This means that in this way, one cannot differentiate whether the lipid transfer protein isolated is a native LTP1 or LTP1b or a mixture of both. However, the intrinsic tyrosine fluorescence emission spectrum revealed a marked difference between LTP1 and LTP1b (Figure 2). Both spectra exhibited a typical maximum fluorescence at 305 nm, but the intensity for

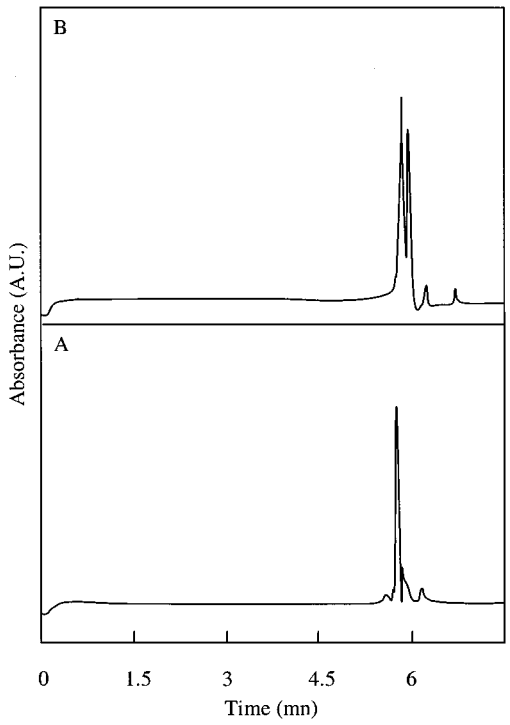

Figure 3. Capillary electrophoregram of LTP1 (A) and LTP1b (B) in 50 mM tetraborate (pH 10).

LTP1b was 2-fold lower that of LTP1. This is somewhat surprising because the binding of a molecule is rather associated with an increase of fluorescence emission (*12*, *13*). Tyrosine emission fluorescence can then be used to afford the differentiation between native LTP1 and LTP1b but necessitates a reference such as native LTP1 for which the purity has been previously determined.

LTP1 and LTP1b were submitted to capillary electrophoresis using 50 mM Tris-HCl (pH 10), and both proteins were eluted at the same time (not shown). However, when proteins were solubilized in tetraborate buffer (pH 10), the electropherogram showed a single peak in the case of LTP1 eluted at 5.74 mn (Figure 3A), whereas two peaks with about the same intensity were observed for LTP1b at a retention time slightly higher than that of native LTP1 (Figure 3B). These results suggest that LTP1b and, probably, the bound adduct itself interact with the tetraborate but not with Tris buffer, by modifying the overall structure and/or charge of the protein. This showed that capillary electrophoresis can also be used to afford the differentiation between native LTP1 and LTP1b but do not necessitate any reference as in case of tyrosine fluorescence.

Relation with Isoforms Isolated from Different Cultivars. We have clearly shown that identification of LTP1b along the purification procedure is far from trivial. As a consequence, our present proposal is to correlate our finding with the different isoforms that have been isolated in other plants. As mentioned above, isoforms of LTP1 have been isolated in various cultivars. These isoforms have not been fully characterized, so no information of their exact molecular mass or sequence is available. The most striking parallelism that can be found with our present work is the case of gravepine. Four isoforms have been separated by hydrophobic interaction chromatography (*20*). Of these four proteins, two were shown to possess the same 40 amino acids showing differences only in molecular masses and hydrophobic characteristics. Unfortunately, the exact mass of proteins is not given in this reference, so it is not possible to know whether an adduct of 294 Da was also found in that case. It must be remembered here that a marked difference was observed in the chromatogram patterns between LTP1s from barley and wheat. This suggests that for other plants, one could expect to obtain various patterns in which LTP1 and LTP1b could be eluted at the same time. In the same way, both proteins could be separated more easily than in the case of wheat (Figure 1). It is then tempting to speculate that among all of the isoforms observed in other plants, some could be analogous to LTP1b.

Attempt To Characterize the Adduct. Because the bound adduct was not released on mass spectrometry and upon extraction with different nonpolar solvents such as hexane, chloroform, and ethyl acetate (results not shown), it appeared to be probable that the adduct was covalently bound to the polypeptide backbone. It could be released by reduction and alkylation (see above) or alkaline pH (not shown), but attempts to recover the adduct after these treatments in the solution by both extraction with nonpolar solvents and by analysis of the extract by LC-MS failed. This revealed that the adduct could be degraded by these chemical procedures. However, the identification of this adduct is beyond the scope of the present paper, and it then clearly appears that its identification will come from structural studies by both multidimensional ^{1}H NMR (M. Ptak, personal communication) or crystallization of LTP1b (E. Pebay-Peroula, personal communication).

Conclusions. We have isolated an isoform-like LTP1 in wheat that exhibits a molecular weight excess of 294 Da compared to the native protein. A deep survey of the isoforms isolated from other plants suggests that this finding could be a general feature in the plant LTP1 family. Moreover, we have shown that mass spectrometry rather than SDS-PAGE should be used to evaluate the final purity of the protein. RP-HPLC, tyrosine fluorescence, and capillary electrophoresis can also be used to identify LTP1b or LTP1. This work opens new perspectives in the world of plant lipid transfer proteins and should contribute to increase our knowledge of this protein family.

ACKNOWLEDGMENT

We are indebted to Claude Desserme for detailed information about capillary electrophoresis.

LITERATURE CITED

(1) Sorensen, S. B.; Bech, L. M.; Muldbjerg, T. B.; Breddam, K. Barley lipid transfer protein 1 is involved in beer foam formation. *MBAA Tech. Q.* **1993**, *30*, 136−145.

(2) Jones, B. L.; Marinac, L. A. Purification, identification, and partial characterization of a barley protein that inhibits green malt endoproteinases. *J. Am. Soc. Brew. Chem.* **1997**, *55*, 58−64.

(3) Evans, D. E.; Hejgaard, J. The impact of malt derived proteins on beer foam quality. Part I. The effect of germination and kilning on the level of protein Z4, protein Z7 and LTP1. *J. Inst. Brew.* **1999**, *105*, 159−169.

(4) Jégou, S.; Douliez, J. P.; Mollé, D.; Boivin, P.; Marion, D. Purification and structural characterization of LTP1 polypeptides from beer. *J. Agric. Food Chem.* **2000**, *48*, 5023−5029.

(5) Douliez, J. P.; Michon, T.; Elmorjani, K.; Marion, D. Structure, biological and technological functions of lipid transfer proteins and indolines, the major lipid binding proteins from cereal kernels. *J. Cereal Sci.* **2000**, *32*, 1−20.

(6) Gincel, E.; Simorre, J. P.; Caille, A.; Marion, D.; Ptak, M.; Vovelle, F. Three-dimensional structure in solution of a wheat lipid transfer protein from multidimensional ^{1}H NMR data—a new folding for lipid carriers. *Eur. J. Biochem.* **1994**, *226*, 413−422.

(7) Shin, D. H.; Lee, J. Y.; Qwang, K. Y.; Kim, K. K.; Suh, S. W. High-resolution crystal structure of the non specific lipid transfer protein from maize seedlings. *Structure* **1995**, *3*, 189−199.

(8) Lee, J. Y.; Min, K.; Cha, H.; Shin, D. H.; Hwang, K. Y.; Suh, S. W. Rice non-specific lipid transfer protein: the 1.6 Å crystal structure in the unliganded state reveals a small hydrophobic cavity. *J. Mol. Biol.* **1998**, *276*, 437−448.

(9) Lerche, M. H.; Kragelund, B. B.; Bech, L. M.; Poulsen, F. M. Barley lipid-transfer protein complexed with palmitoyl CoA: the structure reveals a hydrophobic binding site that can expand to fit both large and small lipid-like ligands. *Structure* **1997**, *5*, 291−306.

(10) Charvolin, D.; Douliez, J. P.; Marion, D.; Cohen-Addad, C.; Pebay-Peyroula, E. The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. *Eur. J. Biochem.* **1999**, *264*, 562−568.

(11) Zachowski, A.; Guerbette, F.; Grobois, M.; Jolliot-Croquin, A.; Kader, J. C. Characterisation of acyl binding by a plant lipid transfer protein. *Eur. J. Biochem.* **1998**, *257*, 443−448.

(12) Douliez, J. P.; Michon, T.; Marion, D. Steady-state tyrosine fluorescence to study the lipid-binding properties of a wheat non-specific lipid-transfer protein (LTP1). *Biochim. Biophys. Acta* **2000**, *1467*, 65−72.

(13) Douliez, J. P.; Jégou, S.; Pato, C.; Mollé, D.; Tran, V.; Marion, D. Binding of two mono-acylated lipid monomers by the barley lipid transfer protein, LTP1, as viewed by fluorescence, isothermal titration calorimetry and molecular modelling. *Eur. J. Biochem.* **2001**, *268*, 384−388.

(14) Neumann, G. M.; Condron, R.; Svensson, B.; Polya, G. M. Phosphorylation of barley and wheat phospholipid transfer proteins by wheat calcium-dependent protein kinase. *Plant Sci.* **1993**, *92*, 159−167.

(15) Neumann, G. M.; Condron, R.; Thomas, I.; Polya, G. M. Purification and sequencing of a family of wheat lipid transfer protein homologues phosphorylated by plant calcium-dependent protein kinase. *Biochim. Biophys. Acta* **1994**, *1209*, 183−190.

(16) Kader, J. C.; Julienne, M.; Vergnolle, C. Purification and characterization of a spinach-leaf protein capable of transferring phospholipids from liposomes to mitochondria or chloroplasts. *Eur. J. Biochem.* **1984**, *139*, 411−416.

(17) Arondel, V.; Vergnolle, C.; Tchang, F.; Kader, J. C. Bifunctional lipid-transfer: fatty acid-binding proteins in plants. *Mol. Cell. Biochem.* **1990**, *98*, 49−56.

(18) Molina, A.; Garcia-Olmedo F. Enhanced tolerance to bacterial pathogens caused by the transgenic expression of barley lipid transfer protein LTP2. *Plant J.* **1997**, *12*, 669−675.

(19) Meijer, E. A.; De Vries, S. C.; Sterk, P.; Gadella, P. W. J., Jr.; Wirtz, K. W. A.; Hendriks, T. Characterization of the non-specific lipid transfer protein EP2 from carrot (*Daucus carota* L.). *Mol. Cell. Biochem.* **1993**, *123*, 159−166.

(20) Couthos-Thevenot, P.; Jouenne, T.; Maes, O.; Guerbette, F.; Grosbois, M.; Le Caer, J. P.; Boulay, M.; Deloire, A.; Kader, J. C.; Guern, J. Four 9-kDa proteins excreted by somatic embryos of grapevine are isoforms of lipid transfer proteins. *Eur. J. Biochem.* **1993**, *217*, 885−889.

(21) Bech, L. M.; Vaag, P.; Heinemann, B.; Breddam, K. Throughout the brewing process barley lipid transfer protein1 (LTP1) is transformed into a more foam-promoting form. *Proc. Eur. Brew. Conv. Congr.* **1995**, 561−568.

(22) Cammue, B. P.; Thevissen, K.; Hendriks, M.; Eggermont, K.; Goderis, I. J.; Proost, P.; Van Damme, J.; Osborn, R. W.; Guerbette, F.; Kader, J. C. A potent antimicrobial protein from onion seeds showing sequence homology to plant lipid transfer proteins. *Plant Physiol.* **1995**, *109*, 445−455.

(23) Tsuboi, S.; Osafune, T.; Sugeki, R. T.; Nishimura, M.; Yamada, Y. Nonspecific lipid transfer protein in castor bean cotyledon cells: subcellular localization and a possible role in lipid metabolism. *J. Biochem.* **1992**, *111*, 500−508.

(24) Fenn, J. B.; Mann, M.; Meng, C.; Wong, S. F.; Whitehouse, C. M. Electrospray ionisation for mass spectrometry of large biomolecules. *Science* **1989**, *246*, 64−71.

(25) Douady, D.; Grosbois, M.; Guerbette, F.; Kader, J. C. Purification of phospholipid transfer protein from maize seeds using a two-step chromatographic procedure. *Physiol. Veg.* **1985**, *23*, 373−380.

(26) Désormeaux, A.; Blochet, J. E.; Pézolet, M.; Marion, D. Amino acid sequence of a non specific wheat phospholipid binding protein and its conformation revealed by infrared and Raman spectroscopy. Role of disulphide bridges and phospholipids in stabilizing the α-helix structure. *Biochim. Biophys. Acta* **1992**, *1121*, 137−152.

(27) Somerharju, P. J.; Van Paridon, P. A.; Wirtz, K. W. Application of fluorescent analogues to studies on phospholipid transfer proteins. *Subcell. Biochem.* **1990**, *16*, 21−43.

Received for review November 7, 2000. Revised manuscript received February 1, 2001. Accepted February 1, 2001.

JF001327M