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Purification of Lipid Transfer Protein 2 (LTP_2) from Iranian rice paddy

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Abstract

Plant nonspecific lipid transfer proteins (nsLTPs) are divided into nsLTP1 and nsLTP2. The existence of an internal hydrophobic cavity, is a typical characteristic of nsLTPs that serves as the binding site for lipid substrates. In this communication a simple, rapid and low-cost alternative method was developed for purification of nsLTP2 from rice paddy. After extracting, final supernatant was loaded on CM-Sepharose column, which had previously equilibrated with 0.05 M Tris-HCl buffer, pH 8. Bounded proteins were separated using a linear gradient of 0-0.5 M NaCl. Solution of separated proteins was dialyzed and applied on a Phenyl-Sepharose column which previously equilibrated with Tris-HCl 0.05 M, ammonium sulfate 1.5 M, EDTA 0.005 M and $NaHSO_3$ 0.3%, pH 8.4. Tris-Tricin SDS-PAGE of separated proteins, obtained from ion-exchange column, showed multiple bands in the range of 2-20 kDa. Further purification using hydrophobic column resulted in single band of nsLTP2 at about 7 kDa, reflecting a purified sample in the gel.

Keywords: purification, plant lipid transfer proteins, cation-exchange chromatography, hydrophobic chromatography

Introduction

Cellular membrane biogenesis depends on the synthesis and transport of lipids. In eukaryotic cells, lipids are synthesized in the endoplasmic reticulum and golgi apparatus and transported to the cellular and organellar membranes. The synthesis of lipids in one organelle and subsequent movement through the cytosol to the cellular membrane of another organelle, suggest that water soluble transport proteins assisted in lipid transportation. These proteins are Lipid Transfer Proteins, LTPs (Cheng et al., 2004). The binding of lipids to LTPs can be both specific and nonspecific. These proteins have a great concern in pharmaceutical applications. They are good candidates for designing efficient drug delivery systems (Pato et al., 2001, Wang et al., 2005). The nonspecific lipid transfer proteins have affinity toward a variety of hydrophobic molecules (Charvolin et al., 1999). Nonspecific LTPs (nsLTPs) are ubiquitous proteins found in bacteria, yeast, plants and animals. They are the major lipid-binding proteins in plants have been isolated from rice, wheat, barley, maize, peaches and apricots (Cheng et al., 2004; Liu et al., 2002). Plant nsLTPs are basic (with pI 8–10) and disulfide-rich proteins divided into two subfamilies; nsLTP1 (molecular weight ~9 kDa) and nsLTP2 (molecular weight ~7 kDa) (Kader, 1996; Lin et al., 2005). All nsLTPs share a common structural fold stabilized by four disulfide bonds. Different nsLTPs have been purified from various plants. Douliez and colleagues, used cation-exchange, size-exclusion chromatography and RP-HPLC methods for purification of nsLTP2 from wheat seeds (Douliez et al., 2001). Other researchers has purified nsLTP2 from rice seeds using Sephadex (C25), 15S cation-exchange chromatography and HPLC methods (Liu et al., 2002). CM-Cellulose chromatography has been used in addition to gel filtration (Sephadex G50) and FPLC for gaining purified nsLTP2 from rice seeds (Dharmaraj et al., 2002). Gel filtration and HPLC as efficient methods for protein purification, require special and expensive equipments, usually are not available in any laboratories and time consuming as well. To this end, access to cost-effective and simple purification method will be a convenient issue. In this study an attempt was conducted to purify nsLTP2 from rice paddy kernels by using cation-exchange and hydrophobic chromatography, in order to develop a simple, low-cost and rapid procedure. Beside the simplicity of the used method, it provides a good opportunity for LTP purification elsewhere.

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Materials and Methods

Materials

Rice kernels were purchased from Lenjan local of Isfahan. CM-Sepharose and G50 Sephadex resins were obtained from Pharmacia. Chemical reagents were of analytical grade and purchased from Merck.

Purification procedure

Rice LTPs were isolated and purified according to slightly modified previous procedures (Yu et al., 1988; Lin et al., 2005). Rice kernels were ground in a blender and its flour (250 g) was suspended in 400 ml of 50 mM sulfuric acid. After it stood at room temperature for 4 h, the particulate matter was removed by centrifugation at 5000 rpm for 30 min and supernatant was passed through a piece of cheesecloth. The pH of solution was adjusted to 8.00 with concentrated NaOH and after standing at 4°C for 12 h, the produced precipitate was removed by centrifugation at 5000 rpm for 30 min. 100 ml of final supernatant was loaded onto a CM-Sepharose column (1.5×20 cm) which had previously been equilibrated with 0.05 M Tris-HCl buffer pH 8.00 with the flow rate of 12 ml/h. Bounded proteins were separated by a linear gradient of 0 to 0.5 M NaCl in the same buffer (50 ml in each reservoir).Protein was monitored by on-line measurements of the absorbance at 280 nm. Solution of separated proteins was concentrated by freeze drying and dialyzed against 0.05 M Tris-HCl pH 8.4. Ammonium sulfate, EDTA and sodium bisulfite in final concentration of 1.5, 0.005 M and 0.3% was added to dialyzed solution, respectively. Final solution was loaded onto a Phenyl-Sepharose column (1.5×10 cm) which previously equilibrated with 0.05 M Tris-HCl, 1.5 M ammonium sulfate, 0.005 M EDTA and 0.3% NaHSO3 pH 8.4, with the flow rate of 40 ml/h. Elution performed by a decreasing non-linear gradient of buffer in five steps at 1.2, 0.9, 0.6, 0.3 and 0 M of ammonium sulfate. The eluted fractions at 1.2 M ammonium sulfate were pooled, desalted and freeze dried.

Tris-Tricin sodium dodecyl sulfate Polyacrylamide gel electrophoresis

It was performed according to a slightly modified method of Schagger and Von Jagow (Schagger and Von Jagow, 1987). SDS-PAGE was carried out using gel with 3% C, 16.5% acrylamide for resolving gel, 10% acrylamide for spacer gel and 4% acrylamide for stacking gel and samples were then loaded onto the 1.0-mm slab gel followed by electrophoresis with 100 V. Gels were fixed in methanol 50%, acetic acid 10% and proteins were visualized by Coomassie Brilliant Blue G250 staining. Mioglobin (16.95 kDa), Mioglobin I+II (14.4 kDa), Mioglobin I (8.16 kDa), Mioglobin II (6.2 kDa) and Mioglobin III (2.5 kDa) were used as molecular weight markers.

ANS Fluorescence measurements

To confirm the presence of hydrophobic surface on nsLTP2, emission spectral changes of ANS was studied during binding to the protein. ANS fluorescence intensity (400μM) was recorded between 400-600 nm after excitation at 380 nm. Then, ANS was added to solutions containing nsLTP2 (4 and 4.2 μM, respectively) in 0.05 M Tris-HCl buffer, pH 8.4 and fluorescence intensity recorded as described above.

Results

LTP2 purification NsLTP2 obtained from rice kernels was purified using a combination of ion exchange and hydrophobic interaction chromatography. A CM-Sepharose column was initially used for separation of basic peptides following extracting. The basic fraction (figure 1, P2 peak) represents the minor protein fraction. The electrophoretic analysis of P2 proteins obtained from cation exchange chromatography showed the presence of two major sets of bands in Tris-Tricin SDS-PAGE. The first set is comprised of low molecular weight proteins with apparent molecular masses between 6 and 8 kDa while the second one shows band with molecular weight of 2.5 kDa (figure 2, lanes 7-10).

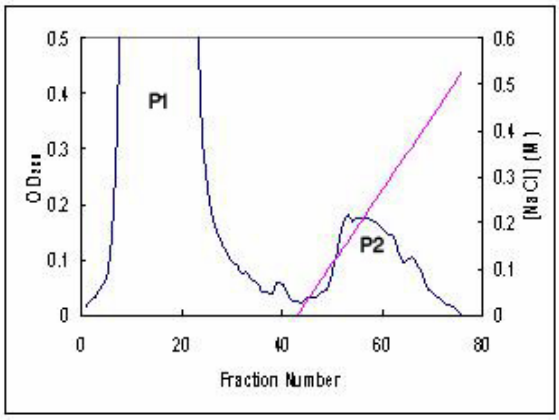

Figure 1. CM-Sepharose chromatography of extraction. The column was equilibrated with 0.05 M Tris-HCl pH 8.0 buffer and eluted with 0-0.5 M NaCl. The flow rate was 12 ml/h.

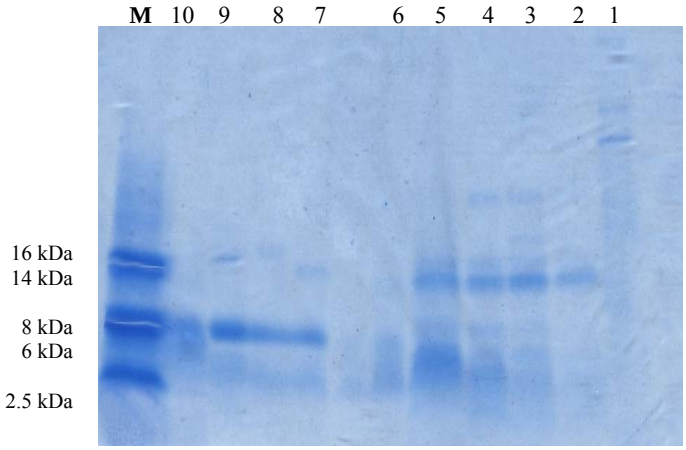

Figure 2. Tris-Tricin SDS-PAGE of P1 and P2 peaks. Lanes 1-6: P1 peak; lanes 7-10: P2 peak. Numbers in lane M refer to molecular mass markers.

For further purification, the P2 peak fractions were also subjected to hydrophobic interaction chromatography on Phenyl-Sepharose column.

The proteins were separated in several peaks (figure 3). Protein patterns were analyzed by Tris-Tricin SDS-PAGE and visualized by coomassie brilliant blue staining. The results showed the presence of a protein with a molecular weight of 7 kDa (figure 4, lanes 4-6).

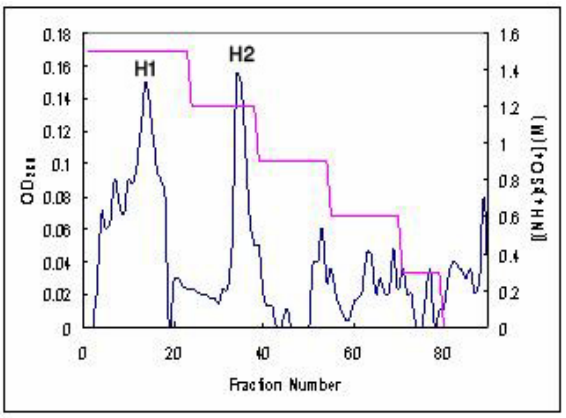

Figure 3. Phenyl-Sepharose chromatography of P2 fractions. The column was equilibrated with 0.05 M Tris-HCl, 1.5 M ammonium sulfate, 0.005 M EDTA and 0.3% NaHSO3 pH 8.4. Flow rate was 40 ml/h.

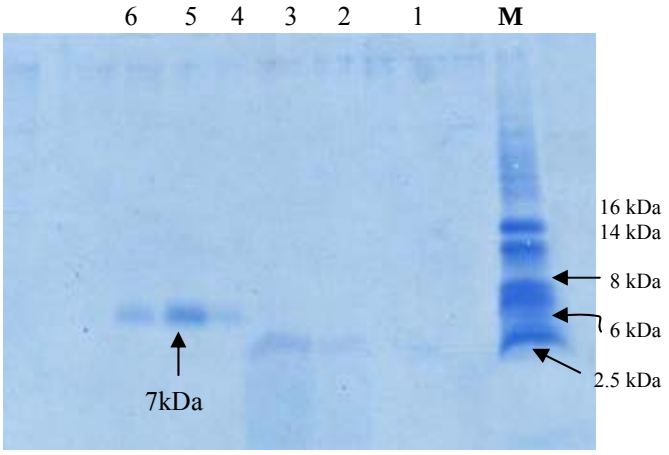

Figure 4. Tris-Tricin SDS-PAGE of protein fractions from Phenyl-Sepharose chromatography. Lanes 1-3: H1 peak; lanes 4-6: H2 peak. Numbers in lane M refer to molecular mass markers.

ANS binding study

ANS is a widely used hydrophobic probe for proteins and membranes characterization. The fluorescence intensity of ANS alone is low in water, while it places in hydrophobic medium its fluorescence intensity increases which is supplementary with a blue shift (Matulis et al., 1998). It has been shown that nsLTP's structure contains a hydrophobic cavity surrounded by four helices connected through disulfide bonds. The hydrophobic cavity is the binding pocket for lipid or fatty acid molecules (Liu et al., 2002). This naturally occurring feature of nsLTP should bring a useful opportunity for checking its presence in relevant solutions, via affinity toward hydrophobic molecules resembling ANS. Fig. 5 shows the ANS emission spectral changes in the absence and presence of nsLTP2 solution. It can be seen that ANS fluorescence intensity is low in the absence of nsLTP2 (curve 1) while its intensity is increased when nsLTP2 is present in the solution (curve 2 and 3), by way of a blue shift due to binding to the hydrophobic patches of the protein.

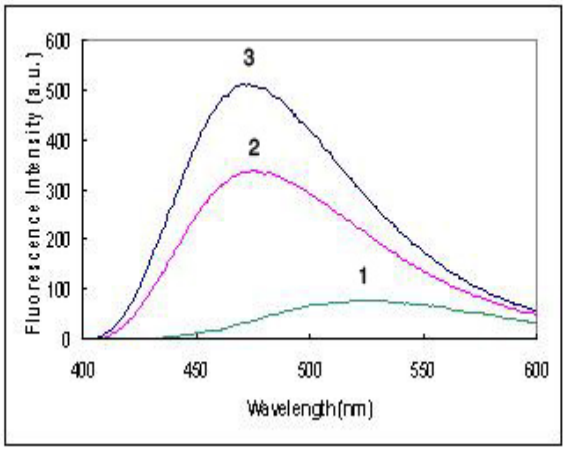

Figure 5. ANS fluorescence intensity (400 µM) in the absence of nsLTP2 (1) and in the presence of nsLTP2 (4µM) (2) and nsLTP2 (4.2 µM) (3).

Discussion

Throughout the study of some articles had been purified LTP2 from various plants, we observed that some of those have loaded output of cation exchange chromatography onto that column in the same conditions and have repeated this stage one or two times over again (Ozaki et al., 1980; Yu et al., 1988). Since there were no sensible reasons for these repeats, we did not recreate this stage more times. Some other researchers have used gel filtration and/or RP-HPLC after ion exchange chromatography (Segura et al., 1993; Liu et al., 2002; Ooi et al., 2005). Using of these methods are

limited for the reason that of being costly and requirements of specific facilities. Consequently, in this study, relative to specific feature of LTP2 protein, presence of a hydrophobic cavity, we applied a hydrophobic chromatography method to separate the protein from rice paddy. The obtained results showed that this type of chromatography is useful and efficient tool for LTP2 purification.

In conclusion, given that other nsLTP2 purification methods have been used to date (Yu et al., 1988; Charvolin et al., 1999; Douliez et al., 2001; Liu et al., 2002) are intricate from the performance view point, due to high-cost, time overwhelming and instrumental dependency, the advantages of the developed procedure introduced in this article make it possible to access LTPs in a rapid and simple route. However, we proposed that ion-exchange chromatography along with hydrophobic interaction chromatography are convenient methods for feasible and rapid purification of nsLTP2.

References

1- Charvolin D. (1999) The crystal structure of a wheat nonspecific lipid transfer protein (ns-LTP1) complexed with two molecules of phospholipid at 2.1 Å resolution. European Journal of Biochemistry 264: 562-568.

2- Cheng H. Ch. (2004) Cheng. Lipid binding in rice nonspecific lipid transfer protein-1 complexes from *Oryza sativa*. Protein Science 13: 2304–2315.

3- Dharmaraj S. (2002) Solution Structure of Plant Nonspecific Lipid Transfer Protein-2 from Rice *(Oryza sativa*). Journal of Biological Chemistry 277: 35267–35273.

4- Douliez J. P. (2001) Disulfide bond assignment, lipid transfer activity and secondary structure of a 7-kDa plant lipid transfer protein, LTP2. European Journal of Biochemistry 268: 1400-1403.

5- Kader J. C. (1996) Lipid transfer proteins in plants. Annul Review of Plant Physiology Plant Molecular Biology 47: 627–654.

6- Lin K. F. (2005) Characterization and structural analyses of nonspecific lipid transfer protein 1 from *Mung Bean*. Journal of Biochemistry 44: 5703-5712.

7- Liu W. J. (2002) Purification and characterization of a novel 7-kDa non-specific lipid transfer protein-2 from rice (*Oryza sativa*). Biochemistry & Biophysics Research Communications 294: 535–540.

8- Ooi L. (2005) Purification and characterization of non-specific lipid transfer proteins from the leaves of Pandanus amaryllifolius (Pandanaceae). Journal of Peptides 62:773-779.

9- Ozaki Y. (1980) Amino acid sequence of a purothionin homolog from barley flour. Journal of Biochemistry 87: 549-555.

10- Pato Ch. (2001) Potential application of plant lipid transfer proteins for drug delivery, Journal of Biochemistry & Pharmacology 62: 555-560.

11- Schagger H. (1987) Tricine-sodium dodecylsulfate polyacrylamide gel electrophoresis for the separation of proteins in the range from 1 to 100 kDa. Analytical Biochemistry 166: 368-379.

12- Segura A. (1993) Purification and antipathogenic activity of lipid transfer proteins (LTPs) from the leaves of *Arabidopsis* and spinach. Journal of FEBS 332: 243-24.

13- Wang Z. h. (2005) Identification of non-specific lipid transfer protein-1 as a calmodulin-binding protein in Arabidopsis, FEBS Letter 579: 1683–1687.

14- Yu Y. G. (1988) Amino acid sequence of a probable amylase/protease inhibitor from rice seeds. Archives of Biochemistry and Biophysics 265: 466-475.