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ScienceDirect
Protein Expression and Purification 51 (2007) 162–169
Protein Expression & Purification
www.elsevier.com/locate/yprep

High-level constitutive expression in *Pichia pastoris* and one-step purification of phospholipase D from cowpea (*Vigna unguiculata* L. Walp)

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Received 26 May 2006, and in revised form 9 July 2006
Available online 1 August 2006

Abstract

Phospholipase D (PLD) is one of the main enzymes involved in signal transduction, vesicle trafficking and membrane metabolism processes. Here we describe the heterologous high-yield expression in the yeast *Pichia pastoris*, one-step purification and characterization of catalytically active PLDα from cowpea (*Vigna unguiculata* L. Walp). Immunoblotting experiments showed that recombinant PLDα is recognized by a polyclonal antibody raised against native soybean PLDα. A single calcium-dependent octyl-Sepharose chromatography step was used to obtain a highly purified recombinant PLDα, as attested by gel electrophoresis, N-terminal amino acid sequence and mass spectrometry data. From 1 L of yeast culture medium, about 8 mg of pure recombinant PLDα was obtained and the specific activity measured on phosphatidylcholine was 27 μmol/min/mg. Contrary to what was observed previously with *Vigna unguiculata* PLDα expressed in insect cells, no proteolytic degradation of the N-terminal calcium-dependent C2 lipid binding domain was observed here. This functional recombinant PLDα should provide a valuable tool for performing detailed studies on the molecular characterization of enzymes as well as structural studies.
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Keywords: Phospholipase D; Octyl-Sepharose; C2 domain; *Pichia pastoris*

Phospholipase D (PLD)[1] is a ubiquitous enzyme that hydrolyzes the terminal phospho diester bond of glycerophospholipids, leading to the formation of phosphatidic acid (PA) and a free polar head group such as choline in the case of phosphatidylcholine (PC). In addition, this enzyme efficiently catalyzes the transphosphatidylation reaction which, in the presence of a primary alcohol, leads to the formation of the corresponding phosphatidyl alcohol [1,2]. One of the PLD products, PA, has been found to serve as a second messenger in several physiological processes occurring in mammalian cells, including the oxidative burst in neutrophils, actin assembly, vesicle trafficking and protein secretion (see [3,4] for reviews). In plants, PLD is known to be involved in many physiological processes, such as seed germination, the growth of seedlings, phosphatidylinositol metabolism in roots, senescence, fruit ripening and stress damage due to cold, water restriction, wounding and pathogenic infection (see [5,6] for reviews). Previous reports indicated that plant PLD was found to be in protein bodies, which are thought of as specialized vacuoles [7] and was associated with plasma and intracellular membranes [8]. During mung bean seed germination and seedling growth, the decrease in phospholipid content has been correlated with an increase in PLD activity [7]. It has been suggested that these activity changes may be due to metabolic reactions involving membrane phospholipids, which are essential to plant growth and development [9].

Various forms of PLD have been cloned and identified in plants. Five types of PLD, known as PLDα, PLDβ, PLDγ,

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[1] *Abbreviations used:* PLD, phospholipase D; PA, phosphatidic acid; PC, phosphatidylcholine.

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doi:10.1016/j.pep.2006.07.018

PLDδ and PLDζ, which differ in their catalytic properties and regulatory processes, have been described in *Arabidopsis thaliana* [6]. The main form, PLDα, is active *in vitro* at millimolar Ca^{2+} concentrations and is phosphoinositide independent. PLDβ, PLDγ and PLDδ, which are most active forms *in vitro* at micromolar Ca^{2+} concentrations, require the presence of phosphoinositide serving as a cofactor [10]. In addition, PLDδ is characterized by the fact that it is activated by oleic acid [11,12], whereas PLDζ activity has been found to be phosphoinositide- and Ca^{2+}-independent [13].

In all the plant PLDα cloned so far, sequence alignments have suggested the presence of a C2 lipid binding domain located at the N-terminus [14,15]. C2 domains often mediate the binding to phospholipids in a Ca^{2+}-dependent manner, and these C2 domains have been identified in a number of enzymes involved in signal transduction and phospholipid metabolism processes [14,16].

Although PLD occurs in plants, bacteria and animals, little structural information is available so far about this enzyme. Only the three-dimensional structure of a bacterial PLD is available to date [17]. This situation is mainly due to the lack of large amounts of purified protein. Up to now, attempts at producing mammalian PLD in high levels have not been successful. The cDNA cloning of PLDα from several plant species opened up the possibility of establishing high-yield PLD expression systems. We previously described the method used to express the PLDα from *Vigna unguiculata* in insect cells and the subsequent purification process. Transient production of recombinant PLDα yielded a protein with a molecular mass of 88 kDa (PLDαa), which was further degraded into 87 kDa (PLDαb), which accumulated in the insect cell culture medium. N-Terminal amino acid sequencing of the PLDαa and PLDαb obtained showed that PLDαb resulted from the proteolytic cleavage of PLDαa at the Gly8–Ile9 peptide bond [18]. This cleavage occurred at the N-terminal of the PLDα C2 domain and was found to change the calcium-dependency of the enzyme [18].

In this study, we overcame the problems associated with this proteolytic cleavage process by expressing *Vigna unguiculata* PLDα gene in the yeast *Pichia pastoris*. High levels of PLDα forming the intracellular protein were expressed and a one-step purification procedure was developed for use with this protein.

Materials and methods

Materials

Egg yolk PA, egg yolk PC (type XI-E), 1,2-dioctanoyl-*sn*-3-phosphocholine (diC8:0 PC), octyl-Sepharose CL-4B, choline oxidase (from *Arthrobacter globiformis*), horseradish peroxidase (type IV) and protease inhibitors were obtained from Sigma Chemicals Company. Soybean phosphatidylethanol (PEtOH) was purchased from Larodan AB (Sweden). Thin layer chromatography (TLC) silica gel 60 F_{254} plates were from Merck.

Construction of the expression plasmids

A cDNA encoding the PLDα from cowpea (*Vigna unguiculata*) in the pVL1392 vector (Invitrogen) was obtained as previously described [18]. The PLDα cDNA (2700 bp) was amplified by PCR with the following primers: forward (5′-CCTGAATTCGCCATGGCGCAAATT CTGCTTC-3′) and reverse (5′-AGTCTAGACCATGCT ATGCTAAC-3′). The forward primer introduced a restriction enzyme site for *Eco*RI, and the reverse primer did so for *Xba*I (underlined). After purification and digestion with *Eco*RI and *Xba*I, the amplified DNA fragment was cloned into the pGAPZB vector (Invitrogen) containing the alcohol oxidase promoter AOX1 and the marker Zeocin. The resulting plasmid harboring the PLDα gene was further amplified in *Escherichia coli* after electroporation of DH5α cells (Life Technologies). Plasmid DNAs were isolated from *E. coli* cell cultures using the alkaline lysis procedure [19] and purified using the Wizard DNA purification system (Promega). Digestion with restriction enzymes and ligation with T4 DNA ligase were performed as recommended by the enzyme supplier (New England Biolabs). The PCRs were carried out using *pfu* DNA polymerase (stratagene). All DNA constructs were checked by performing DNA sequencing (ESGS, France).

Transformation of P. pastoris and selection of PLDα-expressing clones

The *P. pastoris* X33 strain (Invitrogen) was transformed by electroporation with 5 μg of *Avr*II-linearized plasmid (CellJect Electroporation System, Eurogentec), under a charging voltage of 1500 V, a capacitance of 40 μF, and a resistance of 150 Ω. Immediately after the cell transformation, 1 ml of ice-cold 1 M sorbitol was added to the cuvette and aliquots were plated onto YPD (yeast extract peptone dextrose) plates containing 100 μg/ml Zeocin. In negative control assays, *P. pastoris* cells were transformed with pGAPZB not carrying the PLDα gene. After a 3-day incubation period at 30 °C, colonies of transformed *P. pastoris* developed on the plates and transformants harboring the PLDα gene were identified by direct PCR analysis as described previously [20]. Briefly, single colonies isolated from the plates were further grown *in vitro* in 5 ml YPD medium at 30 °C overnight. Aliquots (10 μl) were digested with zymolyase (1 μg/μl, final concentration) for 10 min at 30 °C, immersed in liquid nitrogen for 1 min and used in 50-μl PCRs containing specific primers. The positive clones were further analyzed prior to expression of the recombinant PLDα by enzyme assay and immunoblotting procedures described bellow.

PLDα assay

PLDα activity was assayed spectrophotometrically by measuring the free choline released upon PC hydrolysis, using a continuous method [21] adapted for microplates (96

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