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# Crystallization and preliminary crystallographic study of a recombinant phospholipase D from cowpea (*Vigna unguiculata* L. Walp)

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The plant phospholipase D (PLD) is considered to be a key enzyme involved in various physiological processes such as signal transduction and membrane metabolism. Crystals of the PLD protein from *Vigna unguiculata* have been produced from the recombinant 768 amino-acid protein. The crystals belong to the monoclinic space group $C2$, with unit-cell parameters $a$ = 157.7, $b$ = 65.6, $c$ = 90.2 Å, $\beta$ = 111.5°. There is one molecule in the asymmetric unit. Frozen crystals diffract to at least 1.94 Å resolution using synchrotron radiation. A search for heavy-atom derivatives using ytterbium and tungstate is currently under way in order to solve the three-dimensional structure.

## 1. Introduction

PLD is an ubiquitous enzyme hydrolyzing the terminal phosphodiester bond of glycerophospholipids, leading to the formation of phosphatidic acid (PA) and to a free polar head group such as choline in the case of phosphatidylcholine. This enzyme very efficiently catalyzes a transphosphatidylation reaction which, in the presence of a primary alcohol, leads to the formation of the corresponding phosphatidylalcohol (Eibl & Kovatchev, 1981; Kobayashi & Kanfer, 1987). 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 secretion (for reviews, see Exton, 1999; Frohman *et al.*, 1999; Jones *et al.*, 1999). 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 (for a review, see Pappan & Wang, 1999).

An eukaryotic PLD cDNA was first cloned and sequenced from castor bean (Wang *et al.*, 1994). Subsequently, PLD cDNA was cloned from a wide variety of species ranging from bacteria to humans (for a review, see Liscovitch *et al.*, 2000). Various regions of highly conserved sequences found in PLDs from animal, plant, yeast and bacterial species are also present in bacterial phosphatidylserine synthase, cardiolipin synthase and endonucleases as well as in a variety of genes of unknown function, suggesting the existence of a superfamily (Koonin, 1996; Ponting & Kerr, 1996). All cloned PLDs contain two regions with the invariant charged sequence H$x$K($x$)$_4$D, termed the HKD motif (Hammond *et al.*, 1995; Koonin, 1996; Ponting & Kerr, 1996). It has been proposed that these conserved residues are directly involved in catalysis (Gottlin *et al.*, 1998; Sung *et al.*, 1997). Various mutagenesis studies of PLD from several species have confirmed the importance of these amino acids for *in vitro* catalysis and experimental data has suggested the formation of a covalent phosphohistidine intermediate (Gottlin *et al.*, 1998; Sung *et al.*, 1997). This reaction-mechanism model has been refined and extended through the structural analysis of a member of the PLD superfamily, 16 kDa bacterial endonuclease (Nuc) (Stuckey & Dixon, 1999). Nuc crystallizes as a dimer in which the active site is defined by the HKD motifs of the two monomers.

The crystal structure of a 54 kDa PLD from *Streptomyces* sp. strain PMF has been determined recently to 1.9 Å resolution (Leiros *et al.*, 2000). The monomeric enzyme is folded into two domains related by a pseudo-twofold rotation axis running through the active site and located at the interface of the two domains (Leiros *et al.*, 2000). As for the Nuc protein, the proposed reaction mechanism involves the formation of a covalent phosphohistidine intermediate; this model is likely to be valid for the Nuc and *Streptomyces* PLD as well as for other members of the PLD superfamily (Leiros *et al.*, 2000).

Three forms of plant PLD known as PLD$\alpha$, PLD$\beta$ and PLD$\gamma$ have been described in *Arabidopsis thaliana*. They are encoded by

distinct genes and differ in their catalytic properties and regulatory processes (Pappan *et al.*, 1997, 1998; Qin *et al.*, 1997). PLD$\beta$ and PLD$\gamma$ require phosphoinositide as a cofactor and are active *in vitro* at micromolar calcium concentrations. PLD$\alpha$ is the main and most classical form: it is active *in vitro* at millimolar calcium concentrations and is phosphoinositide independent. The biochemical properties of PLD$\alpha$ from various plant species have been studied and show considerable similarities. The enzyme has been purified from cabbage leaves (Abousalham *et al.*, 1993; Lambrecht & Ulbrich-Hohhmann, 1992), castor bean endosperm (Wang *et al.*, 1993), rice (Ueki *et al.*, 1995), soybean cells (Abousalham *et al.*, 1995) and germinating sunflower seeds (Abousalham *et al.*, 1997).

In all cloned PLD$\alpha$ from plants, sequence alignments have suggested the presence of a calcium/phospholipid-binding C2 domain at the N-terminus (Ponting & Kerr, 1996; Qin *et al.*, 1997). Mammalian, bacterial and yeast PLD lack this C2 domain, which often mediates the binding to phospholipids in a calcium-dependent manner and has been identified in a number of enzymes involved in signal transduction and/or phospholipid metabolism (Chahinian *et al.*, 2000; Nalefski & Falke, 1996; Ponting & Parker, 1996).

We expressed recombinant PLD$\alpha$ (rPLD$\alpha$) from *V. unguiculata* in the baculovirus/insect cell system (El Maarouf *et al.*, 2001). The rPLDa was secreted into the culture medium as an active form and was purified to homogeneity. The availability of baculovirus-derived rPLD$\alpha$ constitutes a valuable source of enzyme. We report here the crystallization conditions and preliminary X-ray data from the rPLD$\alpha$ crystals.

## 2. Results and discussion

### 2.1. rPLD$\alpha$ expression and purification

rPLD$\alpha$ was expressed in the baculovirus/insect cell system as described elsewhere (El Maarouf *et al.*, 2001). Large amounts of baculovirus-derived rPLDa from *V. unguiculata* were secreted into the culture medium of baculovirus-infected insect cells. One-step calcium-dependent octyl-Sepharose chromatography was used to obtain the highly purified rPLDa, as attested by gel electrophoresis, N-terminal amino-acid sequencing and mass spectrometry (El Maarouf *et al.*, 2001). The purified rPLD$\alpha$ yield reached a level of 10 mg per litre of serum-free culture medium.

### 2.2. Crystallization conditions

The rPLDa protein was concentrated to 4.2 mg ml$^{-1}$ in 10 m*M* PIPES buffer pH 6.5 using a centrifugal filter device (Ultrafree Biomax 30K, Millipore, Bedford MA, USA). Precipitation experiments were carried out on the rPLD$\alpha$ protein using various precipitating agents [$(NH_4)_2SO_4$, PEG, NaCl, MPD, ethanol] at various pHs (5, 6, 7, 8, 9). Very small needles were observed from ammonium sulfate precipitation experiments at pH 8. The *SamBA* software (Audic *et al.*, 1997) was then used in order to optimize the crystallization conditions, using ammonium sulfate in association with various salts (sodium chloride, calcium chloride, magnesium chloride and sodium formate) between pH 7.5 and pH 9.0. Crystallization trials were performed at 293 K by hanging-drop vapour diffusion using 24-well culture plates. Each hanging drop was prepared by mixing 0.5 µl of the 4.2 mg ml$^{-1}$ rPLD$\alpha$ with an equal volume of the reservoir solution. The hanging drop on the cover glass was vapour-equilibrated against 500 µl of the reservoir solution in each well of the tissue-culture plate. The best crystals were obtained after one week from a solution containing 1.6–1.7 *M* $(NH_4)_2SO_4$, 0.5 *M* NaCl, 0.1 *M* imidazole buffer pH 8.5

### 2.3. Data collection and processing

A single crystal (0.3 × 0.2 × 0.1 mm) was collected in a Hampton Research 0.5 mm$^3$ loop, flash-frozen to 105 K in a cold nitrogen-gas stream and subjected to X-ray diffraction. The data set was collected on a MAR CCD at the ESRF synchrotron-radiation facility (ID14 EH4) at a wavelength of 0.9465 Å. Data collection was carried out with oscillation angles of 1.0° and a crystal-to-detector distance of 120 mm. The total oscillation range collected was 110°. Space-group determination was performed using the autoindexing option in *DENZO* (Otwinowski, 1993). The crystals belong to the monoclinic space group *C*2, with unit-cell parameters $a$ = 157.72, $b$ = 65.57, $c$ = 90.2 Å, $\beta$ = 111.5°. The packing density for one monomer of rPLD$\alpha$ (87.157 KDa) in the asymmetric unit of the crystals (volume = 867 803 Å$^3$) is 2.49 Å$^3$ Da$^{-1}$, a reasonable value for globular proteins, indicating an approximate solvent content of 50.6% (Matthews, 1968).

The data set was processed using *DENZO* (Otwinowski, 1993); the *SCALA* program from the *CCP*4 package (Collaborative Computational Project, Number 4, 1994) was used for the scaling and data reduction of the native data set. The crystal diffracted to at least 1.94 Å; 261 944 reflections were measured in the resolution range 24.5–1.94 Å. This reduced to a data set of 59 540 unique reflections, with an $R_{sym}$ value of 5.7. It represents a completeness of 94% with a multiplicity of 2.1 and an average $I/\sigma(I)$ of 7.7. For the highest resolution shell, 12 199 reflections were measured in the resolution range 2.01–1.94 Å, corresponding to 5866 unique *hkl*, an $R_{sym}$ value of 28.6, an average $I/\sigma(I)$ of 1.6, a completeness of 94% and a multiplicity of 2.8. In order to solve the rPLD$\alpha$ structure, we are currently investigating various heavy-atom derivatives using both the phospholipid-binding site (tungstate salt) and the two calcium-binding sites (ytterbium salt). We determined that a 1 m*M* sodium tungstate concentration is sufficient to inhibit 40% of the rPLD$\alpha$ activity in 5 min (data not shown). This salt will therefore be used with the MAD method (Hendrickson *et al.*, 1990) to solve the structure. The soaking of rPLDa crystals as well as their co-crystallization with both tungstate and ytterbium are currently being investigated.

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