Phytochemistry 117 (2015) 65–75

Contents lists available at ScienceDirect

Phytochemistry

journal homepage: www.elsevier.com/locate/phytochem

Purification, sequencing and characterization of phospholipase D from Indian mustard seeds

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ARTICLE INFO

Article history:
Received 15 January 2015
Received in revised form 15 April 2015
Accepted 27 May 2015

Keywords:
Phospholipase D
Brassica juncea
Purification
Amino acid sequence
Phosphatidylinositol 4,5-bisphosphate
Substrate specificity
Calcium
Inhibitors

ABSTRACT

Phospholipase D (PLD; E.C. 3.1.4.4) is widespread in plants where it fulfills diverse functions in growth and in the response to stresses. The enzyme occurs in multiple forms that differ in their biochemical properties. In the present paper PLD from medicinally relevant Indian mustard seeds was purified by Ca^{2+}-mediated hydrophobic interaction and anion exchange chromatography to electrophoretic homogeneity. Based on mass-spectrometric sequence analysis of tryptic protein fragments, oligonucleotide primers for cloning genomic DNA fragments that encoded the enzyme were designed and used to derive the complete amino acid sequence of this PLD. The sequence data, as well as the molecular properties (molecular mass of 92.0 kDa, pI 5.39, maximum activity at pH 5.5–6.0 and Ca^{2+} ion concentrations $\geqslant$60 mM), allowed the assignment of this enzyme to the class of α-type PLDs. The apparent kinetic parameters V_{max} and K_m, determined for the hydrolysis of phosphatidylcholine (PC) in an aqueous mixed-micellar system were 356 ± 15 μmol min^{-1} mg^{-1} and 1.84 ± 0.17 mM, respectively. Phosphate analogs such as $NaAlF_4$ and Na_3VO_4 displayed strong inhibition of the enzyme. Phosphatidylinositol 4,5-bisphosphate had a strong activating effect at 2–10 mM $CaCl_2$. PLD was inactivated at temperatures >45 °C. The enzyme exhibited the highest activity toward PC followed by phosphatidylethanolamine and phosphatidylglycerol. PCs with short-chain fatty acids were better substrates than PCs with long fatty acid chains. Lyso-PC was not accepted as substrate.

1. Introduction

Phospholipase D (PLD, E.C. 3.1.4.4) is a phospholipid-cleaving enzyme widespread in both prokaryotic and eukaryotic organisms (Selvy et al., 2011). It catalyzes the hydrolysis of glycerophospholipids such as phosphatidylcholine (PC), phosphatidylethanolamine (PE), or phosphatidylglycerol (PG) at the terminal phosphodiester bond releasing phosphatidic acid (PA) and the corresponding alcohol. This reaction plays an important role in phospholipid metabolism as well as in many cellular processes such as signaling, intracellular trafficking, senescence and membrane remodeling (Wang, 2000; Bargmann and Munnik, 2006; Exton, 2002). In the last decade, the PLD product PA has been shown to be an important signaling lipid in plants; it plays a role in the response to diverse forms of biotic and abiotic stresses (Testerink and Munnik, 2011). PA might also be crucial for the regulation of cell expansion in pollen tubes and root hairs (Testerink and Munnik, 2011), as well as in anisotropic plant growth (Gardiner and Marc, 2013). In seeds where PLD activity increases during germination (Novotna et al., 2000; Oblozinsky et al., 2003; Abdelkafi and Abousalham, 2011) it might be involved in phospholipid metabolism that supports

Abbreviations: BSA, bovine serum albumin; Ca^{2+}-HIC, Ca^{2+}-mediated hydrophobic interaction chromatography; EDTA, ethylenediamine tetraacetic acid; HEPES, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid; lyso-PC, lysophosphatidylcholine; MES, 2-(N-morpholino)ethanesulfonic acid; PA, phosphatidic acid; PC, phosphatidylcholine; 8:0/8:0-PC, 1,2-dioctanoyl-*sn*-glycero-3-phosphocholine; 10:0/10:0-PC, 1,2-didecanoyl-*sn*-glycero-3-phosphocholine; 12:0/12:0-PC, 1,2-dilauroyl-*sn*-glycero-3-phosphocholine; 14:0/14:0-PC, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine; 16:0/16:0-PC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; 18:0/18:0-PC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; 16:0/18:1 PC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; 18:1/18:1-PC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; 1,3-PC, 1,3-diacylglycero-2-phosphocholine; 1,3-8:0/8:0-PC, 1,3-dioctanoyl-glycero-2-phosphocholine; 1,3-12:0/12:0-PC, 1,3-dilauroyl-glycero-2-phosphocholine; 1,3-14:0/14:0-PC, 1,3-dimyristoyl-glycero-2-phosphocholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PIPES, piperazine-1,4-bis(2-ethanesulfonic acid; *Pp*NP, phosphatidyl-*p*-nitrophenol; TRIS, tris(hydroxymethyl)aminomethane; PLD, phospholipase D; SDS, sodium dodecylsulfate; SDS–PAGE, SDS–polyacrylamide gel electrophoresis.

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http://dx.doi.org/10.1016/j.phytochem.2015.05.022
0031-9422/

the growth of seedlings. On the other hand, high levels of PLD in Arabidopsis seeds were found to be detrimental to seed quality and oil stability (Devaiah et al., 2007).

In addition to the hydrolytic reaction, most PLDs efficiently catalyze the transfer of the phosphatidyl moiety to acceptor alcohols if an appropriate alcohol is present. This transphosphatidylation activity of PLD has been widely utilized for many years in the synthesis of phospholipids in the laboratory as well as on an industrial scale (Ulbrich-Hofmann et al., 2005; De Maria et al., 2007; Damnjanovic and Iwasaki, 2013). The search for new PLDs is therefore a challenging aim in basic as well as in applied research.

Plant PLDs represent the largest group within the PLD superfamily (Selvy et al., 2011). Although also monomeric, their domain structures and genomic organization are much more diverse than those of other organisms. Thus, the 12 PLDs in *Arabidopsis thaliana* have been classified into six types based on gene architecture, sequence similarity, domain structure, and their biochemical properties as PLDα (3), β (2), γ (3), δ, ε and ζ (2) (Li et al., 2009). Ten of the 12 Arabidopsis PLD isoenzymes (α-, β-, γ-, δ-, ε-types) contain one C2 domain in their N-terminal region and are designated as Ca^{2+}-dependent or C2-PLDs, whereas the two PLDs of the ζ-type (PLDζ1 and PLDζ2) contain N-terminal PX and PH domains like the mammalian PLDs and are designated as Ca^{2+}-independent or PH/PX-PLDs. Furthermore, 17 PLD members, divided into six types: α(8), β(2), δ(3), ζ(2), κ, and φ, were identified in rice (Li et al., 2007), while 18 putative PLD genes were grouped into six types, α (3), β (4), γ, δ (5), ε (2), and ζ (3), in soybean (Zhao et al., 2012). Eighteen and 11 PLD genes, which could be classified into 6 subgroups (α, β/γ, δ, ε, ζ, and φ) (Liu et al., 2010b), were described in poplar and grape, respectively. Five PLDs (α (3), β (2)) were discovered in tomato (*Lycopersicon esculentum*) (Laxalt et al., 2001). In poplar, grape and rice, the PLD genes encoding proteins with *N*-terminal signal peptides, which replace the C2 or PX/PH domains, have been identified (Liu et al., 2010b; Li et al., 2007).

The α-type is the most prevalent and best studied PLD class in plants. Members of this class have been purified to apparent homogeneity from various plant sources (review in Wang, 2000). The genes encoding several PLDα isoenzymes have been cloned and heterologously expressed, such as from castor beans (Wang et al., 1994), cabbage (Kim et al., 1999; Schäffner et al., 2002), poppy seedlings (Lerchner et al., 2005), tomato (Whitaker et al., 2001; Laxalt et al., 2001), tobacco (Lein and Saalbach, 2001), strawberry (Yuan et al., 2005), peanut seeds (Nakazawa et al., 2006), *Jatropha curcas* (Liu et al., 2010a) and sunflower (Moreno-Pérez et al., 2010). The prevalence of PLDαs in plant cells suggests a catabolic rather than a signaling function of these enzymes (Laxalt et al., 2001); however, a great number of diverse responses to cellular stresses such as water loss, drought, freezing, wounding etc. have been reported (review in Li et al., 2009).

In poppy seedlings (*Papaver somniferum* L.), where lipid metabolism and alkaloid synthesis are closely linked, five isoenzymes with different substrate and hydrolysis/transphosphatidylation selectivities have been detected hitherto (Oblozinsky et al., 2003, 2005; Lerchner et al., 2005; Oblozinsky et al., 2011). Interestingly, the two PLDs that were isolated from the cytosol of poppy seedlings (Oblozinsky et al., 2003) were different from the common α-type PLDs, which are usually predominant in plants. In a recent study, PLD activity was identified in medicinally important Indian plant sources known for their production of secondary metabolites, such as *Brassica juncea* (Indian mustard) seeds, *Zingiber officinale* (ginger) rhizomes, *Allium sativum* (garlic) bulbs and *Azadirachta indica* (neem) leaves (Khatoon et al., 2007). As the crude extract of mustard seeds also showed very high PLD activity with indications of uncommon properties, we were stimulated to purify the PLD from *B. juncea* seeds and to characterize it biochemically in more detail. In addition to the biochemical characterization, the amino acid sequence of the enzyme was derived from the corresponding genomic DNA. In contrast to the results obtained with PLDs from poppy seedlings, the PLD isolated and purified from Indian mustard seeds belongs to the common α-type enzymes but shows a high specific activity and specific substrate selectivity.

2. Results

2.1. Purification of mustard PLD

The PLD from medicinally relevant Indian mustard seeds was purified to homogeneity to enable a thorough biochemical analysis of the enzyme with high activity and uncommon properties previously observed in crude extracts (Khatoon et al., 2007). Crude extracts of dry mustard seeds were prepared using a modification of the protocol described for the purification of PLD from peanut seeds (Nakazawa et al., 2006). The purification protocol comprised three steps: (i) ammonium sulfate fractionation; (ii) Ca^{2+}-mediated hydrophobic interaction chromatography (Ca^{2+}-HIC; Fig. 1A); and (iii) anion exchange chromatography (Fig. 1B). As successfully used in the purification of other α-type PLDs (Abdelkafi and Abousalham, 2011; Lambrecht and Ulbrich-Hofmann, 1992; Novotna et al., 1999; Oblozinsky et al., 2003), Ca^{2+}-HIC proved to be the crucial step in the removal of contaminating proteins. However, this procedure on Octyl-Sepharose CL-4B was only successful if performed at room temperature and not at 4–5 °C, as commonly used. No PLD activity was bound to the column material

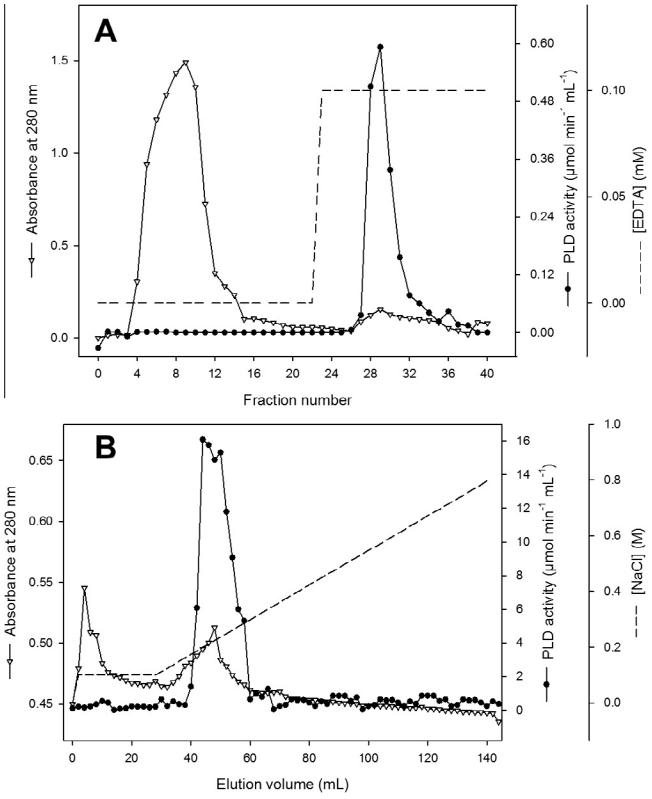

Fig. 1. Purification of PLD from mustard seeds by Ca^{2+}-mediated hydrophobic interaction chromatography (A) and anion exchange chromatography (B). The dialyzed enzyme solution after ammonium sulfate precipitation was applied onto Octyl-Sepharose CL-4B in the presence of 0.1 M $CaCl_2$ at room temperature and eluted with 0.1 M EDTA in 5 mM PIPES buffer, pH 7.0, as described in Section 4.2. (A). The active fractions were applied onto Source 15Q and eluted with a linear NaCl gradient in 10 mM PIPES buffer, pH 7.0, as described in Section 4.2. (B).