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Substrate specificity in phospholipid transformations by plant phospholipase D isoenzymes

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ABSTRACT

Phospholipase D (PLD) catalyzes the hydrolysis and transesterification of glycerophospholipids at the terminal phosphodiester bond. In many plants, several isoforms of PLD have been identified without knowing their functional differences. In this paper, the specificities of two PLD isoenzymes from white cabbage (*Brassica oleracea* var. *capitata*) and two ones from opium poppy (*Papaver somniferum* L.), which were recombinantly produced in *Escherichia coli*, were compared in the hydrolysis of phospholipids with different head groups and in the transphosphatidylation of phosphatiylcholine with several acceptor alcohols. In a biphasic reaction system, consisting of buffer and diethyl ether, the highly homologous isoenzymes are able to hydrolyze phosphatidylcholine, -glycerol, -ethanolamine, -inositol and – with one exception – also phosphatidylserine but with different individual reaction rates. In transphosphatidylation of phosphatidylcholine, they show significant differences in the rates of head group exchange but with the same trend in the preference of acceptor alcohols (ethanolamine > glycerol ≫ L-serine). For L- and D-serine a stereoselectivity of PLD was observed. The results suggest a physiological relevance of the different hydrolytic and transphosphatidylation activities in plant PLD isoenzymes.

1. Introduction

Phospholipases D (PLDs) occurring in bacteria (Carrea et al., 1995), yeast (Waksman et al., 1996), plants (Pappan and Wang, 1999) and mammals (Frohman et al., 1999) catalyze the hydrolysis of the terminal phosphate diester bond in glycerophospholipids releasing phosphatidic acid (PA) and the corresponding alcohol (Fig. 1). This reaction is important for many processes such as membrane degradation and reorganization, cell regulation, transport and signalling (Exton, 2002; McDermott et al., 2004; Bargmann and Munnik, 2006). In addition to their hydrolytic function, most PLDs efficiently catalyze the transesterification of glycerophospholipids if an appropriate acceptor alcohol is present (Fig. 1). This transphosphatidylation reaction is exploited for the synthesis of phospholipids in chemical laboratories and industry (Ulbrich-Hofmann et al., 2005; De Maria et al., 2007), whereas its biological function is hitherto unknown (Yu et al., 1996).

PLDs from different sources share some characteristics in their molecular organization and constitute – together with some other evolutionarily related proteins – the PLD superfamily (Pointing and Kerr, 1996). The most important common feature of PLDs is the presence of two so-called HKD motifs, which form the catalytic site as concluded from the crystal structure of PLD from *Streptomyces* sp. strain PMF (Leiros et al., 2000). PLDs from eukaryotes are characterized by further typical structural regions with putative regulatory functions, such as the N-terminal Phox and Pleckstrin domains in mammalian and yeast PLDs (Frohman and Morris, 1999) or the C2 domain in most plant PLDs (Pappan et al., 2004). Interestingly, plant species are characterized by a high number of different PLD isoenzymes. While no PLD isoenzymes are known in microorganisms and only two types of mammalian PLDs have been described (Kim et al., 2007), 12 PLD isoforms grouped into five types (α-, β-, γ-, δ-, and ζ-type PLD) could be identified in *Arabidopsis thaliana* (Qin and Wang, 2002). Most plant PLDs studied so far belong to the α-type such as two isoenzymes from white cabbage (*Brassica oleracea* var. *capitata*) (Schäffner et al., 2002), called cPLD1 and cPLD2 in the following, and the two isoenzymes from opium poppy (*Papaver somniferum* L.) (Lerchner et al., 2005), called pPLD1 and pPLD2 in the present paper. *In vitro* these so-called conventional PLDs need Ca^{2+} concentrations in the millimolar range (Wang, 2000; Lerchner et al., 2005).

PLD isoenzymes show differences in their substrate specificities which are assumed to correspond to the differences in membrane phospholipids. Thus, PLDα from *Arabidopsis* hydrolyzes vesicles of pure phosphatidylcholine (PC), phosphatidylethanolamine (PE) and phosphatidylglycerol (PG), whereas PLDβ and PLDγ need PE or phosphatidylinositol 4,5-bisphosphate (PIP_2) for cleaving PC and PG (Pappan et al., 1998). Contrary to PLDα, PLDβ and PLDγ also

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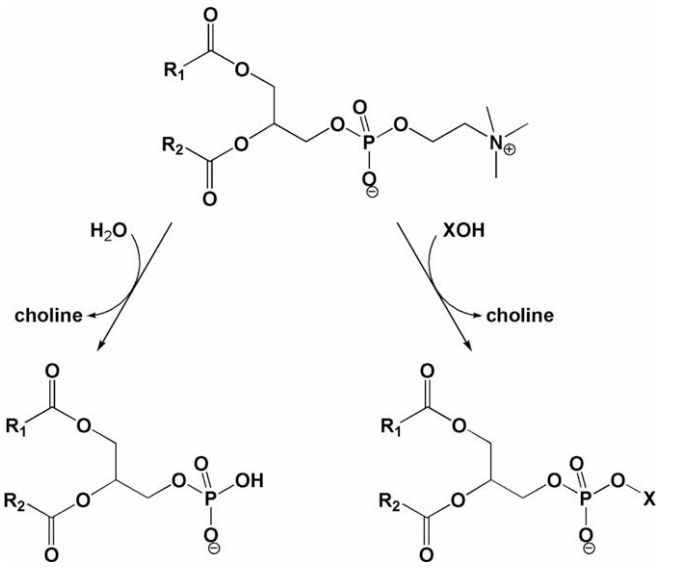

Fig. 1. Scheme of the PLD-catalyzed transformation of phosphatidylcholine. The terminal phosphodiester bond can be hydrolyzed yielding phosphatidic acid (left) or transesterified by substitution of the choline moiety by an acceptor alcohol (XOH) (right).

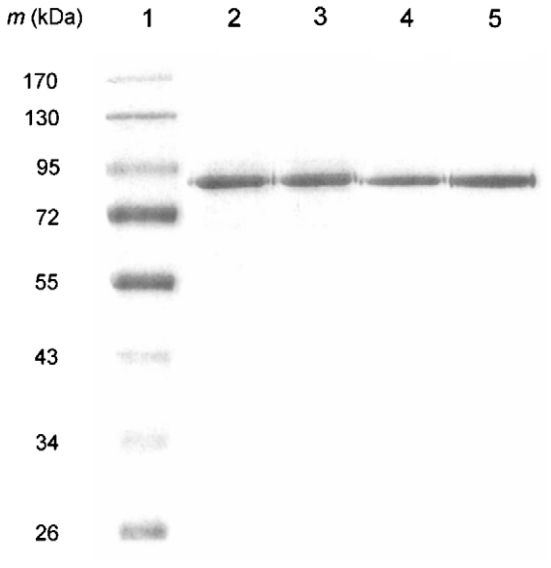

Fig. 2. SDS–PAGE of PLD isoenzymes. The analyses were performed as described in Section 3. Lane1: molecular markers; lane 2: cPLD1, lane 3: cPLD2, lane 4: pPLD1, and lane 5: pPLD2.

hydrolyze phosphatidylserine (PS). The three PLD forms isolated from castor beans also differ in their hydrolytic activities to phosphatides (Dyer et al., 1994). For these enzymes, however, PE was a better substrate than PC, whereas PI and PS were not cleaved. In contrast, two PLDs isolated from poppy seedlings were inactive to PE but cleaved also PS and PI in addition to PC and PG (Oblozinsky et al., 2005), and four PLD isoenzymes from *Catharantus roseus* cells catalyzed the hydrolysis of PI only (Wissing et al., 1996). Besides the head groups in the phospholipids, also the fatty acid components of the substrate may play a role in substrate specificity as reported for PLDα from sunflower (Abousalham et al., 1997).

In contrast to the specificity of PLD isoenzymes with respect to the leaving alcohol (head group) structure, their specificity concerning the acceptor alcohols in the transphosphatidylation reaction has not yet been investigated although it might throw light on the supposed role of this reaction in membrane remodelling and is interesting with respect to their biocatalytic application.

In this paper, we analyze the four related, recombinantly produced and well characterized α-type PLD isoenzymes cPLD1, cPLD2, pPLD1, and pPLD2 with respect to differences in their hydrolytic activities toward natural phospholipids with different head groups (PC, PE, PG, PS, and PI) as well as their transphosphatidylation potential in the head group exchange of PC by glycerol, ethanolamine, L-serine, and *myo*-inositol. Moreover, stereoselectivity with respect to the acceptor alcohol is demonstrated for the introduction of L- and D-forms of serine by cPLD2.

2. Results and discussion

2.1. PLD isoenzymes in phospholipid hydrolysis

Two α-type PLD isoenzymes from white cabbage (cPLD1: NCBI GenBank Accession No. AF113918; cPLD2: NCBI GenBank Accession No. AF113919) and two PLD isoenzymes from opium poppy (pPLD1: NCBI GenBank Accession No. AF451981, pPLD2: NCBI GenBank Accession No. AF451982) were produced and purified to homogeneity (Fig. 2) as described previously (see Section 3). The two enzymes from cabbage consist of 811 (cPLD1) and 813 (cPLD2) amino acid residues, respectively, and differ in 70 positions, while the two enzymes from poppy contain 813 amino acid residues each and differ in 11 positions only.

The four isoenzymes were compared with respect to their hydrolytic activities toward PC, PG, PE, PS, and PI in a biphasic reaction system consisting of an aqueous and a diethyl ether phase (Fig. 3).

All enzymes are able to cleave PC, PE, PG, PI and – with the exception of pPLD1 – also PS (Fig. 3). The graduations in the preference, however, differ. Thus, the order of substrates in the rates of hydrolysis is PC > PG = PE > PI > PS for cPLD1, PE > PG = PC > PI > PS for cPLD2, PE > PC > PG > PI for pPLD1, and PE ≫ PG > PC > PS > PI for pPLD2. In general, cPLD2 is somewhat more active than cPLD1. pPLD2 has the highest activity of all enzymes studied here. Particularly striking is the outstanding activity to PE, which is about fivefold compared to PC (Fig. 3). Also the non-acceptance of PS by pPLD1, in contrast to pPLD2, is remarkable in consideration of the small differences in the amino acid sequences of these two enzymes. Obviously, marginal differences in the isoenzyme structures can cause significant changes in the phospholipid acceptance,

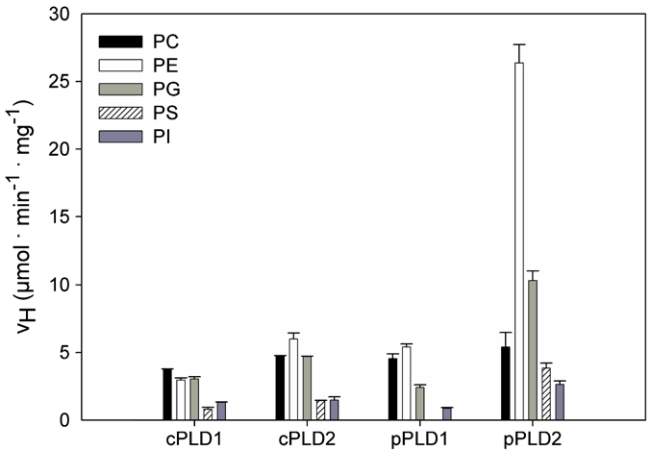

Fig. 3. Initial rates for the hydrolysis of phospholipids with different head groups by PLD isoforms from white cabbage and opium poppy. The reactions were performed and analyzed as described in Section 3. Data show the mean ± SD obtained from two independent experiments.

which presumably has a physiological importance in membrane degradation and remodelling.

In comparison with literature data, the PLD isoenzymes studied in this paper show some similarities to those from other plants such as from castor bean (Dyer et al., 1994), which cleaved PE, PG, and PC with more or less preference. However, these enzymes did not hydrolyze PI and PS. Surprisingly, PI can be hydrolyzed by all isoenzymes from cabbage and poppy in the two-phase reaction system. A small activity was also observed in aqueous medium containing PI in form of mixed micelles (data not shown). Particularly amazing is the comparison of the present results for pPLD1 and pPLD2 with those of two isoenzymes isolated previously from poppy seedlings (Oblozinsky et al., 2003, 2005). The latter enzymes, obviously not identical with the two isoenzymes obtained recombinantly (Lerchner et al., 2005), hydrolyzed phospholipids in the order PC > PG > PS > PI but did not accept PE at all. Therefore, a great variety of substrate specificities are reflected in the multiple forms of PLD in plant kingdom.

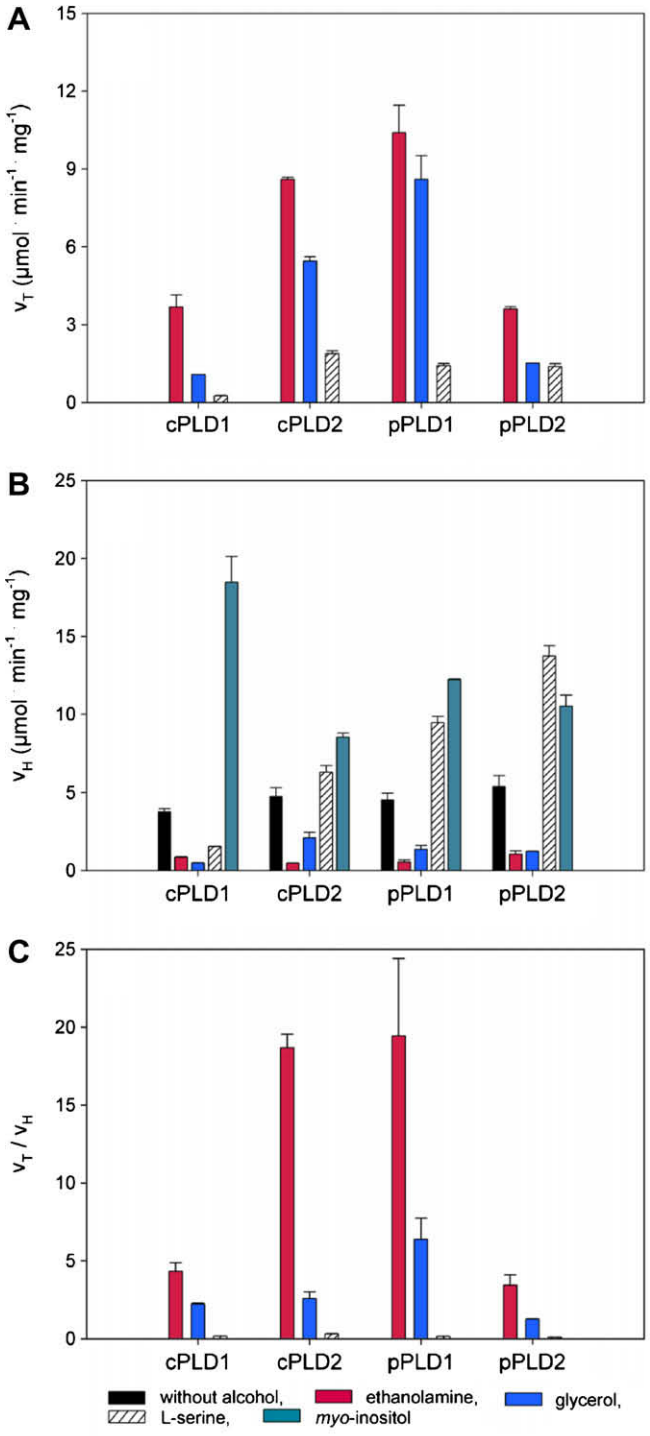

Fig. 4. Conversion of phosphatidylcholine by PLD isoforms from white cabbage and opium poppy in the presence of different acceptor alcohols. A. Initial rates of transphosphatidylation (v_T). B. Initial rates of hydrolysis (v_H). C. Transphosphatidylation potentials (v_T/v_H). The reactions were performed and analyzed as described in Section 3. Data show the mean ± SD obtained from two independent experiments.

Table 1
Initial rates of hydrolysis (v_H) and transphosphatidylation (v_T) of PC by cPLD2 in the presence of L- and D-serine.[a]

Acceptor alcohol	v_H (µmol min^{-1} mg^{-1})	v_T (µmol min^{-1} mg^{-1})	$v_H + v_T$ (µmol min^{-1} mg^{-1})	v_T/v_H
L-Serine	6.30 ± 0.22	1.88 ± 0.01	8.18 ± 0.22	0.30
D-Serine	7.82 ± 0.42	0.21 ± 0.01	8.03 ± 0.42	0.03

[a] The reactions were performed and analyzed as described in Section 3. Data show the mean ± SD obtained from two independent experiments.

2.2. PLD isoenzymes in transphosphatidylation

Starting from PC, the transphosphatidylation capability of the four PLD isoenzymes in comparison with the competing hydrolysis was examined with ethanolamine, glycerol, L-serine and *myo*-inositol as acceptor alcohols (Fig. 4). To probe the enantioselectivity of PLD in the transphosphatidylation activity, the activity of cPLD2 in the substitution of L- and D-serine for choline in PC was compared (Table 1).

In literature, there are no comparative studies on the specificity of different PLD isoenzymes in transphosphatidylation. From the present results it can be concluded that the two pairs of homologous PLDs behave similarly with respect to the acceptance of alcohols introduced into PC, however, with some significant differences in the rates of transphosphatidylation (Fig. 4A) and the competing hydrolysis (Fig. 4B) as well as the ratio of these two reaction rates (Fig. 4C). The graduation in the preference of ethanolamine > glycerol > L-serine as acceptor alcohol is in accordance with the results of early studies on PLD isolated from savoy cabbage (Yang et al., 1967). The high transphosphatidylation potential of ethanolamine compared to glycerol and L-serine may be caused by its positive charge at the reaction conditions used. The binding of the uncharged glycerol or the zwitterionic L-serine to the active site of the enzymes may be weaker due to missing electrostatic attraction.

Myo-inositol cannot be introduced into the phosphatide by cPLD2, cPLD2, pPLD1 or pPLD2, although PI can be cleaved by all these enzymes (Fig. 3). Therefore, the alcohol of the phosphatidyl donor and the acceptor seem to occupy different binding positions in the active site. Possibly, free *myo*-inositol with a molecular volume of 150.9 Å^3 (http://www.molinspiration.com) cannot be accommodated in the active site when the phosphatidyl residue is bound. As concluded from the synthesis of phospholipids with different ethanolamine derivatives (Dippe et al., 2008), head group alcohols > 129 Å^3 are poorly accepted by cPLD2. In contrast, the PLD isoenzyme with transphosphatidylation activity from poppy seedlings (Oblozinsky et al., 2005) was able to substitute *myo*-inositol for choline in PC, even with a higher rate than L-serine.

The striking activation of PC hydrolysis by the non-reactive *myo*-inositol or the low-reactive L-serine (Fig. 4B) is probably induced by effects on the physical properties of the phospholipid interfaces as studied previously for the role of aliphatic alcohols in transphosphatidylation (Hirche et al., 1997).

Although the evaluation of transphosphatidylation potentials of PLDs is complicated by the involvement of the acceptor alcohols into the phase behaviour of the phospholipid interfaces and the results may be influenced by the solvent system used (Hirche and Ulbrich-Hofmann, 1999; Oblozinsky et al., 2005), the observed differences of the PLD isoenzymes in the acceptor alcohol preferences suggest that transphosphatidylation is a reaction of physiological relevance. This hypothesis is strongly supported by the stereoselectivity of cPLD2-catalyzed transphosphatidylation with serine. Thus, the transphosphatidylation rate with the natural enantiomer L-serine is 10 times higher than that with D-serine (Table 1) while the total conversion of PC ($v_H + v_T$) is constant. Obviously, plants express several PLD isoenzymes with subtle structural modifications, which are adapted to the cellular requirements of transformations of special membrane lipids. In these processes transphosphatidylation is assumed to play a significant role comparable with hydrolysis.

3. Experimental

3.1. Materials

PC (98%), PA (98%), PG (98%) and PE (98%), all from soybean, were obtained from LIPOID GmbH, Germany. PS from soybean (98%), ethanolamine (99%), *myo*-inositol (99%), anhydrous diethyl ether (99.9%), L-serine and D-serine (both 98%) were products of Sigma, USA. Glycerol (99.5%) was received from Merck, Germany. PI (50%) from Sigma, USA was purified by HPLC as described in Dippe et al. (2008). All other chemicals were of highest purity commercially available.

3.2. PLD isoenzymes

cPLD1 and cPLD2 from white cabbage (*B. oleracea* var. *capitata*) were produced by expression in *Escherichia coli* and purification by Ca^{2+}-mediated hydrophobic interaction chromatography and an anion exchange chromatography as described in Schäffner et al. (2002) and Stumpe et al. (2007). pPLD1 and pPLD2 from opium poppy (*P. somniferum* L.) were obtained in similar way according to Lerchner et al. (2005).

The purity of the enzymes was checked by sodium dodecyl sulphate polyacrylamide gel elecrophoresis (SDS–PAGE) according to Laemmli (1970) using 10% polyacrylamide gels and silver staining (Blum et al., 1987). The molecular markers PageRuler were from Fermentas, Canada.

3.3. Protein concentration

Protein concentrations were determined by means of the Micro BCA protein assay kit from Pierce, USA, according to the manufacturers' instructions.

3.4. Reaction rates

Reactions were performed in a biphasic reaction system as described in Hirche and Ulbrich-Hofmann (1999). Forty-eight microliters of enzyme solution (10 μg/ml PLD in 300 mM sodium acetate buffer, pH 5.5, containing 120 mM $CaCl_2$) were incubated with 372 μl of phospholipid solution (0.75 μmol phospholipid in diethyl ether). In the transphosphatidylation assays the aqueous phase additionally contained 57.6 μmol of acceptor alcohols. The ionic alcohols (ethanolamine, L- and D-serine) were added from stock solutions adjusted by hydrochloric acid to pH 5.5. Reaction mixtures were shaken at 400 rpm and 30 °C in 1.5 ml HPLC screw flasks closed by polytetrafluoroethylene septa (Roth, Karlsruhe, Germany). After several periods of time (0–220 min), samples of the organic phase (15 μl), which contain the phospholipids nearly completely, were air-dried, and the residuals were dissolved in 25 μl of toluene.

The analyte solution (1.5 μl) was applied to silica 60 plates (Merck, Germany) and analyzed by HPTLC. After phospholipid staining with $CuSO_4/H_3PO_4$, the resulting phospholipid bands were quantified by densitometry according to Aurich et al. (1999). For calculation of phospholipid concentrations, standards containing PC, PA, and PG, PE, PS or PI were used. Initial rates of hydrolysis (v_H) and transphosphatidylation (v_T) were obtained from the fit of the amount of reaction products as a function of the reaction time. All experiments were performed in duplicate.

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