J. Org. Chem. **1986**, *51*, 226.
13. Cha, J. S.; Kim, E. J.; Kwon, O. O.; Kim, J. M. *Synlett* **1995**, 331.
14. (a) Cha, J. S.; Kim, E. J.; Kwon, O. O.; Kwon, S. Y.; Seo, W. W.; Chang, S. W. *Org. Prep. Proced. Int.* **1995**, *27*, 541. (b) Cha, J. S.; Kim, E. J.; Kwon, O. O.; Kim, J. M. *Bull. Korean Chem. Soc.* **1995**, *16*, 691. (c) *Idim. Ibid.* **1996**, *17*, 50.
15. Brown, H. C.; Kramer, G. W.; Midland, M. M. *Organic Synthesis via Boranes*; Wiley-Interscience: New York, 1975.

Substrate Specificity of Cabbage Phospholipase D with Phospholipids Having Different Head Groups

Jieun Lee and Myung-Un Choi*

Department of Chemistry and Center for Molecular Catalysis, Seoul National University, Seoul 151-742, Korea
Received June 5, 1996

A substrate specificity of cabbage phospholipase D (PLD) was studied using the synthetic phospholipids having different head groups. The phospholipids were synthesized from phosphatidylcholine and appropriate bases by transphosphatidylation of PLD. The bases used were ethanolamine, serine, ethanol and γ-hydroxybutyric acid. The phosphatidic acid, the product of PLD, was separated in TLC and measured densitometrically. The kinetic parameters were estimated for each substrate and the effects of pH, SDS, Ca^{2+} and other metal ions were examined. V_{max} values found were 3.75, 2.36, 5.59, 1.63, 2.30 nmol/min/μg protein for phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylethanol, and phosphatidylburytic acid, respectively. These results indicate a broad specificity of cabbage PLD toward phospholipids with different head groups. Particularly phosphatidylserine was most easily hydrolyzed by PLD and its activity did not depend on Ca^{2+}.

Introduction

Phospholipase D (PLD: phosphatidylcholine phosphosphatido hydrolase : E.C. 3.1.4.4) catalyzes hydrolytic cleavage of terminal diester bond of glycerophospholipids with formation of phosphatidic acid and corresponding base.[1] In addition, PLD also can catalyze a transphosphatidylation reaction in which phosphatidate is transferred to various primary alcohols such as ethanol producing phosphatidylethanol (PEt).[2] PLD was first discovered in carrot roots and spinach leaves[3] and since then has been found to occur widely in plants, microorganisms, and mammalian tissues.[4] In animals a great deal of evidence now points that the hydrolytic pathway(s) of intracelluar PLD is one of major route for receptor-linked phospholipid signal pathways.[5] The physiological role of PLD in plants are not well defined. However several reports indicate a possible involvement of PLD in various processes, such as germination,[6] stress-induced changes in PLD activities[7] and membrane composition.[8] The plant PLDs are purified from peanut seeds,[9] cabbage leaves,[10~12] rice bran,[13] and endosperm of castor bean.[14] Recently cDNA for castor bean PLD has been cloned.[15] Subsequently genes encoding PLD activity are identified from rice,[16] human,[17] and yeast.[18]

Although there is a surge of reports on possible function of PLD in animal cell as well as plant tissue, only a limited amount of information on the line of enzymology of PLD are available. Assay system for PLD is not fully established yet since the complication of interfacial interaction.[19] Inevitably *in vitro* assay system usually include amphiphatic substance like SDS or organic solvent such as diethylether. The requirement of Ca^{2+} for PLD activity has been assumed to be essential. However some of recent data implicate it is not always true. A couple of data suggest that Ca^{2+} is more likely related to a structural stability of PLD rather than catalytic function, for example, Ca^{2+} was a crucial factor for hydrophobic binding[20] and in some model substrate, the activity did not depend on Ca^{2+} concentration.[21] There was no systematic study on substrate-specificity for plant PLD. Generally phosphatidylcholine has been accepted as most favorable substrate for hydrolytic activity of PLD without any extensive study.[22~24] Recently there were several studies for structure-reactivity relationship using model substrates such as alkyl phosphoryl choline[25] and *p*-nitrophenyl phosphoryl derivatives.[21] For effect of acyl chain length, PCs with different acyl chains were examined.[26] However the model substrates examined so far have been limited on the effect of acyl chain length and these substrates were usually complicated by the presence of different amount of detergent in the assay system.

In view of the complication stemmed from different acyl chain length, present substrate-specificity study uses tailored phospholipids with different head groups but has exactly identical acyl chains. This was achieved by exchanging the head groups through transphosphatidylation reaction catalyzed by PLD with same batch of egg PC. This approach would reveal head group specificity of cabbage PLD without any

major shortcomings.

Experimental

Materials. γ-Hydroxybutyric acid, sodium dodecyl sulfate (SDS), 2-[*N*-morpholino]ethanosulfonic acid (MES), octylsepharose CL-4B were purchased from Sigma. Crude egg phosphatidylcholine (PC) from Sigma was purified by aluminium oxide column. Two types of thin layer chromatography sheets of silica gel (Art. 5735 and 5721) were obtained from Merck. All other chemicals were reagent grade commercially available.

Enzyme preparation. Phospholipase D was prepared from inner, yellowish-white leaves of savoy cabbage. The cabbage juice was treated by 55 ℃ heat and the PLD in supernatant precipitated with cold acetone.[11] Further purification method was carried out as described by Abousalham *et al.*[27] The lyophilized acetone powder (2 g) suspended in 20 mL of 30 mM PIPES buffer (pH 6.2) containing 50 mM $CaCl_2$ was applied to an octylsepharose CL-4B hydrophobic column equilibrated in 30 mM $CaCl_2$ at a flow rate of 30 mL/h. The PLD activity was eluted by chelating the Ca^{2+} ions and the PLD recovery was about 50% with a 423-fold increase in specific activity. A silver stained SDS/PAGE of the eluted PLD showed a major single band having molecular weight of 89 kDa.[11,28] The eluent was stored at 4 ℃ in 30% ethylene glycol.

Synthesis of substrates. Phospholipid with different head group was prepared by an enzymatic transphosphatidylation reaction using phospholipase D. The following method was modified from Yang *et al.*[2] and Jung.[29] In the case of phosphatidylserine (PS), 18.4 g of L-serine was dissolved in 40 mL of 0.2 M acetate-acetic acid buffer (pH 5.6) containing 80 mM $CaCl_2$ and 0.2 g of PLD acetone powder. Then the aqueous mixture was poured into diethylether solution containing 0.4 g of purified PC in a total volume of 40 mL. The reaction mixture was vigorously stirred on a magnetic stirrer. Incubation was stopped by addition of 40 mL of 100 mM EDTA solution and lipids were extracted with 160 mL of diethylether : ethanol (4 : 1, v/v). The solution was evaporated by means of vacuum pump and the residue was redissolved in 40 mL of chloroform : methanol (5 : 8, v/v) and then mixed with 1 volume of water and 3.7 volumes of chloroform. After standing for an hour, the lower organic layer was collected and mixed with an equal volume of ethanol, followed by evaporation to dryness under reduced pressure. The residue was dissolved in a desired volume of chloroform and stored at −20 ℃. The synthetic procedure of other phospholipids was same as the method of PS synthesis except the corresponding base used. A volume of 4 mL of ethanol was dissolved in 40 mL of the 0.2 M acetate-acetic acid buffer (pH 5.6) for phosphatidylethanol (PEt), 9.88 mL of ethanolamine was dissolved for phosphatidylethanolamine (PE), and 5 g of γ-hydroxybutyric acid was dissolved for phosphatidylbutyric acid (PB). The synthesized phospholipids were separated and identified by TLC. The developing solvent system for PE and PB was chloroform : methanol : ammonium hydroxide (65 : 30 : 3, v/v) and the R_f was 0.6-0.5. For PS, solvent was chloroform : methanol : acetic acid : water (45 : 20 : 6 : 1, v/v) and R_f was 0.6. For PEt, solvent was ethylacetate : isooctane : acetic acid : water (65 : 10 : 15 : 50, v/v) and R_f was 0.4. The separated phospholipid was scrapped off from TLC and dissolved by addition of methanol : chloroform (1 : 1, v/v).

Assays of phospholipase D. The reaction product phosphatidate was separated by TLC and estimated by a densitometric method. A suitable portion of chloroform solution containing of 0.5 μmol of desired phospholipid was evaporated to dryness under nitrogen gas. The dried phospholipid was suspended in 1 mL of 50 mM MES buffer (pH 6.5) containing 2.5 μmol of SDS by bath-type sonicator for 5 minutes. The sonicated suspension was mixed with 0.1 mL of 125 mM $CaCl_2$ and 10-50 μL of the enzyme solution. The reaction mixture was incubated for 30 minutes at 37 ℃ and terminated by addition of 1 mL of chloroform : methanol (2 : 1, v/v) solution. Then water-soluble product like serine was eliminated by 1 mL of 0.1 M KCl. The extracted phospholipids were separated by thin layer chromatography on a developing solvent system of ethylacetate : isooctane : acetic acid : water (65 : 10 : 15 : 50, v/v) and visualized by sulfuric acid spray (10%, v/v), then the charred band of phosphatidic acid was quantitized by UltroScan XL densitometer. The results were presented as averages of duplicated data. Usually the duplication data fell within less than 10% of each other.

Results and Discussion

Kinetic parameters. The kinetic constants for phospholipids with different head groups were determined by varying the concentration of substrates, in a range of 0-20 mM (Figure 1). The apparent K_m and V_{max} values estimated from Lineweaver-Burk plot and the values were summarized in Table 1. K_m values under the presence of SDS were 2.04, 4.88, 2.55, 1.67, 1.69 for PC, PE, PS, PEt and PB respectively. Relatively the K_m values for phospholipids with different head groups are not so different one another. However the K_m value of PE seems to be two times larger than that of the other phospholipids. V_{max} value of PS was the largest among the tested phospholipids and the efficacy (V_{max}/K_m)

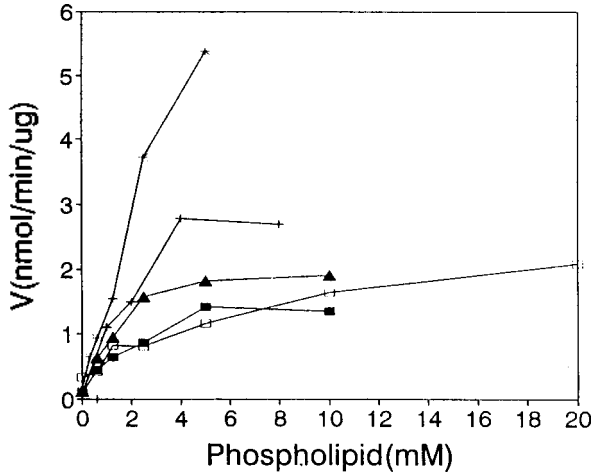

Figure 1. Effect of substrate concentration on the activity of PLD. (+) PC; (·) PE; (*) PS; (■) PEt; (▲) PB.

Table 1. Kinetic parameters for PLD-catalyzed hydrolysis of phospholipids having different head groups

	K_m (mM)	V_{max} (nmol/min·μg protein)
PC	2.04	3.75
PE	4.88	2.36
PS	2.55	5.59
PEt	1.67	1.63
PB	1.69	2.30

Abbreviation of substrates : PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; PEt, phosphatidylethanol: PB, phosphatidylbutyric acid

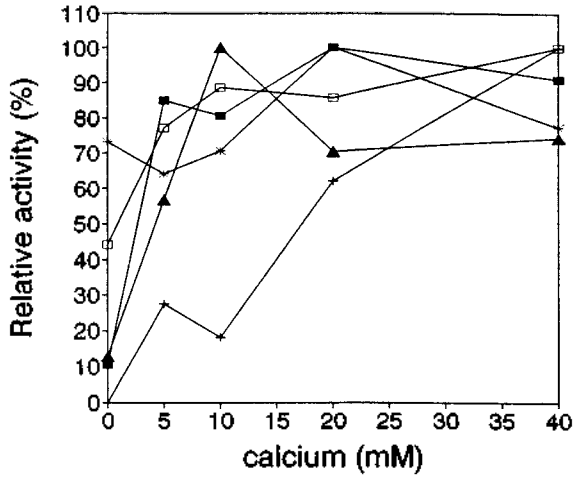

Figure 2. Effect of calcium ion concentration on the PLD activity toward various phospholipids. (+) PC; (□) PE; (*) PS; (■) PEt; (▲) PB.

of enzyme activity decreases in following order : PS>PC> PB>PEt>PE. As a whole the substrate specificity of cabbage PLD seems to be rather broad as the disparity of V_{max} of tested phospholipids is at most three fold. Under this experimental condition the PS is the best substrate for cabbage PLD. Early papers on cabbage PLD reported that PC was better substrate than PE[22,23] and current result could confirm the previous findings. However, an isoenzyme obtained from castor bean PLD revealed that the PE was most favorable substrate and the PS was not hydrolyzed.[6] The different substrate-specificity of castor bean PLD from cabbage PLD indicates that enzymes from different sources may show different substrate-specificity. It has been known that the pH optimum for the hydrolysis of PC is between pH 5.5 and pH 6.5 depending on the physical state of substrate and Ca^{2+} concentration.[24-29] When optimum pH was examined for various phospholipids with different head groups it was found to be in the range of pH 5.5-6.5 (data not shown).

Effect of metal ions. Since it has been known that Ca^{2+} is required for the catalytic activity of PLD, we examined the effects of Ca^{2+} ion on the hydrolytic rate of different substrates (Figure 2). The PLD activities increased as the concentration of Ca^{2+} increased except the PS. The PS whose base has zwitter ion in it showed no activation effect by Ca^{2+} ion. Additionally other metal ions such as Mg^{2+}

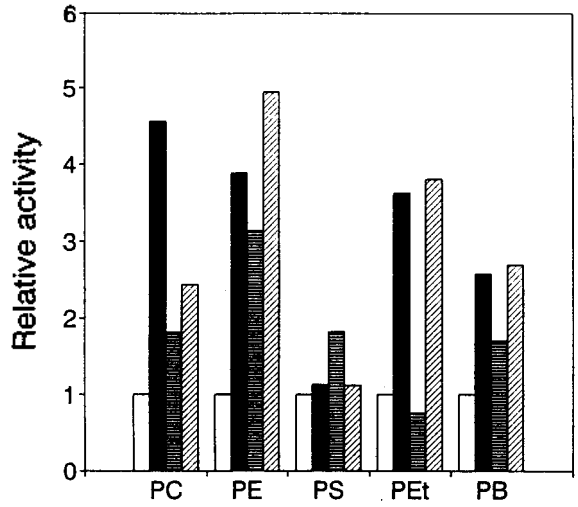

Figure 3. Effect of some metal ions on the PLD activity toward various phospholipids. The concentration of metal chloride were 10 mM. The PLD activity were shown relativley to the activity obtained with 4 mM EDTA. (□) EDTA; (■) Ca; (▤) Mg; (▨) Mn.

and Mn^{2+} ions were examined for their effectiveness for activation of PLD with different phospholipids (Figure 3). It turned out that the effect of metal ions were very diversified and seemed to depend on the head groups. PS was not affected by metal ions as significantly as observed with Ca^{2+} ion. The other phospholipids showed different patterns. Mn^{2+} is the most effective activator for PE hydrolysis, while Ca^{2+} is the best activator for PC. Mn^{2+} is almost as effective as Ca^{2+} ion for either PB or PEt. Mg^{2+} is not so effective as the other metal ions, but PB and PE were partially activated by Mg^{2+}. These observations again reinforce the notion that Ca^{2+} ion is not necessarily essential for the catalytic function of PLD.[21]

Effects of SDS and diethylether. In order to obtain reliable PLD assay, one must decide which physical form of substrate to employ in the assay. Here we employed the SDS-PC micellar system for substrate-specificity study. In this respect we extended this study further to diethylether-water interfacial system to confirm whether an alteration of physical state of substrate affect the specificity. In Figure 4(a) and (b) the two assay systems were compared to each other. Although the observed optimum activity of the ether system was in average 15% smaller than that of the SDS system, the relative activity of each phospholipids tested were comparable (data not shown). By the same token, the following trends were similar in both systems. Without added Ca^{2+} ion the both assay systems did not work well for most substrates. However without SDS or ether but with added Ca^{2+} the activity was moderately observed in all substrates, particularly the substrate PE, PEt and PB exerted almost their full activity with only Ca^{2+} ion system. As pointed out above when PS was used as a substrate, the PLD activity could reach its full activity by any combination of assay system except the SDS alone system which gave about 40% of the optimum activity.

Collectively the substrate-specificity of cabbage PLD to-

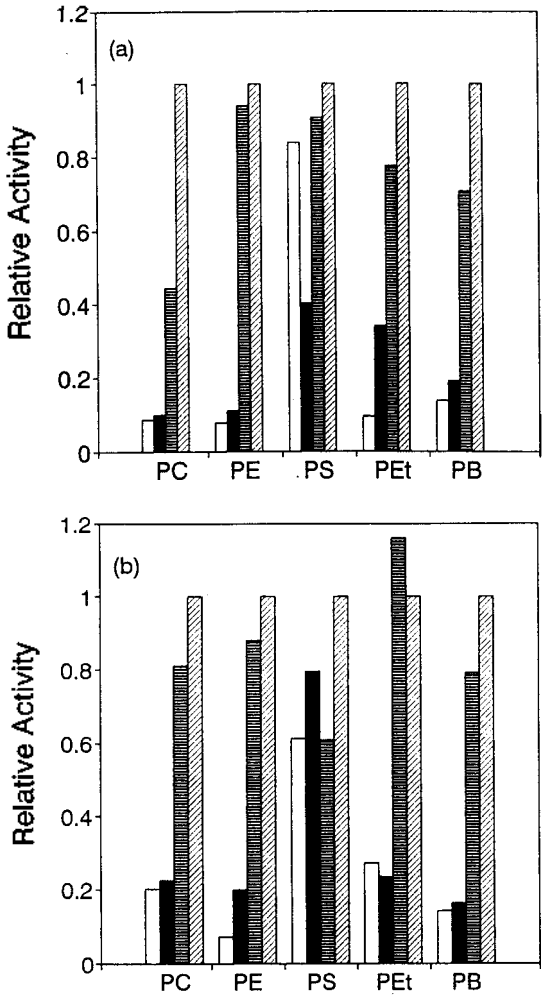

Figure 4. (a) Effect of SDS and calcium ion on the PLD activity. (□) none; (■) SDS; (▤) Ca; (▨) Ca+SDS. (b) Effect of ether and calcium ion on the PLD activity. (□) none; (■) ether; (▤) Ca; (▨) Ca+ether.

ward phospholipid with different head groups reveals a broad specificity. The broad but subtle difference of specificity, PS and PC are more favorable than PE, PEt, and PB, might originate from differences in size and/or charge of head groups. However the specificity observed show not enough discrimination against head groups to allow any further differentiating explanation. Contrarily the broad specificity implies rather a loose substrate-binding site of cabbage PLD for the head groups. Nonetheless PS was the most active substrate and the hydrolytic activity did not depend on Ca^{2+} ion. Therefore it is plausible that Ca^{2+} is not necessarily required for the catalytic activity of cabbage PLD.

Acknowledgment. This study was supported by Korean Ministry of Education through a research fund from the Basic Science Research Institute (BSRI-94-3418).

References

1. Holbrook, P. G.; Pannell, L. K.; Daly, J. W. *Biochim. Biophys. Acta.* **1991**, *1084*, 155.
2. Yang, S. F.; Freer, S.; Benson, A. A. *J. Biol. Chem.* **1967**, *242*, 477.
3. Hanahan, D. J.; Chikoff, I. L. *J. Biol. Chem.* **1947**, *68*, 233.
4. Heller, M. *Adv. Lipid Res.* **1978**, *16*, 267.
5. Liscovitch, M. *Signal-acivated phospholipases*; R. G. Landes Co. Austin, 1994.
6. Dyer, J. H.; Ryu, S. B.; Wang, X. *Plant Physiol.* **1994**, *105*, 715.
7. Ryu, S. B.; Wang, X. *Plant Physiol.* **1995**, *108*, 713.
8. Cheour, F.; Arul, J.; Makhlouf, J.; Willemot, C. *Plant Physiol.* **1992**, *100*, 1656.
9. Heller, M.; Mozes, N.; Peri, I.; Maes, E. *Biochim. Biophys. Acta* **1974**, *369*, 397.
10. Allyger, T. T.; Wells, M. A. *Biochemistry* **1979**, *18*, 5348.
11. Lee, H.; Choi, M.; Koh, E. *Korean Biochem. J.* **1989**, *22*, 487.
12. Lambert, R.; Ulbrich-Hoffmann, R. *Biol. Chem. Hoppe-Seyler* **1992**, *373*, 81.
13. Lee, M. H. *Plant Sci.* **1989**, *59*, 25.
14. Wang, X.; Dyer, H. J.; Zheng, L. *Arch. Biochem. Biophys.* **1993**, *306*, 2186.
15. Wang, X.; Xu, L.; Zheng, L. *J. Biol. Chem.* **1994**, *269*, 20312.
16. Veki, J.; Morika, S.; Komari, L.; Kumashiro, T. *Plant Cell Physiol.* **1995**, *36*, 903.
17. Hammond, S. M.; Altshuller, Y. M.; Sung, T.; Rudges, S. A.; Rose, K.; Engebrecht, J.; Morris, A. J.; Frohman, M. A. *J. Biol. Chem.* **1995**, *270*, 29640.
18. Waksman, M.; Eli, Y.; Liscovitch, M.; Gerst, J. E. *J. Biol. Chem.* **1996**, *271*, 2361.
19. Dennis, E. A. *Meth. Enzymol.* **1991**, *197*, Section I.
20. Lambert, R.; Ulbrich-Hoffmann, R. *Biotech. Bioeng.* **1993**, *41*, 833.
21. Cha, J.; Lee, J.; Koh, E., Choi, M. *Bull. Korean Chem. Soc.* **1994**, *11*, 1001.
22. Davison, F. M.; Long, C. *Biochem. J.* **1958**, *69*, 458.
23. Lands, W. E. M.; Hart, P. *Biochim. Biophys. Acta.* **1965**, *98*, 532.
24. Quarles R. H.; Dawson, R. M. C. *Biochem. J.* **1969**, *112*, 795.
25. Ohno, Y.; Okazaki, M.; Hara, I. *Biochem. Cell Biol.* **1990**, *68*, 376.
26. Koh, E.; Park, I. *J. Korean Chem. Soc.* **1996**, *40*, 630.
27. Abousalham, A.; Riviere, M.; Teissere, M.; Verger, R. *Biochim. Biophys. Acta* **1993**, *1158*, 1.
28. Lim, J. *Master thesis*; Dept. of Chem., Seoul National Univ. 1994.
29. Jung, K.; Koh, E.; Choi, M. *Bull. Korean Chem. Soc.* **1989**, *10*, 595.