was added rapidly and then a solution of fluorene (16.0 g, 0.1 mole) in DMSO (100 ml) was added during 30 sec period with stirring in an ice bath. After 5 min stirring, the reaction mixture was neutralized with dil. HCl, added 400 ml of water and then extracted with ethyl acetate. The organic layer was separated, washed with water then dried over anhyd. $Na_2SO_4$. The yellow oily residue, obtained by evaporation of solvent, was triturated with benzene. The resulting colorless precipitate was collected and recrystallized from benzene to produce 16.1 g (74%) of crystalline solid; mp. 144-145℃ (lit[9]. 144-145℃); IR (KBr) 3300 $cm^{-1}$ (OH stretching); $^1$H-NMR ($CDCl_3$) 7.48 (s, 8H, ArH), 4.85 (t, 2H, OH), 3.33 ppm (s, 4H, $CH_2$).

**3,9-Di(9-fluorenylidenyl)-1,4,6,9-tetraoxa-5,5-undecane 8.** A mixture of compound 7 (5.0 g, 22 mmole) and *n*-butyltin oxide (6.05 g, 24 mmole) in toluene (100 ml) was refluxed for 12 h to collect water in Dean-Stark moisture trap. After collection of water the moisture trap was removed and then 5 ml of carbon disulfide was added dropwise and heating was extended overnight at 100℃. The yellow oily residue, resulted upon removal of solvent, was triturated with methanol to give white solid. A recrystallization from methylene chloride and methanol gave 4.81 g (94%) of colorless crystalline solid; mp. 212-213℃; IR (KBr) 1230 and 1140 $cm^{-1}$ (C-O stretching); $^1$H-NMR (DMSO $d_6$) 7.88 (m, 16H, ArH), 4.31 ppm (s, 8H, $CH_2$).

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# Large Unilamellar Phospholipid Vesicles as a Model Substrate for Phospholipase D

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The hydrolytic susceptibility of large unilamellar vesicle (LUV) toward cabbage phospholipase D (PLD) was studied. The activity of PLD was determined by pH stat titration method. Using phosphatidylcholine LUV as substrate a pH optimum of 6.96 was observed. For maximal activity the optimal temperature of 31℃ and 10 mM of $Ca^{2+}$ were required. The apparent $K_m$ value estimated was 2.5 mM. The hydrolytic activity of PLD toward PC LUV was somewhat high despite the absence of activator in assay system and this high susceptibility of PC LUV may be attributed to the structural properties of LUV. The effect of amphiphatic substances such as dicetyl phosphate and phosphatidic acid on the enzyme activity were also examined in mixed LUVs.

## Introduction

Since the discovery of the spherically closed structure of amphiphatic substances, phospholipid vesicles have been used as model systems for various biological membranes.[1] And at the same time various methods have been developed to form morphologically different vesicles such as multilamellar vesicle, small unilamellar vesicle, and large unilamellar vesicle (LUV).[2] For some lipolytic enzymes, lipid vesicle was selectively used as substrate. Since the water insoluble lipid substrates form aggregates in aqueous media, any hydrolytic enzymes acting upon lipid substrates react with lipids at a water-lipid interface and hence are affected by the physical properties of the interface.[3-5]

The hydrolytic activity of phospholipase D (PLD) toward various aggregation forms of substrates have also been studied. Phospholipase D (phosphatidylcholine phosphatidohydrolase, EC 3.1.4.4.) hydrolyzes ester bond between the phosphatidic acid and alcohol moiety of phospholipids and also mediates a transfer reaction by which the phosphatidic acid moiety of phospholipid substrate is transferred to an alcohol acceptor.[6] The substrate structures such as monomer,[7] phospholipid-detergent mixed micelle,[8,9] vesicle,[10] and monolayer at air/water interface[11] have been used to illustrate the interfacial properties of PLD. Other properties of macromolecular substrates such as gel to aqueous crystalline phase transition and curvature of vesicle can be considered as factors which were correlated with the hydrolytic activity of PLD.[12,13]

In this study, the hydrolytic susceptibility of egg lecithin LUV prepared by reverse phase evaporation method was studied as a model substrate for phospholipase D. General properties of PLD toward phosphatidylcholine (PC) LUV were examined and compared to the previouse results obtained from ultrasonicated vesicles.[10]

## Experimental

**Materials.** Choline iodide, bovine serum albumin, and dithiothreitol were purchased from Sigma Chemical Company. 2-(N-Morpholino) ethane sulfonic acid (MES), alumina, and dicetyl phosphate were purchased from Aldrich Chemical Company. TLC plastic sheets of silica gel 60F254 were purchased from Merck Company. Crude egg phosphatidylcholine (PC) from Sigma was purified by aluminium oxide column.[14] Phosphatidic acid (PA) was prepared from egg PC by enzymatic synthesis as described by Jung[10] and purified further by silicic acid column. All other chemicals were reagent grade commercially available.

**Preparation of Phospholipase D.** PLD was partially purified from soluble proteins of savoy cabbage by heat treatment, acetone precipitation, and Sephadex G-200 gel filtration as described by Lee *et al.*[15] The enzyme was stored in 5 mM phosphate buffer (pH 6.5), 10% (w/w) inositol solution at 4℃.

**Preparation of Lipid Vesicles.** Large unilamellar vesicles were prepared by reverse-phase evaporation procedure. This procedure was first introduced by Szoka and Papahadjopoulos[16] and the detailed process was described previously by Lim and Choi.[17] An aliquot of chloroform solution containing 66 μmol of lipid component was dried on the wall of a glass vial. Then the lipid was redissolved in 3 ml of diethyl ether and 1 ml of distilled water was added. The resulting two-phase system was sonicated under $N_2$ at 0℃ for about 5 minutes with ultrasonicator (microtip, S & M VC500 sonicator, power output 2 and 50% duty cycle). After sonication ether was removed by blowing of $N_2$ with vortexing at 37℃. As the majority of diethyl ether was removed the mixture formed a viscous gel and subsequently became an aqueous suspension. To exclude the possible effect of trace ether on the PLD activity, the aqueous suspension was then placed under reduced pressure (water aspirator) for 45 minutes. This suspension was diluted with 7 ml of distilled water solution and centrifuged at 80,000 g for 10 minutes. The supernatant was used as substrate for enzyme assay. The concentration and composition of phospholipid vesicles were determined by assay of phosphorus. The phosphorus was assayed as described by Chen *et al.*[18] The composition of mixed phospholipid vesicles were determined after separation of the components by thin layer chromatography on silica gel plate.

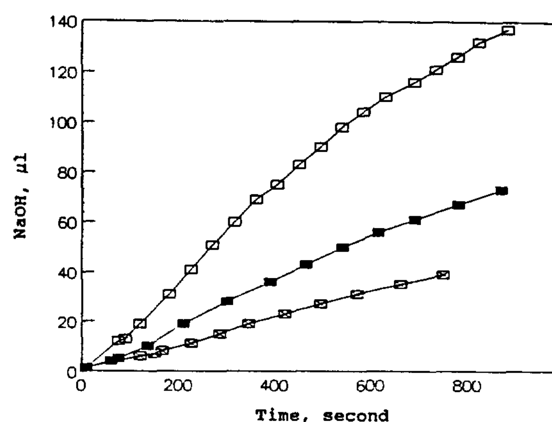


**Figure 1.** Time dependence of the amount of NaOH added in the hydrolysis of PC LUV system by PLD. The incubation medium contained 10 mM $CaCl_2$, $10^{-5}$ M DTT, and 2.64 mM PC. □, 7.5 μg of protein; ■, 3.9 μg of protein; □, 1.9 μg of protein.

**Assay of Phospholipase D.** The change in $H^+$ ion concentration induced by the enzymatic cleavage of the terminal phosphate ester bond of PC was followed by pH stat titration using a autotitroprocessor (Metrohm, 670 Titroprocessor). NaOH solution of 0.015 normal was used as standard base solution. In pH stat assay of the phospholipase D activity toward PC LUV, 2.2 ml of 3 mM PC vesicle solution was mixed with 0.1 ml of 250 mM $Ca^{2+}$ solution, and 0.1 ml of $2.5\times10^{-5}$ M dithiothreitol. Then 0.1 ml of enzyme solution was added and the pH of the reaction mixture was adjusted to pH 6.96 and maintained at that value by addition of the NaOH solution.

## Results and Discussion

**Assay Condition for pH Stat Method.** In this study, the essential factors that influence the enzyme activity against PC LUV were studied and compared with the effects of these factors on other forms of substrate. The enzyme activity was measured by pH stat titration method. In every assay the titration was performed for 10 minutes and initial slope (μl of NaOH added/minut) was used as a unit of enzyme activity. Duplicate assay results were reproducible within 10%. Dithiothreitol (DTT) was routinely included in assay as a stabilizer at the concentration of $10^{-5}$ M. At the concentration range of $10^{-6}$-$10^{-4}$ M DTT the curve of titration rate versus time shows a consistent pattern as shown in Figure 1. However in the absence of DTT it was observed that the curve was irregular and the titration rate was gradually decreased with time compared to the titration rate determined at the concentration of $10^{-5}$ M DTT. Figure 1 also reveals that after 15-20 μl of NaOH was added the curve turned up regardless of the amount of enzyme. The slope of the curve after this turning up point seemed to be proportional to it's initial slope. This phenomenon of the transient increase of the enzyme activity along incubation time is simi-

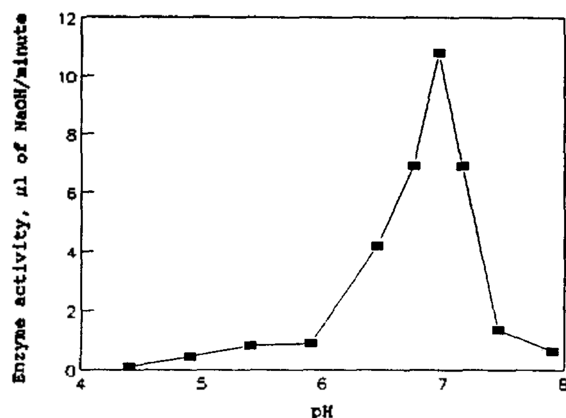


**Figure 2.** Effect of pH on the activity of PLD toward PC LUV. Initial slope (µl of NaOH added/minute) in titration is used as a unit of the enzyme activity.

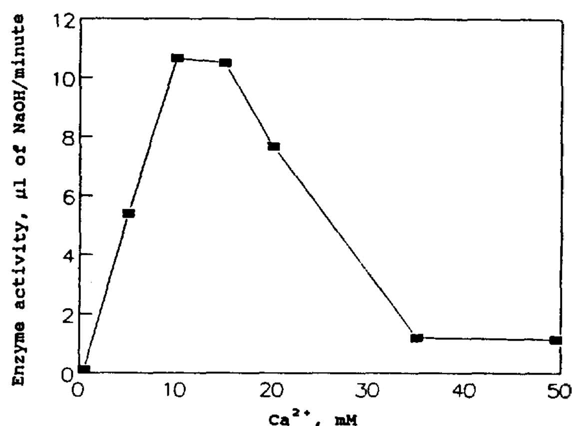


**Figure 3.** The effect of $Ca^{2+}$ concentration on the activity of PLD.

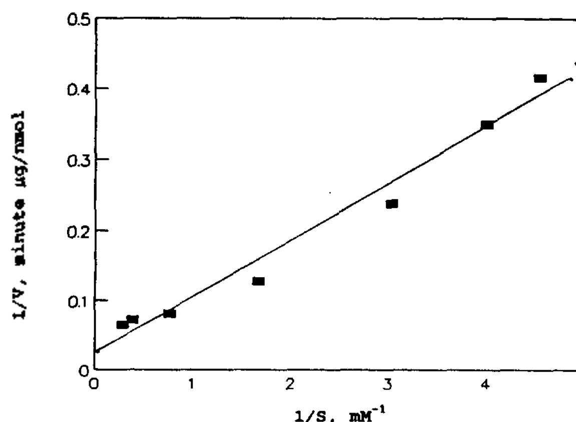


**Figure 4.** Lineweaver-Burk plot of the substrate dependence on the PLD activity.

lar to the fact of PC-SDS mixed micelle system.[10]

Up to now, the acidimetric assay of phospholipase D that follows the change of $H^+$ concentration by pH stat titration or color change of indicator has not been used much except only with short chain PC present as monomer. Because there have been a skeptical view about the applicability of acidimetric assay with long chain PC which forms aggregation in aqueous solution.[13] However, this result shows that the hydrolytic activity of phospholipase D against long chain PC can be assayed by pH stat titration method.

**General Properties of PLD Toward PC LUV.** In order to obtain the pH optimum, NaOH and HCl was used to adjust pH before starting pH stat titration. The pH profile curve showed rather sharp peak and optimum pH was around pH 6.96 as shown in Figure 2. This is somewhat higher than the values determined using other aggregation forms of PC, *i.e.*, the pH optimum of 4.9 for ultrasonically treated PC particle and pH 6.3 for PC-SDS mixed micelle at a molar ratio of 1 : 0.5.[6,10] The effect of protein amount was examined at substrate concentration of 2.65 mM. Linearity of activity with protein concentration was obtained up 15 µg/tube. When the effects of incubation temperature on the enzyme activity were examined, the optimum temperature was found out to be 31℃. It is known that $Ca^{2+}$ is essential for cabbage PLD activity and the optimal range of $Ca^{2+}$ concentration was also known to be dependent upon the conditions of the reaction such as pH, the purity of enzyme, the physical state of substrate, and in some cases substrate concentration.[6,10] In this case the optimum concentration of $Ca^{2+}$ was obtained at 10 mM as shown in Figure 3.

When the effect of substrate conentration was examined with 7.5 µg phospholipase D, substrate saturation was observed above 2 mM PC concentration. The kinetic parameter was determined from Lineweaver-Burk plot as shown in Figure 4. The rate of PC hydrolysis was obtained from the initial slope multiplied by the normality of NaOH (0.015 N). The $V_{max}$ value for initial activity was about 16 nmol/min/µg protein and $K_m$ value for PC LUV was about 2.5 mM. The $K_m$ value determined in this experiment was higher than that determined in the presence of anionic amphiphiles like SDS ($K_m$ value of 0.67 mM) and PA ($K_m$ value of 1.43 mM) as an activator.[10] When the $V_{max}$ was compared to the specific activity of 50-60 nmol/min/µg protein for PC SDS mixed micelle system determined under the same condition, the activity of PC LUV system is approximatly one fourth of the PC-SDS system. In ultrasonically treated PC-SDS system, however, SDS has been known to stimulate the enzyme activity up to 160 fold.[8] With respect of this fact, the hydrolytic susceptibility of PC LUV was remarkably high, despite the absence of any activator. This result suggests that the LUV is highly susceptible that means very good substrate to the PLD hydrolysis probably because of it's structural property.

**Hydrolytic Activity Toward Mixed Vesicles.** The mixed vesicles of PC and PA and of PC and dicetyl phosphate were prepared to investigate the effect of anionic amphiphiles on the PLD activity in LUV system. These anionic amphiphiles were incorporated into LUV only at a limited amount. The mixed vesicle of PC-PA with the molar ratio of more than 0.4 : 1 (PA : PC) and the mixed vesicle of PC-dicetyl phosphate with the molar ratio of more than 0.05 : 1 (dicetyl phosphate : PC) can not be prepared by reverse phase evaporation method. These results may be attributed to the net anionic charge of these amphiphiles. The enzyme activity toward mixed LUV was assayed at a fixed concentration of PC (1.7 mM) with various molar ratios of PA or dicetyl phosphate. The effect of pH on the enzyme activity

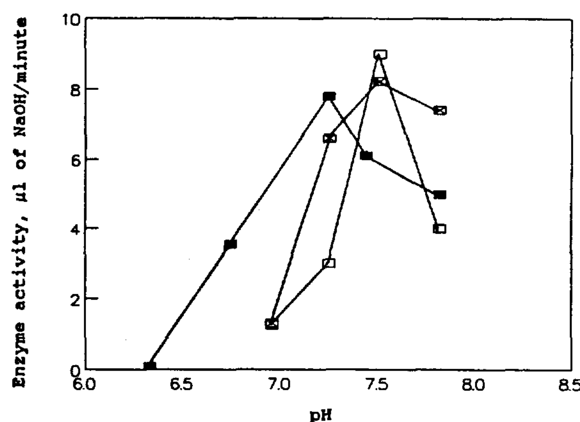


**Figure 5.** Effect of pH on the activity of phospholipase D toward the mixed LUV. The assay medium contained 10 mM $CaCl_2$, $10^{-5}$ M DTT, 1.7 mM PC with various molar ratio of amphiphiles, and 7.5 µg of protein. □, PC-Dicetyl phosphate (1 : 0.05); ■, PC-PA (1 : 0.16); □, PC-PA (1 : 0.3).

against mixed vesicle was determined by measuring the initial slope in pH stat titration at the protein amount of 7.5 µg. In the presence of PA and dicetyl phosphate, the optimum pH was shifted about 0.3-0.5 units higher from the optimum value of 6.96 determined with PC LUV system as shown in Figure 5. At a molar ratio of 1 : 0.05 of PC-dicetyl phosphate mixed LUV, the initial slope was 9 µl/minute at optimum pH of 7.5 and at molar ratios of 1 : 0.16 and 1 : 0.3 of PC-PA the initial slope at optimum pH was about 8 µl/minute. These values are comparable to that of the PC only LUV system. This means that the PA and dicety phosphate do not act as activators in LUV system. It has been reported that PA is a good activator for the phospholipase D when ultrasonically treated with PC.[8,10] The shift in optimum pH due to anionic amphiphiles like PA and SDS was also described.[11] The effect of substrate structure on the lipolytic enzyme activity has been studied mainly in view of the capability of enzyme-substrate complex formation which is influenced by surface charge, exposure of each lipid molecule to surface, or the phase state of the membrane, *i.e.*, gel phase versus liquid crystalline phase. The high susceptibility of LUV to PLD may be attributed to the highly exposed lipid molecules to surface in the unilamellar vesicle. The present study shows clearly the LUV as an excellent model substrate for PLD. Nevertheless further understanding of LUV structure is required to correlate the enzme activity with phase state of vesicle which can be determined by the curvature of bilayer or the presence of other chemicals.

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