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Characterization of Phosphatidylcholine-Hydrolyzing Phospholipase D in the Scuticociliate Parasite, *Uronema marinum*

Jung Soo Seo[*], Moo-Sang Kim[**], Na-Young Kim[**], Sang Jung Ahn[***], Bo Young Jee[*], Sung Hee Jung[*], Jin Woo Kim[*], Ki-Hong Kim[**], Hyung-Ho Lee[***] and Joon-Ki Chung[†]**

[*]Patholgy Division, National Fisheries Research and Development Institute, 408-1 Silang, Gijang, Busan 619-902, Korea
[**]Department of Aquatic Life Medicine, Pukyong National University, Busan, 608-737, Korea
[***]Faculty of Food Science and Biotechnology, Pukyong National University, Busan, 608-737, Korea

We report the existence of new type of phosphatidylcholine-hydrolyzing phospholipase D (PLD), which has been characterized and partially purified in the scuticociliate, *Uronema marinum*. The enzyme from partial purification showed that it was existed in membrane fraction and was a neutral PLD, which catalyzed both transphosphatidylation and hydrolysis reaction. The activity of partially purified membrane-bound PLD was also found to be optimal at pH 7.0-7.5 for 2 hours at 37℃ and depended strictly on the presence of Ca^{2+} (2.5 mM) and Mg^{2+} (1.6 mM). Immunoblot analysis indicated that the enzyme was distinct from hPLD1 (human PLD1) and hPLD2 (human PLD2) because it was not recognized by a polyclonal antibody raised to the 12 terminal amino acid of these enzymes. We also found that the membrane-bound PLD is a PIP_2-dependent PLD and that GTP-binding proteins are not implicated in the regulation of this enzyme: This enzyme activity is markedly stimulated by phosphatidylinositol 4,5-bisphosphate (PIP_2) but not by the small G-protein Arf and GTPrS. In addition, this enzyme was capable of hydrolyzing phosphatidylcholine (PC) but not phosphatidylethanolamine (PE), implying that PC was a preferred substrate.

Key words: Phospholipase D, *Uronema marinum*, Phosphatidylcholine, Phosphatidylinositol 4,5-bisphosphate

Introduction

Phospholipase D (PLD) is an enzyme that plays important roles in signal transduction in many types of cells. PLD catalyzes the hydrolysis of phosphatidylcholine (PC), the major phospholipid of membranes, to phosphatidic acid (PA) and choline in response to a variety of hormones, neurotransmitters, growth factors, cytokines, and chemoattractants (Exton, 1997; Liscovitch et al. 2000). PA has been implicated as a biologically active molecule that can affect the activities of protein kinases, a protein-tyrosine phosphatase, GTPase-activating proteins of small G proteins and phospholipase Cr1 (Amsterdam et al. 1994; English, 1966; Munnik, 2001). PA can be further metabolized by PA phosphohydrolase to yield diacylglycerol, a protein kinase C (PKC) activator, and by phospholipase A_2 to form the intercellular messenger lysophosphatidic acid (Lopez, et al. 1995; Waggoner, 1999). PLD also catalyzes a phosphatidyl transfer reaction in which a primary alcohol serves as nucleophilic

†Corresponding Author : J. K. Chung, Tel : 82-51-620-6142
Fax : 82-51-628-7430, E-mail : jkchung@pknu.ac.kr

acceptor in place of H_2O (Kanfer, 1994; Massenburg et al. 1994). The resulting production of phosphatidyl alcohol represents a specific hallmark of PLD activity in vivo and vitro assay systems. It has been demonstrated that PLD is activated via multiple pathways involving heterotrimeric and small G proteins, PKC, protein-tyrosine kinase, Ca^{2+}, and unsaturated fatty acids (Billah, 1993; Brown et al. 1993; Lopez et al. 1995; Massenburg et al. 1994; Natarajan et al. 1996). Since PLD activity was first characterized from carrot extracts (Hanahan and Chaikoff, 1947), PLD has been found in many sources and has recently been cloned in bacteria, yeast, plants, and mammalian sources (Exton, 1997; Morris et al. 1996). These PLDs exhibit sequence similarities that are part of a PLD superfamily (Banno et al. 1997; Koonin, 1996; Morris et al. 1996; Ponting and Kerr, 1996). Different types of PLD have different substrate specificity and requirements for divalent cations, suggesting for the existence of PLD isozymes (Cockcroft, 2001; Exton, 2002).

In parasite systems, it is also known that PLD plays important roles in the development of parasite and the parasite-host cell association (Connelly and Kierszenbaum, 1984 and 1985; Rasmussen and Rasmussen, 1999; Wang et al. 2001). PLD activity in protozoa was first characterized from eukaryotic protozoan *Tetrahymena pyriformis* (Wang et al. 2001). PLD in *Tetrahymena* was associated with microsomal membranes and was a neutral phospholipase which catalyzed both transphosphatidylation and hydrolysis reactions. This enzyme activity was stimulated by PIP_2 but was insensitive to phorbol 12- myristate 13-acetate (PMA) and guanosine 5'-3-O-(thio)triphosphate (GTPrS), and did not require Ca^{2+} for maximal activity.

Several scuticociliate species belonging to the genera *Uronema*, *Miamiensis* and *Philasterides* are facultative histophagous parasites in marine fish (Cheung et al. 1980; Iglesias et al. 2001; Sterud et al. 2000; Thompson and Moewus, 1964; Yoshinaga and Nakazoe, 1993). These ciliates are characterized by their high potential for invading systemically and destroying fish tissues, leading to high mortalities in fish culture process (Kwon et al. 2002). However, very little is known to date about the evasive mechanisms of the parasite. Connelly and Kierszenbaum (1985) reported a crucial role of PLD in the initial stages of host cell infection by *Tetrahymena cruzi*, although it is other protozoan. It can be therefore assumed that PLD may be also involved in the evasive mechanism of the scuticociliate against host immune system. Unfortunately, so far the presence of the evolutionally related PLD(s) has not been clearly identified in the scuticociliate species. Here we report the presence and some properties of PLD activity in scuticociliate, *Uronema marinum*, a pathogenic parasite in marine fishes. This is, to our knowledge, the first report in which the PLD activity has been reported in this organism.

Materials and Methods

Materials

Phosphatidylcholine (PC), phosphatidylethanolamine (PE), GTPrS were purchased from Sigma (St. Louis, MO); $PI(4,5)P_2$ were obtained from Roche; [2-*pamitoyl*-9,10-^{3}H](pam)$_2$PC (89 Ci/mmol) and [*choline-methyl*-^{3}H](pam)$_2$PC (50 Ci/mmol) were purchased from DuPont NEN.

Isolation and culture of parasite

The scuticociliate parasites, *Uronema marinum*, which were identified by morphological characteristics (Jee et al., 2001) and homology of 18S rRNA gene sequence (Kim et al., 2004) were aseptically

isolated from the brain of naturally infected olive flounder, *Paralichthys olivaceus*. The isolated parasites were washed three times in Hank's balanced salt solution (HBSS, Sigma) by centrifugation at 1,000 × g for 5 min at 4℃ and inspected microscopically to ensure an artificial contaminant. A pure culture of the isolated parasite was derived from a single organism and maintained by weekly subculture in 15 ㎖ of minimum essential medium (MEM, Sigma) containing 1% fetal bovine serum, penicillin (100 ㎍/㎖, Sigma) and streptomycin (100 U/㎖, Sigma) at 15℃. For the enzymatic analyses, parasites were cultured as described previously (Seo et al., 2005). Briefly, 10 ㎖ (about 1 × 10^5 cells) of the stock culture were axenically inoculated into 1 ℓ of MEM containing 1% fetal bovine serum, penicillin (100 ㎍/㎖, Sigma) and streptomycin (100 U/㎖, Sigma) and grown for three days at 20℃. The parasite growth was estimated daily by counting parasites in a Neubauer chamber. At the late exponential phase after inoculation (3 days), the parasites were harvested into 1 ㎖ of MEM culture medium by centrifugation at 1,000 × g for 10 min at 4℃. The harvested parasites were washed 3 times in HBSS by centrifugation at 1,000 × g for 5 min at 4℃ and stored at -75℃ until sufficient material was obtained for purification.

Extraction and partial purification of PLD from *U. marinum* membrane

All procedures were performed at 4℃. The frozen parasites (approximately 2 × 10^8) were thawed and suspended in 20 ㎖ of homogenization buffer containing 20 mM Hepes (pH 7.0), 1 mM EGTA, 0.1 mM dithiothreitol (DTT), 1.5 mM phenylmethylsulfonyl fluoride (PMSF), and leupeptin and aprotinin (each at 1.5 ㎍/㎖). The parasites were then disrupted 3 times for 30 s with sonicator (Vibra cell, Sonics & materials Inc, USA) at a setting of 40%. The homogenate was centrifuged at 1,000 × g for 20 min and then the supernatant was collected. The pellet was further homogenized in homogenization buffer and recentrifuged at 1,000 × g for 20 min. The supernatants from two homogenization steps were pooled and then centrifuged at 100,000 × g for 1 h. The resulting supernatant was saved for use as the cytosolic fraction. The pelleted membrane was stirred for 1 h with homogenization buffer containing 1% Triton X-100 and recentrifuged at 100,000 × g for 1 h. The supernatant was saved for use as the solubilized membrane fraction and insoluble material was discarded. The membrane fraction (16 mg of protein in 10 ㎖) was applied to a TSK-gel Heparin-5PW column (7.5 × 75 mm) that had been equilibrated with Buffer A containing 20 mM Hepes (pH 7.0), 1 mM EGTA, leupeptin and aprotinin (each at 1.5 ㎍/㎖), and 0.1% Triton X-100. Proteins were eluted from the column with a 60-㎖ linear gradient of 0 to 1.0 M NaCl in Buffer A. Fractions of 1 ㎖ were collected at a flow rate of 1 ㎖/min and assayed for PLD activity.

Assay of PLD activity

PLD activity was assayed, as described previously (Chung et al. 1997) with a minor modification, by measuring the generation of either ^{3}H-labeled PA and phosphatidylethanol (PEtOH) from [2-*pamitoyl*-9,10-^{3}H](pam)$_2$PC in the presence of ethanol or ^{3}H-labeled choline from [*choline-methyl*-^{3}H](pam)$_2$PC. Briefly, 25 ㎕ of lipid vesicles containing PE, PI(4,5)P_2, and PC in molar ratio of 16:1.4:1 with either 0.16 ㎛M [2-*palmitoyl*-9,10-^{3}H](pam)$_2$PC(total ~10^6 cpm/assay) or [*choline-methyl*-^{3}H](pam)$_2$PC (total 200,000 cpm/assay) were added to 125 ㎕ of a mixture containing PLD source, 50 mM Hepes-NaOH (pH 7.5), 3 mM

EGTA, 80 mM KCl, 2.5 mM $MgCl_2$, and 2 mM $CaCl_2$. The final concentration of PC was 3.4 μM, and the free Ca^{2+} concentration was calculated to be 300 nM. The assay mixture for the measurement of PA and PEtOH also contained 0.65% (v/v) ethanol. The reaction mixture was incubated at 37℃ for 1 hr. The reaction was terminated by the addition of 1 ㎖ of stop solution containing $CHCl_3$, CH_3OH, and concentrated HCl (50:50:0.3, v/v) and 0.35 ㎖ of 1 M HCl containing 5 mM EGTA. After separation of the aqueous and organic phases by centrifugation, the released [^{3}H]choline in 0.5 ㎖ of the aqueous phase was quantitated by liquid scintillation counting. For measurement of [^{3}H]PA and [^{3}H]PEtOH, 0.4 ㎖ of the organic phase was dried under the vacuum speed centrifugation. Lipids were resuspended in 20 $\mu\ell$ of solvent containing $CHCl_3$, CH_3OH, and CH_3COOH (65:15:5, v/v), applied to the thin-layer chromatography plate (TLC; silica gel 60 F-254, Merck), and developed with the same solvent. Radioactive spots were quantified with Cyclone Phosphor Imager (Packard. Co. U.S.A).

Results

Identification and partial purification of PLD in scuticociliate

To investigate whether PLD is present in scuticociliate, PLD activity was determined in the cytosol and membrane fraction of a whole scuticociliate extract (Table 1). Determination of PLD activity in the crude scuticociliate extract was not possible as there was significant inhibition of the assay. However, following fractionation of the scuticociliate

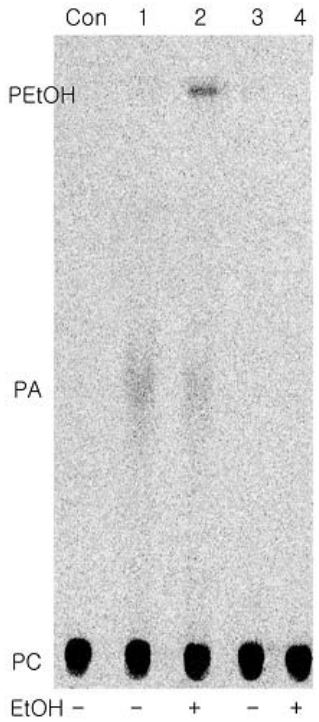

Fig. 1. **Reaction products from hydrolysis of phosphatidylcholine.** The particulate and cytosol fraction were incubated with phospholipids vesicles in reaction mixture without (Con, lane 1, lane 3) and with (lane 2, lane 4) 0.67% (v/v) ethanol. The substrate vesicles comprised of PE, PIP_2, and PC in a molar ratio of 16:1.4:1 with [2-*palmitoyl*-9,10-^{3}H]dipalmitoyl PC to yield 200,000 cpm per assay. After incubation for 1 h at 37℃, the lipid products were analyzed by TLC and visualized by Cyclone Phosphor Imager. The positions of PC, PA, and PEtOH are indicated. Lane C represents control (substrate alone). Lane 1 and 2 represent particulate fraction (7 $\mu\ell$). Lane 3 and 4 represent cytosol fraction (7 $\mu\ell$).

Table 1. Distribution of phospholipase D (PLD) activity in subcellular fractions of scuticociliates.

Subcellular preparation	Total protein (mg)	Specific activity (pmol/mg/min)	Total activity (pmol/min)	% of PLD activity
Membrane	16	42.8	685.1	68.9
Cytosol	32	8.7	332.1	31.1

Scuticociliate extracts were fractionated into particulate (membrane) and cytosol fractions and assayed for PLD activity as described under "MATERIALS AND METHODS". The results are from one typical data out of triplicate assays. PLD activity in each fraction is expressed as a percentage of the sum of the individual subcellular fractions.

extract it was able to estimate the enzyme activity. The production of [^{3}H]PA or [^{3}H]PEtOH was found in the membrane fraction but not in the cytosol fraction, indicating the localization of the PLD activity to the membrane fraction (Fig. 1). For further characterization of PLD activity, the membrane fraction was applied to a Heparin 5-PW column and eluted with a linear NaCl gradient (Fig. 2). All the PLD activity was absorbed to the column, and eluted as two peaks (designated peaks "A" and "B") in the fractions containing about 210 mM and 450 mM NaCl, respectively.

Identification of the reaction products

The release of [^{3}H]choline from [*choline-methyl*-^{3}H](pam)$_2$PC in a water-soluble form can result from phospholipase C activity as well as phospholipase D activity (Massenburg et al., 1994). Therefore we analyzed the reaction products produced by the two active peaks (peak A and B) from Heparin 5-PW column to identify that these active peaks were due to PLD. Two active fractions (peak A and B) from Heparin 5-PW HPLC column were incubated with [2-*palmitoyl*-9,10-^{3}H](pam)$_2$PC in the presence and absence of 0.67% ethanol under the standard assay condition, and the radioactive lipids were separated by TLC. PLD attacks PC to form a transient phosphatidyl-PLD intermediate. With water as an acceptor, PA is product; however, in the presence of ethanol, the phosphatidyl moiety is also transferred to the alcohol, generating PEtOH. This transphosphatidylation activity is a hallmark of PLD and is an established criterion for demonstrating the specific action of PLD (Pai et al. 1988). As shown in Fig. 3, whereas peak A fraction did not produce either PA

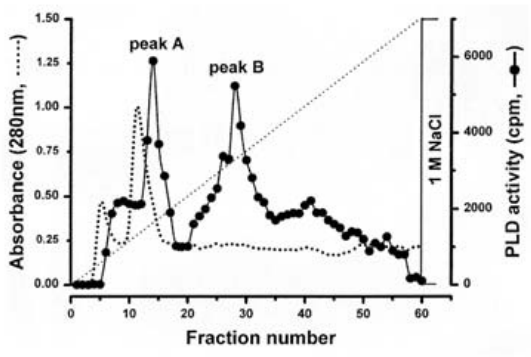

Fig. 2. **Chromatography of PLD activity through Heparin-5PW HPLC column.** Membrane from *U. marinum* were prepared, extracted, and processed as described under "MATERIALS AND METHODS". Eluted fractions (1 ml) were assayed for protein (-) and PLD activity in the absence of GTPrS and Arf (●).

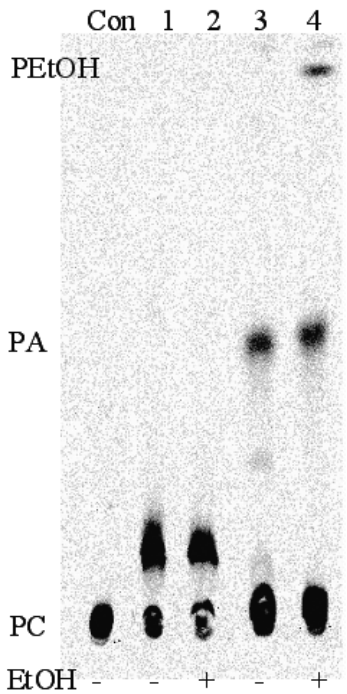

Fig. 3. **Reaction products of phosphatidylcholine-hydrolyzed phospholipase D.** The active fraction from a Heparin-5PW HPLC column was incubated with phospholipids vesicles in reaction mixture without (Con, lane 1, lane 3) and with (lane 2, lane 4) 0.67% (v/v) ethanol. The substrate vesicles comprised PE, PIP$_2$, and PC in a molar ratio of 16:1.4:1 with [2-*palmitoyl*-9,10-^{3}H]dipalmitoyl PC to yield 200,000 cpm per assay. After incubation for 1 h at 37℃, the lipid products were separated by TLC and visualized by Cyclone Phosphor Imager. The positions of PC, PA, and PEtOH are indicated. Lane C represents control (Con; substrate alone). Lane 1 and 2 represent membrane peak A (7 $\mu\ell$), Lane 3 and 4 represent membrane peak B (7 $\mu\ell$).

or PEtOH in the absence and presence of ethanol (lane 1 and 2), peak B fraction produced only PA in the absence of ethanol and PEtOH, as well as PA, in the presence of alcohol (lane 3 and 4). This result clearly shows that the activity of peak B, but not of peak A, is due to PLD.

Characterization of PLD activity

PLD activity increased at a linear rate for up to 120 min (Fig 4A), and showed a temperature-dependent increase with maximum activity at 40℃ (Fig. 4B). The effect of pH on PLD activity was also examined using pH values ranging from 4.0 to 9.0 under the standard assay conditions. PLD activity showed a rather sharp pH profile, with optimum activity at pH 7.0-7.5 (Fig. 4C). However, PLD activity was not affected by the small G-protein, ADP-ribosylation factor (Arf), in the presence of GTPrS, indicating its independence of small GTP-binding protein (data not shown).

It has been reported that mammalian PLD requires divalent cation such as Ca^{2+} and Mg^{2+} for the enzyme activity (Cockcroft, 2001; Exton, 2002). We have therefore examined the effects of these divalent cations on PLD activity from scuticociliate. For this study, free Ca^{2+} and Mg^{2+} in the reaction mixture were controlled using Ca^{2+}/Mg^{2+}-EGTA buffers at pH 7.5. PLD activity gradually increased with increasing concentration of Ca^{2+} and Mg^{2+} and reached a peak at 1.6 and 2.5 mM, respectively, suggesting that PLD activity was more sensitive to Ca^{2+} than Mg^{2+}. However, at higher concentrations (3 and 5 mM) of Ca^{2+} and Mg^{2+}, PLD activi-

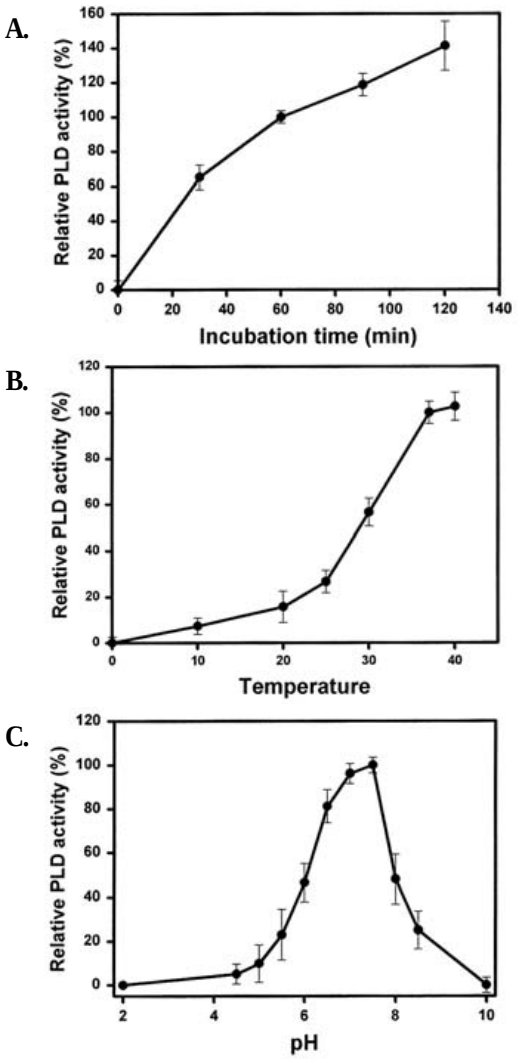

Fig. 4. **Effects of incubation time (A), temperature (B) and pH (C) on PLD activity.** A membrane peak B fraction from a preparative Heparin-5PW HPLC column was incubated with phospholipid substrate vesicles in reaction mixture under various conditions. Results are the mean ± S.D. from four independent experiments.

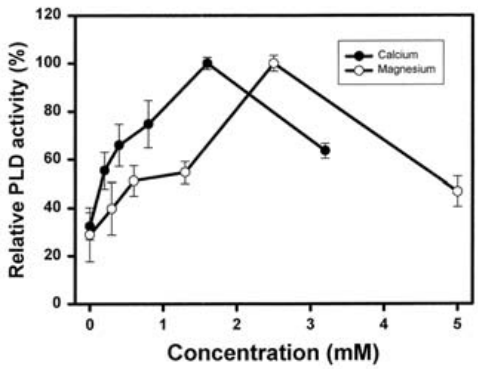

Fig. 5. **Effects of Mg^{2+} (A) and Ca^{2+} (B) on PLD activity.** A membrane peak B fraction from a preparative Heparin-5PW HPLC column was measured in the presence of six different concentrations of Mg^{2+} or Ca^{2+}. Results are the mean ± S.D. from four independent experiments.

ty markedly decreased (Fig 5).

We next examined the effects of various phospholipids substrates on PLD activity from scuticociliate (Fig. 6). Various concentrations of each phospholipid were added to the reaction mixture containing 0.16 μM [*choline-methyl*-^{3}H](pam)$_2$PC (total 200,000 cpm/assay) as radioactive substrate. PC (up to 3.4 mM) did not affect PLD activity and higher concentrations were inhibitory (Fig. 6A). PE (up to 53.6 mM) slightly increased PLD activity, but it also showed an inhibitory effect at higher concentrations (Fig. 6B). PLD activity was dependent on PIP_2, and its maximal activity occurred at 4.7 mM and sustained up to 9.4 mM (Fig. 6C).

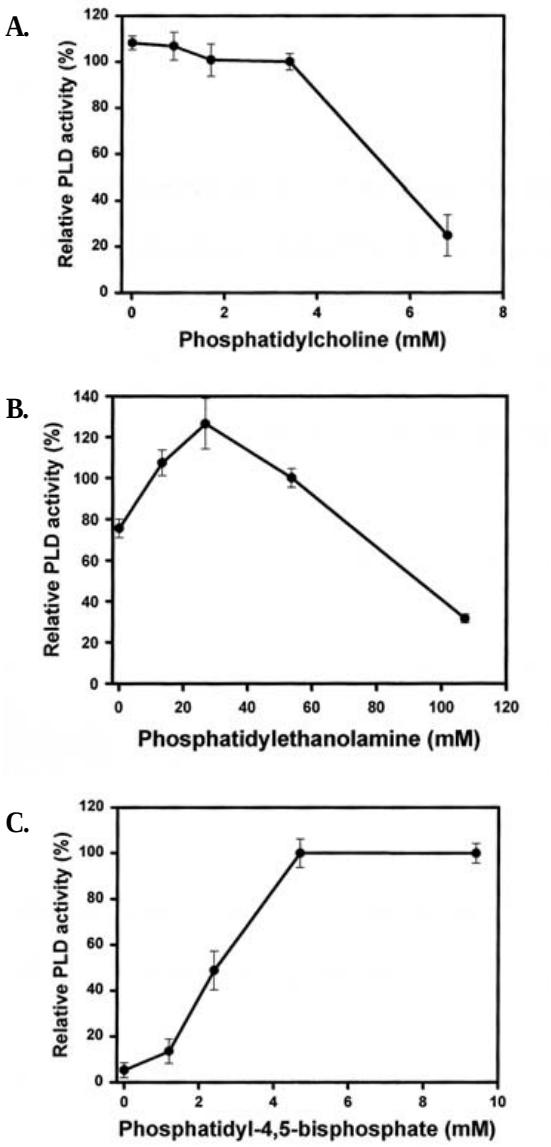

Fig. 6. **Effects of substrate concentration on PLD activity.** A membrane peak B fraction from a preparative Heparin-5PW HPLC column was measured in the presence of five different concentrations of PC (A), PE (B) and PIP_2 (C). Results are the mean ± S.D. from four independent experiments.

Discussion

The present results provide the existence of the PIP_2-dependent PLD activity in *Uronema marinum*. A wide range of PLD's have been reported with differing localizations and molecular weights as well as differences in their responses to Ca^{2+}, Mg^{2+}, PIP_2, sodium oleate, detergents, pH, small G-proteins, PKC, and substrate. The biochemical characterization of this PLD activity revealed that it was membrane-associated enzyme which showed a neutral pH optimum and Ca^{2+}-Mg^{2+}, dependency. The enzyme was also stimulated by PIP_2, utilizing phosphatidylcholine as a preferential substrate. However, *Uronema* PLD activity was not affected by PMA, sodium oleate, GTPrS and small G-protein (Arf), suggesting that this PLD possesses a distinct difference in reaction profies to the already reported PLDs.

Most mammalian PLD have been reported that it found in association with membranes and appeared to be specific for phosphatidylcholine (PC) (Martin and Michaelis, 1989; Brown et al. 1993; Siddiqui and Exton, 1992; Kanfer et al. 1994) and have a high affinity for anionic phospholipids, and all appear to require Ca^{2+} for catalytic activity (Exton, 1997). Unlike in mammalian, it has been little reported for PLD characterization and purification in protozoan. A few studies have investigated PLD in *Tetrahymena* and reported that PLD have con-

cerned with cell growth, proliferation, death and signal transduction (Kovacs et al. 1996, 1997; Rasmussen and Rasmussen, 1999; Wang et al. 2001). In addition, PLD in *Trypanosoma cruzi* have increased parasite-host cell association and these appearances suggested that PLD play a role in the initial stages of host cell infection by parasite (Connelly and Kierszenbaum, 1985).

In this study, we found that membrane and cytosol fraction in *U. marinum* highly appeared PLD activity (Table 1 and Fig. 1). Transphosphatidylation, a unique reaction in which primary alcohols serve as substrates for the production of phosphatidylalchols (PtdOH), is catalyzed by PLD. Therefore, stimulation of PLD activity can be assayed by measuring PEtOH formation in alcohols (Van et al. 1993). Through purifying particulate fraction with Heparin-5PW HPLC, we separated two PLD activity peaks from membrane in *U. marinum* (Fig. 2). Under transphosphatidylation reaction of these fractions, it was visualized the formation of both PA and PEtOH products in membrane-bound peak B but not in membrane-bound peak A. In addition, for transphosphatidylation reaction, we demonstrated that membrane-bound peak A and cytosol fraction were not PC-hydrolyzing PLD but other phospholipase subtype in *U. marinum*, and differed functional with membrane-bound PLD. We have previously reported that the cytosol fraction in *U. mariunm* was the phosphatidylcholine-hydrolyzing phospholipase C (Seo et. al., 2005).

Membrane-bound PLD for peak B fraction in the Fig. 4 showed maximum activity under condition of pH (7.0-7.5), temperature (37℃) and incubation time (120 min). In Fig. 5 described also that the activity of membrane-bound PLD in *U. marinum* depended on the presence of Mg^{2+} (2.5 mM) and Ca^{2+} (1.6 mM). Generally, PLD forms interact tightly with the lipid bilayer through direct binding to anionic lipids (Stieglitz et al. 2001). While Ca^{2+} lessens the affinity of PLD for anionic lipids such as PA, it also clusters the anionic lipids on the membrane surface.

It has been suggested that PLD1 and PLD2 isozymes act through similar catalytic mechanisms since both enzymes have an absolute requirement for phosphatidylinositol 4,5-bisphosphate (PIP_2) for activation (Cockcroft, 2001; Exton, 2002). Through substrate specificity, a substrate molar of PC: PE: PIP_2 (3.4:13.4:4.7) was found to be required for optimal stimulation of PLD. The membrane-bound PLD was also stimulated by PIP_2 in a dose-dependent manner, a fact which may indicate a more widespread occurrence of this phenomenon. It should be noted that hPLD1 and hPLD2 antibodies are known to cross-react with PLD from human, mouse, yeast, and plant species (Blum et al. 2001). However, in this study, immunoblot analysis indicated that the enzyme was distinct from hPLD1 or hPLD2 isozymes, under not recognized by antibodies of these enzyme (data not shown).

In conclusion, this study is the first report describing the characterization of PLD from *U. marinum*. The membrane-bound PLD was stimulated by PIP_2, Ca^{2+} and Mg^{2+} but not require GTPrS and Arf. The optimum activity was found at pH 7.0-7.5 for 2 hr at 37 ℃. The membrane-bound PLD was highly activity by optimal molar of PC: PE: PIP_2 (3.4:13.4:4.7) on substrate composition, and demonstrate that it was PIP_2-dependent PLD. Therefore, the membrane-bound PLD presented in *U. marinum*, demonstrate to new PLD type.

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