Substrate recognition mechanism of *Streptomyces* phospholipase D and enzymatic measurement of plasmalogen

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Running title: Substrate recognition mechanism of phospholipase D

ABSTRACT

The substrate recognition mechanism of phospholipase D and enzymatic measurement of choline plasmalogen were investigated. A phospholipase D (PLD₆₈₄) from *Streptomyces* sp. strain NA684 was purified 184-fold from the culture supernatant with 23.7% recovery. Maximum activity for L- -lysophosphatidylcholine (LPC) hydrolysis was found at pH 5.0 and 80°C. The hydrolytic activity was remarkably affected by the concentration of Triton X-100 in the reaction mixture. In the presence of 0.05 0.5% and 0.1 0.2% (wt/vol) Triton X-100, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC) and choline plasmalogen were efficiently hydrolyzed by PLD₆₈₄, respectively. Hydrolysis of LPC and choline lysoplasmalogen did not require Triton X-100; rather, the hydrolytic activity was inhibited by more than 0.05% (wt/vol) Triton X-100. The enzyme preferred mixed micelle substrates to liposomal substrates and hydrolyzed 98.4% of mixed micelle POPC in 1 h. Kinetic analysis showed that the rate-limiting steps of hydrolysis of mixed micelle POPC and emulsified LPC by PLD₆₈₄ were the bulk step and the surface step, respectively. These results suggest that PLD₆₈₄ has at least two substrate recognition mechanisms to recognize various phospholipids that have considerably different physical properties derived from their head and tail groups. Understanding of how PLD₆₈₄ recognizes substrate forms will be useful for elucidating roles of lipolytic proteins in nature. Moreover, we report an enzymatic measurement of choline plasmalogen using PLD₆₈₄ and phospholipase B. This is the first enzymatic method for measuring choline plasmalogen.

Keywords: *Streptomyces* sp.; phospholipase D; kinetics; functional expression; substrate recognition; plasmalogen determination

INTRODUCTION

Phospholipase D (PLD) (EC 3.1.4.4) catalyzes the hydrolytic cleavage of phosphodiester

1 bonds of glycerophospholipids, forming phosphatidic acid and the corresponding alcohol. Some PLDs
2 have transphosphatidylase activity. For example, phosphatidylcholine (PC) is converted into
3 phosphatidylethanolamine (PE) in the presence of ethanolamine (1). Several PLDs from
4 streptomycetes have been sequenced, including PLDs from *Streptomyces antibioticus* (2),
5 *Streptomyces cinnamomeum* (3), *Streptomyces halstedii* (4), *Streptomyces septatus* (5) and the
6 *Streptomyces* sp. PMF strain (6, 7). These PLDs show significant sequence similarity and belong to the
7 PLD superfamily containing two HxKxxxD sequences, called HKD motifs, (8). Leiros *et al.*
8 determined the crystal structure of a PLD from *Streptomyces* sp. PMF (PLD_{PMF}; UniProt accession no.
9 P84147; Protein Data Bank code: 1V0W) (7). PLD_{PMF} is a monomeric protein consisting of two
10 domains with similar topology. The crystal structure and mutational studies revealed the substrate
11 binding pocket (1). However, recognition of the micelle-forming substrates by PLD has not been
12 determined, and it is only known that PLDs are generally more active toward aggregated substrates
13 than free substrates (8). Previous studies have demonstrated that the activities of PLDs depend on the
14 substrate forms, such as liposomal, emulsified, or mixed micellar substrates (8). PLD activity has been
15 shown to be highest at the interface with micelle-forming substrates, and this activity is drastically
16 reduced toward phospholipid vesicles compared with micelle-forming and monomeric substrate (8).
17 Thus, the form of the associated substrates is a crucial determinant of the enzymatic activity, yet how
18 PLDs recognize their substrate form remains unresolved. To understand the substrate recognition
19 mechanism provides a new perspective on roles of lipolytic proteins.

20 In the present study, we report the hydrolytic activity of PLD₆₈₄ from *Streptomyces* sp.
21 NA684 toward liposomal, emulsified, and mixed micelle substrate. Moreover, we demonstrate two
22 distinct substrate recognition mechanisms for emulsified LPC and mixed micelle PC with Triton
23 X-100, and establish an enzymatic method to measure choline plasmalogen.

24

25 MATERIALS AND METHODS

1 *Materials*– L- α -Lysophosphatidylcholine, Egg (LPC),
2 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC),
3 1-*O*-1'-(*Z*)-octadecenyl-2-hydroxy-*sn*-glycero-3-phosphocholine (choline lysoplasmalogen; LPLS-PC),
4 1-(1*Z*-octadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphocholine (choline plasmalogen; PLS-PC),
5 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphoethanolamine (POPE),
6 1-*O*-1'-(*Z*)-octadecenyl-2-hydroxy-*sn*-glycero-3-phosphoethanolamine (ethanolamine
7 lysoplasmalogen; LPLS-PE), 1-(1*Z*-octadecenyl)-2-arachidonoyl-*sn*-glycero-3-phosphoethanolamine
8 (ethanolamine plasmalogen; PLS-PE), and
9 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phospho-(1'-*rac*-glycerol), monosodium salt (POPG) were
10 purchased from Avanti Polar Lipids Inc. (Alabaster, AL, USA). L- α -lysophosphatidylethanolamine
11 (LPE) was purchased from Doosan Serdary Research Laboratories (Toronto, Canada).
12 L- α -phosphatidylinositol (PI) and sphingomyelin from chicken egg yolk (SM) were purchased from
13 Sigma-Aldrich Japan Co., LLC. (Tokyo, Japan). *sn*-Glycero-3-phosphocholine was purchased from
14 Bachem AG (Hauptstrasse, Bubendorf, Switzerland). Tryptic soy broth (TSB) and Bacto-peptone were
15 purchased from Becton, Dickinson and Company (Franklin Lakes, NJ, USA). Toyopearl Phenyl-650M
16 was purchased from Tosoh Co. (Tokyo, Japan). HiTrap Q HP, RESOURCE PHE and Mono S columns
17 were purchased from GE Healthcare Japan (Tokyo, Japan). Choline oxidase (COD) from *Arthrobacter*
18 *globiformis* was gifted from Asahi Kasei Pharma Corp. (Tokyo, Japan). Peroxidase (POD) was
19 purchased from Oriental yeast Co., Ltd. (Tokyo, Japan). 4-Aminoantipyrine was purchased from
20 Nacalai Tesque Inc. (Kyoto, Japan). *N,N*-Bis(4-sulfobutyl)-3-methylaniline, disodium salt (TODB)
21 was purchased from Dojindo Laboratories (Kumamoto, Japan). Recombinant phospholipase B
22 (PLB₆₈₄) was purified by the method reported previously and used for this work (9). All other
23 chemicals were of the highest or analytical grade.

24 *Bacterial strains, plasmids and culture conditions*– *Streptomyces* sp. NA684 was isolated from a
25 soil sample of Fukushima, Japan (9). Strain NA684 was incubated in 5 mL of 3% (wt/vol) TSB at

28°C for 48 h with shaking (160 strokes per min). One milliliter of the 48-h culture was transferred into a 500-mL flask containing 100 mL of 3% (wt/vol) TSB and then incubated at 28°C for 48 h with shaking (180 rpm).

E. coli HST08 premium competent cells (Takara Bio Inc., Shiga, Japan) were used as a host for molecular cloning. The pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA) and the pMD20-T vector (Takara Bio Inc., Shiga, Japan) were used as cloning vectors. Recombinant *E. coli* cells were cultured on Luria-Bertani (LB) agar plates at 37°C; if necessary, ampicillin, isopropyl- β -D-thiogalactopyranoside and X-Gal were used as supplements in the agar. *Streptomyces lividans* 1326 (NBRC15675) obtained from the NITE Biological Resource Center (Chiba, Japan) was used as a host for expression of PLD₆₈₄. The pUC702 was used as an expression vector (9). Recombinant *S. lividans* cells were cultured in 3% (wt/vol) TSB containing 5 μ g/mL thiostrepton at 28°C for 48 h with shaking (180 rpm).

Purification of PLD₆₈₄— All procedures were performed at 4°C. The culture supernatant was obtained by centrifugation ($18,800 \times g$, 10 min) after 48 h of culturing. The resulting culture supernatant was placed in an ammonium sulfate solution at 80% saturation containing buffer A (20 mM Tris-HCl buffer, pH 8.0). After incubation at 4°C for two hours, the precipitant was collected by centrifugation at $18,800 \times g$ for 10 min and the pelleted material suspended in buffer A. The enzyme sample was centrifuged ($21,800 \times g$, 10 min) to remove insoluble materials. To the obtained supernatant, ammonium sulfate was added to a final concentration of 1 M and this sample was loaded onto a Toyopearl Phenyl-650M column (2.5×3.5 cm) equilibrated with buffer A containing 1 M $(\text{NH}_4)_2\text{SO}_4$. The column was washed with three column volumes (CV) of 1 M $(\text{NH}_4)_2\text{SO}_4$ /buffer A at a flow rate of 10 mL/min (2 cm/min) and the protein was eluted with a linear gradient (10 CV) of 1 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in buffer A at a flow rate of 6 mL/min (1.2 cm/min). The active fractions were pooled and the buffer was exchanged to buffer B (20 mM Tris-HCl, pH 9.0) using Vivaspin 20 10,000 MWCO (Vivaspin 20 10K; Sartorius Co., Inc., Göttingen, Germany) followed by loading onto a

1 Mono Q column (1 mL) equilibrated with buffer B. After column washing (3 CV), the protein was
2 eluted with a linear gradient (20 CV) of 0 to 1 M NaCl in buffer B at 1 mL/min (5 cm/min). The active
3 fractions were pooled, and the buffer was exchanged to the 1 M $(\text{NH}_4)_2\text{SO}_4$ /buffer A using Vivaspin
4 20 10K, followed by loading onto a RESOURCE PHE column (1 mL) equilibrated with 1 M
5 $(\text{NH}_4)_2\text{SO}_4$ /buffer A. After column washing (3 CV), the protein was eluted with a linear gradient (40
6 CV) of 1 to 0.2 M $(\text{NH}_4)_2\text{SO}_4$ in buffer A at 2 mL/min (6 cm/min). The active fractions were pooled
7 and the buffer was exchanged to buffer C (20 mM MES-NaOH, pH 6.0) using Vivaspin 20 10K
8 followed by loading onto a Mono S column (1 mL) equilibrated with buffer C. After column washing
9 (3 CV), the protein was eluted with a linear gradient (25 CV) of 0 to 0.5 M NaCl in buffer C at 1
10 mL/min (5 cm/min). Fractions exhibiting high specific activity were pooled and used for further
11 investigations.

12 *PLD activity assay*— The standard assay mixture (50 μL) containing 50 mM acetate buffer (pH
13 5.6), 0.8 mM LPC and 10% (vol/vol) enzyme solution was incubated at 65°C for 5 min. The enzyme
14 reaction was stopped by heating at 100°C for 5 min. After centrifugation ($21,800 \times g$, 1 min), 200 μL of
15 the colorimetric solution containing 0.03% (wt/vol) 4-aminoantipyrine, 0.02% (wt/vol) TODB, 0.75
16 U/mL COD and 5 U/mL POD was added into the enzyme reaction mixture to determine the
17 concentration of choline released by the enzyme reaction. One unit (U) of enzyme activity was defined
18 as the amount of enzyme that released 1 μmol of choline from phospholipid substrates per min.

19 *Effect of pH and temperature on PLD activity*— Each buffer (acetate buffer, BisTris-HCl,
20 Tris-HCl and glycine-NaOH) was used to investigate the effect of pH on enzyme activity and stability.
21 The optimum pH was examined by incubation at 37°C for 5 min with 0.8 mM LPC in 50 mM of each
22 buffer. The pH stability was assayed by incubating the enzyme at 4°C for 12 h in 45 mM of each
23 buffer solution. The residual enzyme activity was measured by incubating at 60°C for 5 min with 0.8
24 mM LPC in 50 mM acetate buffer, pH 5.0. The optimum temperature was determined by measuring
25 the enzyme activity at each temperature for 5 min with 0.8 mM LPC in 50 mM acetate buffer, pH 5.0.

The thermal stability was determined by incubating the enzyme in 50 mM HEPES-NaOH (pH 7.5) at each temperature for 60 min, and then the residual activity was measured by incubating at 60°C for 5 min with 0.8 mM LPC in 50 mM acetate buffer, pH 5.0. All experiments were carried out three times independently.

Substrate specificity— The substrate specificity of the enzyme toward choline phospholipids (POPC, LPC, PLS-PC and LPLS-PC) was investigated in the presence of various Triton X-100 concentrations. The enzyme activity was assayed by incubation at 37°C for 5 min with 0.8 mM substrates in 50 mM acetate buffer (pH 5.0) containing each concentration of Triton X-100.

The effect of the head group of phospholipids (POPC, POPE, POPG, and PI) on the hydrolytic activity was investigated toward two substrate forms of mixed micelle with Triton X-100 and liposome by HPLC analysis. Multilamellar vesicles of phospholipids were prepared according to hydration method (9). The enzymatic reaction was performed at 37°C for 60 min with 609 ng-protein/mL (2.27 mU) of the purified PLD₆₈₄ (372 U/mg-protein for LPC) and 5 mM mixed micelle substrates containing 15.9 mM Triton X-100 or liposomal substrates in 50 mM acetate buffer (pH 5.0). Residual substrate in the reaction mixture (100 μ l) was extracted by 400 μ l of a chloroform-methanol (2:1, vol/vol) solvent. The chloroform phase was evaporated and the residual materials were dissolved in 20 μ l of the chloroform-methanol (4:1, vol/vol) solvent. Phospholipids in the extracts were separated using an Inertsil NH₂ column (GL Science Co., Tokyo, Japan; 5 μ m, 150 $\times$ 3.0 mm I.D.) and detected at 215 nm by the HPLC system (Hitachi LaChrom Elite, Hitachi Co. Ltd., Tokyo, Japan). Elution was achieved using ethanol, CH₃CN and 10 mM NH₄H₂PO₄ (pH 5.8), (50/40/10, vol/vol). The column was kept at 40°C and the pump was operated at a flow rate of 0.4 mL/min. The hydrolytic rate is exhibited as percentage of substrate hydrolysis by the enzyme reaction. All experiments were carried out three times independently.

Dynamic light scattering analysis— Substrates such as POPC and LPC form a mixed micelle with Triton X-100 or emulsion. The size distribution of the mixed micelle or emulsion substrates was

1 measured with a particle size analyzer (Nano Partica SZ-100Z, Horiba Ltd., Kyoto, Japan), based on
2 dynamic light scattering (DLS) for the reaction mixture. DLS measurements were conducted at 37°C,
3 and the micelle size distributions were analyzed by algorithms equipped with the SZ-100Z instrument.

4 *Steady-state kinetics*– The purified enzyme was used for steady-state kinetic analysis. For POPC
5 hydrolysis, the initial velocity of the enzymatic reaction was determined at each concentration of
6 POPC/Triton X-100 that was constant molar ratio, 1:3. The enzyme concentration was constant at 60.9
7 ng-protein/mL (1.13 nM). The concentration of POPC ([POPC]) was calculated using a molecular
8 weight of 760.07. For LPC hydrolysis, the initial velocity of the enzymatic reaction was determined at
9 each concentration of LPC without Triton X-100. The enzyme concentration was constant at 244
10 ng-protein/mL (4.52 nM). The concentration of LPC ([LPC]) was calculated using a molecular weight
11 of 503.33. The enzyme reaction was performed at 37°C for 5 min in 50 mM acetate buffer (pH 5.0).
12 The kinetic parameters, K_m , V_{max} and k_{cat} , were determined by nonlinear fitting of the data on the initial
13 velocity at different [POPC] and [LPC] (0.426 to 2.13 mM and 0.1 to 1.6 mM, respectively) to a
14 Michaelis-Menten plot (KaleidaGraph, Synergy Software, Reading, PA, USA). The k_{cat} value of
15 PLD₆₈₄ was calculated using a molecular weight of 54,000 as a monomeric protein and one catalytic
16 site. All experiments were carried out three times independently.

17 *Protein analyses*– The protein concentration was determined with a bicinchoninic acid protein
18 assay reagent kit (Thermo Fisher Scientific Inc., Rockford, IL, USA) with BSA as the standard. The
19 molecular weight of the purified PLD₆₈₄ was estimated by size exclusion chromatography and blue
20 native polyacrylamide gel electrophoresis (BN-PAGE; Life Technologies Corporation, Carlsbad, CA,
21 USA) analyses. Size exclusion chromatography was performed using a Superdex 200 10/300 GL
22 column (1.0 × 30 cm; GE Healthcare Japan) at a flow rate of 0.5 mL/min (37.5 cm/h) with 20 mM
23 Tris-HCl (pH 8.0) containing 0.15 M NaCl. The column was calibrated using a standard protein gel
24 filtration calibration kit (GE Healthcare Japan). BN-PAGE and SDS-PAGE analyses were carried out
25 according to the manufacturer's instructions and the Laemmli method (10), respectively. For internal

amino acid sequencing, the proteins excised from the SDS-PAGE gel were digested by trypsin (Sequencing Grade Modified Trypsin, Promega Corporation, Madison, WI, USA) and the obtained peptides were extracted according to the method of Shevchenko *et al.* (11). The extracted peptides were analyzed using a nano ACQUITY UPLC[®] BEH130 C18 column (75 $\mu\text{m} \times 150 \text{ mm}$, 1.7 μm) and a Xevo QTOF MS (Waters Corp., Milford, MA, USA), according to the method reported previously (9).

Cloning of the PLD₆₈₄ gene—Chromosomal DNA of *Streptomyces* sp. NA684 was purified by salting out according to Pospiech and Neumann (12). Two oligonucleotide primers (S1; 5'-cccacccscayctg-3' and A1; 5'-ccasgcsgggtagaggttc-3') were designed and synthesized based on the internal amino acid sequences (ADTPPTPHLD and KNLYPAWLQD) of PLD₆₈₄. The first PCR reaction mixture (50 μL) contained 1 $\times$ PCR buffer for KOD FX Neo, 20 nmol dNTPs, 15 pmol of each primer, 1 U of KOD FX Neo DNA polymerase (Toyobo Co., Ltd., Osaka, Japan) and $\sim 30 \text{ ng}$ of the chromosomal DNA as a template. The two-step PCR was performed with 30 cycles of 98°C, 10 s and 68°C, 1.3 min. The obtained DNA fragment (1.3 kbp) was cloned into the pGEM-T Easy Vector, and the resulting recombinant plasmid was called pPAT. To reveal the complete sequence of the ORF coding PLD₆₈₄, two primers for inverse PCR, the sense primer IS1 (5'-ggggatcacattgaggtgatagagggcgc-3') and the antisense primer IA1 (5'-atctcgcagcaggacatgaacgcgacctgc-3'), were designed based on the partial sequence of the PLD₆₈₄ gene in pPAT. The genomic DNA (2.4 μg) was digested with *Sac* I and self-ligated. The inverse PCR reaction mixture (50 μL) contained 1 $\times$ PCR buffer for KOD FX Neo, 20 nmol dNTPs, 15 pmol of each primer, 1 U of KOD FX Neo DNA polymerase and $\sim 100 \text{ ng}$ of the *Sac* I-digested and self-ligated DNA. The inverse PCR was done with 20 cycles of 98°C, 10 s and 68°C, 4 min. The obtained DNA fragment (3 kbp) was cloned into the pGEM-T Easy Vector, and the resulting recombinant plasmid was called pINV. To clone the complete PLD₆₈₄ gene, two cloning primers were used: the sense primer SE1 (5'-gcgggagccgataccttctg-3') and the antisense primer AN1 (5'-gcgccccgcgccccgtccgc-3'), were

designed based on the sequence of the PLD₆₈₄ gene in pINV. The genomic PCR reaction mixture (50 µL) contained 1× PCR buffer for KOD FX Neo, 20 nmol dNTPs, 15 pmol of each primer, 1 U of KOD FX Neo DNA polymerase and ~30 ng of the chromosomal DNA. The obtained DNA fragment was purified and directly sequenced followed by ligation into pMD20-T vector, and the resulting recombinant plasmid was called pPLD.

Nucleotide and peptide sequence accession number– The nucleotide sequences of the 16S rDNA of *Streptomyces* sp. strain NA684 and the PLD₆₈₄ gene, designated *pld*, were deposited in the DDBJ database under the accession number AB738936 and AB771745, respectively.

Expression and purification of recombinant PLD₆₈₄– *S. lividans* 1326 (NBRC15675) possessing no PLD activity was used as a host for PLD₆₈₄ extracellular production. The PCR was carried out using the following primers: 5'-ctagctagcgcggacaccccgccac-3' (NheIpld) and 5'-gaagatcttcagccggcccggtgc-3' (pIbBgl2), containing the *N*-terminal codon (*Nhe* I, single underline; Ala, italics) and the stop codon (*Bgl* II, single underline; stop codon, italics) of the mature PLD₆₈₄, respectively. The PCR reaction mixture (50 µL) contained: 1× PCR buffer for KOD FX Neo, 20 nmol dNTPs, 15 pmol of each primer, 1 U of KOD FX Neo DNA polymerase and ~30 ng of pPLD as a template. The thermal cycling parameters were 94°C for 2 min, followed by 25 cycles of 98°C for 10 s and 68°C for 1.5 min. The obtained fragment was purified and digested with *Nhe* I and *Bgl* II, and then subcloned into the *Nhe* I and *Bgl* II sites of pUC702 carrying the promoter, signal peptide sequence and the terminator region of PLD from *Streptomyces cinnamomeum* (13). The obtained recombinant plasmid was sequenced and designated as pUC702/pld. The transformation techniques for *S. lividans* followed the methods of Bibb *et al.* (14) and Thompson *et al.* (15). Transformants carrying pUC702/pld were screened using lecithin-emulsified nutrient plates (16). Clones displaying a cloudy halo were collected and the clone exhibiting the highest activity in the 5-mL cultivation was selected. The recombinant PLD₆₈₄ produced by the transformed *S. lividans* was purified from 72-h culture supernatant by ammonium sulfate precipitation (80% saturation), and a Toyopearl Phenyl-650M

column chromatography (2.5×3.0 cm, a linear gradient (16 CV) of 1 to 0 M $(\text{NH}_4)_2\text{SO}_4$ in buffer A, with 10 mL/min) and a RESOURCE Q column chromatography (1 mL, a linear gradient (40 CV) of 0 to 0.2 M NaCl in buffer B, with 2 mL/min).

Homology modeling of PLD₆₈₄— From the sequence information, PLD₆₈₄ has the highly conserved catalytic HKD motifs and shows 73.0% identity to PLD_{PMF} whose structural features have been studied (7). Based on a template (PLD_{PMF}, Protein Data Bank code: 1V0W), the homology model of PLD₆₈₄ was created using HHPRED (<http://toolkit.tuebingen.mpg.de/hhpred>) and Modeller 9.11 (<http://toolkit.tuebingen.mpg.de/modeller>). VERIFY3D (http://nihserver.mbi.ucla.edu/Verify_3D/) was used to assess the reliability of the predicted model and showed that 72.6% of the amino acid residues had an average 3D–1D score of more than 0.2.

Enzymatic measurement of plasmalogen using PLD₆₈₄ and PLB₆₈₄— Initially, phospholipid mixtures containing POPC, LPC, SM and PLS-PC were hydrolyzed by PLB₆₈₄ as follows. The reaction mixture (35 μL) containing 1.14 mM POPC, 1.14 mM LPC, 1.14 mM SM, 0.571 mM PLS-PC, 0.357% (wt/vol) Triton X-100, 28.6 mM Tris-HCl (pH 8.0) and 11.2 U of PLB₆₈₄ (specific activity: 800 U/mg-protein) was incubated at 37°C for 10 min. After heat inactivation (100°C, 5 min), 10 μL of 0.2 M acetate buffer (final 40 mM, pH 5.0) and 2.35 U of PLD₆₈₄ (specific activity: 384 U/mg-protein) were added to the above reaction mixture (total 50 μL), and the PLD reaction was performed at 37°C for 10 min. The concentration of the released choline was determined by the above-mentioned PLD activity assay method.

RESULTS

Purification of PLD₆₈₄— An extracellular PLD₆₈₄ was purified to electrophoretic homogeneity from the 48-h culture supernatant (Figure S1). Table 1 summarizes the purification result of PLD₆₈₄. The purified PLD₆₈₄ with specific activity of 372 U/mg-protein was obtained and the total amount of the purified PLD₆₈₄ was 244 μg from the culture supernatant (265 mL).

Effect of pH and temperature on PLD activity As shown in Fig. 1A and B, the highest enzyme activity for LPC hydrolysis was found at 80°C and pH 5.0 in the absence of Triton X-100. Enzyme activity was maintained between pH 4.0 and 10.5 at 4°C for 12 h (Fig. 1A). The enzyme was stable between 4 and 55°C for 60 min at pH 5.0 (Fig. 1B).

Protein analyses SDS-PAGE analysis showed that the purified enzyme appeared as a single band of 54 kDa (Figure S1). Size exclusion chromatography and native PAGE analyses demonstrated that PLD₆₈₄ behaves as a monomeric protein (data not shown). Twenty-five peptides were determined by LC-MS/MS analysis containing *de novo* sequencing. Two peptide sequences, ADTPPTPHLD and KNLYPAWLQD, were selected to design oligonucleotide primers for PCR.

Substrate specificity The substrate specificity toward choline phospholipids was determined in various Triton X-100 concentrations (Fig. 2 and Table 2). In the presence of 0.05–0.25% (wt/vol) Triton X-100, POPC and PLS-PC were efficiently hydrolyzed, but not at <0.05% (wt/vol). Interestingly, the lysophospholipids, LPC and LPLS-PC, were efficiently hydrolyzed at ≤0.005% (wt/vol) Triton X-100, but not >0.005% (wt/vol). Triton X-100 concentration remarkably affected the hydrolytic activity. Each type of phospholipid substrates had the optimum Triton X-100 concentrations to be hydrolyzed. Conversely, PLD₆₈₄ exhibited no activity toward SM and GPC in any Triton X-100 concentration tested (data not shown). The hydrolytic rate on various diacylglycerophospholipids was determined (Fig. 3). PLD₆₈₄ preferred mixed micelle substrates to liposomal substrates. PLD₆₈₄ exhibited the highest hydrolytic rate toward mixed micelle POPC (98.4% of the hydrolytic rate).

Steady-state kinetics For POPC or LPC as substrates, the enzyme reaction followed normal Michaelis-Menten kinetics (Fig. 4). The apparent K_m ($K_{m(app)}$), V_{max} ($V_{max(app)}$) and k_{cat} ($k_{cat(app)}$) values for POPC and LPC hydrolysis (37°C, pH 5.0) were determined to be 0.271 and 0.0513 mM; 4.44 and 0.270 mmol min⁻¹ mg-protein⁻¹; 4.0×10^3 and 2.43×10^2 s⁻¹, respectively. PLD₆₈₄ showed about 3-fold higher catalytic efficiency ($k_{cat(app)}/K_{m(app)} = 1.48 \times 10^4$ s⁻¹ mM⁻¹) for mixed micelle POPC than that ($k_{cat(app)}/K_{m(app)} = 4.74 \times 10^3$ s⁻¹ mM⁻¹) for emulsified LPC.

1 *Nucleotide sequence of the PLD₆₈₄ gene* The nucleotide sequence of the gene encoding PLD₆₈₄
2 (*pld*) was determined from the sequence of the 1.7 kbp PCR product obtained by the genomic PCR.
3 The ORF of *pld* consisted of 1,617 bp encoding a protein of 538-amino-acid residues and started at
4 ‘ttg’, a rare start codon, which was located, downstream of the stop codon ‘tga’ in the same frame and
5 ribosome binding site, gaagg (Figure S2). SignalP prediction (<http://www.cbs.dtu.dk/services/SignalP/>)
6 showed that the *N*-terminal amino acid of the active form of PLD₆₈₄ certainly starts at Ala1 of the
7 deduced amino acid sequence, indicating that the preceding 30-amino-acid residues represent a Sec
8 signal peptide sequence required for secretion (Figure S2). Homology searches performed with the
9 BLAST algorithm indicated that the amino acid sequence of the mature PLD₆₈₄ shows 89% identity to
10 that of the PLD of *S. antibioticus* (SaPLD; UniProt accession no. Q53728) (2).

11 *Expression, purification and characterization of PLD₆₈₄* High efficiency extracellular
12 production of PLD₆₈₄ was successfully achieved in *S. lividans* cells transformed with the expression
13 vector pUC702/*pld*. The specific PLD activity in the culture supernatant (9.6 U/mg-protein) was
14 4.8-fold higher than that (2.0 U/mg-protein) of the wild-type strain. High specific activity (384
15 U/mg-protein) and a large amount (4.89 mg-protein) of recombinant PLD₆₈₄ was purified to
16 electrophoretic homogeneity from 265 mL of the culture supernatant by simple purification steps
17 (Table 3).

18 *Enzymatic measurement of plasmalogen in phospholipid mixture* As shown in Fig. 5, the
19 choline concentration related to PLS-PC concentration ([PLS-PC]) in the reaction mixture was
20 detected, and two calibration curves were linear between 0–0.4 mM PLS-PC in the presence and in the
21 absence of phospholipid mixture and their regression lines were defined by equations $y = 0.00231x +$
22 0.169 ($R^2 = 1$) and $y = 0.00200x + 0.0830$ ($R^2 = 0.992$), respectively. The calibration curve may be
23 suitable for the crude sample like the blood sample and the sensitivity of detection is likely suitable to
24 detect PLS-PC in plasma, whose concentration has been reported to be 50–100 μ M by a HPLC
25 method using 125 I (17). This enzymatic method can easily measure plasmalogen and is able to screen

blood samples in high-throughput.

DISCUSSION

The result of enzyme purification shows that PLD₆₈₄ accounts for high ratio contents of total protein in the culture supernatant. *Streptomyces* sp. NA684 is a good producer of an enzymatically active PLD. Furthermore, we have successfully achieved the efficient extracellular production of PLD₆₈₄ using *S. lividans* cells. The production of PLD₆₈₄ reached 16.3 mg/L-culture.

For the hydrolytic activity of LPC, the optimum pH of PLD₆₈₄ is pH 5.0 and this pH optimum matches those of other *Streptomyces* PLDs (pH 5.5–6.0) (3, 18, 19). The observed maximum temperature of enzyme activity (~80°C) is much higher than that of other *Streptomyces* PLDs (~60°C) investigated using PC/Triton X-100 mixed micelle (3, 18, 20). LPC was emulsified without Triton X-100 and could maintain emulsion state. However, Triton X-100 has a cloud point of 63–69°C, in the mixed micelle solution, the substrate state must be affected by temperature above the cloud point. Thus, under our experimental conditions, the cloud point of Triton X-100 has no relation to the enzyme activity. It was reported that PLDs from *Streptomyces tendae* and *Streptomyces olivochromogenes* were stable at 70 and 75°C, respectively (20, 21). On the other hand, PLD₆₈₄ is stable between pH 4.1 and 10.5 for 12 h at 4°C, and pH 7.5 for 1 h at 55°C; however, incubation at pH 7.5 and 65°C for 1 h decreases the relative activity to <1%, demonstrating that the enzyme is relatively unstable to heat.

Substrate specificities of phospholipase Cs toward liposomal substrates have been reported previously (22, 23). On the other hand, the enzyme activity of *Streptomyces* phospholipases is generally stimulated by Triton X-100 (9, 19–21, 24–27). For example, phospholipase B activity is elevated in the presence of Triton X-100 and the highest activity was shown at 0.75% (wt/vol) Triton X-100 (9). PLD₆₈₄ was also activated by Triton X-100 and showed maximal hydrolytic activity toward POPC in the presence of 0.15% (wt/vol) Triton X-100, and the molar ratio of POPC and Triton X-100

1 was 1:3 (Fig. 2). As shown in Figure S3, PLD₆₈₄ seems to prefer 10-55 nm of the POPC/Triton X-100
 2 mixed micelle diameter and the shape of the mixed micelle is probably oblate ellipsoid or sphere (28).
 3 We previously reported that sphingomyelinase C from *Streptomyces griseocarneus* showed high
 4 activity toward 27.6-31.6 nm of SM/Triton X-100 mixed micelle in 0.5 to 1.5 M MgCl₂ (29). These
 5 results suggest that the micelle size is probably an important factor on the substrate recognition by
 6 streptomycete phospholipases. As shown in Fig. 3, the results describing the hydrolytic rates of
 7 diacylphospholipids demonstrate that PLD₆₈₄ prefers mixed micelle substrates to liposomal substrates
 8 for hydrolysis reaction. However, Uesugi *et al.* reported that dissociate constant (K_D) value of
 9 TH-2PLD (73% identity to PLD₆₈₄) for liposomal POPC was 5.3 ± 0.9 nM, suggesting that the enzyme
 10 has high affinity for liposomal POPC (30). Therefore, PLD₆₈₄ would also have affinity toward
 11 liposomal POPC; however, the step after forming an enzyme-liposome complex would be rate-limiting.
 12 On the other hand, Hirano *et al.* reported that the V_{max} on PC/glycerol monooleate mixed liposome
 13 hydrolysis for *Streptomyces* sp. PLD (containing HKD motif, Sigma-Aldrich Japan Co.) was enhanced
 14 20-fold by glycerol monooleate in a glycerol monooleate content-dependent manner, suggesting the
 15 enhancement of the PLD-PC (ES) complex formation on a membrane (31). In the present study,
 16 PLD₆₈₄ efficiently hydrolyzed diacylphospholipid/Triton X-100 mixed micelle, suggesting
 17 PLD₆₈₄-POPC (ES) complex formation was also enhanced by Triton X-100, and the hydrolysis
 18 reaction was following the surface binding model (32). In the surface binding model, a soluble enzyme
 19 (E) first associates with mixed micelle POPC (M) to form an enzyme-mixed micelle complex (EM,
 20 bulk step). Conversely, for emulsified LPC micelle hydrolysis, the enzyme specifically binds to a
 21 surface LPC molecule in the phospholipid binding model. In both cases, in a subsequent step (surface
 22 step), the enzyme associated with the micelle scoots on the micelle surface and then binds a
 23 phospholipid substrate molecule in its catalytic site and subsequently hydrolyzes a phospholipid
 24 substrate molecule.

25 The catalytic mechanism of PLD has been studied; it is well-known that two histidine residues

1 of HKD motifs act as catalytic residues (1). Thus, POPC and LPC are catalytically hydrolyzed by the
2 two histidine residues through the same catalytic mechanism after forming ES complexes. However,
3 PLD generally prefers aggregated substrates, such as mixed micelle, emulsion, and liposome, to free
4 ones (8). Therefore, it's very important to investigate the sequential catalytic reaction step that PLD
5 forms EM or EL complex and ES complex as shown in Fig. 6; nevertheless, the affinity between PLD
6 and mixed micelle or emulsified phospholipid and the ES forming rate have not been investigated in
7 detail. And also there is no report on the rate-limiting step. In this study, we have determined $K_{m(app)}$
8 and $k_{cat(app)}$ values by steady-state kinetic analysis. We assumed that the $k_{cat(app)}$ values exhibited whole
9 velocity on the surface step. As compared with kinetics parameters ($K_{m(app)} = 0.271$ mM, $k_{cat(app)} = 4.0 \times$
10 10^3 s⁻¹) for mixed micelle POPC hydrolysis, those ($K_{m(app)} = 0.0513$ mM, $k_{cat(app)} = 2.43 \times 10^2$ s⁻¹) for
11 emulsified LPC hydrolysis were lower, demonstrating that PLD₆₈₄ possesses a higher affinity and
12 much lower turnover number toward emulsified LPC than mixed micelle POPC. From these results,
13 the rate-limiting step for POPC/Triton X-100 mixed micelle hydrolysis would be the bulk step;
14 whereas, the surface step would be rate-limiting for emulsified LPC hydrolysis (Fig. 6). In addition,
15 PLD₆₈₄ recognizes mixed micelle POPC as the surface binding model and emulsified LPC as the
16 phospholipid binding model. Accordingly, we conclude that PLD₆₈₄ recognizes mixed micelle POPC
17 and emulsified LPC by distinct mechanism. Since POPC and LPC have considerably different
18 physical properties such as micelle conditions even in nature, so that PLD₆₈₄ may exhibit at least two
19 substrate recognition mechanisms to recognize phospholipid substrates. This approach would be
20 helpful to investigate affinities and ES forming rate toward various substrate forms.

21 The signal peptide of PLD₆₈₄ had a typical Sec signal sequence (a basic amino-terminus, a
22 central apolar core, and a carboxy-terminal region containing the signal peptidase recognition site),
23 suggesting PLD₆₈₄ should be secreted via the secretory pathway (Sec-system secretion) (33). In
24 addition, there is no R-R-x-φ-φ 'twin-arginine' amino acid motif required for secretion via the
25 translation pathway (34, 35). Most *Streptomyces* phospholipases are secreted via the Sec-system

pathway (2, 3, 9, 24), except phospholipase Cs and C-type enzymes secreted via the Tat-system pathway (36-38).

It is known that PLDs from yeasts, plants and mammals have also two HxKxxxxD motifs (1, 8). PLD₆₈₄ also contained two HxKxxxxD motifs (¹⁶⁸HSKLLVVD¹⁷⁵ and ⁴⁴¹HHKLVSVD⁴⁴⁸). These conserved residues have been suggested to form the catalytic site involved in the hydrolysis of a phosphoester bond and the transphosphatidylation (1, 8). The deduced amino acid sequence of PLD₆₈₄ shows 89% identity to that of SaPLD and relatively high identity (>70%) to those of other *Streptomyces* PLDs (2, 3, 7, 39, 40). Moreover, homology modeling study demonstrated that the structure of PLD₆₈₄ was closely resembled to that of PLD_{PMF} (Figure S4). As regard to protein sharing >30% sequence identity, it is well-known that the structure and function are similar to each other. In fact, the pH profile of PLD₆₈₄ was similar to those of other PLDs. Therefore, we think the substrate recognition mechanism would be conserved, so that those known PLDs may prefer mixed micelle POPC/Triton X-100 and emulsified LPC like PLD₆₈₄. Unfortunately, the substrate recognition mechanisms of other PLDs have not been reported.

Recently, PLD attracts attention as a diagnostic agent enzyme. For example, Morita *et al.* reported the phosphatidylserine determination method using PLD from *Streptomyces chromofuscus* (41). Moreover, it has been reported that plasmalogen level is correlated with dementia and cancer, so that plasmalogen can be a biomarker for them (42). At present, plasmalogen concentration in a blood sample can be determined by the ¹²⁵I-HPLC method and LC-MS analysis; however, these methods need complicated procedures and extensive analysis time. Thus, new method that can easily and rapidly determine plasmalogen is required for development of the diagnostic agent. We have achieved the measurement of PLS-PC in phospholipid mixtures by the following enzymatic method. First, PLB₆₈₄ hydrolyzed POPC and LPC to *sn*-glycero-3-phosphocholine and fatty acids, and then choline was released from the residual PLS-PC by PLD₆₈₄. The concentration of the released choline was determined by the conventional colorimetric assay using COD and POD. Although the linear

1 calibration curves (in the presence and the absence of POPC, LPC and SM) for PLS-PC were obtained
2 by the enzymatic method, y-intercept of the curve in the presence of phospholipid mixture was
3 significantly higher than that of in the absence of one. However, both curves showed the almost same
4 slopes and A_{550} values corresponding to [PLS-PC]. As POPC hydrolysis by PLB₆₈₄ was certainly
5 incomplete, extra choline would be released from residual POPC by PLD₆₈₄. However, we believe that
6 the two calibration curves may be useful for the determination of [PLS-PC] even in the crude sample.
7 Our enzymatic method without ^{125}I and expensive analyzers can be applied to high-throughput
8 screening for the diseases concerned with PLS-PC. This is the first report describing the enzymatic
9 measurement of PLS-PC and this method can easily assay plasmalogen as a biomarker for dementia
10 and cancer, and may contribute to early detection of these diseases.

11

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FOOTNOTE

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FIGURE LEGENDS

Figure 1. Effect of pH and temperature on LPC hydrolytic activity and stability. (A) The solid line indicated the enzyme activity assayed at 37°C for 5 min with 0.8 mM LPC in 50 mM of each buffer. The buffers were: acetate buffer (pH 4.1–5.6; open circles), bisTris-HCl (pH 5.6–7.2; open squares) and Tris-HCl (pH 7.2–8.8; open triangles). The dotted line indicated the residual activity after incubating at 4°C for 12 h in 45 mM of each buffer: acetate buffer (pH 4.1–5.6; closed circles), bisTris-HCl (pH 5.6–7.2; closed squares), Tris-HCl (pH 7.2–8.8; closed triangles), and glycine-NaOH (pH 8.8–10.5; closed diamonds). The residual activity was assayed at 60°C (below cloud point of Triton X-100) for 5 min with 0.8 mM LPC in 50 mM acetate buffer (pH 5.0). (B) The solid line indicated the enzyme activity assayed at each temperature in 50 mM acetate buffer (pH 5.0). The dotted line indicated the residual activity after incubating at each temperature for 60 min in 50 mM HEPES-NaOH (pH 7.5). The residual activity was assayed at 60°C for 5 min with 0.8 mM LPC in 50 mM acetate buffer (pH 5.0). The experiments were performed in triplicate and the data presented represent the means $\pm$ the standard deviations.

Figure 2. Effect of Triton X-100 concentration in the reaction mixture on the enzyme activity. The enzyme activity of PLD₆₈₄ was assayed by incubation with 0.8 mM substrate (closed circles, POPC; open circles, LPC; closed triangles, PLS-PC; open triangles, LPLS-PC) at 37°C for 5 min in 50 mM

1 acetate buffer (pH 5.0) containing each percentage (wt/vol) of Triton X-100. The experiments were
2 performed in triplicate and data represent the means $\pm$ standard deviations.

3
4 **Figure 3.** Liposomal and mixed micellar phospholipids hydrolytic rates by the catalytic reaction of
5 PLD₆₈₄. The enzyme reaction was carried out by incubation at 37°C for 60 min with 5 mM of the
6 mixed micelle substrate containing 15.9 mM Triton X-100 or the liposomal substrate in 50 mM acetate
7 buffer (pH 5.0). The hydrolytic rates of liposomal substrate and micellar substrate presented represent
8 open and oblique bar, respectively.

9
10 **Figure 4.** Michaelis-Menten plots of the steady-state kinetics of PLD₆₈₄ activity. The initial velocity of
11 POPC (A) and LPC (B) hydrolysis by the purified PLD₆₈₄ was determined by incubation at 37°C for 5
12 min with various substrate concentrations in 50 mM acetate buffer (pH 5.0) containing 3:1 molar ratio
13 of Triton X-100 to POPC.

14
15 **Figure 5.** The calibration curve of PLS-PC using an established enzymatic method. After the
16 phospholipase B reaction, the concentration of PLS-PC in the presence (open circles) and in the
17 absence (open triangles) of POPC, LPC and SM was determined using recombinant PLD₆₈₄.

18
19 **Figure 6.** Illustration describing the substrate recognition by PLD₆₈₄. Surface binding model and
20 phospholipid binding model are shown: M, mixed micelle POPC/Triton X-100; and L, emulsified LPC.
21 Phospholipid molecules are depicted in yellow, Triton X-100 molecules in black and brown, and the
22 enzyme in blue. E, PLD₆₈₄; S, substrate; P, product

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

A) Surface binding model

B) Phospholipid binding model

Table 1. Purification of PLD₆₈₄ from *Streptomyces* sp. NA684

Purification step	Activity ^a (U/ml)	Sample (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Fold
48-h culture supernatant	1.44	265	0.711	189	2.0	382	100	1.0
80% ammonium sulfate	9.51	37	4.59	170	2.1	352	92.2	1.0
Phenyl-650M	2.07	108	0.517	55.8	4.0	224	58.6	2.0
Mono Q	57.7	3.5	0.985	3.45	58.6	202	52.9	28.9
RESOURCE PHE	26.6	7.0	0.0864	0.605	308	186	48.7	152
Mono S	45.3	2.0	0.122	0.244	372	91	23.7	184

^a PLD activity was assayed at 65°C using the reaction mixture containing 50 mM acetate buffer (pH 5.6) and 0.8 mM LPC.

Table 2. Substrate specificity toward various choline-type phospholipids.

Substrate	Triton X-100 ^a (mM)	Molar ratio ^b	Specific activity ^c (U/mg)	Relative activity ^d (%)
POPC	2.39	1 : 3	2254	100
LPC	0.0398	20 : 1	219	9.7
PLS-PC	3.18	1 : 4	878	39.0
LPLS-PC	0.0398	20 : 1	58.0	2.6

^a The concentration of Triton X-100 for maximal PLD activity. ^b Molar ratio of substrate to Triton X-100.

^c Specific PLD activity was assayed at 37°C using the reaction mixture containing 50 mM acetate buffer (pH 5.0), 0.8 mM substrate, and each optimum concentration of Triton X-100. ^d Relative activity was defined the activity for POPC as 100% at the maximal condition (pH 5.0, 37°C).

Table 3. Purification of the recombinant PLD₆₈₄ produced by *S. lividans*

Purification step	Activity ^a (U/ml)	Sample (ml)	Protein (mg/ml)	Total protein (mg)	Specific activity (U/mg)	Total activity (U)	Yield (%)	Fold
72-h culture supernatant	11.7	265	1.22	324	9.6	3094	100	1.0
80% ammonium sulfate	140	21	5.24	110	26.7	2937	94.9	2.8
Phenyl-650M	24.3	105	0.294	30.8	82.8	2555	82.6	8.7
RESOURCE Q	469	4.0	1.22	4.89	384	1877	60.6	40.2

^a PLD activity was assayed at 65°C using the reaction mixture containing 50 mM acetate buffer (pH 5.6) and 0.8 mM LPC.