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Molecular Cloning of the Phospholipase D Gene from *Streptomyces* sp. YU100 and Its Expression in *Escherichia coli*

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The gene for phospholipase D (PLD) of *Streptomyces* sp. YU100 was cloned from λ phage library and heterologously expressed in *Escherichia coli*. Using an amplified gene fragment based on the consensus sequences of streptomycetes PLDs, λ phage library of *Streptomyces* sp. YU100 chromosomal DNA was screened. The sequencing result of *Bam*HI-digested 3.8 kb fragment in a positive phage clone revealed the presence of an open reading frame of a full sequence of PLD gene encoding a 540-amino acid protein including 33-amino acid signal peptide. The deduced amino acid sequence showed a high homology with other *Streptomyces* PLDs, having the highly conserved 'HKD' motifs. The PLD gene excluding signal peptide sequence was amplified and subcloned into a pET-32b(+) expression vector in *E. coli* BL21(DE3). The recombinant PLD was purified by nickel affinity chromatography and compared the enzyme activity with wild-type PLD. The results imply that the recombinant PLD produced by *E. coli* had the nearly same enzyme activity as PLD from *Streptomyces* sp. YU100.

***Keywords*:** phospholipase D, *Streptomyces*, gene cloning, heterologous expression

The phospholipase D (PLD) catalyzes two reactions: one is the hydrolysis of lecithin (phosphatidylcholine, PC) to phosphatidic acid and choline (Bian and Roberts, 1992), and the other is the transphosphatidylation of lecithin to functional phospholipids by interconversion of polar headgroups with serine, ethanolamine or glycerol (Dawson, 1967; Ulbrich-Hofmann, 2000). Thus PLD is an industrially important enzyme for the production of functional phospholipids including phosphatidylserine (PS), phosphatidylethanolamine, and phosphatidylinositol from lecithin, a byproduct in soy-oil productions (Ousley and Morell, 1992).

Those functional phospholipids have some benefits for age-associated memory and mood enhancement from stress due to structural and functional changes in the lipid composition of the neuronal membranes in the human brain (Rapallino *et al*., 1990; Crook *et al*., 1991; Brambilla *et al*., 1996). In particular, significant improvements have been reported in the brain functions of elderly patients with Alzheimer's disease (Amaducci, 1988), Parkinson's disease (Toffano and Battistin, 1980), dementia (Delwaide *et al*., 1986), epilepsy (Loeb *et al*., 1987), and geriatric depression (Maggioni *et al*., 1990).

PLD is the widely distributed in animals, plants, and microorganisms (Morris *et al*., 1996). Even though cabbage PLD is the most abundant in nature, yet it shows a very low transphosphatidylation activity (Wang, 2000). In contrast, microbial PLD, especially from *Streptomyces* sp., is known to have a higher transphosphatidylation activity than those enzymes from other organisms (Imamura and Horiuti, 1979; Yu *et al.*, 1996). Recently, several *Streptomyces* species have been investigated and/or cloned for their industrial application in the production of functional phospholipids (Okawa and Yamaguchi, 1975; Imamura and Horiuti, 1979; Iwasaki *et al*., 1994; Ogino *et al*., 1999; Hatanaka *et al*., 2002a; Moon *et al.*, 2006). As a result, it has been known that PLD from *S. antibioticus*, *S. cinnamoneus*, *S. halstedii*, *S. septatus*, and *Streptomyces* sp. PMF strain show significant sequence similarity and similar enzymatic properties including high transphosphatidylation activity, relatively high optimum reaction temperatures, and Ca^{2+}-independent activity (Juneja *et al*., 1988; Hatanaka *et al*., 2002b).

Previously, *Streptomyces* sp. YU100 having a high transphosphatidylation activity was screened from Korean soil sample, and its PLD was purified and characterized by our research group (Lim *et al*., 2002a; Lim *et al*., 2002b). In this paper, cloning of PLD gene from this strain and its heterologous expression in *E. coli* BL21(DE3) are reported.

Materials and Methods

Bacterial strains, plasmids, and culture conditions

Streptomyces sp. YU100 was grown on tryptic soy broth (TSB) medium, and cultured at 28°C for 72 h on a shaking incubator at 200 rpm.

E. coli BL21(DE3) and pET-32b(+) were purchased from Novagen (USA). *E. coli* strains were grown in Luria-Bertani (LB) medium (1% tryptone, 0.5% yeast extract, 1% NaCl) at 37°C. For selection of clones ampicillin was added in a final concentration of 50 μg/ml to the medium. *E. coli* ER1647 (Novagen, USA) was grown in LB supplemented with 0.2% (w/v) maltose and 10 mM $MgSO_4$ for the propagation of λ phage.

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Preparation of genomic DNA and gene amplification
Genomic DNA was prepared from *Streptomyces* sp. YU100 using cetyltrimethylammonium bromide (CTAB) method (Kieser *et al*., 2000) with slight modification. For the preparation of PLD probe, synthetic oligonucleotides were designed at the conserved region in several streptomycetes PLD genes: forward primer PLD-F; 5'-AAGGA CGACTAC CTCGACAC-3' and reverse primer PLD-R; 5'-TTGTTGG AGATCTG GATGTG-3'. PCR for gene amplification was performed in a GeneAmp PCR System 2400 thermal cycler (Perkin-Elmer, USA) in 50 μl reaction mixtures containing 20 ng/ml genomic DNA as the template, 0.5 pmole/μl primers, 0.1 mM dNTPs, 0.02 U/μl *EF-Taq* DNA polymerase (Solgent, Korea) and 1× *EF-Taq* DNA polymerase reaction buffer. PCR conditions were denaturation: 94°C for 5 min; 35 cycles of amplification: 94°C for 45 sec, 55~60°C for 45 sec and 72°C for 50~70 sec; additional elongation: 72°C for 7 min. The amplified PLD fragment was cloned into T&A vector (RBC, Taiwan).

In order to express PLD gene in *E. coli* host cells, PLD gene excluding signal peptide sequence was amplified using forward primers PLD-S; 5'-CCGCATCCATGGACACGGG GGCCACCCCGGC-3' having *Nco*I site and reverse primer PLD-A; 5'-CAATCACTCGAGGTCCTGGCAGATTCCGC GTT-3' having *Xho*I site. The amplified gene by the same procedure as above was subcloned into pET-32b(+) vector.

Construction and screening of the genomic library of *Streptomyces* sp. YU100
Genomic DNA of *Streptomyces* sp. YU100 was partially digested with *Bam*HI and size-fractionated on an agarose gel. DNA fragments with size ranging from 7 to 25 kb were isolated from the gel, ligated to *Bam*HI-digested λBlueSTAR arms (Novagen, USA), packaged into phages using packaging extracts (PhageMaker System, Novagen), and transfected into *E. coli* ER1647. The recombinant phage library (approximately 6×10^3 plaques) was screened by plaque hybridization using the amplified PLD fragment which was labeled with [α-^{32}P]dCTP by the DecaLabel DNA labeling kit (Fermentas, USA). Firstly, the phage plaques were transferred to Nytran N nylon membranes (Schleicher & Schuell, Germany) and processed for hybridization. The hybridization was done at 42°C for 10 h, washed with 0.5× SSC (0.15 M NaCl and 15 mM sodium citrate) and 0.1% SDS at 60°C for 10 min followed by 0.1× SSC and 0.1% SDS, and autoradiographed. Phage DNA of the positive clone was purified using the lambda mini kit (QIAGEN, Germany), digested with *Bam*HI, *Not*I or *Sac*II and subjected to Southern hybridization using the same PLD probe.

Analysis of *Streptomyces* PLD gene
The phage DNA from a positive phage clone was digested with *Bam*HI, and subjected to Southern hybridization for the confirmation of a positive fragment, The *Bam*HI-digested fragment giving a positive signal was subcloned into pGEM-3Zf(+) vector and the nucleotide sequence determination was entrusted to Genotech (Korea). Open reading frame (ORF) and deduced protein sequence was determined by BLAST (Altschul *et al*., 1990) and ExPasy translate tool, respectively. Multiple alignments were performed using CLUSTAL W program (Thompson *et al*., 1994) and signal peptide analysis was done by SignalP 3.0.

Heterologous expression and purification of recombinant PLD
PCR-amplified PLD gene (1.5 kb) for heterologous expression was double digested with *Nco*I and *Xho*I, ligated to pET-32b(+) digested by the same enzyme, and transformed into *E. coli* BL21(DE3). A single colony of *E. coli* BL21 (DE3) cells harboring the plasmid pET-PLD was grown at 37°C with shaking in 5 ml of LB medium containing 50 μg/ml ampicillin overnight. An aliquot (2 ml) of this culture was inoculated into 200 ml of the same medium and grown at 32°C with shaking. When cells reached an OD_{600} of 0.5~0.6, expression was induced by adding isopropyl-β-*D*-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. After growth was continued for another 4 h at 25°C, cells were harvested by centrifugation at 8,000×g for 10 min at 4°C. The cell pellet was lysed by incubation in 20 ml lysis buffer (20 mM Tris-HCl; pH 8.0, 0.5 M NaCl, 0.3% Sarcosyl, 0.3% Nonidet P-40, 0.4 mg/ml lysozyme) on ice for 1 h, and sonicated on ice for 15 min using Ultrasonic processor (Heat Systems, USA). The suspension was centrifuged at 13,000×g for 20 min and the supernatant representing the soluble cell fraction was collected.

The soluble cell fraction was dialyzed against a 100-fold volume of binding buffer (20 mM Tris-HCl; pH 8.0, 0.3 M NaCl, 5 mM imidazole) for 4 h at 4°C and applied onto 5 ml Ni^{2+}-nitrilotriacetic acid (Ni-NTA) agarose (Peptron, Korea) equilibrated with binding buffer. The column was washed with 30 ml of the same buffer until the UV absorption returned to the baseline. Then the recombinant PLD protein was washed with 50 ml of washing buffer (20 mM Tris-HCl; pH 8.0, 0.3 M NaCl, 50 mM imidazole) and eluted with 20 ml of elution buffer (20 mM Tris-HCl; pH 8.0, 0.3 M NaCl, 150 mM imidazole). The eluted fractions were pooled, dialyzed against water, frozen at -70°C, and lyophilized. Protein concentration was determined by the Bradford method (Bradford, 1976).

Electrophoresis and Western hybridization
The protein samples were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE). For Western hybridization, the separated proteins on gel were transferred to Protran nitrocellulose membrane (Schleicher & Schuell, Germany) at 200 mA for 1 h. The membrane was blocked with 5% skim milk in TBST buffer (20 mM Tris-HCl, 140 mM NaCl, 0.1% Tween 20, pH 7.6) for 1 h at room temperature. The membranes were immunoblotted with anti-(His tag) mouse monoclonal antibody (IG Therapy, Korea) for 2 h. After washing 3 times with TBST, the membrane was incubated with anti-(mouse IgG) antibody conjugated with horseradish peroxidase (HRP) as secondary antibody (Santa Cruz Biotechnology, USA) for 1 h. After three successive washing with TBST, His-tagged recombinant protein was exposed to X-ray film (Kodak, USA) using a chemiluminescence assay kit (Pierce, USA).

Transphosphatidylation activity assay
PLD samples were mixed with 5 mM phosphatidylcholine

(PC) and 20 mM *L*-serine in a reaction buffer (0.1 mM $CaCl_2$, n-butyl acetate saturated with 0.1 M sodium citrate buffer, pH 6.0) and incubated at 35°C, 150 rpm for 24 h. Transphosphatidylation activity was assayed by HPLC using Shimadzu LC-10AT gradient-controlled HPLC system equipped with Shimadzu photodiode array detector (Model SPD-M10A) and dual channel UV detection at 205 nm. Reaction mixture was eluted with a 5 μm XTetra® Silica gel 60 column (4.6×250 mm, Waters, USA) using solvent A (isopropanol/hexane/water/14.8 M NH_4OH, 57.8: 40: 2: 0.2, by vol.) and solvent B (isopropanol/hexane/water/14.8 M NH_4OH, 51.8: 40: 8: 0.2, by vol.). The elution profile was as follows: 0 min, B=0%; 0-10 min, B increased to 100%; 10~22 min, B= 100%; 22~25 min, decreased to 0%.

Hydrolytic activity assay

The hydrolytic activity of PLD was measured using Amplex red assay system (Molecular Probes, USA). In this system choline hydrolyzed from PC is oxidized in the presence of choline oxidase with liberating H_2O_2, which reacted with 10-acetyl-3,7-dihydrophenoxazine (Amplex red reagent) by horse radish peroxidase (HRP) to give fluorescence. Briefly, PLD samples were prepared with Amplex red reagent (25 μM 10-acetyl-3,7-dihydrophenoxazine, 0.5 U/ml HRP, 0.05 U/ml choline oxidase, 0.125 mM PC) in the reaction buffer (50 mM Tris, 5 mM $CaCl_2$, pH 8.0) and incubated at 37°C for 1 h. Fluorescence intensity was measured with a fluorescence microplate reader (FLUOstar Optima, BMG Labtechnologies, Germany) using excitation at 540 nm and detection at 590 nm.

Results

Cloning of PLD gene from *Streptomyces* sp. YU100 chromosomal DNA

PLD gene fragment was amplified from *Streptomyces* sp. YU100 chromosomal DNA by using primers for the conserved sequences of streptomycetes PLD genes. The sequencing result of the amplified 440-bp DNA fragment showed a high homology with other streptomycetes PLD genes.

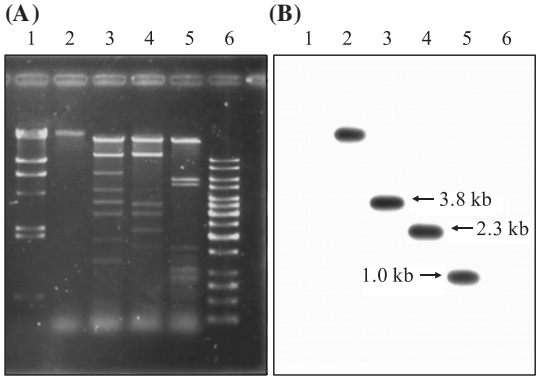

Fig. 1. Confirmation of a positive plaque by Southern hybridization with PLD probe. A positive λ phage DNA was purified by QIAGEN lambda mini kit, digested with *Bam*HI, *Not*I, and *Sac*II, and run on the agarose gel. (A) agarose electrophoretic pattern, (B) Southern hybridization with PLD probe. Lanes: λ DNA/*Hin*dIII marker; 2, a positive λ phage DNA from positive plaque; 3, a positive λ DNA/*Bam*HI; 4, a positive λ DNA/*Not*I; 5, a positive λ DNA/*Sac*II; 6, 1 kb DNA ladder.

A λ phage library of *Streptomyces* sp. YU100 chromosomal DNA was constructed by inserting chromosomal DNA partially digested with *Bam*HI and size-fractionated (7~25 kb), into λBlueSTAR arms. After *in vitro* packaging, the library was transfected into *E. coli* ER1647, and the phage library was screened with ^{32}P-labeled PLD gene fragment. One plaque giving strong signal was found and the inserted PLD gene was confirmed by PCR amplification and Southern blotting. In Southern analysis, *Bam*HI-, *Not*I- and *Sac*II-digested phage DNA gave strong signals in 3.8, 2.3, and 1.0 kb fragments bound with PLD probe, respectively (Fig. 1). Three of them, 3.8 kb fragment of *Bam*HI-digested DNA was subcloned in pGEM-3Zf(+) vector and transformed into *E. coli* JM109.

Sequencing analysis

The nucleotide sequence of insert DNA in pGEM-3Zf(+) vector was determined. Among 3,709 nucleotides determined, one ORF was found by BLAST ORF finder. The deduced amino acid sequence of ORF was compared by multiple alignment with those of other streptomycetes PLDs using CLUSTAL W program (Fig. 2). The comparison of *Streptomyces* PLD proteins showed around 70~75% homology with each other (Table 1). Among them, PLD of *Streptomyces* sp. YU100 showed the highest homology with that of *S. septatus*. For the extracellular secretion, the signal peptide was deduced using SignalP 3.0 program. The cleavage site by membrane secretory system was presumed to be between 33rd and 34th amino acids (ASA-DT). The finally secreted PLD may be comprised of 507 amino acids, to give the molecular weight of 54 kDa and the pI value of 5.8. In matured PLD protein of this strain, two copies of the HxKxxxxD (HKD) motif was also found, which is conserved in all members of PLD superfamily.

The sequence of the PLD gene from *Streptomyces* sp. YU100 was submitted to GenBank under the accession number of EU369918.

Heterologous expression in *E. coli* and enzyme purification

The whole PLD ORF excluding signal peptide was amplified for heterologous expression in *E. coli*. A forward primer having *Nco*I site and a reverse primer having *Xho*I site were employed for gene amplification, and the amplified gene in size of 1.5 kb was inserted into pET-32b(+) vector for the expression of a His-tag fused protein. The plasmid DNA from positive transformants was confirmed by restriction digestion (Fig. 3). The selected clone of pET-PLD plasmid was cultured in LB broth supplemented with 50 μg/ml ampicillin, incubated at 32°C until the OD_{600} reached 0.6 and agitated further for additional 4 h at 25°C in the presence of 0.2 mM IPTG. The His-tagged-PLD protein was well overexpressed with a molecular mass of ~70 kDa in SDS-PAGE and Western hybridization analysis (Fig. 4).

The recovered cell pellets were disrupted by ultrasonication and then soluble fraction was separated by centrifugation

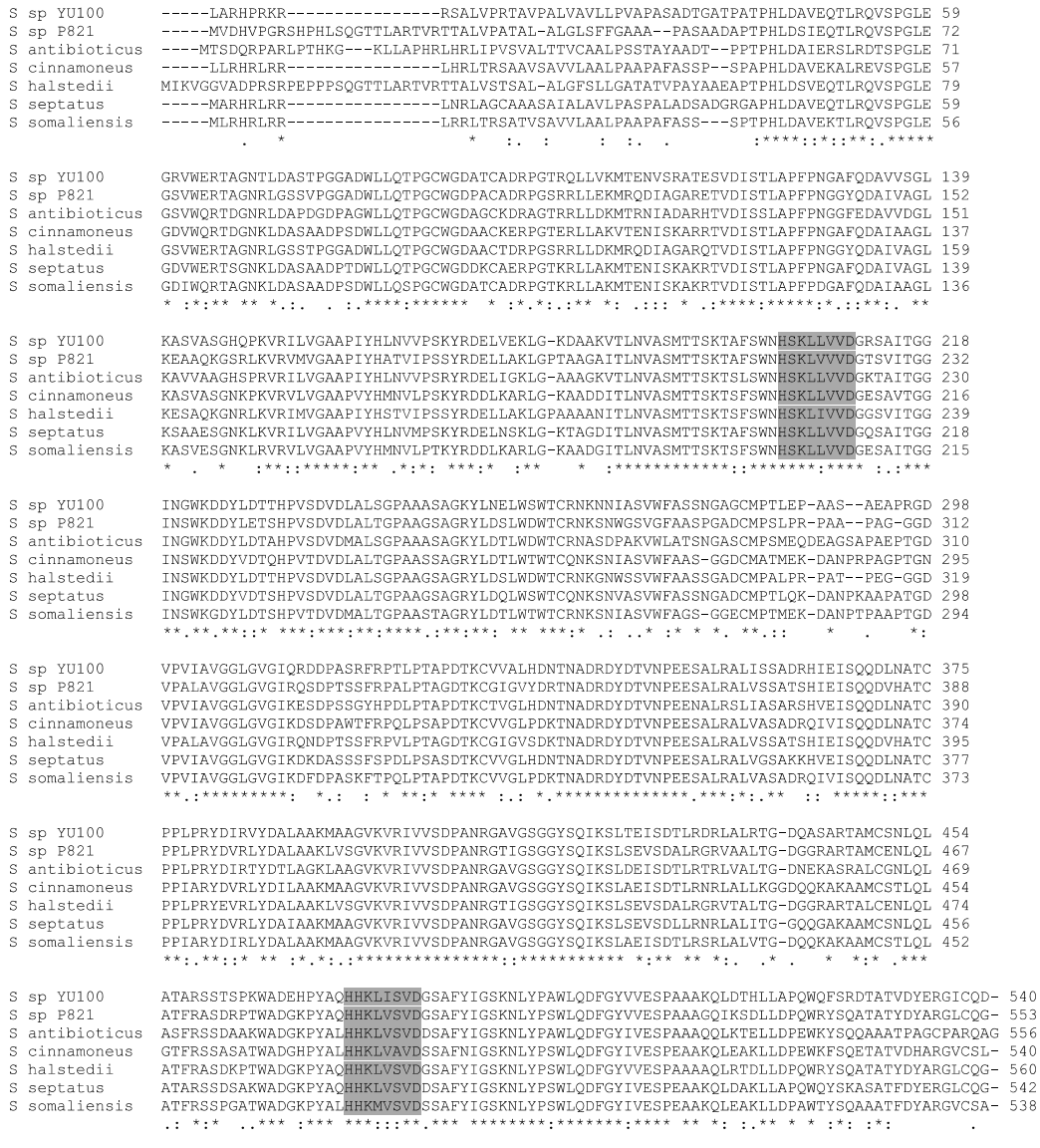

Fig. 2. Multiple alignment of amino acid sequences of streptomycetes PLD enzymes. Identical amino acid residues are indicated by asterisks, and similar residues are indicated by dots. Light grey regions denote conserved 'HKD' motifs (HxKxxxD).

Table 1. Homology comparison of streptomycetes PLDs

	GenBank Number	Length (AA)	Homology in amino acid sequence (%)						References
			Streptomyces sp. P821	*S. antibioticus*	*S. cinnamoneus*	*S. halstedii*	*S. septatus*	*S. somaliensis*	
Streptomyces sp. YU100	EU369918	540	70	69	72	70	75	72	This work
Streptomyces sp. P821	AAN41662	553		63	65	89	70	67	Moon *et al.* (2006)
S. antibioticus	BAA03913	556			68	64	68	69	Iwasaki *et al.* (1994)
S. cinnamonus	BAA75916	540				65	78	89	Ogino *et al.* (1999)
S. halstedii	BAB72230	560					69	67	Hatanaka *et al.* (2002b)
S. septatus	BAB69062	542						79	Hatanaka *et al.* (2002a)
S. somaliensis	CAF28888	538							Jeong *et al.* (2004)

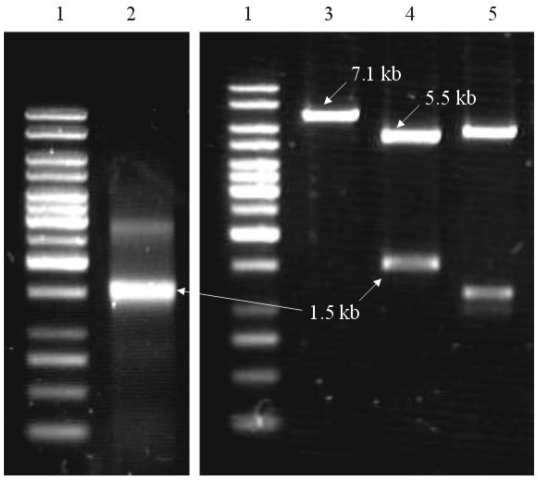

Fig. 3. Subcloning of PLD gene into pET-32b(+) vector. The PLD gene was amplified by PCR using the primers containing restriction sites of *Nco*I and *Xho*I and subcloned into pET-32b(+) expression vector. Lanes: 1 kb DNA ladder; 2, amplified PLD gene; 3, pET-PLD/*Nco*I; 4, pET-PLD/(*Nco*I + *Xho*I); 5, pET-PLD/*Bgl*II.

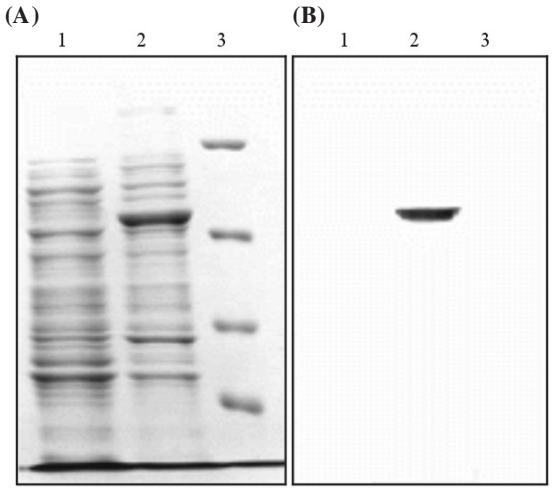

Fig. 4. Heterologous expression of recombinant PLD in *E. coli* BL21(DE3) cells. The recombinant PLD expressed in *E. coli* BL21 (DE3) harboring the pET-PLD expression vector was analyzed by 12% SDS-PAGE (A) and Western hybridization using anti-(His-tag) antibody (B). Lanes: total cell extract without induction; 2, total cell extract after induction with 0.2 mM IPTG; 3, protein molecular weight marker.

and loaded on Ni-NTA resin to purify the recombinant PLD protein. SDS-PAGE analysis revealed that recombinant PLD expressed in *E. coli* showed a quantity of soluble protein (Fig. 5). Fractions containing the His-tagged fusion protein were pooled and used for the activity assay.

Enzyme activity of recombinant PLD

To check the enzyme activity of recombinant PLD protein, the hydrolytic activity and the transphosphatidylation activity were measured. Transphosphatidylation activity was confirmed by incubating the purified recombinant PLD protein

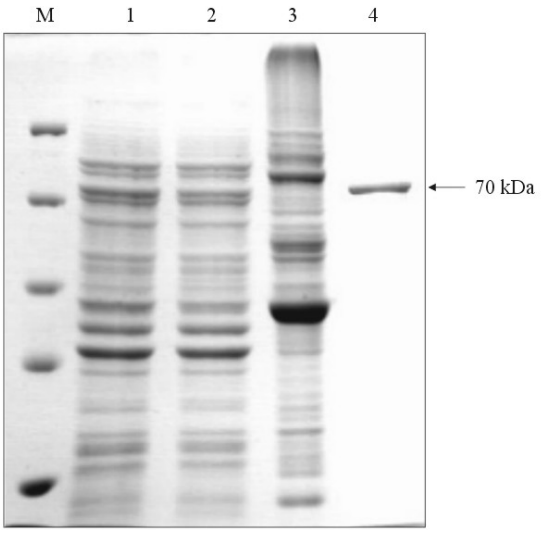

Fig. 5. Purification of recombinant PLD by Ni-affinity column chromatography. The recombinant PLD expressed in *E. coli* BL21 (DE3) harboring pET-PLD was purified by Ni-affinity column chromatography. Lanes: M, protein molecular weight marker; 1, before loading on the column; 2, flow-through with binding buffer (5 mM imidazole); 3, Wash with washing buffer (50 mM imidazole); 4, eluate with elution buffer (150 mM imidazole).

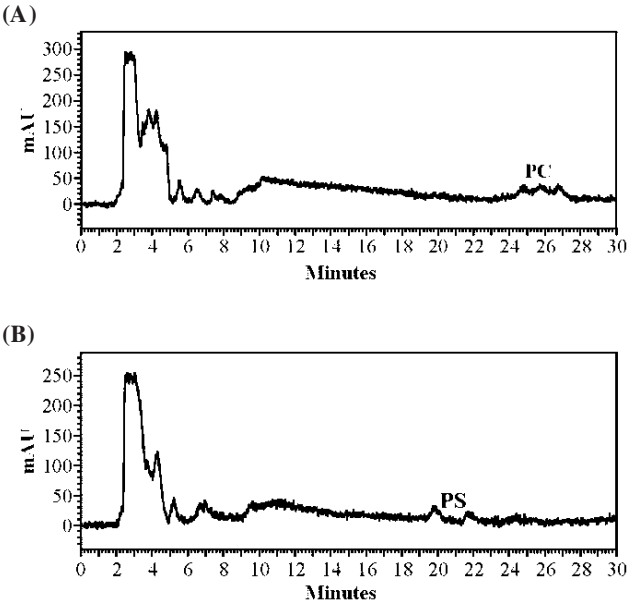

Fig. 6. Determination of transphosphatidylation activity of recombinant PLD by HPLC analysis. The reaction mixtures were subjected to HPLC using UV detection at 205 nm for the determination of transphosphatidylation activity. (A) The chromatogram before transphosphatidylation reaction. (B) The chromatogram of reaction mixture for 24-h transphosphatidylation. The chromatogram showed the production of PS by transphosphatidylation reaction.

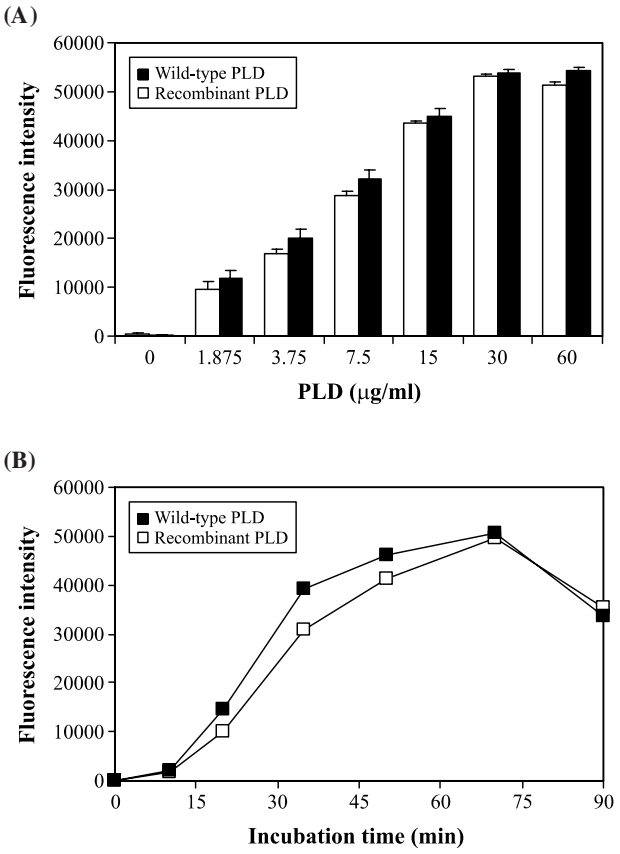

Fig. 7. Comparison of hydrolytic activities of wild-type PLD and recombinant PLD. (A) The hydrolytic activities depending on the amount of PLD enzymes. Various concentration of wild-type and recombinant PLD from 1.875 to 60 μg/ml was subjected to the hydrolytic reaction of PC for 60 min, and determined by Amplex red reagent kit. (B) Time course of hydrolytic conversion of PC by 30 μg/ml of wild-type and recombinant PLD. The maximal conversion was achieved at 70 min when determined by Amplex red reagent kit (■) *Streptomyces* PLD, (□) recombinant PLD.

with PC and *L*-serine at 35°C for 24 h, and analyzed by HPLC. The chromatogram showed the presence of PS produced by transphosphatidylation at the retention time around 20 min (Fig. 6).

The assay for hydrolytic activity was also determined based on the measurement of choline that is released during the hydrolysis reaction. When PLD concentration for hydrolytic reaction was varied from 1.875 μg/ml to 60 μg/ml, the full hydrolysis of PC was achieved after 50 min-reaction with above 30 μg/ml of wild-type and recombinant PLDs (Fig. 7).

Discussion

In this study, the cloning of PLD gene from *Streptomyces* sp. YU100 and its heterologous expression in *E. coli* was attempted. First of all, synthetic oligonucleotides primers were designed based on the consensus sequences of streptomycetes PLDs to amplified PLD gene fragment. Using the PCR product of 440 bp as a probe, λ phage DNA library of *Streptomyces* sp. YU100 chromosomal DNA was screened and a positive phage plaque was selected. Among the restriction fragments of a selected phage DNA, 3.8 kb *Bam*HI-digested fragment was subcloned into pGEM-3Zf(+) plasmid and subjected to nucleotide sequence determination.

The sequencing result revealed an open reading frame representing a full gene for PLD. The deduced 540-amino acid sequence of PLD showed 70∼75% similarity with those of other *Streptomyces* PLDs. Especially the highest similarity was found with that of *S. septatus* PLD (Hatanaka *et al.*, 2002a).

All members of PLD superfamily contain one or two copies of the conserved HxKxxxxD (HKD) motif, which was purposed to mediate catalysis (Liscovitch *et al*., 2000). Two separated copies of this motif, embedded in surrounding sequences that are also conserved but less rigorously duplicated, are found in all PLD homologs, including two phospholipid synthesis enzymes, cardiolipin synthase and phosphatidylserine synthase (Koonin, 1996; Ponting and Kerr, 1996). In the cloned PLD gene, two copies of this conserved 'HKD' motif were also found.

Because PLD enzyme of *Streptomyces* sp. YU100 is produced extracellularly, the signal peptide sequence was presumed by SignalP 3.0 program. It was found that membrane secretory system cleaves between 33rd and 34th amino acids (ASA-DT). The diversity of amino acid sequences at N-terminal depending on producers may reflect the different membrane secretory system recognizing signal peptide. Thus, the finally secreted PLD comprised of 507 amino acids may have the molecular weight of 54 kDa and the pI value of 5.8.

Using the primers having *Nco*I and *Xho*I sites, the cloned PLD gene excluding signal peptide sequence was subcloned into pET-32b(+) vector for heterologous expression in *E. coli* BL21(DE3). The gene induction was achieved with 0.2 mM IPTG when the OD_{600} of host *E. coli* cells reached 0.6. The expressed protein was purified by affinity chromatography on Ni-NTA resin and the purified recombinant PLD protein was found to be 70 kDa in SDS-PAGE analysis.

Because PLD catalyzes two reactions, the hydrolysis of PC and the transphosphatidylation to functional phospholipids like PS, both enzyme activities of recombinant PLD were checked. In HPLC analysis of transphosphatidylation reaction mixture of recombinant PLD, the produced phosphatidylserine was observed at the retention time around 20 min. When the hydrolytic activity was determined, it was also observed that the recombinant PLD shows nearly the same enzyme activity as wild-type *Streptomyces* PLD.

The heterologous expression of *Streptomyces* PLD in *E. coli* is important for its industrial application, because of short culture time as well as its production in large quantity. Further work should be done for the secretion of PLD from recombinant *E. coli.*

Acknowledgements

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