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Gene Cloning of *Streptomyces* Phospholipase D P821 Suitable for Synthesis of Phosphatidylserine

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Abstract A strain, P821, with phospholipase D activity was isolated from soil and identified as a *Streptomyces* species. The phospholipase D enzyme was purified from a culture broth of the isolated strain using ammonium sulfate precipitation and DEAE-Sepharose, phenyl-Sepharose, and Superose 12 HR column chromatographies. The purified enzyme exhibited an optimum temperature and pH of 55°C and 6.0, respectively, in the hydrolysis of phosphatidylcholine and remained stable up to 60°C within a pH range of 3.5–8.0. The enzyme also catalyzed a transphosphatidylation reaction to produce phosphatidylserine with phosphatidylcholine and serine substrates. The optimum conditions for the transphosphatidylation were 30°C and pH 5.0, indicating quite different optimum conditions for the hydrolysis and transphosphatidylation reactions. The gene encoding the enzyme was cloned by Southern hybridization and colony hybridization using a DNA probe designed from the conserved regions of other known phospholipase D enzymes. The resulting amino acid sequence was most similar to that of the PLD enzyme from *Streptomyces halstedii* (89.5%). Therefore, the enzyme was confirmed to be a phospholipase D with potential use in the production of phosphatidylserine.

Key words: Phospholipase D, *Streptomyces*, phosphatidylserine, transphosphatidylation

Phospholipase D (PLD, EC 3.1.4.4) is an enzyme that catalyzes the hydrolysis of the ester bond between phosphatidic acid (PA) and the alcohol moiety of phospholipids [6]. PLD is also an efficient catalyst for the synthesis of phospholipids [14], which is achieved via a transphosphatidylation reaction: the PA moiety of a phospholipid substrate is transferred into an acceptor alcohol [21]. Based on this reaction, highly abundant phospholipids, such as phosphatidylcholine, can be used to synthesize relatively rare phospholipids, such as phosphatidyldiethyleneglycol, phosphatidylserine (PS), phosphatidylglycerol, and phosphatidylnucleosides, which have many applications in the pharmaceutical and food industries [10].

Among these synthesized phospholipids, PS is particularly important in the pharmaceutical industry, and has been extensively studied as regards its effect on the brain, including the enhancement of Na^+, K^+-ATPase activity in the brain [19], an increased brain glucose concentration [1], and a recovery effect on age-associated memory impairment in experimental animals [4, 22]. Delwaide *et al.* [5] reported that the oral administration of bovine brain cortex-derived PS (BC-PS) to patients with senile dementia improved their cognitive disorders. However, for safety reasons, BC-PS is not suitable for use in food because of the risk of infectious encephalopathies. Thus, to overcome this problem, a transphosphatidylation reaction using PLD has been applied to produce PS from soybean lecithin [15, 18]. The PLD from white cabbage leaves was initially used for the transphosphatidylation reactions. However, after it was demonstrated that certain *Streptomyces* PLDs have a higher transphosphatidylation activity, *Streptomyces* PLDs have since been used for various transphosphatidylation reactions [3].

Accordingly, this study purified a PLD enzyme from *Streptomyces* sp. P821 and characterized its optimum conditions for hydrolysis and transphosphatidylation reactions.

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MATERIALS AND METHODS

Materials

The L-α-phosphatidylcholine, L-α-phosphatidyl-DL-glycerol, L-α-phosphatidylinositol, L-α-phosphatidyl-L-serine, L-serine, choline oxidase, and peroxidase were all purchased from Sigma-Aldrich Co. (St. Louis, MO, U.S.A.), and the other enzymes and chemicals were from commercial companies.

Isolation of PLD-Producing *Streptomyces* Strain

A soil sample collected near Taejon, Korea, was dried at 55°C for 10 h. The dried soil sample (1 g) was then suspended in a sterile 0.85% NaCl solution (10 ml) and spread on a humic acid-vitamin agar plate (0.1% humic acid, 0.05% Na_2HPO_4, 0.171% KCl, 0.005% $MgSO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.002% $CaCO_3$, 1.5% agar, and trace amount of a vitamin mixture) containing nalidixic acid (20 μg/ml) and cycloheximide (50 μg/ml). After incubation at 28°C for 2 weeks, about 200 colonies were selected, inoculated into 10 ml of a GSS medium (1% soluble starch, 2% glucose, 2.5% soybean meal, 0.1% beef extract, 0.4% yeast extract, 0.2% NaCl, 0.025% K_2HPO_4, 0.2% $CaCO_3$, pH 6.8), and cultured at 28°C for 72 h using a shaking incubator. The culture supernatants were then tested for their PLD activity.

Purification of PLD P821

The *Streptomyces* sp. P821 was cultured at 28°C for 72 h in a GMYB medium (0.4% glucose, 1% malt extract, 0.5% yeast extract, pH 7.0). The culture supernatant (12 l) was then collected and the ammonium sulfate precipitate (40–85% fraction) recovered by centrifugation (10,000 ×g, 20 min). Thereafter, the protein precipitate was resuspended and dialyzed against a potassium phosphate buffer (20 mM, pH 7.0), and the protein solution loaded onto a DEAE-Sepharose column (30×150 mm). Although some of the proteins bound with the resin, the PLD enzyme passed through the column. The active fraction was then dialyzed against a 20 mM Tris-HCl, 1.5 M $(NH_4)_2SO_4$ buffer (pH 7.5) and reloaded onto a phenyl-Sepharose column (30×150 mm). The bound proteins were eluted with a decreasing concentration of ammonium sulfate, and the PLD enzyme eluted with a 0.5 M ammonium sulfate solution. The active fraction was then dialyzed against a 50 mM Tris-HCl, 150 mM NaCl buffer (pH 7.0) and loaded onto a Superose 12 HR 10/30 column equipped with an FPLC system (Pharmacia LKB, Uppsala, Sweden). Finally, the active fraction was collected and the purified enzyme analyzed by SDS-PAGE (12% gel).

PLD Assay

The PLD activity was assayed as follows [2, 12, 13]: 100 μl of soybean phosphatidylcholine (1.5%) in a 30 mM Tris-HCl, 15 mM $CaCl_2$, and 3.4% Triton X-100 buffer (pH 7.0) was mixed with 50 μl of the enzyme solution. After being incubated for 30 min at 37°C, the reaction was stopped by the addition of 50 μl of 50 mM EDTA. The amount of choline released was analyzed colorimetrically as follows [9]: 400 μl of a 10 mM 4-aminoantipyrine, 16 mM phenol, and 10 mM Tris-HCl buffer (pH 7.0), and 400 μl of choline oxidase (6 units) and peroxidase (0.4 units) in a 10 mM Tris-HCl buffer (pH 7.0) were added to the reaction mixture (200 μl). After incubation for 20 min at 37°C, the absorbance was measured at 500 nm. One PLD unit was defined as the amount of enzyme releasing 1 μmol of choline from the phosphatidylcholine per min.

Effect of Temperature and pH on Activity of PLD P821

To identify the optimum reaction temperature, the hydrolytic activity of the enzyme toward the phosphatidylcholine was measured at various temperatures (20–80°C) and pH 7.0. The thermal stability was estimated by measuring the residual activity at 37°C and pH 7.0 after heating the enzyme for 30 min at different temperatures (20–70°C).

Meanwhile, to determine the optimum pH, the hydrolytic reaction rate was measured at various pHs (pH 3.5–9.0) and 37°C. The enzyme was incubated for 1 h in different pH buffers (pH 2.0–9.0) and the residual activity measured at 37°C and pH 7.0. A GTA buffer (100 mM 3,3-dimethylglutaric acid, 100 mM Tris, 100 mM 2-amino-2-methyl-1,3-propanediol) was used as the universal pH buffer [16].

Reaction Properties of PLD P821

To identify the substrate specificity, 100 μl of various phospholipids (1.5%) in a 30 mM Tris-HCl, 15 mM $CaCl_2$, and 3.4% Triton X-100 buffer (pH 7.0) were mixed with 50 μl of the enzyme solution. After incubation for 30 min at 37°C, the reaction was stopped by the addition of 50 μl of 50 mM EDTA. Following chloroform/methanol extraction, the reaction products were analyzed using thin layer chromatography. Chloroform/methanol/ammonia (28%) (v/v/v, 65:25:5) was used as the developing solvent. The spot on the plate was visualized with 10% $CuSO_4$ in 8% phosphoric acid. The amount of phosphatidic acid was analyzed using a Gel Doc 2000 image system (Bio-rad, Hercules, CA, U.S.A.) with the Quantity one program.

To perform a transphosphatidylation reaction, 50 μl of the soybean phosphatidylcholine (1 mg/ml in diethyl ether) and 100 μl of 1.5 M serine in a 100 mM sodium acetate buffer (pH 4–6) were mixed [11], and then 50 μl of the enzyme (6.6 units/ml) was added and the reaction allowed to continue for 1 h. Following chloroform/methanol extraction, the amount of phosphatidylserine was analyzed using the TLC method, as described previously.

Cloning of PLD P821 Gene

Many PLD enzymes from *Streptomyces* have been found to include several conserved regions in their protein

sequences. Therefore, a PCR reaction was performed using chromosomal DNA isolated from *Streptomyces* sp. P821 and two primers designed based on the conserved regions.

Primer 1: 5'-ACC(G)AACGCC(G)GACCGC(G)GAC-TAC-3'

Primer 2: 5'-TAC(G)CCGAAGTCCTGG(C)AGCCA-3'

The PCR product (0.5 kb DNA) was then used as a DNA probe in a Southern hybridization experiment. A positive signal was detected a PstI-digested chromosomal DNA fragment of about 3.0 kb. The DNA fragment was extracted from the gel, ligated with a pUC118 vector, and transformed into *E. coli* XL1-Blue. When colony hybridization was performed using the same DNA probe, a positive colony was detected and the recombinant plasmid purified and identified.

RESULTS AND DISCUSSION

Isolation of PLD-Producing *Streptomyces* Strain

Among the 200 colonies isolated from the soil sample, the bacterial strain P821 exhibiting the greatest PLD activity was screened. The strain was found to be Gram-positive and the vegetative cells produced a branched mycelium (data not shown). The strain also produced a melanoid pigment, along with gray and smooth-surfaced spores arranged in a spiral chain (data not shown). Therefore, these morphological and physiological results indicated a *Streptomyces* strain [8]. Its 16S rRNA sequence was then used to draw a phylogenetic tree with other *Streptomyces* species (Fig. 1), which revealed a more than 99% identity with certain *Streptomyces* strains, including *St. lavendulae*, *St. subrutilus*, and *St. virginiae*. As such, the strain was designated as *Streptomyces* sp. P821. Thereafter, since there have been no previous reports on PLD enzymes from closely related species, an attempt was made to purify and characterize the PLD enzyme.

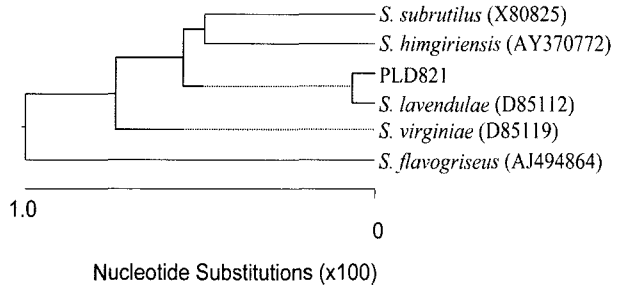

Fig. 1. Phylogenetic tree of several *Streptomyces* species closely related to *Streptomyces* sp. P821.
The 16S rRNA sequence was compared with those of several *Streptomyces* species and their phylogenetic relationship drawn based on the ClustalW method using the DNASTAR program.

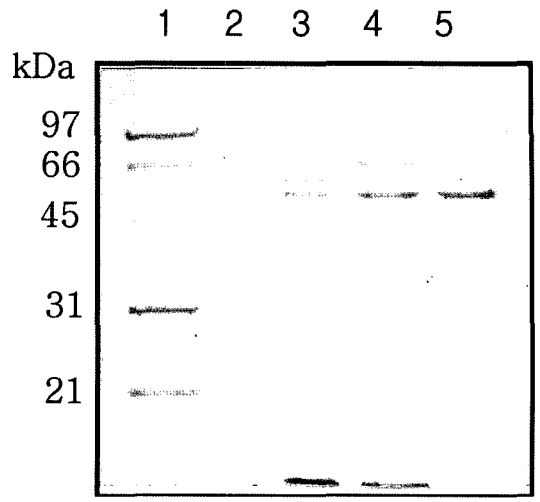

Fig. 2. SDS-PAGE of PLD P821 enzyme.
Lane 1 shows the protein size markers, lane 2 shows the ammonium sulfate 40–85% fraction, and lanes 3, 4, and 5 show the active fractions obtained from the DEAE-Sepharose, phenyl-Sepharose, and Superose 12 HR column chromatographies, respectively.

Purification of PLD

The *Streptomyces* sp. P821 was cultured at 28°C for 72 h in a GMYB medium, and then the PLD enzyme was purified from the culture broth using ammonium sulfate precipitation and DEAE-Sepharose, phenyl-Sepharose, and gel filtration chromatographies, as described previously. The PLD activity in the culture broth (12 l) was 15,500 U, whereas that of the finally purified PLD was 449 U. The specific activity of the purified enzyme was measured to be 2,250 U/mg protein, and the final purification fold was 78.8.

The purified enzyme was homogeneous on an SDS-PAGE gel with a molecular mass of 57 kDa (Fig. 2). The N-terminal amino acid sequence of the protein band was determined as NH_2-ADAPTPHLDSIEQTL, which is similar to that of the PLD enzyme from *S. halstedii* with an 87% homogeneity (GenBank-id, BAB72230) [7], suggesting that the 57-kDa-sized protein band on the gel was a PLD enzyme.

Biochemical Properties of PLD P821

The optimum temperature for the purified enzyme as regards the hydrolysis of the phosphatidylcholine was 55°C, and the enzyme remained stable up to 60°C (Fig. 3A). The thermostability of the enzyme was different from that of the closely related *St. halstedii* PLD, which is only stable up to 45°C.

The optimum pH was pH 6.0 and the enzyme remained fairly stable within a pH range of 3.5–8.0 (Fig. 3B). Among the various phospholipids tested as substrates, PC was hydrolyzed most rapidly, whereas phosphatidylserine and phosphatidylglycerol were hydrolyzed slowly, and phosphatidylinositol was not hydrolyzed (Fig. 4A). Therefore,

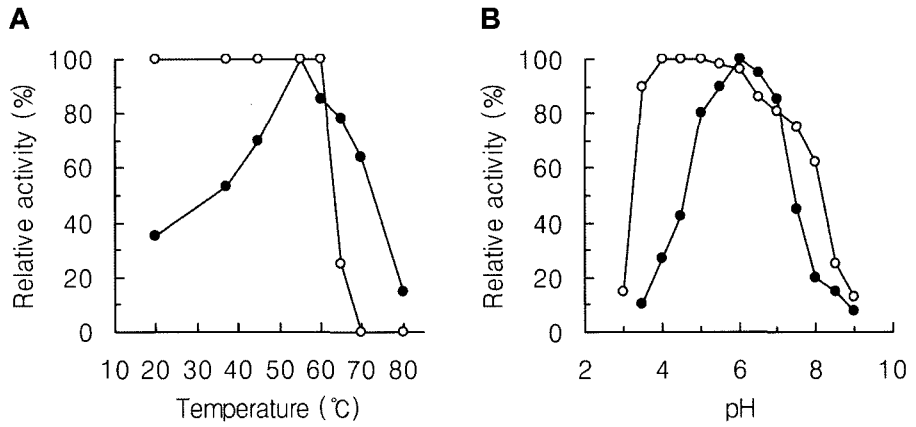

Fig. 3. Effect of temperature and pH on the activity of PLD P821 enzyme.
A. The hydrolytic activity of the PLD P821 enzyme was measured at different temperatures (●). The enzyme was heated for 30 min at different temperatures, and then the residual activity was measured (○). **B.** The hydrolytic activity was also measured at different pHs (●). The enzyme was incubated for 1 h at different pHs, and then the residual activity was measured (○).

the enzyme exhibited a quite specific hydrolytic activity according to the alcoholic moieties of the phospholipids.

In addition, the enzyme catalyzed a transphosphatidylation reaction: PS was made by an enzyme-mediated reaction using PC and serine as the substrates. As shown in Fig. 4B, when the serine concentration was increased above 0.3 M, the transphosphatidylation reaction rate increased abruptly. The optimum temperature for the reaction was measured

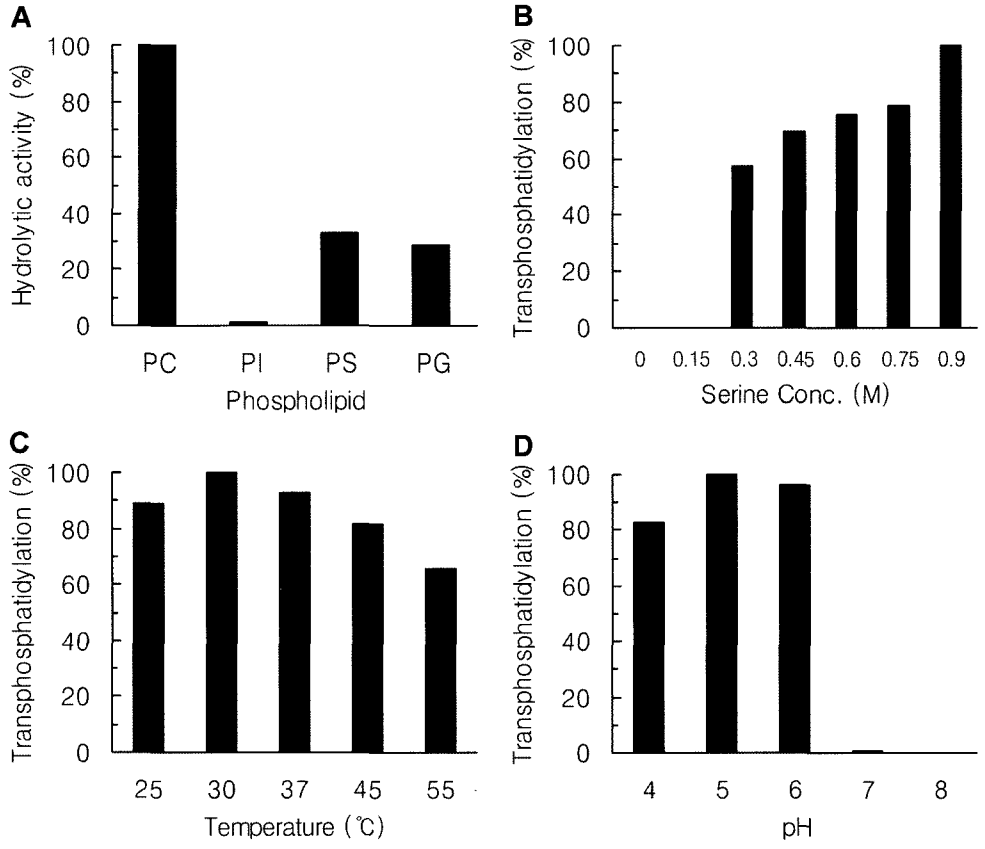

Fig. 4. Hydrolysis and transphosphatidylation activity of PLD P821 enzyme.
The hydrolytic activity of the enzyme toward various phospholipids was measured (**A**). The transphosphatidylation activity of the enzyme was measured at different serine concentrations (**B**), temperatures (**C**), and pHs (**D**).

to be 30°C (Fig. 4C) and the reaction only proceeded under acidic conditions (pH 4.0–6.0) (Fig. 4D). Therefore, the optimum conditions for this transphospatidylation reaction were determined to be 30°C and pH 5.0.

As a result, the enzyme was found to perform a transphosphatidylation and phosphohydrolysis reaction quite differently with respect to the temperature and pH (Figs. 3 and 4). Such discrepancies were also recently observed with other PLDs [20].

Comparison of Protein Sequences

To compare the enzyme with other PLD enzymes on a molecular level, a gene cloning experiment was performed, as described previously. The PLD gene was obtained using a DNA probe for Southern blotting and colony hybridization. An *E. coli* transformant harboring the corresponding PLD gene was identified, and the recombinant plasmid isolated from the cell contained a 1,662-bp-sized open reading frame (Fig. 5). The gene included the two primer sequences (primer 1 and primer 2) used to make the DNA probes, plus the same protein sequence as the N-terminal sequence determined previously from the purified enzyme. The gene had a signal sequence of 49 amino acids and a mature enzyme (504 amino acids)

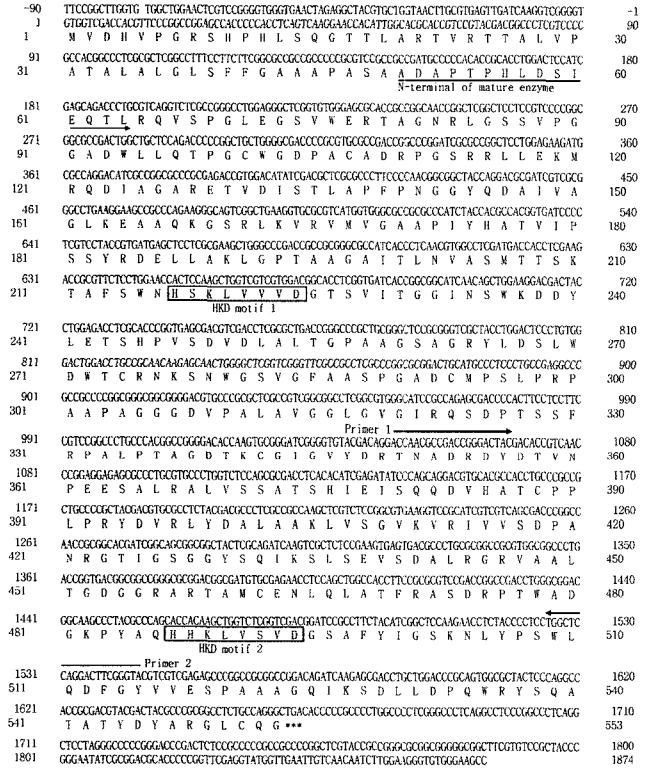

Fig. 5. Nucleotide and amino acid sequences of the enzyme.
The numbering of the nucleotides starts at the 5' end of the PLD gene. The N-terminal of the mature protein and nucleotide sequences used to make the primers are marked with arrows. The two HKO motifs are boxed.

with a molecular mass of 53,174 Da. Two HKD motifs were also found, suggesting a PLD protein [17]. The sequence was submitted to the GenBank under accession number AAN 41662.

When the amino acid sequence was compared with those for other proteins in the Swissprot database using the Blast Search program, a similarity was found with the PLD enzymes from *St. halstedii* (BAB72230, 89.5%), *Streptomyces* sp. PMF (1V0YA, 74.3%), and *St. septatus* (BAB69062, 70.8%). Therefore, the enzyme turned out to be similar to the *St. halstedii* PLD.

Transphosphatidylation is usually carried out in a biphasic system consisting of water-insoluble organic solvents. Thus, the thermostability of the PLD is important when using such a system. Hatanaka *et al.* [7] recently investigated the thermostability of the PLD enzymes from *St. halstedii* and *St. septatus* and found that the latter was relatively more thermostable; whereas the *St. halstedii* enzyme lost its activity at 45°C, the *St. septatus* enzyme remained stable even at higher temperatures. Based on a chimeric mutagenesis experiment, it was then suggested that the Asp346 residue was very important for thermostability.

Interestingly, the PLD P821 enzyme exhibited the same high optimum temperature and thermostability as the enzyme from *St. septatus*, despite its higher sequence similarity to the *St. halstedii* enzyme. This thermostability may also be explained by the presence of the Asp346 residue in the C-terminal part of the PLD P821 enzyme (Fig. 6). Therefore, this result suggests that the PLD enzyme can be effectively used for transphosphatidylation carried out in a biphasic system.

In conclusion, a *Streptomyces* PLD enzyme was purified and the optimum conditions for a transphosphatidylation reaction characterized. Since PS is known to have a recovery effect on memory impairment, PLD-mediated transphosphatidylation has become a very important process for the large-scale production of PS. Accordingly, the PLD P821 enzyme has potential industrial application in the production of the PS molecule.

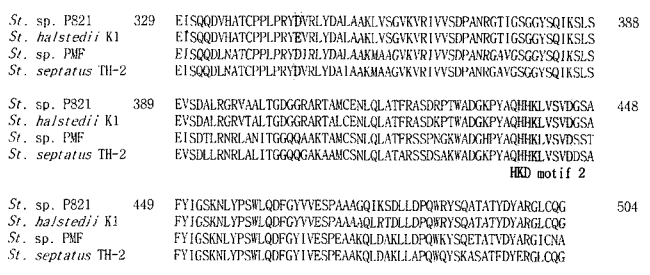

Fig. 6. Comparison of primary structures of the C-terminal region among *Streptomyces* sp., including *Streptomyces* sp. P821, *St. halstedii* K1, *Streptomyces* sp. PMF, and *St. septatus* TH-2.
The asterisk indicates the Asp (Glu) 346 residue in the *Streptomyces* PLDs. The HKD motif 2 is gray-shadowed.

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REFERENCES

1. Bruni, A., G. Toffano, A. Leon, and E. Boarato. 1976. Pharmacological effects of phosphatidylserine liposomes. *Nature* **260:** 331–333.
2. Chung, Y. J., J. R. Jeong, B. C. Lee, J. Y. Kim, Y. I. Park, and J. Y. Ro. 2003. Phospholipase D in guinea pig lung tissue membrane is regulated by cytosolic ARF proteins. *J. Microbiol. Biotechnol.* **13:** 897–905.
3. Comfurius, P., E. M. Bevers, and R. F. A. Zwaal. 1990. Enzymatic synthesis of phosphatidylserine on small scale by use of a one-phase system. *J. Lipid Res.* **31:** 1719–1721.
4. Crook, T. H., J. Tinklenberg, J. Yesavage, W. Petrie, M. G. Nunzi, and D. C. Massari. 1991. Effects of phosphatidylserine in age-associated memory impairment. *Neurology* **41:** 644–649.
5. Delwaide, P. J., A. M. Gyselynck-Mambourg, A. Hurlet, and M. Ylieff. 1986. Double-blind randomized controlled study of phosphatidylserine in senile demented patients. *Acta Neurol. Scand.* **73:** 136–140.
6. Exton, J. H. 1998. Phospholipase D. *Biochim. Biophys. Acta* **1436:** 105–115.
7. Hatanaka, T., T. Negishi, M. Kubota-Akizawa, and T. Hagishita. 2002. Study on thermostability of phospholipase D from *Streptomyces* sp.. *Biochim. Biophys. Acta* **1598:** 156–164.
8. Holt, J. G., R. N. R. Krieg, and P. H. A. Sneath. 1994. Genus *Streptomyces*, pp. 605–671. *In* W. R. Hensyl (ed.), *Bergey's Manual of Determinative Bacteriology*, 9th Ed. Lippincott Williams & Wilkins Co., Baltimore.
9. Imamura, S. and Y. Horiuti. 1979. Purification of *Streptomyces chromofuscus* phospholipase D by hydrophobic affinity chromatography on palmitoyl cellulose. *J. Biochem.* **85:** 79–95.
10. Iwasaki, Y., H. Nakano, and T. Yamane. 1994. Phospholipase D from *Streptomyces antibioticus*: Cloning, sequencing, expression, and relationship to other phospholipases. *Appl. Microbiol. Biotechnol.* **42:** 290–299.
11. Juneja, L. R., T. Kazuoka, N. Goto, T. Yamane, and S. Shimizu. 1989. Conversion of phosphatidylcholine to phosphatidylserine by various phospholipases D in the presence of L- or D-serine. *Biochim. Biophys. Acta* **1003:** 277–283.
12. Lim, S. K., J. W. Choi, E. T. Lee, Y. H. Khang, S. D. Kim, and D. H. Nam. 2002. Isolation of *Streptomyces* sp. YU100 producing extracellular phospholipase D. *J. Microbiol. Biotechnol.* **12:** 71–76.
13. Lim, S. K., J. W. Choi, M. H. Chung, E. T. Lee, Y. H. Khang, S. D. Kim, and D. H. Nam. 2002. Production and characterization of extracellular phospholipase D from *Streptomyces* sp. YU100. *J. Microbiol. Biotechnol.* **12:** 189–195.
14. Rich, J. O. and Y. L. Khmelnitsky. 2001. Phospholipase D-catalyzed transphosphatidylation in anhydrous organic solvents. *Biotechnol. Bioeng.* **72:** 374–377.
15. Sakai, M., H. Yamatoya, and S. Kudo. 1996. Pharmacological effects of phosphatidylserine enzymatically synthesized from soybean lecithin on brain functions in rodents. *J. Nutr. Sci. Vitaminol.* **42:** 47–54.
16. Shimbo, K., Y. Iwasaki, T. Yamane, and K. Ina. 1993. Purification and properties of phospholipase D from *Streptomyces antibioticus*. *Biosci. Biotech. Biochem.* **57:** 1946–1948.
17. Stuckey, J. A. and J. E. Dixon. 1999. Crystal structure of a phospholipase D family member. *Nat. Struct. Biol.* **6:** 278–284.
18. Suzuki, S., H. Yamatoy, M. Sakai, A. Kataoka, M. Furushiro, and S. Kudo. 2001. Oral administration of soybean lecithin transphosphatidylated phosphatidylserine improves memory impairment in aged rats. *J. Nutr.* **131:** 2951–2956.
19. Wheeler, K. P, and R. Whittam. 1970. ATPase activity of the sodium pump needs phosphatidylserine. *Nature* **225:** 449–450.
20. Yang, H. and M. F. Roberts. 2003. Phosphohydrolase and transphosphatidylation reactions of two *Streptomyces* phospholipase D enzymes: Covalent versus noncovalent catalysis. *Protein Sci.* **12:** 2087–2098.
21. Yang, S. F., S. Freer, and A. A. Benson. 1967. Transphosphatidylation by phospholipase D. *J. Biol. Chem.* **242:** 477–484.
22. Zanotti, A., L. Valzelli, and G. Toffano. 1989. Chronic phosphatidylserine treatment improves spatial memory and passive avoidance in aged rats. *Psychopharmacology* **99:** 316–321.