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Review

Phospholipase D from *Streptoverticillium cinnamoneum*: protein engineering and application for phospholipid production

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Dedicated to Professor Dr. Kenji Soda in honor of his 70th birthday

Abstract

This review is focusing on an industrially important enzyme, phospholipase D (PLD), exhibiting both transphosphatidylation and hydrolytic activities for various phospholipids. The transphosphatidylation activity of PLD is particularly useful for converting phosphatidylcholine (PC) into other phospholipids. During the last decade, the genes coding for PLD have been identified from various species including mammals, plants, yeast, and bacteria. However, detailed basic and applied enzymological studies on PLD have been hampered by the low productivity in these organisms. Efficient production of a recombinant PLD has also been unsuccessful so far. We recently isolated and characterized the PLD gene from *Streptoverticillium cinnamoneum*, producing a secretory PLD. Furthermore, we constructed an overexpression system for the secretory enzyme in an active and soluble form using *Streptomyces lividans* as a host for transformation of the PLD gene. The *Stv. cinnamoneum* PLD was proven to be useful for the continuous and efficient production of phosphatidylethanolamine (PE) from phosphatidylcholine. Thus, the secretory PLD is a promising catalyst for synthesizing new phospholipids possessing various polar head groups that show versatile physiological functions and may be utilized in food and pharmaceutical industries.

Keywords: Phospholipase D; Transphosphatidylation; Hydrolysis; Phospholipid synthesis; *Streptomyces* sp.

1. Introduction

Phospholipase D (PLD; EC 3.1.4.4) is an enzyme that occurs ubiquitously in various organisms including mammals [1], plants [2], yeast [3] and bacteria [4–6]. The enzyme catalyzes two types of reactions (Fig. 1): hydrolysis of phosphatidylcholine (PC), a major substrate of PLD, to phosphatidic acid (PA) and choline (hydrolytic reaction), and transfer of polar head groups to others (transphosphatidylation reaction). For example, in the presence of ethanol, PLD exchanges the choline moiety of PC

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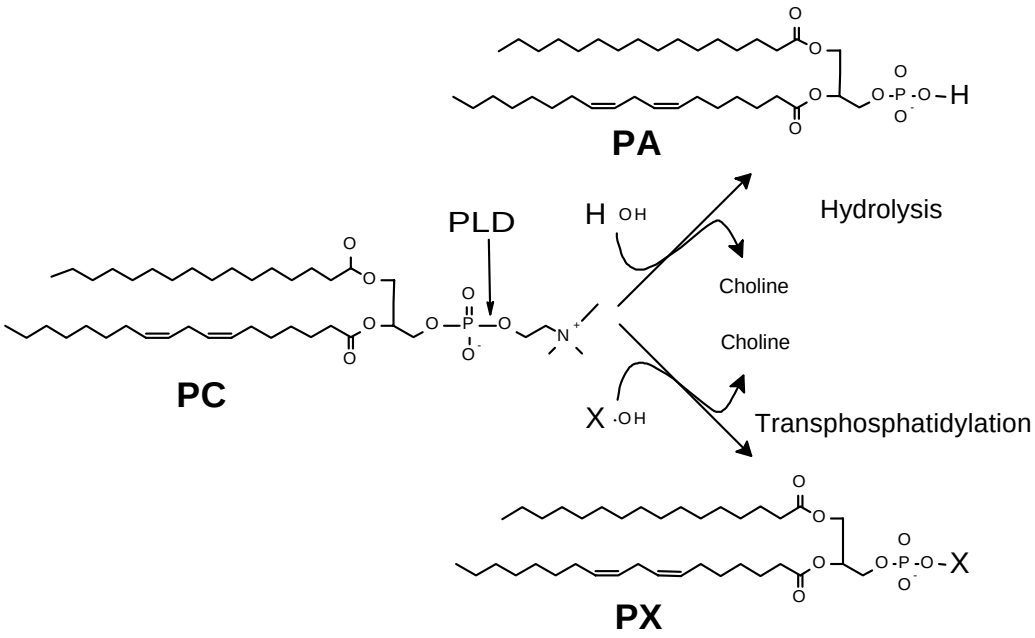

Fig. 1. Schematic representation of PLD reactions.

by ethanol forming phosphatidylethanol (PEt). The enzymes from eukaryotic sources have generally the hydrolytic activity much higher than the transphosphatidylation activity. In mammalian cells, PC and phosphatidylethanolamine (PE) are the most and less favored substrates, respectively, for the hydrolytic activity of PLD. The hydrolytic product PA and its metabolite diacylglycerol are known to activate many cell-signaling enzymes [7–9] and thereby induce cell proliferation and differentiation through the activation of protein kinase C (PKC). In yeast cells, PLD is implicated in spore formation [10]. These eukaryotic PLDs are mainly localized in membrane fractions, and their activities are regulated by various intracellular factors (e.g. small GTP binding proteins and protein kinases) [11]. In bacteria, the PLD activity was discovered mainly in the culture medium of *Actinomycetes* [4–6]. The constitutively active enzymes usually show both hydrolytic and transphosphatidylation activities and prefer PC to other phospholipids as a substrate [12,13].

The hydrolytic activity of PLD can be assayed by measuring the amount of a liberated polar head group from PC (choline) or artificial substrates (e.g. *p*-nitrophenol from phosphatidyl-*p*-nitrophenol) [14–16] spectrophotometrically or with a radioactive substrate [1,17], while the transphosphatidylation activity can be assayed by measuring the amount of a newly synthesized phospholipid from either a fluorinated or radioactive substrate by thin-layer chromatography equipped with an imaging analyzer [18].

Phospholipids, as an abundant lipid component in nature, possess glycerol structure with a polar head moiety and two fatty acid chains [19]. This amphiphilic property facilitates the application of phospholipids in various industrial fields such as pharmaceuticals, cosmetics, and foods [20–23]. For example, phosphatidylserine (PS) is used as a liposome for drug delivery systems (DDS), a surfactant, an emulsifier for cosmetics, and a dietary supplement for foods. Additionally, PS is known to improve memory performance in patients suffering from age-associated memory impairment [24] or Alzheimer's disease [25]. Other phospholipids are also used in cosmetics [26]. As for the availability of phospholipids, PC can be easily extracted from various natural materials, which makes it the most popular phospholipid used in industries. However, other phospholipids are usually

obtained by a series of purification steps from raw materials, which makes them expensive. These situations only allow the industrial use of PC among various phospholipids.

The enzymes from *Actinomycetes* show higher transphosphatidylation activity than those from other sources and exhibit broad substrate specificity to phospholipids. It is therefore expected that the *Actinomycetes* PLD is applicable for synthesizing various phospholipids from inexpensive PC in industrial fields. So far, many researches have been conducted to isolate bacterial strains producing high amounts of PLD. Our group found that, among various bacteria examined, an *Actinomycetes* strain, *Streptoverticillium cinnamoneum* (IFO 12852), releases the highest activity of PLD in the medium [13]. Subsequently, the enzyme was purified from the culture medium of *Stv. cinnamoneum*, and the nucleotide sequence of the PLD gene was also determined [27]. More recently, an overexpression system for the secretory PLD was constructed by using *Streptomyces lividans* as a host and an *E. coli*/*S. lividans* shuttle vector (Ogino et al., unpublished results). We further accomplished the continuous and efficient production of PEt from PC using the recombinant enzyme.

In this review, we describe the reaction mechanism of PLD, gene and protein structures, and mutational studies, focusing on the *Stv. cinnamoneum* PLD. For application of this enzyme, we also state the establishment of an overexpression system for the secretory PLD and discuss its potentiality for the production of novel phospholipids from PC.

2. Reaction mechanism of PLD

PLD is generally defined as an enzyme that catalyzes the hydrolysis of PC to choline and PA [28]. In addition, in the presence of a primary alcohol, most enzymes of PLD catalyze the transphosphatidylation reaction, transferring the polar head group of PC (choline) to the alcohol (Fig. 1). When PLD was first identified [29], it was unclear whether the hydrolysis of PC proceeds via P–O or C–O bond cleavage of phospholipids, although the cleavage of a C–O bond is chemically unfeasible. An analysis of the hydrolytic reaction in ^{18}O-labeled water has shown that the ^{18}O atom is incorporated into PA, indicating that PLD cleaves the P–O bond but not the C–O bond [30]. The mechanisms of the two catalytic reactions of PLD, hydrolysis and transphosphatidylation, consist of two steps (Fig. 2); the first step is the release of choline, forming a common intermediate (E–P, the phosphatidyl group covalently attached to the enzyme)

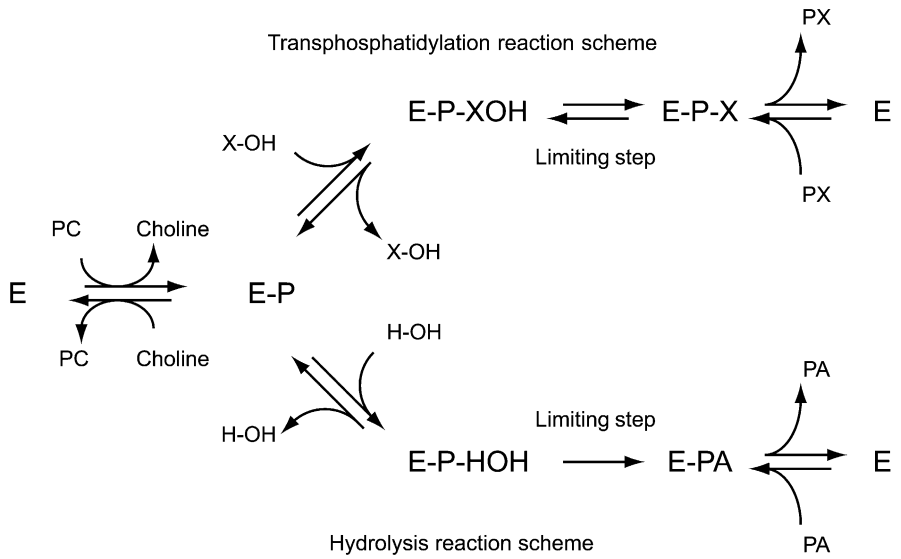

Fig. 2. PLD reactions based on a ping-pong mechanism.

and the second step involves the hydrolysis of E–P to PA or the transfer of the phosphatidyl moiety to a primary alcohol [31]. By using *Streptomyces antibioticus* PLD and fluorescence-labeled PC as substrate, a His residue in the HKD motif contained in the C-terminal half has been shown to be involved in the formation of the covalent phosphatidyl–enzyme complex [32].

In the application of *Actinomycetes* PLD to phospholipid synthesis, phospholipids and the enzyme are usually dissolved in a two-phase solvent system consisting of an organic solvent and water. This solvent system enables easy separation of the phospholipid product from the reaction mixture. The reaction mechanism of PLD was therefore investigated by using the two-phase solvent system (diethyl ether/water) with *Stv. cinnamoneum* PLD [33]. The enzyme converts PC into other phospholipids and vice versa by the transphosphatidylation reaction. In contrast, PA is not utilized as a substrate by the hydrolysis reaction. It was shown that the transphosphatidylation and the hydrolysis reaction are reversible and irreversible, respectively. Thus, both reactions were postulated to proceed by a ping-pong mechanism as shown in Fig. 2. Step (I): PLD forms an enzyme–substrate complex with PC, and the PLD–PC complex is converted into the PLD–phosphatidyl complex releasing choline as the first product. Step (II): a nucleophilic acceptor molecule including primary alcohols (for transphosphatidylation reaction) and water (for hydrolytic reaction) associates with the complex. Step (III): the phospholipid or PA dissociates from the complex as the final product. Based upon this mechanism, the steady-state kinetic constants for each step were calculated by a parameter-fitting program. In the transphosphatidylation reaction, the kinetic constant at step (I) is independent of the second substrate and differs solely with phospholipid substrates, whereas the kinetic constants at steps (II) and (III) depend on the nucleophilicity of the acceptor. In the hydrolytic reaction, the constants are unaffected by the presence of the second substrate at any step. Adaptation of these constants determined from the steady-state kinetic analysis to the estimation of the amount of the final product (phospholipid) led to a good agreement with the experimental values, which were independent of the acceptor concentration.

3. Primary structures of PLD

During the last decade, the genes coding for PLD were cloned from bacteria, plants, yeast, and mammals, and their nucleotide sequences were determined [34,35]. Human cDNAs for the two major isoforms (hPLD1 and hPLD2) of PLD have also been cloned, together with their spliced variants. The amino acid identity between hPLD1 and hPLD2 is approximately 50%, and their mRNAs are expressed in all tissues examined [34]. The *SPO14* gene involved in mitosis and spore formation in yeast has been shown to encode a PLD enzyme [36]. The bacterial genes encoding PLD were cloned from the chromosomal DNA of various *Actinomycetes* strains [27,37,38]. The polypeptide length of bacterial enzymes (500–600 amino acid residues) are usually shorter than those of the eukaryotic enzymes (>1000 amino acid residues). The amino acid sequence similarity among bacterial enzymes is about 70% [39]. The similarity between the *Streptomyces* PLD and eukaryotic enzymes is less than 30%. As shown in Fig. 3A, the primary structures of eukaryotic PLD contain four highly conserved regions (regions I–VI). Bacterial PLD enzymes lack region III, which is postulated to possess a regulatory function for eukaryotic enzymes [34]. Within regions I and IV, a highly conserved sequence, HxKxxxxD (x represents any amino acid residue; designated as an HKD motif), was found (Fig. 3B). Either a GG or GS sequence was also identified at the seven amino acids downstream of each HKD motif, both of which were designated as GG/S motifs. The HKD motif was found not only in PLD isoforms but also in the PLD superfamily including bacterial PS and cardiolipin synthases, bacterial endonucleases, poxvirus envelope protein, and *Yersinia pestis* toxin [40]. The PLD superfamily consists of eight classes: class I, eukaryotic PLD including yeast and plant PLD; class II, bacterial PLD; class III, PS synthases; class IV, cardiolipin synthases; class VII, endonuclease Nuc containing only one HKD motif, which forms a homodimer unlike other members of the PLD superfamily; and classes V, VI, and VIII, HKD motif-containing proteins of unknown functions [40]. The function of the GG/S motifs in PLD isoforms and the PLD superfamily is discussed below.

Unlike the conventional PLD isoforms, PLD-like enzymes exclusively possessing a hydrolytic activity for PE and PS rather than PC were found in yeast

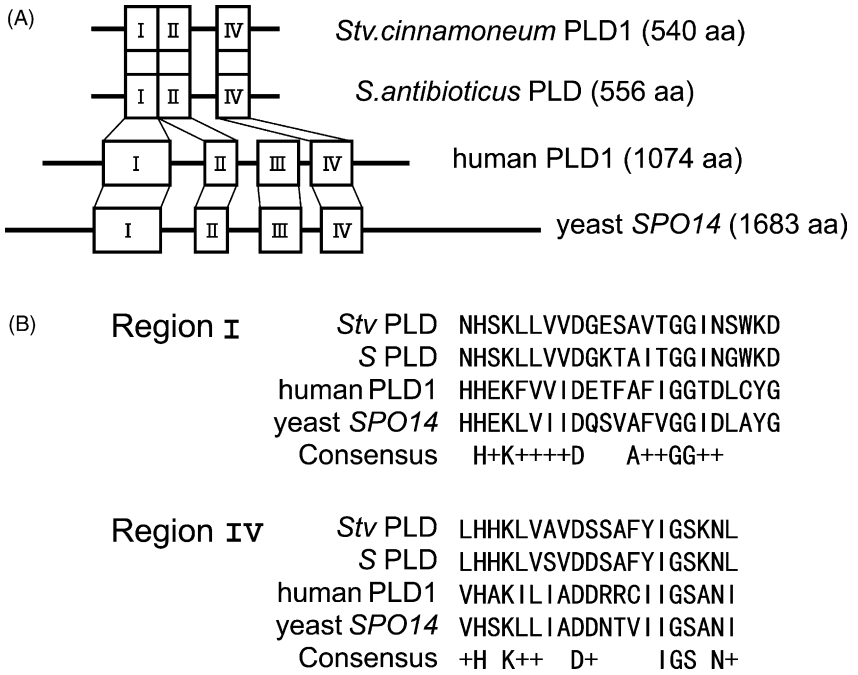

Fig. 3. Comparison of various PLD sequences. (A) Schematic representation of the PLD sequences. (B) Sequence alignment of homologous regions I and IV. Abbreviations: *Stv* PLD: *Stv. cinnamoneum* PLD; *S* PLD: *S. antibioticus* PLD. Identical residues are indicated in the consensus sequence, and similar residues are also indicated with + signs. The GenBank accession numbers for the compared sequences are AB007132 (*Stv. cinnamoneum*); D16444 (*S. antibioticus*); U38545 (human PLD1) and L46807 (yeast *SPO14*: *Saccharomyces cerevisiae* PLD1).

[41,42] and *Stv. cinnamoneum* [43]. There is no enzyme showing similar substrate specificity in the PLD superfamily. Neither purification nor sequence determination of these enzymes have been accomplished. Although the nucleotide sequence of the yeast genome has been already determined, no putative PLD gene other than the *SPO14* gene has been identified. This fact suggests that these enzymes possess a unique structure distinct from the PLD superfamily.

4. Crystal structures of PLD

Crystal structure of the PLD superfamily was first determined with a bacterial endonuclease Nuc [44]. Nuc is a protein of small molecular mass (16 kDa) having only one HKD motif and one GS motif, and forms a homodimer unlike other members of the PLD superfamily. Each HKD motif is positioned within two loops, and they associate with each other to form a single active site. The crystal structure was then obtained with the PLD enzyme of *Streptomyces* sp. strain PMF [39]. This enzyme is a monomer and consists of two domains, which contain two HKD motifs and two GG/S motifs (see Fig. 4). Both the crystal structures show a similar topology, strongly suggesting that all enzymes in the PLD superfamily share a similar structure. The postulated active site is located in the bottom of the center cleft of the molecule, and is surrounded by two HKD and two GG/S motifs. One molecule of phosphate was found in the active site of the crystal structure of PLD [39], which suggested the interaction of the phosphate polar head group of phospholipids with the HKD motifs in the active site.

Alignment of the primary structures of PLD from *Streptomyces* sp. strain PMF and *Stv. cinnamoneum* showed a high identity (>81%) in the overall sequence. Therefore, the 3-D structure of *Stv. cinnamoneum* PLD was predicted by a homology modeling method using the PLD structure of *Streptomyces* sp. strain PMF as a

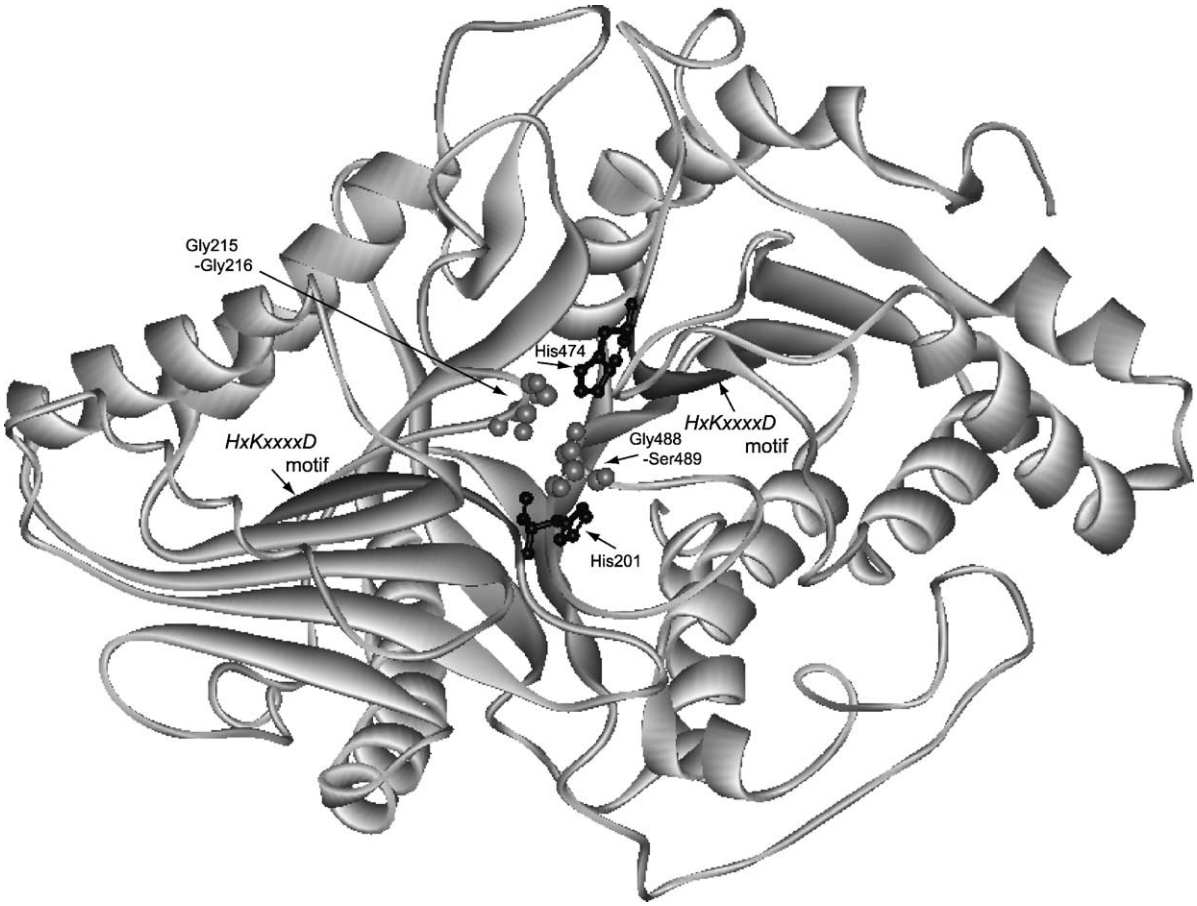

Fig. 4. 3-D structure of *Stv. cinnamoneum* PLD predicted by MODELLER. The twice repeated HKD motifs in the amino and carboxyl-terminal regions are both indicated by dark portion. The GG (G1y215-Gly216) and GS (Gly488-Ser489) in the GG/S motifs are represented with dark balls for all atoms. The histidine residue in each HKD motif is shown by a black ball-and-stick model.

template and a MODELLER 6 version 2 program [45]. The predicted structure of *Stv. cinnamoneum* PLD was very similar to the template structure (Fig. 4), corroborating that the active site of the *Stv. cinnamoneum* PLD is also surrounded by two HKD and two GG/S motifs.

5. Mutational studies on the active site residues

Site-specific mutagenesis studies on a mammalian PLD [46] and the *Y. pestis* toxin [47] showed that the two HKD motifs are indispensable for the catalytic activity of the PLD superfamily. The HKD motifs are presumed to work as a catalytic center, and the histidine residue in the HKD motif in region IV was shown to form a phosphatidylhistidine intermediate [46,48]. However, it is intriguing that the *S. chromofuscus* PLD possessing no HKD motif shows similarity of the overall structure to other bacterial enzymes and has both activities [49]. On the other hand, we found another sequence, a GG/S motif, conserved in the PLD superfamily. The two GG/S motifs surround the putative active site in a symmetric manner (see Fig. 4). Each glycine residue in the GG/S motifs of *Stv. cinnamoneum* PLD was substituted by a serine residue, and the serine residue also by a glycine residue (Ogino et al., unpublished results). As shown in Fig. 5, the G215S and G216S mutants exhibited approximately 9- and 16-folds higher transphosphatidylation activity,

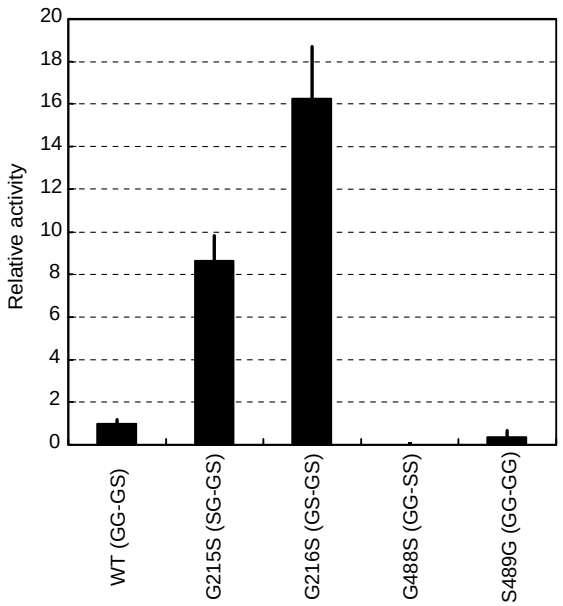

Fig. 5. Transphosphatidylation activity of mutant enzymes of *Stv. cinnamoneum* PLD. The transphosphatidylation activity was assayed by thin-layer chromatography (Ogino et al., unpublished results).

respectively, than the wild type PLD. On the contrary, the S489G mutant showed reduced activity and G488S lost the activity completely. It was concluded that the serine residue in each motif is preferable for the PLD activity, though dispensable itself. Based on the predicted structure, it was suggested that the GG/S motifs play an important role in the catalytic activity of PLD.

6. Production of PLD

Attempts to produce a substantial amount of eukaryotic PLD have failed so far. These eukaryotic enzymes were expressed either as an inactive or a membrane-bound form (see Section 1). Endogenous PLD enzymes of *Actinomycetes* are released into the medium and exhibit good stability against heat and organic solvents, which allows us to use the enzyme for phospholipid synthesis in an industrial scale. Many researchers have thus tried to isolate *Actinomycetes* secreting a large amount of PLD into the culture medium [4–6,13]. Our group succeeded in identifying *Stv. cinnamoneum* that secretes the largest amount of PLD among the bacterial strains examined [13]. However, the productivity of secretory PLD was not high enough even after improvement of the culture conditions (being less than 2 mg/l). Bacterial PLD genes were then transformed into *E. coli* for producing the recombinant enzyme. The transformant produced an insoluble form of PLD called an inclusion body [37] or a soluble form with low activity in the cytoplasmic fraction [50]. These situations prompted us to change the host cell from *E. coli* to *S. lividans* (Ogino et al., unpublished results). A DNA fragment containing the structural gene for *Stv. cinnamoneum* PLD and a terminator sequence was inserted into the *E. coli*/*S. lividans* shuttle vector pUC702 [51], and transformed into *S. lividans*. The transformant released about three-folds higher amount of active PLD into the medium than did the wild type strain (approximately 6 mg/l) (Fig. 6B). Additional insertion of a promoter sequence of the *Stv. cinnamoneum* PLD gene (Fig. 6A) was found to significantly enhance the secretion of active PLD (approximately 42 mg/l) (Fig. 6B). Since the *S. lividans* transformant can grow in the synthetic medium, the culture medium was shown to contain almost exclusively the recombinant PLD by SDS-PAGE analysis. We further examined the fed-batch culture to establish the mass-scale production of active PLD using the *S. lividans* transformant carrying the expression plasmid pUC702–promoter–PLD. The transformant finally released a markedly large amount of active PLD (about 118 mg/l) into the medium 60 h after the cultivation (Ogino et al., unpublished results). Cultivation of immobilized recombinant cells using biomass support particles (BSPs) [52], which make it easier to separate the culture medium from the cells, was also examined. The use of BSPs conferred the continuous and efficient production of PLD (about 53 mg/l for three batches). The system consisting of the *S. lividans* transformant, the fed-batch culture, and BSPs appears to be most suitable for producing the active PLD enzyme for phospholipid synthesis in an industrial scale, as described below.

7. Enzymatic phospholipid synthesis with PLD

As mentioned before, phospholipids have been recently recognized as an important and promising

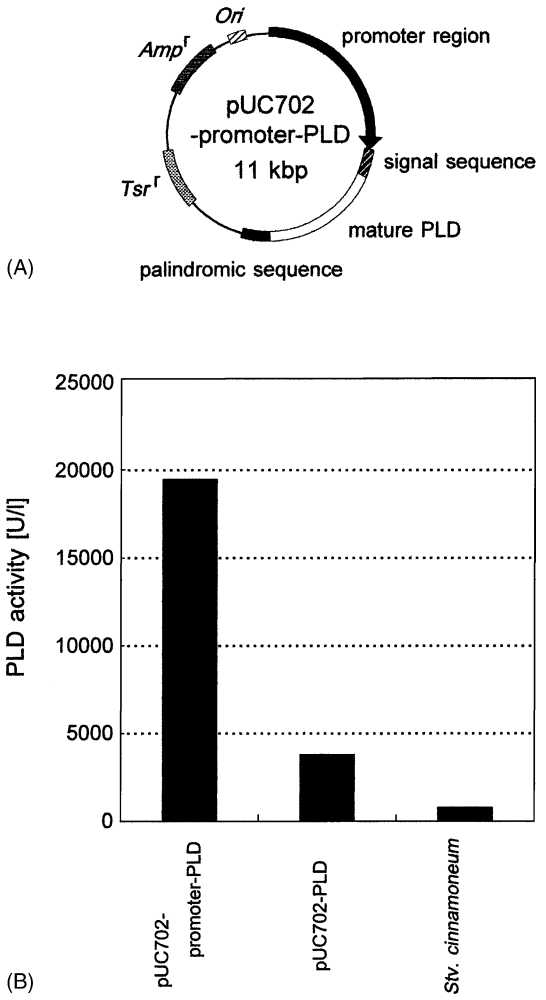

Fig. 6. Overexpression of *Stv. cinnamoneum* PLD by *S. lividans*. (A) Expression plasmid pUC702–promoter–PLD; *Amp*^{r}: ampicillin resistant gene; *Tsr*^{r}: thiostrepton resistant gene. (B) Comparison of the expression level of PLD by the transformed *S. lividans* and the wild type strain of *Stv. cinnamoneum*, all cultured for 30 h.

material for pharmaceuticals, cosmetics, and food staffs (see reference [53] for a detailed review covering many papers on enzymatic synthesis of phospholipids reported before 2000). Especially, the demand for pure phospholipids is increasing in various industries because of their inherent physiological activities [54] (see Section 1). The *Actinomycetes* PLD utilizes the inexpensive phospholipid, PC, for the transphosphatidylation activity and generates other phospholipids possessing various polar head groups in the presence of primary and secondary alcohols (as an acceptor molecule) (see reference [55]). As described above, PLD can catalyze the transphosphatidylation reaction in the two-phase solvent system [56,57]. We used the culture medium of *Stv. cinnamoneum* as an enzyme source and investigated the production of PE from PC and ethanolamine in the two-phase solvent system. For repetitive use of the enzyme solution, only the organic solvent phase (diethyl ether) was collected after each reaction. Even after 20 times repetition of the reaction, the conversion rate from PC to PE was maintained at more than 80% [13]. This result indicated that the culture medium of *Stv. cinnamoneum* is an ideal enzyme source for phospholipid synthesis in an industrial scale.

8. Conclusions

PLD catalyzes two distinct reactions, hydrolysis and transphosphatidylation, by a ping-pong mechanism. Although the two HKD and two GG/S motifs are important for the enzyme activity, the detailed reaction mechanism has still been unclear. The combination of extensive mutational analyses and crystal structure determinations will shed light on it. On the other hand, various phospholipids have been recognized as useful materials for pharmaceuticals, cosmetics, foods, and so on. Establishment of a mass-production technology for phospholipids has been awaited for a long time. We developed, for the first time, an efficient expression system for the active PLD and demonstrated its potentiality for phospholipid synthesis in an industrial scale. The use of the mutant enzymes of PLD (G215S and G216S) possessing 9- and 16-folds higher activity than the wild type will further improve the phospholipid synthesis. Our PLD-production system should promote both basic studies on PLD and its application to industrial phospholipid synthesis.

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