Purification, Characterization, and Sequence Determination of Phospholipase D Secreted by *Streptoverticillium cinnamoneum*[1]

Chiaki Ogino,* Yukinari Negi,* Toshiko Matsumiya,* Koichi Nakaoka,* Akihiko Kondo,† Shun'ichi Kuroda,‡,2 Shinji Tokuyama,§ Ushio Kikkawa,‡ Tsuneo Yamane,‖ and Hideki Fukuda*,3

*Graduate School of Science and Technology, †Faculty of Engineering, and ‡Biosignal Research Center, Kobe University, Kobe 657-8501; §Faculty of Agriculture, Shizuoka University, Shizuoka 422-8529; and ‖Graduate School of Bio- and Agro-Sciences, Nagoya University, Nagoya 461-8601

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Phospholipase D (PLD), secreted into the culture medium of an actinomycete, *Streptoverticillium cinnamoneum*, has been purified to homogeneity and characterized. The *Stv. cinnamoneum* PLD efficiently catalyzes both the hydrolysis and transphosphatidylation of various phospholipids, including phosphatidylethanolamine (PE), phosphatidylcholine (PC), and phosphatidylserine (PS). However, the substrate specificity differs between the two reactions; PE serves as the most preferred substrate for the hydrolysis, but PC and PS are better substrates than PE for the transphosphatidylation. In addition, the transphosphatidylation but not the hydrolysis of PE and PC is markedly activated on the addition of metal ions, especially Al^{3+}. Nucleotide and amino acid sequence determination of the *Stv. cinnamoneum* PLD revealed the presence of common structural motifs identified in all PLD sequences from various species.

Key words: phospholipase D (PLD), *Streptoverticillium cinnamoneum*, substrate specificity, transphosphatidylation.

Phospholipase D (PLD) is an enzyme occurring ubiquitously in various organisms (see Ref. *1* for a recent review). Its major substrate, phosphatidylcholine (PC), is hydrolyzed to phosphatidic acid (PA) and choline. PLD also catalyzes the interconversion of the polar headgroups of phospholipids through its transphosphatidylation activity; for example, PC is converted into phosphatidylethanol (PEt) in the presence of ethanol. Two major isozymes of PLD (PLD1 and PLD2) have so far been found in mammalian cells. The activity of PLD1 is extensively regulated by many cellular factors, such as phosphatidylinositol 4,5-bisphosphate (PIP_2), protein kinase C (PKC), and the small GTP-binding proteins, ARF and RhoA (*2*). PLD2 is constitutively active in the presence of PIP_2 and is not affected by any other cellular factors (*3*). These mammalian PLDs are postulated to alter the properties of cellular membranes by changing the lipid composition and hence induce many effects *in vivo*. Additionally, PA, the major product of mammalian PLDs, is known to activate many enzymes *in vivo* and is further converted to either diacylglycerol (a PKC activator) by phosphatidate phosphohydrolase or lysophosphatidic acid (a major extracellular signal) by phospholipase A_2 (*4*). Thus, mammalian PLDs have been proposed to be key enzymes in many cellular functions.

Recently, genes coding for PLD have been cloned from various sources including mammalian cells (*5-7*). Several regions of the deduced amino acid sequences, which are presumably essential for the PLD activity, are well conserved from bacteria to mammals (*8*). Furthermore, Secundo *et al.* (*9*) demonstrated by chemical modification that a Lys residue is essential for the PLD activity. Sung *et al.* (*10*) also demonstrated by site-directed mutagenesis that some residues (including a Lys residue) in human PLD1 are involved in the catalytic activity. However, unequivocal elucidation of the catalytic functions of mammalian PLDs must await further detailed structural analyses involving, for example, NMR and X-ray crystallography with a sufficient amount of the purified enzyme.

Certain bacteria belonging to the *Actinomycetes* are known to secrete PLD into the culture medium (*6*), and we recently found that an actinomycete, *Streptoverticillium cinnamoneum*, secretes the enzyme most efficiently among various *Actinomycetes* species examined (*11*). The amount of secreted enzyme (about 3 mg/liter of culture broth) was even higher than those produced on heterologous expression of recombinant enzymes from other sources (*12*).

[1] The nucleotide sequence data reported in this paper will appear in the DDBJ, EMBL, and GenBank nucleotide sequence databases under accession number AB007132.

[2] Present address: Department of Structural Molecular Biology, Institute of Scientific and Industrial Research, Osaka University, 8-1 Mihogaoka, Ibaraki, Osaka 567-0047.

[3] To whom correspondence should be addressed: Division of Molecular Science, Graduate School of Science and Technology, Kobe University, 1-1 Rokkodai-cho, Nada-ku, Kobe, Hyogo 657-8501. Tel: +81-78-803-1164, Fax: +81-78-803-1171, E-mail: fukuda@appchem.chme.kobe-u.ac.jp

Abbreviations: PA, phosphatidic acid; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PEt, phosphatidylethanol; PI, phosphatidylinositol; PIP_2, phosphatidylinositol 4,5-bisphosphate; PKC, protein kinase C; PLD, phospholipase D; PS, phosphatidylserine.

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Thus, the *Stv. cinnamoneum* PLD appears to be more suitable for structural studies on this class of enzymes than mammalian PLDs, whose purification is difficult due to their tight association with cellular membranes (*7, 8*). In this paper, we report the purification and catalytic properties of the enzyme secreted by *Stv. cinnamoneum*, with emphasis on its different substrate specificities in the hydrolysis and transphosphatidylation reactions, and the aberrant activation of the transphosphatidylation activity by Al^{3+} ions. The nucleotide sequence of the *Stv. cinnamoneum* PLD gene has also been determined.

MATERIALS AND METHODS

Materials—PC, PA, PEt, phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI) used as substrates for PLD were obtained from Doosan Serdary Research Laboratories (Englewood Cliffs, NJ, USA).

Spectrophotometric Assay for Hydrolysis Activity—A spectrophotometric assay for the hydrolysis activity of PLD (*13*) was routinely used in the enzyme purification. The reaction mixture (total volume, 100 μl) comprised an appropriate amount of enzyme, 0.5% (w/v) PC, 0.1% (v/v) Triton X-100, and 40 mM Tris-HCl (pH 7.4). After incubation at 37°C for 10 min, the reaction was terminated by the addition of 50 μl of a solution comprising 50 mM EDTA and 100 mM Tris-HCl (pH 7.4). The reaction mixture was cooled to room temperature, mixed with 500 μl of 20 mM potassium phosphate buffer (pH 7.6) containing 10.5 μg of phenol, 0.295 μg of 4-aminoantipyrine, 170 μg of choline oxidase, and 1 μg of peroxidase, and then incubated at 37°C for 5 min to allow color development. The absorbance of the reaction mixture was measured at 505 nm. A calibration curve was obtained by adding a standard solution of choline chloride to the reaction mixture instead of the enzyme solution. One unit (U_{hyd}) of hydrolysis activity was defined as the amount of enzyme which liberated 1 μmol of choline per min.

Thin Layer Chromatographic (TLC) Assay for the Hydrolysis and Transphosphatidylation Activities—The substrate specificity of the enzyme and the effects of metal ions were examined by means of a TLC assay (*14*). The reaction mixture (total volume, 100 μl), comprising an appropriate amount of enzyme, 100 μg of a phospholipid, 20% ethanol (only added for measurement of the transphosphatidylation activity), and 40 mM Tris-HCl (pH 7.4) was incubated at 37°C for 10 min, and the reaction products were extracted with 800 μl of an ice-cold solution of chloroform/methanol (1:1). The chloroform phase was lyophilized and then spotted onto a TLC plate (Silica gel 60 F_{254}; Merck KGaA, Darmstadt, Germany). The plate was developed with an upper phase of a mixture of ethyl acetate/2,2,4-trimethylpentane/acetic acid/water (13:2:3:10). Spots on the plate were visualized by staining with Coomassie Brilliant Blue R-250, and the intensity of each spot was quantitated with NIH image software (National Institutes of Health, Bethesda, MD, USA). The spots corresponding to PA and PEt produced on hydrolysis and transphosphatidylation, respectively, were identified by comparison with the PA and PEt standards. One U_{hyd} was defined as the amount of enzyme which produced 1 μmol of PA per min from phospholipid. On measurement of the hydrolysis of PC, the activity determined with the spectrophotometric assay was the same as that with the TLC assay. For the transphosphatidylation activity, one unit (U_{trans}) was defined as the amount of enzyme which produced 1 μmol of PEt per min from phospholipid.

Enzyme Purification—Unless otherwise mentioned, all operations were carried out at 4°C. *Stv. cinnamoneum* (IFO 12852) was cultivated in 5 liters of medium comprising 1% (w/v) glucose, 0.75% meat extract, 0.75% polypeptone, 0.3% NaCl, and 0.1% $MgSO_4 \cdot 7H_2O$ (MPD medium) with shaking at 30°C for 72 h. Ammonium sulfate (2,360 g) was added to the culture broth (final concentration, 70% saturation), and the mixture was kept at 4°C overnight. The precipitate was collected by centrifugation, and then redissolved in 20 mM Tris-HCl (pH 8.0) containing 1 mM EDTA (buffer A). The enzyme solution was loaded in several portions onto a Sephadex G-100 column (Pharmacia Biotech AB, Uppsala, Sweden; 22×450 mm) equilibrated with buffer A. The active fractions were combined and then applied to a hydroxyapatite column (Nacalai Tesque, Kyoto; 15×57 mm) equilibrated with buffer A. After washing the column with 100 ml of buffer A, the enzyme was eluted isocratically with 500 mM potassium phosphate in buffer A and then dialyzed against buffer A. To the enzyme solution (100 ml) was added NaCl (11.7 g), followed by application to a Phenyl-Sepharose CL-4B column (Pharmacia Biotech AB, Uppsala, Sweden; 15×28 mm) equilibrated with 2 M NaCl in buffer A. After washing the column with 50 ml of 2 M NaCl in buffer A, the enzyme was eluted with a reverse linear gradient (2.0-0 M) of NaCl in buffer A at the flow rate of 1 ml/min. The active fractions were pooled and then dialyzed against 20 mM acetate buffer (pH 5.8) containing 1 mM EDTA (buffer B). The enzyme solution (50 ml) was applied to a Macro-Prep High S column (Pharmacia Biotech AB, Uppsala, Sweden; 15×28 mm) equilibrated with buffer B. After washing the column with 50 ml of buffer B, the enzyme was eluted with a 280-ml linear gradient (0-0.3 M) of NaCl in buffer B. The active fractions were pooled and dialyzed thoroughly against 20 mM Tris-HCl (pH 8.0), and then used as the purified enzyme for subsequent experiments.

Amino Acid Sequence Determination—The purified enzyme (35 μg, 614 pmol) was pyridylethylated with 4-vinylpyridine, and then digested with 3 μg of lysyl endopeptidase (Wako Pure Chemical, Osaka) at 37°C overnight in 100 μl of 20 mM NH_4HCO_3, 10% (v/v) acetonitrile, and 0.1% (v/v) Triton X-100. The peptides were separated with a Perkin Elmer-Applied Biosystems model 173A liquid chromatography system (Foster City, CA, USA), and then analyzed with a Perkin Elmer-Applied Biosystems model 492 automated amino acid sequencer.

Gene Cloning and Nucleotide Sequence Determination—Chromosomal DNA of *Stv. cinnamoneum* was prepared according to the method of Hopwood *et al.* (*15*), and partially digested with *Sau*3AI. A *Stv. cinnamoneum* DNA library was constructed by subcloning the *Sau*3AI-digested fragments (2-5 kbp) into the *Bam*HI site of a cloning vector, pUC8. Because the partial amino acid sequences determined for the *Stv. cinnamoneum* PLD showed high similarity with the sequence of the *Streptomyces antibioticus* enzyme (*6*), as described later (see Fig. 5), a pair of PCR primers was synthesized based on the *S. antibioticus* sequence: 5′-CATCTGGACGCCATCGAG-3′ (primer A)

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and 5′-CGGCACCACGTTGAGGTG-3′ (primer B), corresponding to the sequences from His-55 to Glu-60 and His-174 to Pro-179, respectively, of the *S. antibioticus* PLD. PCR was performed using the *Stv. cinnamoneum* DNA library, as a template, primers A and B, and an Advantage-GC™ genomic PCR kit (CLONTECH Laboratories, Palo Alto, CA, USA). An amplified DNA fragment (375 bp) was labeled with [α-^{32}P]dCTP, and then used as a probe for colony hybridization screening of the *Stv. cinnamoneum* DNA library under highly stringent conditions. The nucleotide sequences of positive clones were determined with a Perkin Elmer-Applied Biosystems model 373S automated DNA sequencer. Since the positive clones encoded the 3′-truncated form of the *Stv. cinnamoneum* PLD gene, PCR was performed to amplify the 3′-flanking region with primer C (5′-TCATCTCCCAGCAGGACCT-GA-3′), corresponding to the nucleotide sequence 1295-1315 of the *Stv. cinnamoneum* PLD gene, and primer F (5′-GAGCGGATAACAATTTC-3′), corresponding to nucleotide sequence 40-56 5′-upstream from the *Bam*HI site of pUC8, using the *Stv. cinnamoneum* DNA library as a template. The nested 2nd PCR was performed with primer D (5′-CGCCCGCTACGACGTCCGCC-3′), corresponding to nucleotide sequence 1335-1354 of the *Stv. cinnamoneum* PLD gene, and primer E (5′-CAGGAAACAGCTATGAC-3′), corresponding to nucleotide sequence 20-38 5′-upstream from the *Bam*HI site of pUC8, using the product of the 1st PCR as a direct template. A DNA fragment (about 1 kbp) generated in the 2nd PCR was subcloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA, USA), and then analyzed as to the nucleotide sequence.

RESULTS AND DISCUSSION

Purification of Stv. cinnamoneum PLD—PLD excreted by *Stv. cinnamoneum* was purified from the culture medium by four column chromatographic steps (Table I). The active fractions eluted on the final chromatography gave a single band of a 54-kDa protein on SDS-PAGE analysis (Fig. 1, lane 5). Starting from 5 liters of the culture medium, 530 μg of the purified PLD with specific hydrolytic activity of 468 U_{hyd}/mg was obtained, which showed specific transphosphatidylation activity of about 1,600 U_{trans}/mg. These specific activities of the *Stv. cinnamoneum* PLD are similar to those of other actinomycete PLDs (*16*). Although the final yield of the purified enzyme is low and the purification method must be improved, *Stv. cinnamoneum* is probably the most efficient source for producing a substantial amount of enzymatically active PLD. The hydrolytic activity was optimum at pH 6 and around 50°C (Fig. 2), and the transphosphatidylation activity showed a similar optimum pH and temperature (data not shown). When the enzyme was incubated at 55°C and pH 7.4 for 24 h, both activities were completely lost, showing that the enzyme is rather unstable as to heat.

Substrate Specificity for Phospholipids—The phospholipid specificity of the *Stv. cinnamoneum* PLD was investigated with various phospholipids (PC, PE, PS, and PI) as substrates. As shown in Fig. 3A, the hydrolytic activity was about 3-fold higher for PE than for PC and PS, and very low for PI (PE > PC = PS ≫ PI). PLDs from other sources, such as mammals and plants, all hydrolyze PC, but are inactive toward PE and PS (*1*), while only the yeast PLD2 hydrolyzes PC, PE, and PS (*17*). On the other hand, the transphosphatidylation activity was about 2-fold higher for PC and PS than for PE (Fig. 3B), but the enzyme was almost inactive toward PI (PC = PS > PE ⋙ PI ≒ 0). Thus, the *Stv. cinnamoneum* PLD is a unique enzyme that has a broader phospholipid specificity than those from other sources, showing distinct substrate preferences in the hydrolysis and transphosphatidylation reactions.

Effects of Metal Ions—PLDs from various sources were reported to be activated by certain metal ions (*e.g.*, Ca^{2+} and Mg^{2+}) (*1, 2, 18*). The effects of metal ions on the activity of the *Stv. cinnamoneum* PLD were investigated by adding various metal salts (1 mM each of $CaCl_2$, $MgCl_2$, $MnCl_2$, $ZnSO_4$, and $AlCl_3$) to the reaction mixture. As a control, EDTA was added at 10 mM to deplete metal ions from the reaction mixture. When the hydrolysis of PC and PE was measured, the activity was not affected by metal

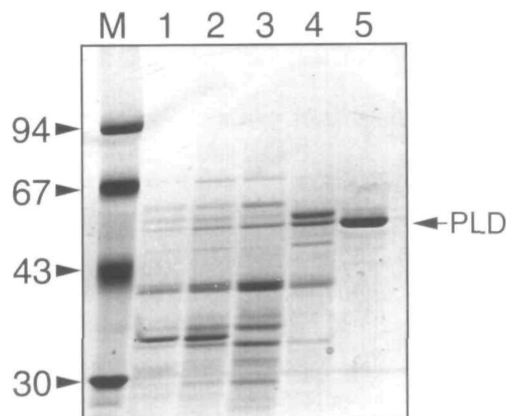

Fig. 1. **Purification of *Stv. cinnamoneum* PLD.** Enzyme samples from each purification step (total protein, about 5 μg) were analyzed by SDS-PAGE (12.5%) and stained with Coomassie Brilliant Blue R-250. Lane M, size marker proteins (kDa); lane 1, the culture medium of *Stv. cinnamoneum*; lane 2, the eluate from Sephadex G-100; lane 3, the eluate from hydroxyapatite; lane 4, the eluate from Phenyl Sepharose CL-4B; and lane 5, the eluate from Macro-Prep High S.

TABLE I. **Summary of the purification of *Stv. cinnamoneum* PLD.**

Purification steps	Total protein[a] (mg)	Total activity[b] (U_{hyd})	Specific activity (U_{hyd}/mg)	Purification (-fold)	Yield (%)
Culture medium	332	3,830	11.5	1.0	100
Sephadex G-100	90.5	1,760	19.4	1.7	46
Hydroxyapatite	32.4	1,070	32.9	2.9	28
Phenyl Sepharose CL-4B	10.7	927	87.0	7.6	24
Macro-Prep High S	0.53	250	468	41	6.5

[a]Protein was measured by the Bradford method according to the manufacturer's protocol (Bio-Rad Laboratories, Richmond, CA, USA). [b]Hydrolytic activity was assayed with PC as the substrate by means of a spectrophotometric assay (*13*).

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ions (Fig. 3A). In contrast, the transphosphatidylation activity for PC was enhanced by Mn^{2+} ions (about 1.8-fold) and Al^{3+} ions (about 2.5-fold) (Fig. 3B). The transphosphatidylation of PE was also enhanced markedly (about 6-fold) by Al^{3+} ions. Low concentrations of Al^{3+} ions (<5 μM) had no effect on the transphosphatidylation of PC (data not shown), while 5 mM Al^{3+} ion enhanced the activity most significantly (about 4.4-fold). Again, the hydrolytic activity was unaffected by the addition of various concentrations of Al^{3+} ions (up to 5 mM). High concentrations of Al^{3+} ions above 5 mM completely inhibited both activities (hydrolysis and transphosphatidylation), but the activities were recovered on removal of the Al^{3+} ions by dialysis (data not shown). These results suggest that Al^{3+} ions are bound reversibly to the enzyme and specifically activates the transphosphatidylation of PC and PE, the mechanism of which remains to be elucidated. It is unknown whether metal ions (including Al^{3+} ions) affect both activities for PS and PI or not.

It is unlikely that Al^{3+} ions, particularly at such high concentrations as above 1 mM, are of physiological significance for actinomycetes. Therefore, the marked activation by Al^{3+} ions of the transphosphatidylation is presumably a fortuitous phenomenon. However, it should be noted that the accumulation of Al^{3+} ions in the human brain has been suggested to be a possible cause of Alzheimer's disease, by altering the structure and function of the amyloid β protein, and also by inhibiting the class of enzymes (metalloproteases) associated with the processing and degradation of amyloid β (*19*). Thus, Al^{3+} ions may also participate in some physiological functions by interacting with PLDs in mammals, although the activation of mammalian PLDs by Al^{3+} ions has not been reported.

Gene Cloning and Nucleotide Sequence Determination—To obtain internal amino acid sequence information, the purified PLD was subjected to digestion with lysyl endopeptidase, and the peptides separated by reversed phase liquid chromatography were analyzed with an automated amino acid sequencer. Because the N-terminal (N1) and nine internal (I1-I9) amino acid sequences determined (a total of 151 amino acid residues; see Fig. 4) showed high homology (identity, >60%; similarity, >80%) with the relevant regions of the *S. antibioticus* PLD sequence (*6*), the PCR-amplified 375-bp DNA fragment corresponding to the sequence from His-55 to Pro-179 of the *S. antibioticus* PLD was used as a probe for gene cloning of the *Stv.*

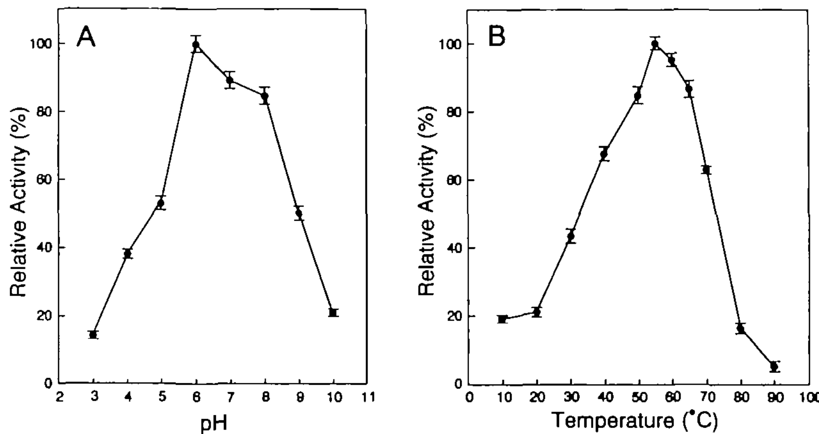

Fig. 2. **Effects of pH and temperature on the hydrolytic activity.** Panel A, the hydrolytic activity for PC was measured at 37°C in buffers of various pHs (pH 3, Gly-KCl; pH 4, citrate-HCl; pH 5, acetate-NaOH; pH 6-7, 3-morpholinopropanesulfonate; pH 8-9, Tris-HCl; and pH 10, Gly-NaOH), and is shown as percent activity relative to the activity at pH 6 taken as 100%. Panel B, the hydrolytic activity for PC was measured at various temperatures at pH 7.4 (adjusted to 25°C and uncorrected for different temperatures), and is shown as percent activity relative to the activity at 55°C taken as 100%. Error bars represent SD of three measurements.

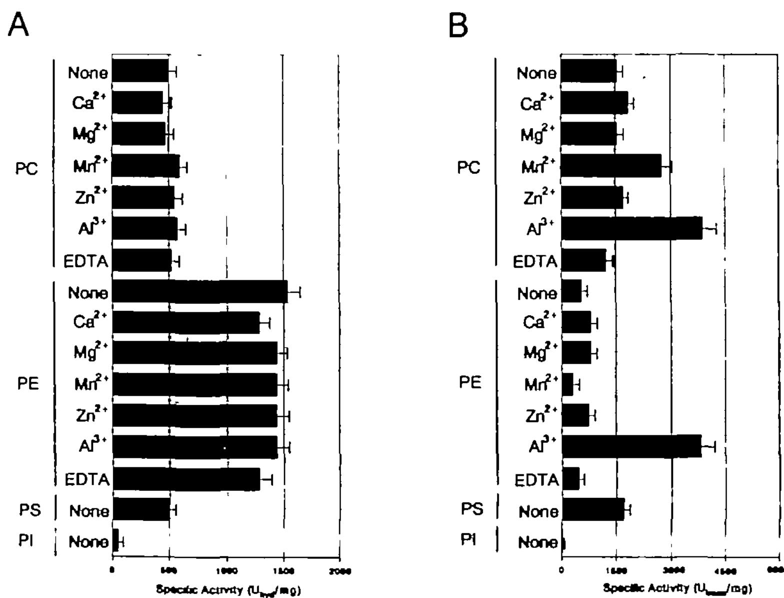

Fig. 3. **Substrate specificity and effects of metal ions.** The hydrolytic (panel A) and transphosphatidylation (panel B) activities were measured with PC, PE, PS, or PI as the substrate in the absence or presence of a metal ion (1 mM) or EDTA (10 mM), as described under "MATERIALS AND METHODS." Error bars represent SD of three measurements.

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cinnamoneum PLD. On colony hybridization screening of a genomic DNA library, four positive clones were isolated from among about 10^5 transformants. Because these clones were found to carry 3′-truncated forms of the PLD gene (three clones harboring nucleotides 1-1,290, and one harboring nucleotides 1-1,458, see Fig. 4), the 3′-terminal region of the PLD gene was obtained by the nested-PCR method as described under "MATERIALS AND METHODS." Finally, the nucleotide sequence (1,868 nucleotides) containing the full-length *Stv. cinnamoneum* PLD gene was determined (Fig. 4). The full-length sequence of the *Stv. cinnamoneum* PLD gene was similar to that of the *S. antibioticus* PLD gene (about 79%).

Judging from the N-terminal sequence (N1) determined for the purified PLD, the N-terminal residue (Ser) is encoded by an AGC codon (nucleotides 307-309). There are two in-frame stop codons, TAA (nucleotides 193-195) and TGA (nucleotides 1825-1827). In the region from the TAA stop codon to the AGC Ser codon (nucleotides 193-309), there is no ATG initiation codon. Some genes in Gram-positive bacteria including actinomycetes are known to utilize a TTG codon as an initiation codon [*e.g.*, the protein A gene (*20*) from *Staphylococcus aureus*]. In addition, there is a potential ribosome binding site (Shine-Dalgarno sequence) at nucleotides 193-200 (5′-TAAGGATG-3′). The TTG codon found at nucleotides 205-207 was therefore assumed to act as an initiation codon for the *Stv. cinnamoneum* PLD gene. Thus, the open reading frame was predicted to be 1,620-nucleotides long, starting at nucleotide 205 and ending at nucleotide 1824, and to encode a PLD precursor consisting of 540 amino acid residues (Fig. 4). As the secreted PLD (mature enzyme) has Ser at it N-terminus, it is considered that the precursor polypeptide possesses a cleavable N-terminal signal peptide (34 amino acid residues). The sequence of the predicted signal peptide meets the criteria for prokaryotic signal peptides (*21*). The calculated molecular weight of the mature protein (506 amino acid residues) is 53,879, which agrees well with the observed molecular mass of the purified PLD (54 kDa; see Fig. 1, lane 5).

Amino Acid Sequence Comparison—The deduced amino acid sequence of the *Stv. cinnamoneum* PLD shows the highest similarity with that of the *S. antibioticus* enzyme (identity, 71%; similarity, 85%), and relatively high similarity (>50%) with those of other bacterial PLDs. With the eukaryotic enzymes (yeasts, plants, and mammals), the *Stv. cinnamoneum* PLD exhibits low similarity (<30%). As reported for other PLDs (*10*), the *Stv. cinnamoneum* PLD also contains two HxKxxxxD motifs (x represents any amino acid residue) and a Ser/Thr residue (Ser-489) at the 8th position on the C-terminal side from the second HxKxxxxD motif (see Fig. 4). These conserved residues have been suggested to form the catalytic site involved in the hydrolysis or formation of a phosphoester bond (*10*).

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CCGTCCCGTGCCGCCCTGCCGGTGATCACGAAGCTATGGCCCGGACCTATCCGCTGAAATCCCTCTCGGAGGCGGCCTGCCGTACCTCTG    90
AGGACGGGTGGCCGAAGACCGTGTCATCCGTGAGATGTACGGCTCCCGGGAGCTGATAGCTTCTCCGCGTTGATCTTCCGTTCGCGGAAT   180
CGTTACTTCGTTTAAGGATGCACATTGCTCCGCCACCGGCTCCGCCGTTTACACCGTCTGACCCGCAGTGCGGCGGTCTCGGCCGTCGTC   270
            rbs        M  L  R  H  R  L  R  R  L  H  R  L  T  R  S  A  A  V  S  A  V  V     22

CTGGCCGCCCTGCCCGCGGCTCCGGCCTTCGCGAGCAGCCCCTCGCCCGCCCCGCACCTGGACGCCGTGGAGAAGGCGCTGCGCGAGGTC   360
 L  A  A  L  P  A  A  P  A  F  A  S  S  P  S  P  A  P  H  L  D  A  V  E  K  A  L  R  E  V    52
                                   N1                                  I1
TCACCGGGGCTGGAGGGTGACGTCTGGCAGCGCACCGACGGCAACAAGCTGGACGCCTCCGCCGCGGACCCCTCCGACTGGCTGCTGCAG   450
 S  P  G  L  E  G  D  V  W  Q  R  T  D  G  N  K  L  D  A  S  A  A  D  P  S  D  W  L  L  Q    82
                                                 I2
ACCCCCGGTTGCTGGGGCGACGCCGCGTGCAAGGAGCGTCCCGGCACCGAGCGCCTGCTCGCCAAGGTGACGGAGAACATCTCCAAGGCC   540
 I  P  G  C  W  G  D  A  A  C  K  E  R  P  G  T  E  R  L  L  A  K  V  T  E  N  I  S  K  A   112
                                                                                         I3
AGGCGCACGGTGGACATCTCCACGCTCGCGCCCTTCCCGAACGGTGCGTTCCAGGACGCGATAGCCGCCGGCCTCAAGGCGTCGGTCGCG   630
 R  R  T  V  D  I  S  T  L  A  P  F  P  N  G  A  F  Q  D  A  I  A  A  G  L  K  A  S  V  A   142

TCCGGCAACAAGCCGAAGGTCCGCGTCCTGGTCGGCGCCGCGCCGGTCTACCACATGAACGTACTGCCCTCGAAGTACCGGGACGACCTC   720
 S  G  N  K  P  K  V  R  V  L  V  G  A  A  P  V  Y  H  M  N  V  L  P  S  K  Y  R  D  D  L   172
                   I4
AAGGCCCGGCTCGGCAAGGCCGCCGACGACATCACGCTGAACGTCGCGTCGATGACGACGTCGAAGACCAGCTTCTCCTGGAACCACTCC   810
 K  A  R  L  G  K  A  A  D  D  I  T  L  N  V  A  S  M  T  T  S  K  T  S  F  S  W  N  H  S   202
                                                                                      #
AAGCTCCTCGTCGTGGACGGCGAGTCGGCCGTCACCGGTGGCATCAACAGCTGGAAGGACGACTACGTCGACACCCAGCACCCGGTGACC   900
 K  L  L  V  V  D  G  E  S  A  V  T  G  G  I  N  S  W  K  D  D  Y  V  D  T  Q  H  P  V  T   232
 #              #
GACGTGGACCTGGCGCTGACCGGCCCCGCCGCGAGCTCCGCCGGCCGCTACCTGGACACGCTCTGGACGTGGACGTGCCAGAACAAGAGC   990
 D  V  D  L  A  L  T  G  P  A  A  S  S  A  G  R  Y  L  D  T  L  W  T  W  T  C  Q  N  K  S   262

AACATCGCCAGTGTGTGGTTCGCGGCCTCGGGCGGCGACTGCATGGCCACGATGGAGAAGGACGCGAACCCCAGGCCCGCCGGGCCCACG  1080
 N  I  A  S  V  W  F  A  A  S  G  G  D  C  M  A  T  M  E  K  D  A  N  P  R  P  A  G  P  T   292
                                                             I5
GGCAACGTCCCCGTGATCGCCGTGGGCGGCCTCGGCGTCGGCATCAAGGACTCCGACCCCGCCTGGACGTTCCGCCCGCAGCTGCCCTCC  1170
 G  N  V  P  V  I  A  V  G  G  L  G  V  G  I  K  D  S  D  P  A  W  T  F  R  P  Q  L  P  S   322
                                                 I6
GCCCCGGACACCAAGTGCGTCGTCGGCCTGCCCGACAAGACCAACGCCGACCGTGACTACGACACGGTCAACCCCGAGGAGAGCGCCCTG  1260
 A  P  D  T  K  C  V  V  G  L  P  D  K  T  N  A  D  R  D  Y  D  T  V  N  P  E  E  S  A  L   352
                 I7
CGGGCCCTGGTGGCCAGCGCCGACCGCCAGATCGTCATCTCCCAGCAGGACCTGAACGCCACCTGCCCGCCCATCGCCCGCTACGACGTC  1350
 R  A  L  V  A  S  A  D  R  Q  I  V  I  S  Q  Q  D  L  N  A  T  C  P  P  I  A  R  Y  D  V   382

CGCCTCTACGACATCCTCGCCGCCAAGATGGCGGCCGGGGTGAAGGTGCGCATCGTCGTCAGCGACCCCGCCAACCGCGGCGCGGTCGGC  1440
 R  L  Y  D  I  L  A  A  K  M  A  A  G  V  K  V  R  I  V  V  S  D  P  A  N  R  G  A  V  G   412
                                                I8
AGCGGCGGCTACTCGCAGATCAAGTCCCTGGCCGAGATCAGCGACACGCTCCGCAACCGTCTCGCCCTGCTCAAGGGCGGCGACCAGCAG  1530
 S  G  G  Y  S  Q  I  K  S  L  A  E  I  S  D  T  L  R  N  R  L  A  L  L  K  G  G  D  Q  Q   442

AAGGCCAAGGCGGCCATGTGCTCCACCCTCCAGCTGGGGACCTTCCGCAGCTCCGCGAGCGCCACGTGGGCCGACGGGCACCCCTACGCC  1620
 K  A  K  A  A  M  C  S  T  L  Q  L  G  T  F  R  S  S  A  S  A  T  W  A  D  G  H  P  Y  A   472

CTGCACCACAAGCTGGTGGCGGTCGACAGCTCCGCCTTCAACATCGGCTCCAAGAACCTCTACCCCTCGTGGCTGCAGGACTTCGGCTAC  1710
 L  H  H  K  L  V  A  V  D  S  S  A  F  N  I  G  S  K  N  L  Y  P  S  W  L  Q  D  F  G  Y   502
    #     #           #                       #        I9
ATCGTGGAGAGCCCGGAGGCCGCCAAGCAGCTTGAGGCCAAGCTCCTCGACCCCGAGTGGAAGTTCTCGCAGGAGACCGCGACGGTCGAC  1800
 I  V  E  S  P  E  A  A  K  Q  L  E  A  K  L  L  D  P  E  W  K  F  S  Q  E  T  A  T  V  D   532

CACGCGCGGGGCGTCTGCTCGCTCTGAGACGACTGAGCGCCCGGACGTTCCGGGGCGCATGACGGGGG                         1868
 H  A  R  G  V  C  S  L  *                                                                    540
```

Fig. 4. **Nucleotide and deduced amino acid sequences of the *Stv. cinnamoneum* PLD gene.** Nucleotide and amino acid numbers are indicated at the right of each lane. The N-terminal (N1) and internal (I1-I9) amino acid sequences determined with the purified enzyme and lysyl endopeptidase-digested peptides, respectively, are underlined. A potential ribosome binding site (rbs) is also underlined. Two HxKxxxxD motifs and a Ser residue (Ser-489) conserved in all known PLD sequences are indicated by # marks. The sequence corresponding to the probe encoding His-55 to Pro-179 of the *S. antibioticus* PLD is boxed.

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Recently, eukaryotic PLDs were noted to share at least four homologous regions (I, II, III, and IV; Fig. 5A) (*8*). Bacterial PLDs including the *Stv. cinnamoneum* enzyme have three of these four regions (I, II, and IV; Fig. 5B). Because a cluster of aromatic amino acids in region III has been proposed to serve as the binding site for the choline portion of PC (*10*), the loss of region III in bacterial PLDs may contribute to their broader specificities for phospholipid substrates. Further comparison of the *Stv. cinnamoneum* enzyme with other PLDs revealed another homologous region (X) conserved in the N-terminal regions of bacterial and mammalian PLDs, but not in those of yeast and plant enzymes (Fig. 5A). The consensus sequence of region X (15 amino acid residues) shows no similarity to other protein motifs with known functions. In the corresponding region of plant PLDs, there is a Ca^{2+}/phospholipid-binding C2 domain (CalB domain) necessary for their Ca^{2+}-dependent activation (*18*). Therefore, it is speculated that region X of bacterial and mammalian PLDs also serves as a metal binding site (Ca^{2+} and Mg^{2+} for mammalian PLDs, and Al^{3+} for the *Stv. cinnamoneum* enzyme).

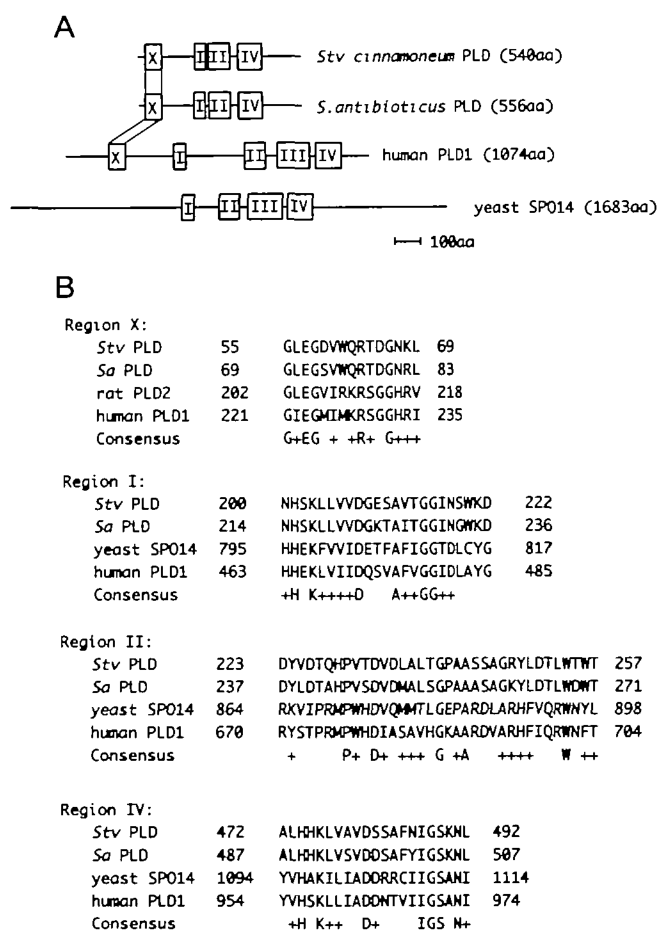

Fig. 5. **Sequence alignment of PLDs from various sources.** Panel A, schematic representation of the PLD sequences modified from Ref. *8*. The homologous regions including newly identified region X are indicated by open boxes. The GenBank accession numbers for the compared sequences are AB007132 (*Stv. cinnamoneum* PLD), D16444 (*S. antibioticus* PLD), U38545 (human PLD1), and L46807 (yeast SPO14 = *Saccharomyces cerevisiae* PLD1) (aa, amino acid residues). Panel B, sequence alignment of homologous regions X, I, II, and IV. Abbreviations: *Stv* PLD, *Stv. cinnamoneum* PLD; *Sa* PLD, *S. antibioticus* PLD. Residue numbers are shown on both sides. Identical residues are indicated in the consensus sequence and similar residues are also indicated by + signs.

Concluding Remarks—PLD is widely distributed in nature and catalyzes physiologically important reactions, *i.e.* the hydrolysis and transphosphatidylation of phospholipids. However, the structure-function relationship of the enzyme has been hardly studied due to the difficulty in the purification of a substantial amount of the enzyme. In this paper, we described the purification, characterization, and sequence determination of PLD secreted into the culture medium of *Stv. cinnamoneum*. Although the purification procedures need further improvements to increase the yield of the purified enzyme, *Stv. cinnamoneum* seems to be an ideal source of PLD for structural studies on PLD.

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