The Study on Phosphatidylinositol-Specific Phospholipase C from *Bacillus thuringiensis*: Synthesis of Homogeneous Substrates, Substrate Specificity and Other Properties

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The properties of phosphatidylinositol-specific phospholipase C (PI-PLC) from *Bacillus thuringiensis* were studied in detail. The enzyme was extremely thermostable in 0.1% bovine serum albumin and retained 73% of its activity at 100 °C for 10 min, while it was labile in the absence of albumin. The enzymatic activity was inhibited by $HgCl_2$ or *p*-chloromercuriphenylsulfonic acid and restored by dithiothreitol. The kinetic parameters (K_m and V_{max}) of PI-PLC were determined for the mixed micelle of yeast phosphatidylinositol (PI)/Triton X-100 or sodium deoxycholate. Four PIs having different acyl chains: dilauroylphosphatidylinositol (DLPI), dimyristoylphosphatidylinositol (DMPI), dipalmitoylphosphatidylinositol (DPPI) and dioleoylphosphatidylinositol (DOPI) were synthesized from yeast PI through the processes of deacylation and reacylation, identified by infrared (IR) and Fourier transform nuclear magnetic resonance (FT-NMR) spectra, and subjected to the action of PI-PLC. All the synthetic PIs were hydrolyzed by this enzyme, with DLPI and DMPI being the best substrates. PI-PLC did not catalyze the hydrolysis of the phosphatidylnucleosides 5′-phosphatidylcytidine, 5′-phosphatidyluridine, 5′-phosphatidylthymidine, 5′-phosphatidyladenosine and 5′-phosphatidyl-2′-deoxyadenosine.

Keywords phosphatidylinositol (PI); PI specific-phospholipase C; synthetic PI; substrate specificity; molecular property; *Bacillus thuringiensis*

Phosphatidylinositol (PI)-specific phospholipase C (PI-PLC), (EC 3.1.4.10) has been reported to be produced by several gram-positive bacteria.[2–7)] The bacterial PI-PLC s are now being used to investigate glycosylphosphatidylinositol (GPI)-anchored antigens and enzymes, since most of these membranous proteins have been shown to be released by the action of bacterial PI-PLCs.[4,8,9)]

PI-PLC from *Bacillus thuringiensis* catalyzes the hydrolysis of PI and lysophosphatidylinositol (lysoPI).[7)] The enzyme hydrolyzes PI to yield diacrylglycerol and *myo*-inositol 1,2-cyclic phosphate. Recently, complementary deoxyribonucleic acid (cDNA) cloning of this enzyme disclosed its amino acid sequence.[10,11)] We have been investigating the properties of this PI-PLC and its actions on biomembranes.[5,7)]

PI exists as a minor component in the biomembranes, where PI content is only 5—10% of total phospholipids. However, PI is one of the important phospholipids in biomembranes, since oligosaccharide-linked PI is used as a novel protein anchor, GPI-anchor. Also, PI is related to signal transduction such as PI-turnover and shown to modulate the properties of receptors or enzymes in the membranes. For example, the binding property of opiate receptor on the surface of rat brain membrane is changed by PI-PLC treatment, without significant release of the receptor.[12)] PI-PLC from *B. thuringiensis* is now widely used in biomembrane studies, although its properties have not yet been completely clarified. Thus, in this study we examined the heat stability, the effects of thiol reagents on PI-PLC activity and the substrate specificity for synthetic phospholipids.

Materials and Methods

Preparation of Yeast and Synthetic PIs PI of natural origin was purified from an autolysate of baker's yeast by batch operation with diethylaminoethyl (DEAE)-cellulose and silicic acid column chromatography according to Trevelyan's method.[13)] Dilauroylphosphatidylinositol (DLPI), dimyristoylphosphatidylinositol (DMPI), dipalmitoylphosphatidylinositol (DPPI) and dioleoylphosphatidylinositol (DOPI) were synthesized as follows: As the starting material, natural PI was purified from baker's yeast by DEAE-Sepharose CL-6B column chromatography instead of DEAE-cellulose batch treatment or silicic acid column chromatography. To 7.2 mmol of natural PI in 500 ml $CHCl_3$ in an ice bath were added 2.1 mol of 3,4-dihydro-α-pyran and then 8.1 mmol of anhydrous *p*-toluenesulfonic acid, in order to protect hydroxyl groups in the inositol moiety of PI. After stirring the mixture for 30 min, the chloroform layer was washed twice with a saturated aqueous solution of Na_2CO_3, dried over anhydrous Na_2SO_4 and concentrated by evaporation *in vacuo*. Thereafter, the concentrate was applied to silicic acid column which was then eluted with $CHCl_3$–CH_3OH (9 : 1). From the eluate was obtained 5.2 g (5.8 mmol) of modified PI whose inositol-hydroxyl groups were tetrahydropyranylated.

The preparation of modified PI was dissolved in 300 ml of $CHCl_3$ and mixed with 120 ml of 0.33 N KOH in CH_3OH. Then the mixture was stirred for 20 min under cooling in an ice bath, neutralized with 26 ml of ethyl formate and partitioned according to Folch *et al.*[14)] by addition of $CHCl_3$ and water ($CHCl_3 : CH_3OH : H_2O = 2 : 1 : 0.9$). The lower phase was withdrawn, washed with 10% aqueous $NaHCO_3$, dried over anhydrous Na_2SO_4 and concentrated by evaporation *in vacuo*. The concentrate was applied to silicic acid column and the column was eluted with $CHCl_3$–CH_3OH (7 : 3). From the eluate, 1.8 g (2.6 mmol) tetrahydropyranyl-3-glycerophosphoinositol was obtained.

Using fatty acid anhydrides prepared by the method of Selinger and Lapidot,[15)] tetrahydropyranyl-3-glycerophosphoinositol was acrylated in the presence of 4-(dimethylamino)-pyridine. The product was then purified by chromatographies with Amberlite IR-200C and silicic acid, and deprotected by removal of tetrahydropyranyl groups with *p*-toluenesulfonic acid under stirring in an ice bath. The synthetic PIs thus obtained were purified by silicic acid column chromatography and characterized by infrared (IR) spectrum, Fourier transform nuclear magnetic resonance (FT-NMR) and fast atom bombardment mass spectrometry (FAB-MS) using JASCO IR-810, JEOL FX-90Q and JEOL JMS-DX303, respectively.

PI-PLC and Other Reagents According to the method reported previously,[5)] PI-PLC was purified from the culture broth of *B. thuringiensis* IAM 12077 in a homogeneous state as indicated by polyacrylamide gel electrophoresis. The phosphatidylnucleosides 5′-phosphatidylcytidine (P-Cyd), 5′-phosphatidyluridine (P-Urd), 5′-phosphatidyladenosine (P-Ado), 5′-phosphatidylthymidine (P-Thd) and 5′-phosphatidyl-2′-deoxyadenosine (P-dAdo),[16)] were kindly supplied by Dr. S. Shuto, Research Laboratories, Toyo Jozo Co., Ltd. All other chemicals used were of analytical reagent grade unless otherwise stated.

The Assay of PI-PLC Activity The assay of PI-PLC activity was carried out by the method reported previously.[5)] The standard reaction mixture

for the enzyme assay contained 40 mM borate–NaOH (pH 7.5), 0.02% bovine serum albumin (BSA), PI-PLC and 2 mM PI with 0.16% sodium deoxycholate (SDC). After incubation of the mixture at 37 °C for 20 min, the reaction was stopped with $CHCl_3$–CH_3OH–HCl (66 : 33 : 1) and the mixture was centrifuged at $3000 \times g$ for 5 min. Then, aliquots were withdrawn from the upper layer of the mixture and subjected to phosphate analysis according to the method of Eibl and Lands,[17] after oxidation of organic phosphate by the method of Fiske and Subbarow.[18] One unit of enzyme activity was defined as the amount which catalyzed the hydrolysis of 1 μmol yeast PI per min at pH 7.5 and 37 °C.

Substrate Specificity of PI-PLC The reaction mixtures containing 40 mM borate–NaOH (pH 7.5), 0.02% BSA, 10—50 munits of PI-PLC and 2 mM synthetic or yeast PI with 0.2% Triton X-100 or 0.16% SDC, were incubated at 37 °C for 15 min. At the same time, the reaction mixtures without detergents were also incubated as the control runs. After incubation, the hydrolysis of PI was estimated using the same procedures an in the routine PI-PLC assay mentioned above.

Estimation of Protein Protein contents were determined according to the method of Lowry *et al.*[19] with BSA as standard.

Treatment with Thiol Reagents To investigate the effects of thiol reagents, PI-PLC was preincubated in the presence of 0.05% BSA, 0.05—5 mM $HgCl_2$, *p*-chloromercuriphenylsulfonic acid (PCMBS) or dithiothreitol (DTT) at 25 °C for 30 min, prior to the activity measurements.

Results

Unique Thermostability of *B. thuringiensis* PI-PLC First, *B. thuringiensis* PI-PLC was diluted with 0.1% fresh BSA and heated immediately at various temperatures for 5 or 10 min (Fig. 1a). In general, the enzyme activity was extremely stable in the presence of BSA: after heating at 70 °C, PI-PLC retained 60% of its activity, which was the minimum. It must be noted that 80—90% of the activity was reserved after heating at 80—90 °C, and that 73% still remained even after heating at 100 °C for 10 min. When PI-PLC was diluted with freezed-and-thawed BSA, however, the enzyme became thermolabile and lost its activity almost completely at 80 °C, although the activity was partially restored at 100 °C. When PI-PLC was diluted with 40 mM borate–NaOH (pH 7.5) instead of 0.1% BSA, the activity gradually decreased as the heating temperature rose (Fig. 1b). The temperature for half inactivation was approximately 45 °C. In this case also, 12.6% of the activity remained at 100 °C for 5 min.

Effect of Thiol Reagents on PI-PLC Activity As shown in Table I, the activity of PI-PLC was inhibited by treatment with $HgCl_2$ or PCMBS, and the inhibition was nearly complete at 0.5 or 5 mM, respectively. The value of IC_{50} for $HgCl_2$ or PCMBS was 0.13 or 1.95 mM, respectively. On the other hand, the treatment with DTT was essentially without effect. It was interesting that the activity of $HgCl_2$-treated enzyme was completely restored by DTT of 10-fold excess to $HgCl_2$ (Fig. 2). Similarly, the activity of PCMBS-treated PI-PLC was restored by excess DTT (data not shown).

Kinetic Parameters of PI-PLC for PI/SDC and PI/Triton X-100 Mixed Micelles Table II shows the values of K_m and V_{max} of PI-PLC for yeast PI/detergent mixed micelles. Based on the study of PI-PLC action toward micellar PI,[7] the molar ratio of Triton X-100 or SDC to PI in the mixed micelle was kept at 0.8 or 1.93, respectively, where the enzyme activity was maximally stimulated. The values of K_m and V_{max} for PI/Triton X-100 were smaller than those for PI/SDC.

Synthesis of Four Synthetic PIs From purified yeast PI, the PIs DLPI, DMPI, DPPI and DOPI were synthesized by tetrahydropyranylation with 3,4-dihydro-α-pyran, subsequent release of fatty acids with methanolic KOH and reacylation of glycerolic hydroxyl groups with acyl

Table I. Effects of Thiol Reagents

Reagent	Concentration	Relative activity (%)	IC_{50}
Control		100	
PCMBS	5 mM	2.7	
	500 μM	90	1.95 mM
	50 μM	96	
$HgCl_2$	5 mM	0	
	500 μM	1.2	0.13 mM
	50 μM	78	
DTT	5 mM	135	
	500 μM	125	
	50 μM	120	

Table II. Kinetic Parameters of PI-PLC

Detergents	K_m (mM)	V_{max} (μmol/min mg protein)
SDC	5.0	3280
Triton X-100	2.4	574

The determination of parameters was carried out using Woolf-Augstinsson-Hofstee's plot.

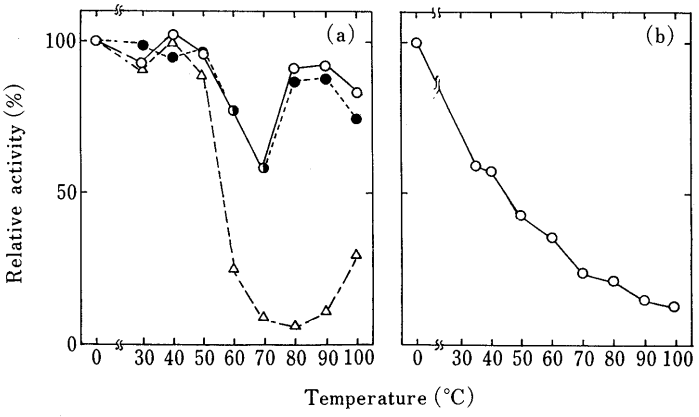

Fig. 1. Heat Stability of PI-PLC from *B. thuringiensis*

The enzyme activity was determined after heating at the temperatures indicated as in (a) and (b), and expressed as percentages of the activity after standing at 0 °C for 5 or 10 min (unheated control). (a) The enzyme solution (0.1 unit PI-PLC/ml), obtained by dilution with fresh (○, ●) or (△) freezed-and-thawed 0.1% BSA, was heated for 5 min (○, △) or 10 min (●). (b) The enzyme solution (0.1 unit PI-PLC/ml), obtained by dilution with 40 mM borate–NaOH (pH 7.5), was heated for 5 min.

TABLE III. Properties of Four Synthetic PIs

PI	IR	FT-NMR	FAB-MS	Recovery (%)
DLPI	3390, 2940, 1740, 1460	0.87 (t), 1.25 (m)	721 $[M+Na]^+$, 743 $[M+2Na-H]^+$	32
DMPI	3390, 2950, 1740, 1460	0.87 (t), 1.25 (m)	777 $[M+Na]^+$, 799 $[M+2Na-H]^+$	43
DPPI	3360, 2950, 1730, 1450	0.87 (t), 1.27 (m)	833 $[M+Na]^+$, 855 $[M+2Na-H]^+$	37
DOPI	3390, 2950, 1730, 1460	0.87 (t), 1.26 (m)	885 $[M+Na]^+$	34

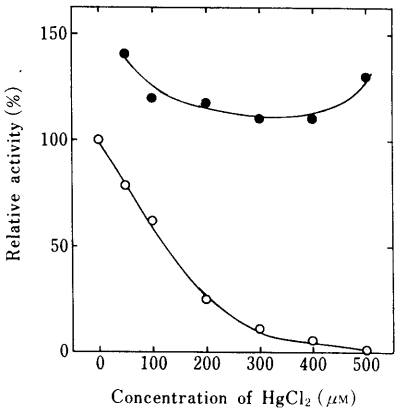

Fig. 2. Effect of DTT on the Activity of Hg^{2+}-Treated PI-PLC

The enzyme (10 munits) in 40 mM borate–NaOH (pH 7.5) was preincubated with 50—500 μM $HgCl_2$ at 25 °C for 30 min, and further incubated for 30 min in the absence (○) or presence (●) of DTT whose concentration was made to be 10 times as much as that of $HgCl_2$. After incubation with the effectors, the enzyme activity was determined as described in Materials and Methods.

anhydrides, followed by removal of tetrahydropyranyl groups from the *myo*-inositol moiety.

As shown in Table III, synthetic PIs were recovered at the rate of 32—43%. Each of them was characterized by IR and FT-NMR spectra as one of the monophosphoinositides (Table III) and finally identified by FAB-MS charts (Fig. 3 and Table III) on the basis of the chain lengths of fatty acids (C12–C18).

The action of PI-PLC toward synthetic PIs and phosphatidylnucleosides. As shown in Table IV, all the synthetic PIs were effectively hydrolyzed by PI-PLC. In the absence of detergents, the enzymatic hydrolysis of DLPI became 5-fold that of purified yeast PI, being the largest among those of vesicular PIs. Both DMPI and DPPI were hydrolyzed at the half rate or less of DLPI hydrolysis. As shown in the degradation of yeast PI,[7] the PI-PLC activity toward micellar, synthetic PIs was significantly stimulated in the presence of the detergents Triton X-100 and SDC, except in the system using DPPI or DOPI/Triton X-100 mixed micelle as the substrate. In spite of the greatest hydrolysis being observed in the absence of the detergents, the enhancement of DLPI hydrolysis was not as high in the presence of Triton X-100, whereas the hydrolysis of yeast PI or DMPI was enhanced by 6- or 2-fold, respectively. Thus, yeast PI and DMPI were hydrolyzed at almost the same rate in the presence of Triton X-100. In the presence of SDC, more extensive stimulation was observed in the action of PI-PLC toward all synthetic PIs as well as yeast PI. Especially, nearly 10-fold enhancement was observed in the enzymatic hydrolysis of yeast PI or DMPI. As the result of this marked stimulation, the hydrolysis of DMPI became almost comparable to that of DLPI. The hydrolysis of the sole unsaturated PI, DOPI, was also stimulated in the

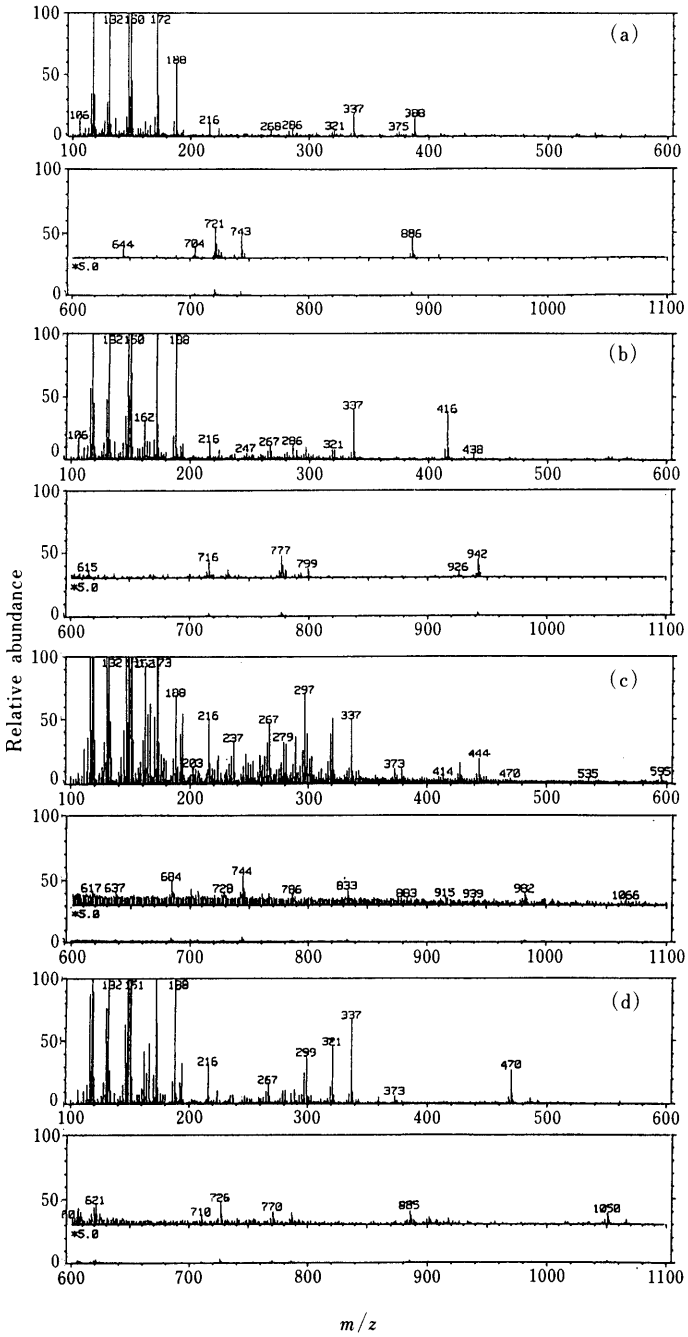

Fig. 3. Positive ion FAB-MS of Synthetic PIs

In (a) DLPI, (b) DMPI, (c) DPPI and (d) DOPI, both m/z 188 and 337 correspond to a triethylamine matrix.

presence of SDC, exceeding that of yeast PI. Among synthetic PIs, DPPI showed the lowest sensitivity to both the enzymatic hydrolysis and its stimulation by SDC.

We also examined the enzymatic hydrolysis of synthetic phosphatidylnucleosides in the presence of SDC, where

TABLE IV. Hydrolytic Activity of PI-PLC toward Synthetic PIs and Phosphatidylnucleosides

	Activity (μmol/min mg protein)		
	No detergent[a]	Triton X-100[b]	SDC[c]
Yeast PI	21.3	123	237
DLPI	115	129	510
DMPI	49.5	101	476
DPPI	38.4	29.7	89.3
DOPI	45.9	46.9	280
P-Cyd	n.d.	n.d.	(—)
P-Urd	n.d.	n.d.	(—)
P-Ado	n.d.	n.d.	(—)
P-Thd	n.d.	n.d.	(—)
P-dAdo	n.d.	n.d.	(—)

a) PI-PLC: 50 munits. *b*) PI-PLC: 20 munits. *c*) PI-PLC: 10 munits. n.d.: not determined.

PI-PLC activity was maximally exhibited. The enzyme activity was first determined by routine assay system, adding 2 mM phosphatidylnucleosides instead of PI. However, none of five phosphatidylnucleosides was hydrolyzed by 10 munits of PI-PLC under the conditions examined, as shown in Table IV. The possibility of enzymatic hydrolysis was pursued in the presence of 100 munits of PI-PLC with prolonged incubation (30 min). However, PI-PLC failed to catalyze the hydrolysis of P-Cyd, P-Urd, P-Ado, P-Thd or P-dAdo.

Discussion

Bacterial PI-PLCs are now extensively used in studies on GPI-anchored proteins and related lipophosphoglycans. PI-PLC from *B. thuringiensis* which we first found,[7] is now commercially available, and is the most widely used PI-PLC in this field. Some properties of this enzyme have been studied.[7,20-22] According to the sequence analysis of recently cloned cDNAs,[10,11,22] the amino acid sequences of PI-PLCs from *B. thuringiensis* and *B. cereus* have recently been predicted to show 97% homology; only 8 of 298 amino acid residues are different. Also, some properties of PI-PLC from *B. cereus* have been reported.[4,20,23-26] Using ^{31}P-NMR, Volwerk *et al.*[25] have unequivocally demonstrated a two-step mechanism for PI-PLC from *B. cereus* involving sequential phosphotransferase and cyclic phosphodiesterase activities, which reasonably explains the production of both *myo*-inositol-1,2-cyclic and 1-phosphates we originally suggested.[4] However, substrate specificities of PI-PLC from *B. cereus* and *B. thuringiensis* have not been as extensively investigated. Both enzymes catalyze the hydrolysis of lysoPI[7,23] as well as PI and GPI anchors from natural sources. Lin *et al.*[26] examined stereospecificity of PI-PLC from *B. cereus* using a mixture of (R_p)- and (S_p)-1,2-dipalmitoyl-*sn*-glycero-3-thiophosphoinositol. According to their observation, R_p isomer is preferentially hydrolyzed by the action of PI-PLC.

In the present study, we succeeded in the synthesis of PIs esterified with the same fatty acids at the 1 and 2 positions of glycerol, utilizing the difference in the conditions of hydrolysis between diacylglycerols and D-*myo*-inositol-tetrahydropyranyl ethers; ester linkages of the former are sensitive to alkaline hydrolysis while ether linkages of the latter are cleaved easily under acidic conditions. From FT-NMR and FAB-MS analyses, we confirmed the success of the synthesis of PIs. The procedures for PI synthesis were reproducible with sufficient recoveries. Thus we investigated the action of PI-PLC on the synthetic PIs differing in their lengths of fatty acyl chains attached to glycerol moiety, and on phosphatidylnucleosides having a phosphodiester linkage between hydroxyl groups of diacylglycerols and D-ribofuranose instead of D-*myo*-inositol. All the synthetic PIs proved to be hydrolyzed by this enzyme, while the enzyme failed to hydrolyze phosphatidylnucleosides. Thus PI-PLC from *B. thuringiensis* preferentially acts on phosphodiesters linked to D-*myo*-inositol ring, but not on those attached to D-ribofuranose ring. The enzyme probably has a spatial or conformational specificity for D-*myo*-inositol.

Effects of the detergents Triton X-100 and SDC on the substrate specificity were rather complicated. Generally, both detergents stimulated the action of PI-PLC toward synthetic and yeast PIs, although the activities were not evenly enhanced. In earlier reports we showed that SDC is much more effective in the stimulation of PI-PLC of *B. thuringiensis* than Triton X-100, in the foregoing reports.[7,27] Also from this study, the activity of PI-PLC proved to be maximally exhibited in the presence of SDC. The anionic charge of micellar PIs is probably more effectively dispersed in the presence of an anionic detergent, SDC. Such a dilution effect might favor the enzymatic breakdown of PIs by PI-PLC, in that the enzyme becomes more accessible to the micellar surface of PIs. From Table IV, it is also concluded that PIs having saturated, short-chain fatty acids (DLPI, DMPI) are generally good substrates in the presence of detergents such as Triton X-100 and SDC. In the presence of SDC in particular, DLPI and DMPI were hydrolyzed by PI-PLC at the highest rate among the synthetic PIs tested. Therefore, DMPI and DLPI seemed to be the best substrates for the assay of PI-PLC from *B. thuringiensis*. Since PIs from natural sources such as yeast and liver are the mixtures of several molecular species differing in constituent fatty acids, they are not ideal substrates for PI-PLC assay. Due to unsaturated fatty acids in the diacylglycerol moiety, natural PIs are rather sensitive to air oxidation, while synthetic, saturated PIs are completely stable and resistant to oxidation. In the usual enzyme assay, the reaction mixture is extracted with $CHCl_3$–CH_3–HCl (66 : 33 : 1) at the end of enzyme reaction, and Phosphate content of the methanol–water phase is estimated by spectrophotometry or by radioactivity measurement. When DLPI was used as a substrate, high blank values were obtained since nearly 10% of the substrate was partitioned to the methanol–water layer on extraction with $CHCl_3$–CH_3OH–HCl. Therefore, DMPI must be the best substrate for PI-PLC assay among the synthetic and yeast PIs tested.

PI-PLC from *B. thuringiensis* exhibited abnormal thermostability in the presence of albumin (Fig. 1). The enzyme was slightly inactivated around 70 °C but reactivated at higher temperatures. Such an anomalous response to heat treatment was also observed in the case of phosphatidylcholine-hydrolyzing phospholipase C (α-toxin) from *Clostridium perfringens*.[28,29] In the absence of albumin, however, the activity of PI-PLC fell with rise in temperature. This suggests that the activity of PI-PLC is extremely

unstable to heat in the dilute solution and that the addition of albumin serves to stabilize the enzyme by supplementing a sufficient concentration of protein.

We reconfirmed the inhibitory effects of thiol blockers such as Hg^{2+} and PCMBS on the activity of PI-PLC as reported earlier,[20,30] and further demonstrated reactivation of the inhibited enzyme by DTT. We initially speculated that could be a cysteine residue which might be involved in the enzyme activity. However, as Low recently pointed out,[31] the single cysteine residue predicted from the nucleotide sequences of cDNA is present in the N-terminal signal peptide and not in the mature protein molecules of PI-PLCs from *B. thuringiensis* and *B. cereus*.[10,11,22] At present, the discrepancy between the amino acid sequences and the behavior of enzyme inhibitors remains unsolved. Also, the results suggest another possibility: that the inhibition of PI-PLC by Hg^{2+} might be caused by the salt formation between C-terminal or side-chain COOH (Glu, Asp) of the enzyme molecule and Hg^{2+}, and that the enzyme activity is restored by DTT simply at the result of the removal of Hg^{2+}.

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