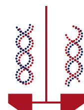

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Salt-induced increase in the yield of enzymatically synthesized phosphatidylinositol and the underlying mechanism

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The purpose of this study was to improve the efficiency of enzymatic synthesis of phosphatidylinositol (PI) from phosphatidylcholine (PC) and *myo*-inositol in a phospholipase D (PLD)-mediated transphosphatidylation. A conventional biphasic reaction system consisting of ethyl acetate and an aqueous buffer afforded PI with a yield of 14 mol%. In contrast, the reaction performed in the presence of high concentration (0.8–4.3 M) of NaCl in the aqueous phase showed improved PI yield in a NaCl concentration-dependent manner. At 4.3 M NaCl, PI yield of as much as 35 mol% was achieved. The increase in the PI yield offered by other tested salts varied; however, we observed that some salts caused inactivation of the enzyme when used at high concentrations. Although NaCl at high concentration increased the apparent hydrolytic activity on aggregated PC, it decreased the activity towards monomeric PC, indicating that high concentration of salt intrinsically inhibits the enzyme. Binding assays revealed that PLD re-localized from the aqueous phase to the solvent–buffer interface, where the enzymatic reaction takes place, in the presence of both, the salt and PC. Hence, we concluded that improvement of the PI synthesis in the presence of salt occurs mainly due to the accumulation of the enzyme at the interface by strengthening the hydrophobic interactions, by which the apparent activation outweighs the salt-induced inhibitory effect. Using this improved system, several PI with defined structures, namely *sn*-1, 2-dioleoyl-PI, *sn*-1-palmitoyl-2-oleoyl-PI, and *sn*-1-stearoyl-2-arachidonoyl-PI, were successfully synthesized with overall yields of 25–37%, and PI isomeric purities of 91–96%.

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[**Key words:** Phospholipase D; Phosphatidylinositol; Salt effect on enzymatic activity; Transphosphatidylation; Enhanced interfacial binding]

Phosphatidylinositol (PI) is a phospholipid species present mainly in eukaryotic organisms. Its biological properties, related to its role in lipid metabolism, make PI an attractive biomolecule for further studies and applications. For example, Sparks's research group reported that PI stimulates reverse cholesterol transport in rabbits (1,2), and that orally administered PI increases high-density lipoprotein cholesterol levels in humans (3), indicating the therapeutic value of PI. In addition, Yanagita's research group demonstrated that supplementary PI decreases triacylglycerol levels in the serum and liver of rats and mice (4,5) and prevents nonalcoholic fatty liver disease in rats (6). A recent report claims that dietary PI influences immune functions and prevents induced pathogenesis and development of liver injury in mice (7). Honda et al. (8) demonstrated that PI has a high level of safety with respect to oral toxicity and genotoxicity. Therefore, the use of PI as a food supplement is of great interest. However, the current industrial production of PI relies solely on its extraction from natural sources, such as soy lecithin.

Phospholipase D (PLD) is an enzyme that hydrolyzes phospholipids into phosphatidic acid (PA) and corresponding head group alcohols. In the presence of an alcohol, PLD also catalyzes transphosphatidylation, during which the polar head group of the phospholipid can be exchanged with the co-existing alcohol to generate different phospholipid species. Thus, PLD-mediated transphosphatidylation of phosphatidylcholine (PC) with *myo*-inositol is an attractive alternative route for PI production.

PLDs from *Streptomyces* strains are preferred for industrial applications because they have high transphosphatidylation efficiency and are easy to prepare (9). However, a critical drawback of the wild-type *Streptomyces* PLDs is their inability to synthesize PI because the bulky *myo*-inositol cannot fit into the active site of the enzyme. Although two plant PLDs have been reported to catalyze PI synthesis (10,11), it has not been completely elucidated whether these plant PLDs can be used for industrial applications.

In our previous studies, we developed a PI-synthesizing PLD by protein engineering approach. Starting from the wild-type *Streptomyces antibioticus* PLD (SaPLD) with no PI-synthesizing activity, site-specific saturation mutagenesis was performed followed by high-throughput screening that resulted in the isolation of PI-synthesizing PLD variants. The obtained PLD variants catalyzed the generation of PI as a mixture of several positional isomers containing phosphatidyl group attached to different positions of the six hydroxyl groups of inositol (12). Since only 1-PI with phosphatidyl group linked to the 1-hydroxyl group of inositol is naturally occurring, PLD variants that predominantly produce 1-PI over other isomers were isolated (13,14). Further fine-tuning by a modification of the substrate-binding pocket afforded a variant with excellent 1-PI specificity (15).

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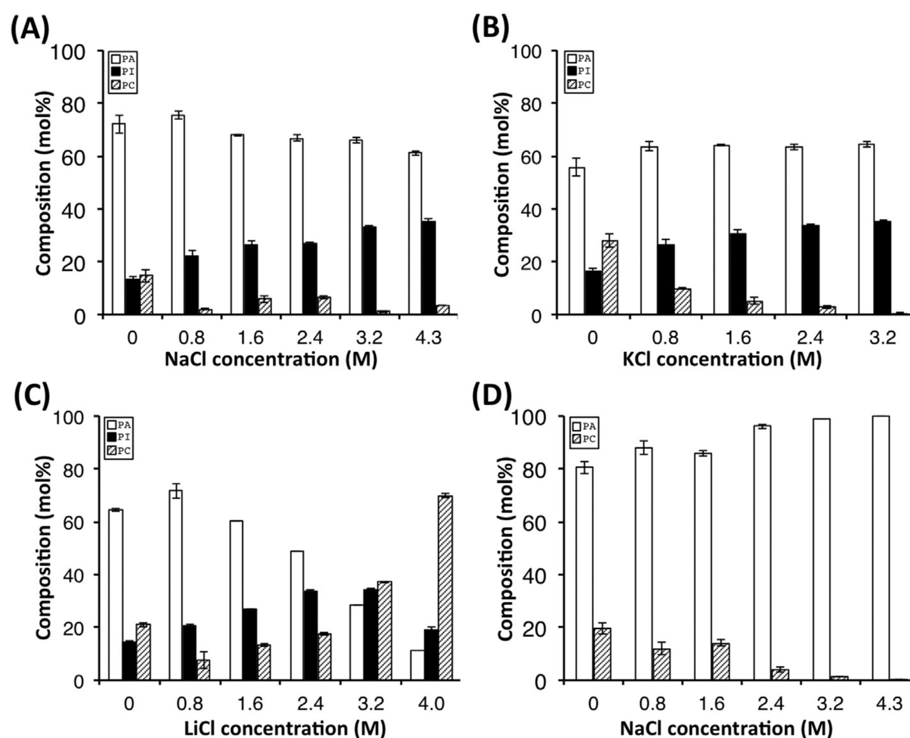

FIG. 1. Effect of salts on PLD-catalyzed reaction in the biphasic system. PI synthesis (A–C) and PC hydrolysis (D) were performed for 24 h in the presence of different concentrations of NaCl (A, D), KCl (B), and LiCl (C). The composition of phospholipids including PA (open columns), PI (closed columns), and PC (hatched columns) was analyzed by HPLC. Data are expressed as the mean ± standard error of two independent HPLC measurements.

Nevertheless, the total yield of PI obtained by the enzymatic synthesis was not satisfactory. To improve PI yield, we developed thermostable PLD variants that can catalyze the reaction at high temperatures. The high temperatures increase the solubility of *myo*-inositol, ensuring its excess in the reaction system, thereby promoting the PI yield (16,17). However, we then recognized another serious limitation: the 1-PI positional specificity of the engineered enzyme was not maintained at high temperatures (15). Hence, a different approach was necessary to improve PI yield without compromising the positional specificity.

Here, we report a simple and, yet, efficient method to improve PI yield by optimization of the reaction conditions. We found that transphosphatidylation in biphasic system in the presence of a salt at extremely high concentrations greatly improves the yield. We concluded that PI yield improvement in the presence of salt can be explained mainly by the enhanced localization of the enzyme at the solvent–water interface. In addition, large-scale enzymatic syntheses afforded several 1-PI species of high purity, with defined chemical structures and in reasonable yields.

MATERIALS AND METHODS

Materials *sn*-1,2-Dioleoyl-PC (DOPC) and *sn*-1-palmitoyl-2-oleoyl-PC (POPC) were purchased from NOF Corporation (Tokyo, Japan). *sn*-1,2-Dihexanoyl-PC (DHPC) and *sn*-1-stearoyl-2-arachidonoyl-PC (SAPC) were from Avanti Polar Lipids (Alabaster, AL, USA). *myo*-Inositol was from Wako Pure Chemical Industries (Osaka, Japan). Choline oxidase (COD) and horseradish peroxidase (HRP) were purchased from Toyobo (Osaka, Japan). 10-Acetyl-3,7-dihydroxyphenoxazine (Amplex Red) was from Cayman Chemical (Ann Arbor, MI, USA).

Enzyme preparation The engineered PLD variant NYR-186T was used throughout this study. This enzyme is a variant of SaPLD with three amino acid substitutions (G186T/W187N/Y385R) and is characterized by the highest 1-PI positional specificity (15). The enzyme was prepared from the recombinant *Escherichia coli* BL21(DE3) strain. Unless stated otherwise, a partially purified enzyme (~60% purity based on SDS-PAGE data) was used. The concentration of the partially purified PLD was estimated by comparing its band density with that of a known amount of a fully purified enzyme on SDS-PAGE. The fully purified PLD (>95% purity) was prepared as described previously (17). Protein concentration of the fully purified PLD was estimated from the absorbance at 280 nm with an extinction coefficient of 1.388 at a concentration of 1 mg/mL, which was calculated using the ProtParam tool (http://web.expasy.org/protparam/).

PI synthesis Transphosphatidylation of PC with *myo*-inositol was performed in a biphasic system consisting of ethyl acetate and acetate buffer. A mixture consisting of 1 mg DOPC dissolved in 100 μL of ethyl acetate, 18 mg of *myo*-inositol, 80 μL of 50 mM sodium acetate buffer (pH 5.6), and NaCl in different concentrations was prepared in a 1.5 mL tube. Twenty microliters of the 0.5 mg/mL PLD solution prepared in 50 mM acetate buffer (pH 5.6) was added to start the reaction. The mixture was incubated at 20°C with shaking at 15,000 rpm using a tube shaker (model M-BR-022UP, TAITEC, Kawagoe, Japan) for 24 h. The reaction was quenched by 10 μL of 1 M HCl. The phospholipids were extracted with 200 μL of chloroform/methanol (2:1, v/v) and stored in a freezer (−25°C) until further analyses.

Large-scale PI synthesis In a glass flask, 100 mg of DOPC was dissolved in 10 mL of ethyl acetate and mixed with 8 mL of NaCl-saturated 50 mM sodium acetate buffer containing 1.8 g *myo*-inositol. After adding 2 mL of the 0.5 mg/mL PLD solution, the mixture was incubated at 20°C with magnetic stirring for 24 h. One milliliter of 1 M HCl was added, and the lipids were extracted with 20 mL of chloroform/methanol (2:1). After removal of the solvent by a vacuum evaporator, the lipids were dissolved in 2 mL of chloroform and loaded on a silica gel column (Wako gel C-200, 3 g). The column was eluted with ~100 mL of chloroform/methanol (9:1) to collect PA, followed by elution with ~120 mL of chloroform/methanol (7:3) to collect PI. The eluate was collected in 6 mL fractions. The fractions containing PI were combined and the solvent was evaporated to afford pure 1,2-dioleoyl-PI (DOPI). A similar procedure was employed for the synthesis of *sn*-1-stearoyl-2-arachidonoyl-PI (SAPI) and *sn*-1-palmitoyl-2-oleoyl-PI (POPI) from SAPC and POPC, respectively.

Lipid analysis The composition of the phospholipid classes (PC, PI, and PA) was analyzed by thin layer chromatography (TLC) and/or normal-phase HPLC as described previously (18).

The composition of PI positional isomers was analyzed as described elsewhere (14). In brief, the PI samples were derivatized into the corresponding *pentakis*-phenylethylcarbamoyl-PI (PI-PEC) followed by the liquid chromatography–electrospray ionization–mass spectrometry (LC–ESI–MS) analysis. The PI-PEC isomers were detected by a single ion monitoring at m/z = 1596.9, 1620.9, or 1578.9 for DOPI-PEC ($C_{90}H_{128}N_5O_{18}P$), SAPI-PEC ($C_{92}H_{128}N_5O_{18}P$), or POPI-PEC ($C_{88}H_{126}N_5O_{18}P$), respectively, and the isomeric purity was calculated from the peak areas.

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